

Macrophage Polarization during Tuberculosis for the Implementation of Host-Directed Therapy by a Novel Immunomodulator

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of Doctor of Philosophy in Sciences*

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Abstract

Tuberculosis, a serious type of infectious disease, is caused by the *Mycobacterium tuberculosis*. The medications against TB are becoming limited due to the XDR-TB and MDR-TB. In this context, host-directed-therapy (HDT) can be a novel approach to treat TB as it targets the TB infected macrophages, instead of the pathogen, through differential macrophage activation i.e. M1/M2 macrophage polarization. In this context, this study has been conducted to investigate macrophage polarization grade during *M. tuberculosis* infection and the role of STK11 isolated from nutshell of *Cocos nucifera* L. on it.

We have found that 6 hrs of H37Ra infection could induce M2 macrophage polarization enhancing the expressions of Arg-1, YM-1, FIZZ-1, CD206, IL-4, & IL-10 significantly ($p < 0.05$). Similarly, H37Rv infection upregulated these M2 markers and diminished M1 markers (iNOS2, iNOS2, CD68, IL-6) in macrophages, while, BCG and heat-killed H37Ra-challenge brought about reverse results. Hence, accomplishment of the cure of *M. tuberculosis* might depend on M1 polarization that could be achieved using a modest immunomodulator.

In this context, we have selected STK11 (46 kDa glycoprotein), isolated from the nutshell of *C. nucifera* L., that earlier proved its potential for macrophage M1 activation. Results indicated that STK11 (200 $\mu\text{g/ml}$) could restrict up to 44.83 % bacilli growth in direct killing assay, while STK11 (10 $\mu\text{g/ml}$) significantly ($p < 0.001$) decreased 62.86 % of intracellular bacilli growth within macrophages highlighting the advantages of HDT using it. Additionally, STK11 boosted M2 markers and reduced M1 markers significantly ($p < 0.05$) in H37Ra-infected macrophages. Hence, STK11 might enforce the M1 polarization to restrict the bacilli growth within the host cells.

Further, STK11 interacted with Ras isoforms and potentiate the nitrite dependent killing of intracellular H37Ra bacilli.

The efficacy of the STK11 have been examined against latent state of *M. tuberculosis* also. Herein, the results indicated that STK11 (200 µg/ml) inhibited the latent H37Ra bacilli by 49.10% in the resazurin assay indicating the direct killing potential of it. STK11 (10 µg/ml) could kill the latent H37Ra bacilli by 73.53% within the macrophages with concomitant nitrite release. This subsequently increased the M1 markers and diminished the M2 markers in latent H37Ra-challenged macrophages. Besides, mRNA expression of TLRs supported the interaction of STK11 with it, which might be involved in STK11-mediated M1 polarization in infected macrophages.

Finally, we have examined the combinatorial effect of STK11 (200 µg/ml) with rifampicin (0.12 µg/ml) against *M. tuberculosis* (H37Ra). This could restrict the growth of H37Ra bacilli by 63.19%. Interestingly, in the presence of STK11 (2.5 µg/ml), rifampicin (0.1 µg/ml) could kill the intracellular bacilli up to 72.20%, which indicated that STK11 could enhance the efficacy of the rifampicin. Further the combinatorial treatment with rifampicin and STK11 also endorsed the M1 markers and suppressed the M2 markers in H37Ra-infected macrophages with prompt nitrite generation, highlighting the effectiveness of combination therapy over their individual effect.

Altogether, STK11 alone or in combination with rifampicin, could be considered as a potent immunomodulator interacting with TLRs and Ras isoforms that might be acquainted for the treatment of tuberculosis through HDT in the near future. However, further studies are required to enlighten this work.

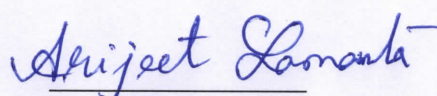
Keywords: *Mycobacterium tuberculosis*; Active TB; Latent TB; Macrophage polarization; STK11; Rifampicin; Host-directed therapy.

DECLARATION

I, **Arijeet Samanta**, a research scholar at **Biodiversity and Environmental Studies Research Centre** affiliated to **Vidyasagar University** at **Midnapore City College**, do hereby declare that the doctoral thesis titled “**Macrophage Polarization during Tuberculosis for the Implementation of Host-Directed Therapy by a Novel Immunomodulator**” is the original research work, carried out by myself under the guidance of **Dr. Santanu Kar Mahapatra**, Associate Professor, Dept. of Paramedical and Allied Health Science, Midnapore City College. This thesis has not been submitted in part or in full to any University for the award of any degree or diploma.

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Abbreviations

ADC	Albumin Dextrose Catalase
AM	Alveolar macrophages
AMK	Amikacin
AMPK family	AMP-activated protein kinase
AMR	Antimicrobial resistance
Arg1	Arginase1
BBB	Blood–Brain Barrier
BCG	Bacillus Calmette-Guerin
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
BTK inhibitor	Bruton's Tyrosine Kinase inhibitor
<i>C. nucifera L.</i>	<i>Cocos nucifera</i> Linn.
CCL	C-C motif chemokine ligand
CD	Cluster of Differentiation
CDC	Centers for Disease Control
CFU	Colony Forming Unit
cGAS	Cyclic GMP-AMP synthase
CLRs	C-type lectin receptors
CPR	Capreomycin
CXCL10	C-X-C motif chemokine ligand 10
CXCL9	Chemokine (C-X-C motif) ligand 9
CYS	Cycloserine
DCIR	Dendritic Cell Immunoreceptor
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment, Short-Course
DRS	Drug Resistance Surveillance
DR-TB	Drug-resistant tuberculosis
EDTA	Ethylene Diamine Tetra acetic Acid

ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Ethambutol
Enoyl-ACP reductase	Enoyl-[acyl carrier protein] reductase
ERK-1/2	Extracellular Signal-Regulated Kinases 1 and 2
ESAT-6	Early Secreted Antigenic Target 6 kDa
ETA	Ethionamide
FASTA	Fast-All
FBS	Fetal Bovine Serum
FFT	Fast Fourier Transform
FIZZ1	Found in Inflammatory Zone 1
Fpr2	Formyl Peptide Receptor 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA-3	GATA binding protein 3
HDT	Host Directed Therapy
HIV	Human immunodeficiency virus
HK-H37Ra	Heat-killed H37Ra
H-Ras	Harvey Rat Sarcoma viral oncogene homolog
HSR	Headspace to volume ratio
IFN- γ	Interferon gamma
IFN- γ R	Interferon-gamma Receptor
IGRA	Interferon Gamma Release Assay
IL	Interleukin
INH	Isoniazid
iNOS2	Inducible nitric oxide synthase 2
IRF	Interferon Regulatory Factor
IRMSD	Interface Root Mean Square Deviation
JAK-STAT	Janus kinase-signal transducer and activator of transcription
JMJD3	Jumonji domain-containing protein-3
JNK-P13/AKT	c-Jun N-terminal kinase (JNK) and Protein Kinase B
KLF-4	Kruppel-like factor 4
KM	Kanamycin
K-Ras	Kirsten rat sarcoma viral oncogene homolog

LPS	Lipopolysaccharide
LTBI	Latent tuberculosis infection
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MDR-TB	Multidrug-resistant tuberculosis
MHC-II	Major Histocompatibility Complex Class II
MIC	Minimum Inhibitory Concentration
MIP1 α	Macrophage Inflammatory Protein 1-alpha
MLP	Mid log phase
MOI	Multiplicity of Infection
MR	Mannose receptor
MRC-I	Macrophage Receptor with C-type Lectin 1
MSR-I	Macrophage Scavenger Receptor 1
MTCC	Microbial Type Culture Collection and Gene Bank
MXF	Moxifloxacin
NDDSs	Novel Drug Delivery Systems
NF- κ B	Nuclear factor kappa-B
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NK cells	Natural killer cells
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NOD-like Receptor family pyrin domain containing 3
NLRs	Nucleotide-binding domain and leucine-rich repeat-containing receptors
NO	Nitric Oxide
NOD	Nucleotide-binding oligomerization domain
N-Ras	Neuroblastoma RAS viral oncogene homolog
NRP	Non-replicating persistence
OA	Osteoarthritis
OD	Optical Density
p65	RELA (REL-associated protein)
PAS	para-Amino salicylic acid
PBCA	Poly butyl cyano acrylate
PDB	Protein Data Bank

PIBCA	Poly isobutyl cyano acrylate
PLG acid	Poly lactic-co-glycolic acid
PPAR	Peroxisome Proliferator-Activated Receptor
PRR	Pattern recognition receptors
PZA	Pyrazinamide
Rif	Rifampicin
RIPA	Radio Immuno Precipitation Assay buffer
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RR-TB	Rifampicin-resistant tuberculosis
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SIRP α	Signal regulatory protein α
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of interferon genes
STK11	Serine Threonine Kinase 11
TAM	Tumour-associated macrophages
TB	Tuberculosis
TE	Tris EDTA
TGF β -SMAD	Transforming Growth Factor beta-SMAD
TGF- β	Transforming Growth Factor beta
Th-1	T helper 1 cells
Th-2	T helper 2 cell
TLR	Toll-like receptor
TNF- α	Tumor Necrosis Factor alpha
TPE	Tuberculous pleural effusion
UIM	Uninfected Macrophages
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis
YM-1	Chitinase-like protein 3 (Chil3)

Chapter 1: Introduction

1.1 General: Tuberculosis

Tuberculosis (TB) is an airborne contagious and perilous infectious disease caused by the bacilli, *Mycobacterium tuberculosis* (Sravanthi et al., 2024). Almost ten (10) million active cases of TB including 1.5 million mortalities annually declares it to be one of the top infectious killers in the world (WHO, 2025). TB cases are reported throughout the world, particularly, low- and middle-income countries, while, most of the cases are reported from India, China, Bangladesh, Nigeria, Indonesia, Philippines, Pakistan, and South Africa (WHO, 2025). It has been reported that about 25% of the world population is infected with different types of *Mycobacterium sp.*; however, all may not results TB, as some may recover from the infection (WHO, 2025). As per the reports, there are 5-10% chance of recurrence also besides the new cases. People with weakened immune systems, and comorbidities like, diabetes, HIV, as well as, risk factors like, malnutrition, tobacco and alcohol intake are more susceptible to the TB (Olmo-Fontánez and Turner, 2022). The common symptoms of tuberculosis are chest pain, prolonged cough, weight loss, fever, weakness or fatigue, and night sweats (Luies and Du Preez, 2020). Based on the mode of infection, TB infections are can be categorised into two types namely: active TB and latent TB. Individuals infected with latent TB do not exhibit any TB symptoms or indicators, however, weaken immune system may progress to active TB (Gengenbacher and Kaufmann, 2012). Healthcare professionals may employ a tuberculin test, chest X-ray with sputum microbiology and NAAT test to confirm the TB infection. They also screen at-risk individuals to rule out active TB (WHO, 2025). Active medications through-typical six-month course of four

antibiotics cure TB. However, the illness may spread and develop drug resistance if the treatment is not thoroughly accomplished (WHO, 2025).

1.2 Epidemiology of TB

TB is placed the highest cause of infectious disease-related death. Unfortunately, in 2023, the WHO stated that most TB cases were originated in the regions of Africa, South-East Asia, Eastern Mediterranean, Western Pacific, Europe, and Americas, with respective percentages of 23%, 46%, 8.1%, 18%, 2.2%, and 3.1%. Worldwide, 87% of cases are attributed from top 30 high TB-burden countries. Among them, 8 nations—India, Indonesia, China, Philippines, Nigeria, Pakistan, Bangladesh, and the Democratic Republic of the Congo—contributed 27%, 10%, 7.1%, 7.0%, 4.5%, 5.7%, 3.6%, and 3%, respectively, contributing to the two-thirds of all cases worldwide. According to the India TB Report 2023, the anticipated incidence of all forms of TB in the year 2020 was 188 per 100,000 people (129 to 257 per 100,000 people). While 19,33,381 events of TB patients (including new cases and relapses) were reported in 2021, which was the 19% higher than the year before. According to Global TB report 2025, India (25%), Indonesia (10%), China (6.5%), Philippines (6.8%), and Pakistan (6.3%) made up the five nations that accounted for 56% of the global total. Reports also indicated that men made for 54% of TB cases in 2024, followed by women (35%), and children and early adolescents (12%). In the year of 2024, TB claimed an estimated 1.23 million death globally, including 1.08 million HIV-negative and 150,000 HIV-positive individuals. A large (1.7%) decline in the global TB incidence rate was observed between 2023 and 2024; the rate returned to 2020 levels, falling to 131 per 100,000 population in 2024. WHO TB strategy included a milestone of reducing TB incidence rates by 20% in 2020, and by 50% in 2025 compared to the 2015 base year.

WHO also target to 80% reduction of TB incidence rate from 2030 and 90% reduction of TB incidence rate from 2035 (Figure 1.1).

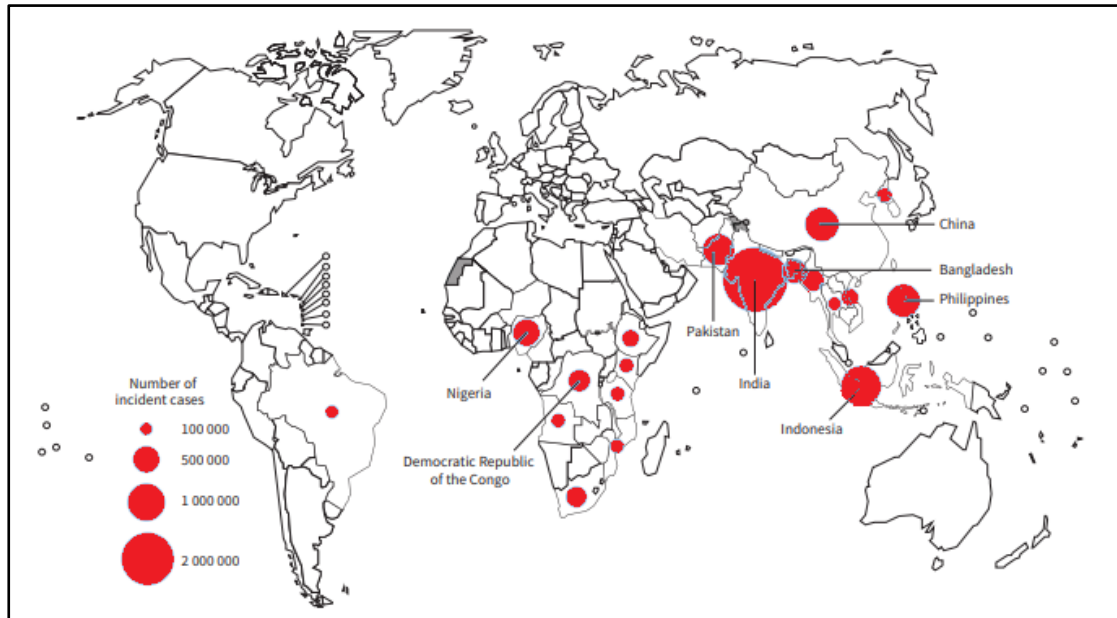


Figure 1.1 Global epidemiology of TB, WHO 2025

1.3 TB pathogenesis

Active TB patients can spread the disease by coughing, sneezing, even while speaking with people, releasing aerosol droplets containing *M. tuberculosis* (Sarkar & Sarkar, 2025). When the new host inhales the *M. tuberculosis* bacilli, then enter the lungs through the respiratory tract. In this stage, alveolar macrophage of the host tries to suppress the *M. tuberculosis* infection. But when the alveolar macrophages unable to kill the bacilli, then the inoculum number increases within the microenvironment, followed by released from it. Then naive alveolar macrophages phagocytose these released bacilli, and the cycle repeats (Leung, 1999). Lymphocytes are also recruited to the site of infection, initiating cell-mediated immune response, in which a cluster of immune cells reaches, attempting to confiscate the bacteria and restrict additional multiplication (Luies & Du Preez, 2020). The host is asymptomatic at this point, and

the TB bacteria may either get eliminated or enter a state of dormancy within the granuloma. However, in weakened immunity, the disease may instantly progress into active TB disease with clinical symptoms (Schluger, 2005).

The fundamental feature of pulmonary TB is the granuloma, which is a shapeless gathering of macrophages and other immune cells pointing to restrict the bacterial spread (Schluger, 2005). Although the granuloma cannot eradicate the pathogen, it confines the bacilli and stops the progression to the disease of active state. Though the bacteria survive by avoiding death, which is achieved by blocking phagolysosome fusion and subverting the host's immune response. This process founds a hospitable niche for *M. tuberculosis* where it can survive for decades and decades, outsmarting the immune system and persisting in a non-replicating or slowly replicating state (Luies & Du Preez, 2020). In this situation, the patient is asymptomatic and non-infectious (latently infected).

Macrophages are transformed into foamy macrophages and other morphotypes as the granuloma matures (Russell et al., 2009). The necrotic lysis of the host immune cells may cause the granuloma's centre to necrotise, resulting in caseum (caseous granuloma) (Huszar et al., 2020). The tubercle bacilli are enclosed and protected by the caseous lesions, which act as reservoirs and keep the bacteria in a latent stage (Grosset, 2003). But in the latter stages of the disease, the caseous core softens and cavitation occurs, which causes the bacteria to be revived. The patient then has active TB, which results in the infectious bacilli spreading to a new host (Russell et al., 2009; Grosset, 2003). This is illustrated in figure 1.2.

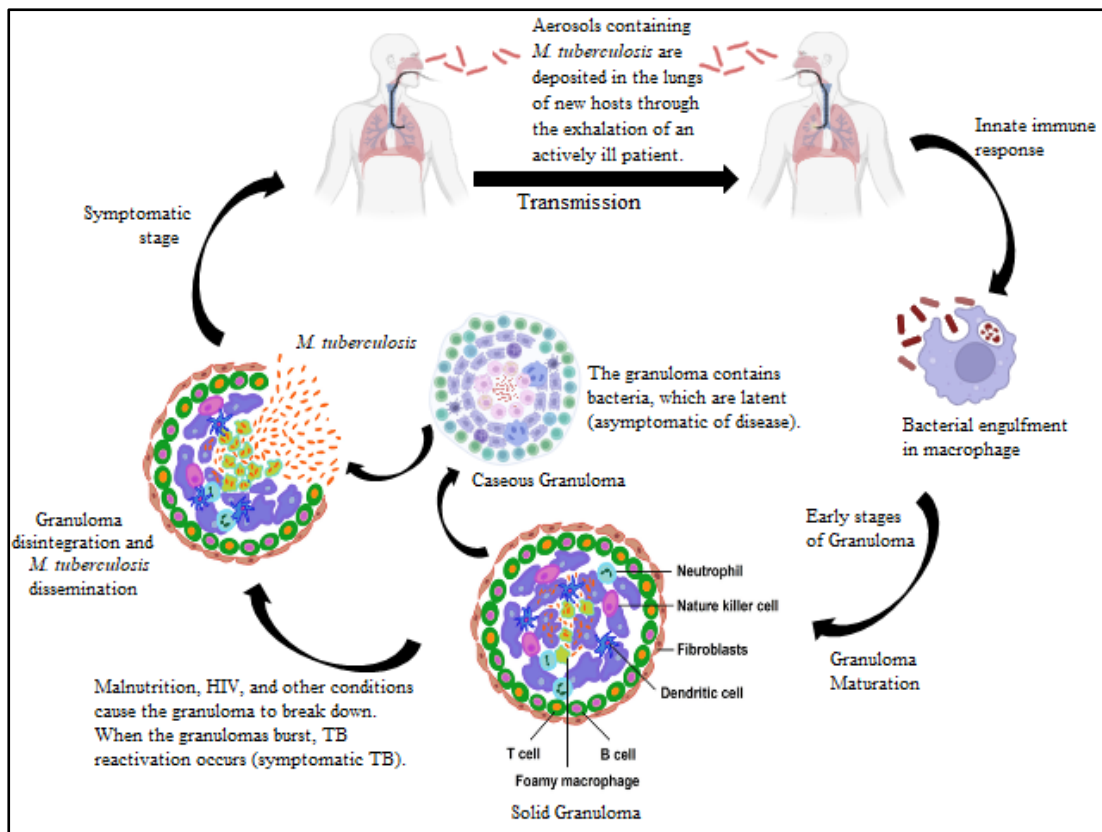


Figure 1.2 Pathogenesis of TB. When the aerosolized form of *M. tuberculosis* enters the lungs through inhalation from the active TB patients, then alveolar macrophages and other immune cells try to engulf the bacilli and form a structure called a granuloma. Granuloma can be the same condition over the decades, without symptoms, and the condition is called the latent stage of TB. But after the different conditions like malnutrition, HIV, etc., the granuloma can be broken down and burst, and TB reactivation occurs, and the cycle repeats.

1.4 Treatment against tuberculosis

Treatment options against TB comprises as stated below:

1.4.1 First-line anti-TB drugs

Using first-line drugs such as isoniazid (INH), pyrazinamide (PZA), rifampicin (Rif), and ethambutol (EMB) for a two-month initial phase, then the continuation with isoniazid and rifampicin for next four months, is the most widely used conventional chemotherapeutic regimen for treating TB (Table 1.1). Due to high resistance rates, streptomycin, a bactericidal antibiotic that affects the synthesis of polypeptides, is no longer considered a first-line drug (Haas & Belknap, 2018; Cohen et al., 2020).

Table 1.1: First-line drug & second-line anti-TB drugs used to treat tuberculosis.

First-line drug & second-line drug	Target	References
Isoniazid (INH)	Enoyl-ACP reductase, mycolic acid elongation	Inturi et al., 2016
Rifampicin (Rif)	DNA-primed RNA polymerase	Bansal et al., 2018
Pyrazinamide (PZA)	Biosynthesis of Fatty acid /depolarization of membrane /RpsA, and the ribosome-sparing translation process	Shi et al., 2011
Ethambutol (EMB)	Deposition of cell wall arabinan	Belanger et al., 1996; Zhang, L et al., 2020
Streptomycin	Inhibition of protein synthesis	He et al., 2025
Amikacin (AMK)	mRNA Misconception and Protein Synthesis Inhibition	Seely et al., 2023
Kanamycin (KM)	Mistranslation and indirect translocation inhibition during protein synthesis.	Vioque, A., & de la Cruz, J., 2003
para-Amino salicylic acid (PAS)	Folic acid biosynthesis inhibition.	Zheng et al., 2013
Cycloserine (CYS)	Bacterial cell wall synthesis inhibition.	Baum et al., 2007
Ethionamide (ETA)	Synthesis of mycolic acid is inhibited by binding to ACP reductase.	Schroeder et al., 2002
Capreomycin (CPR)	Protein synthesis inhibition.	Lin et al., 2014
Ciprofloxacin	Interactions with DNA gyrase, DNA replication, and transcription	Evans-Roberts et al., 2016

1.4.2 Second-line anti-TB drugs

When treating TB in specific conditions like multidrug-resistant tuberculosis (MDR-TB) or extensively drug-resistant tuberculosis (XDR-TB), second-line drugs are advised to use. Different classes of second-line drugs used to treat TB which are listed

in table 1.1. Although, second-line drugs differ from first-line drugs; they may have toxic side effects (like cycloserine), be less effective than first-line drugs (like p-aminosalicylic acid), or not be available in many developing nations (like fluoroquinolones) (Prasad et al., 2021).

1.4.3 Directly observed treatment, short-course (DOTS)

In TB, resistance to drugs is more significant and is a result of insufficient chemotherapeutic management, which extends the course of treatment, increases side effects, and complicates it (Seung et al., 2015). Regardless of sensitivity or resistance to other drugs, MDR refers primarily to the *M. tuberculosis* strains' resistance to both isoniazid and rifampicin. Although treatment resistance can be addressed with alternative second-line or first-line drugs under DOTS Plus. MDR-TB is of concern because it is associated with a significant risk of mortality (Kaona et al., 2004).

Centres for disease control and prevention (CDC) have recently described XDR-TB strains that are resistant to at least 3 of the 6 classes of second-line drugs (fluoroquinolones, polypeptides, aminoglycosides, cycloserine, thioamides, and p-aminosalicylic acid). In certain areas, nearly 20% of MDR-TB cases are identified as XDR-TB, which raises concerns about a potential future epidemic of TB that is nearly incurable (Chin et al., 2024). In spite of these, the best method currently available for TB control is DOTS (WHO, 2025).

There are five main principles form the basis of the WHO's global plans to stop TB are as, (I) political promise to regulate TB (Establish a centralized and selected system for tuberculosis recording, monitoring, and training); (II) case finding by sputum smear microscopy; (III) anti-TB drugs to be administered under the direct supervision of the health care provider/community DOT provider; (IV) a steady, consistent supply of anti-

TB drugs; and (V) a methodical reporting and recording system that enables evaluation of each patient's treatment outcomes for the entire TB control program.

With a success rate of over 95%, DOTS is a controlled chemotherapy that restricts the emergence of new MDR strains of TB (Shah et al., 2007). Health providers must support the associations between private and public practitioners for the program to operate appropriately and efficiently. More than 150 countries have implemented health services to advance public health and safety since 2001. The quality secure laboratory capacity (smear, culture, and drug sensitivity testing), treatment design, long-term adherence to difficult-to-take regimens, drug procurement, side-effect management, recording, reporting, human and financial resource constraints are all critical to the success of DOTS-Plus. Accordingly, DOTS-Plus is far more costly and complicated than DOTS (Singh, A. et al., 2019). MDR-TB can be detected and allayed with the use of drug resistance surveillance data. For the treatment of MDR-TB, WHO now suggests a combination of amikacin (AMK), ethionamide (ETA), fluoroquinolone (such as moxifloxacin, MXF), and PZA (Espinosa-Pereiro et al., 2022). The CDC's primary goal is to control XDR-TB, a type of MDR-TB that is also resistant to fluoroquinolones and at least one injectable second-line drug, such as kanamycin, amikacin, and capreomycin (Seaworth & Griffith, 2017).

1.4.4 Novel drug delivery systems (NDDSs)

The primary cause of drug resistance in TB patients is non-compliance of the patient. Additionally, patients with MDR-TB must take second-line anti-TB drugs for 18 to 24 months. The most promising approach to improve TB therapy seems to be the use of innovative drug delivery devices. NDDSs are biocompatible, release the drug continuously, reduce the frequency of doses, and shorten the overall treatment duration.

As a result, patient compliance improves. Over the past ten years, inhaled drugs for treating both local and systemic diseases have gained significant interest. Table 1.2 provides details on developed drug delivery systems.

Table 1.2: A novel drug delivery system to control TB.

Drug delivery system	Remarks	Reference
Rifampicin-containing mannitol microparticles	Active for the rifampicin delivery to the lung, but Rifampicin is rapidly detached from the lung into the blood circulation.	Garcia-Contreras et al., 2007
Rifampicin + pyrazinamide + isoniazid with poly(lactic-co-glycolic) acid (PLG) nanoparticles	Maintained plasma drug level above MIC (6-9 days).	Mizoe et al., 2008; Pandey et al., 2003
Ethambutol with PLG nanoparticles	Plasma level maintained for 7 days	Pandey et al., 2006
Rifampicin nanoemulsion	99% entrapment efficiency	Ahmed et al., 2008
Clofazimine suspension	Overcomes low solubility	Peters et al., 2000
Rifampicin nanosuspension	Localized delivery to the lungs	Reverchon et al., 2002
Rif, INH, and streptomycin within Poly butyl cyano acrylate (PBCA) and Poly (isobutyl cyano acrylate) (PIBCA) nanocapsules	Longer stability	Shegokar et al., 2011
Isoniazid-poly (ethylene glycol)-poly (aspartic acid) conjugates	5.6-Fold increase in antitubercular activity	Silva et al., 2001
Rifabutin liposomes	Increased therapeutic activity	Gaspar et al., 2008

1.4.5 New anti-TB drugs

As part of the new multidrug approach, several new anti-TB drugs are used alongside existing TB drugs after evaluation for the clinical portfolio. The development of new anti-TB drugs is being actively involved by the WHO, NIAID/NIH, CDC, Wellcome Trust, TDR, Lupin, Bayer, Johnson & Johnson, and their academic partners, and several research institutions. This could help advance the drug-discovery and drug-development process against this disease and offer combination therapy for the treatment of XDR-TB & MDR-TB (Singh & Chibale, 2021).

Most of the anti-TB drugs currently on the market are out-of-date, have major side effects, and are resistant to *M. tuberculosis* bacilli because they were designed approximately 2 to 4 decades ago. Therefore, it is imperative to develop a new generation of anti-TB drugs having a different mechanism of action than current drugs. Numerous new compounds are currently undergoing clinical development as a result of the diligent work of researchers in this regard (Bansal et al., 2018). In this context, another novel approach to combat this disease is host-directed therapy targeting macrophage polarization (Samanta et al., 2024), which is thoroughly described later sections.

1.5 Macrophage polarization and its subtypes, activation, and function

Macrophages are the initial line of defence against specific bacteria through phagocytosis, cytokine secretion, and antigen presentation which differentiate from monocytes and bone marrow hematopoietic stem cells (Matucci et al., 2014; Hirayama et al., 2017). In response to the microenvironment, macrophages exhibited vast functional plasticity, and they polarized into a definite phenotype. M1 macrophages boost the expression of inflammatory cytokines as IL-12, TNF- α , IL-6, iNOS, and

CD86. These have the ability to eradicate intracellular infections and tumour cells, hence enhancing the Th-1 immune response (Kroner et al., 2014; Magdalena & Magdalena, 2022). On the other hand, anti-inflammatory activity is linked with M2 macrophages (alternatively activated) (Zhang et al., 2020). As per different stimuli, M2a, M2b, and M2c are the designations for the three subpopulations of M2 phenotypic macrophages, which differ according to various stimuli. Scavenger receptor, mannose receptor, and arginase-1 (Arg-1) expression are induced in M2a macrophages by IL-4 and IL-13, whereas IL-1R ligands and immune complexes activate M2b macrophages, raising IL-10 production and promoting the response of Th2. Following the released pentraxin 3 and chitinase 3 which play a role in wound healing, IL-10 predominantly activates M2c macrophages (Lawrence & Natoli, 2011).

The human system is highly intricate in its functioning due to the association of many different systems. The immune system is complex, with a network of various immune cells in action. An encounter or challenge with any non-native substance triggers a shot of host immune response, provoking innate and adaptive responses against it. Dendritic cells, macrophages, and natural killer cells are the immune cells connected to the innate immune response. Among the other immune cells, monocyte-derived macrophages are recognized as very versatile. Earlier considered only to be associated with the host defense and apoptotic cells clearance, but now the plethora of literature indicates that macrophages are more than just phagocytic cells and play a pivotal role in many facets due to their dynamicity and their property to orient to the environment they are defied with (Röszer, 2015).

Polarization is often defined as "the activation of the macrophages at a given point in time" (Murray, 2017). Macrophages can be polarized into two different states of activity, known as M1 and M2. These two macrophage subsets have been identified to

evoke a wide range of functionalities (Canton, 2014; Mantovani et al., 2004; Sica & Mantovani, 2012). Secreted cytokines, cell surface indicators, and biological roles of these macrophages vary depending upon the polarization state (Atri et al., 2018). The final result of arginine metabolism is M1/M2 polarity that occurs through two opposite pathways. One of them is the iNOS pathway, which generates nitric oxide and citrulline from arginine, resulting in macrophages that resemble M1 cells. The other is the arginase route, which converts the M2-like macrophage-producing enzyme arginase into urea and ornithine (Lampiasi et al., 2016).

LPS and Th1 cytokines, including IFN- γ and TNF- α , activate macrophages, which then polarize into M1 macrophages. Nonetheless, the transcription factor, T-bet, plays a crucial role in the Th1 cytokines release (Lawrence, & Natoli, 2011). Toll like receptor (TLR) -2, TLR-4, CD80, CD86, and MHC-II are several surface markers that are present on M1 macrophages. These cells secrete cytokines and chemokines, including TNF- α , IL-1, IL-6, IL-12, C-X-C motif chemokine ligand (CXCL) -9, and CXCL10, which have a beneficial effect on normal macrophages, M0 or unpolarized macrophages, and cause them to change into the M1 state. Transcription factors such as NF- κ B, STAT1, STAT5, IRF3, and IRF5 are essential for regulating M1 genes. Additionally, in the case of M1 macrophages, two transcription factors named NF- κ B and STAT1 play a vital role in the M1 macrophages' microbicidal and tumoricidal activities (Murray, 2017; Porta et al., 2015; Wang et al., 2014; Weisser et al., 2013). Pro-inflammatory cytokines are produced against bacterial or viral encounters by macrophages, leading to M1 polarization. Neutrophils, natural killer (NK) cells, and Th1 are engaged by the cytokines, and these compounds increase pro-inflammatory cytokines and kill external pathogens (Malyshev & Malyshev, 2015). Moreover, the activity of killing the M1 phenotype is increased by nitrite production. M1 macrophages

possess antitumoral (inhibiting the development of tumours) activities and anti-angiogenic (reducing the new blood vessels' growth that supports tumour growth rather than blocking the growth of tumour cells themselves) activities (Malyshev & Malyshev, 2015). Unpolarized macrophages enter either M1 (classical activation) or M2 (alternative activation) when the microenvironment changes. Macrophage reprogramming is the term for this trend (Malyshev & Malyshev, 2015).

On the other hand, parasite infection and anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF- β) drive M2 macrophages to become polarized as opposed to unpolarized macrophages (Porta et al., 2015; Wang et al., 2014). The M2 macrophages are only directly activated by IL-4 and IL-13. Other cytokines like IL-33, IL-25 help to produce T-helper type 2 (Th2) cytokines due to activation of GATA-3 in helper T-cells, which increases the activation of M2 macrophages (O'Shea & Paul, 2010). In addition, M2 is classified into 3 subgroups, i.e., M2a, M2b, and M2c. M2 macrophages are known to be "biomedically important" because of their tissue remodelling properties, explained in many inflammatory disease studies, and as pro-resolving inflammation. M2 macrophages are an integral part of the tumour microenvironment as tumour-associated macrophages (TAM) that promote blood vessel formation (angiogenesis), thereby ensuring tumour survival (Röszer, 2015; Chen et al., 2008). M2 macrophages are identified by the expression of some surface markers such as CD206, CD163, CD209, mannitol receptor, FIZZ1, YM 1/2. Chemokines and cytokines, i.e., TGF- β , IL-10, CCL1, CCL17, CCL18, CCL22, and CCL24 (Wang et al., 2014; Gordon & Martinez, 2010) alter unpolarized macrophages or M0 to M2 polarization state (Mulder et al., 2014). Transcription factors, i.e., STAT6, JMJD3, IRF4, and PPAR, control the expression of the M2 genes. Till now, the STAT pathway is the one that activates the

M2 macrophages (Murray, 2017). The M1/M2 polarization signals and their effector functions are summarized in figure 1.3.

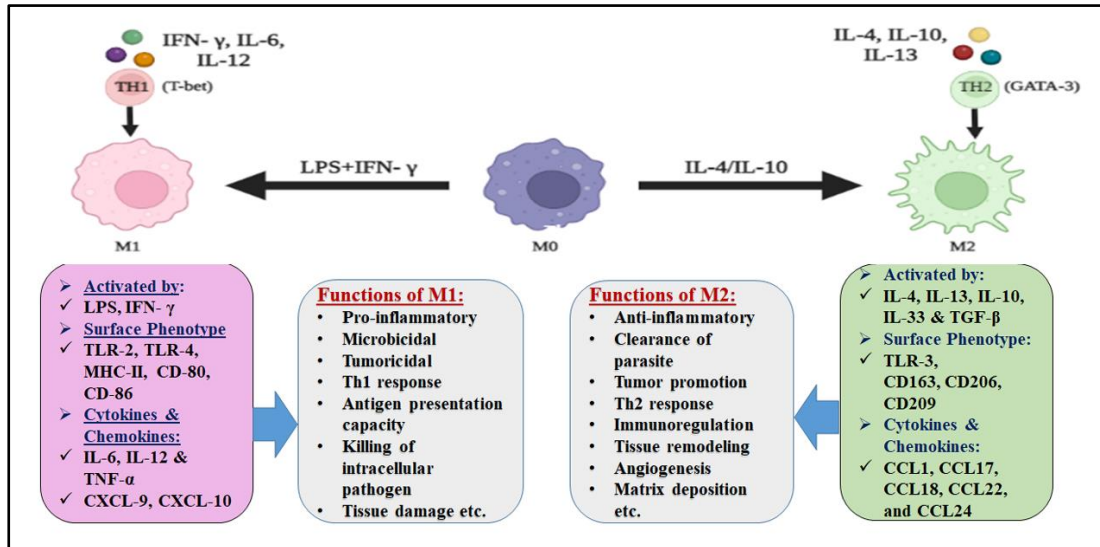


Figure 1.3 Macrophage polarization and specific function of M1 and M2. In the presence of LPS and IFN- γ , M0 macrophages converted to the M1 macrophage state. In the presence of T-bet transcription factor, Th1 cells recruited M1 macrophages and produced IFN- γ , IL-6, and IL-12 cytokines. It produced many surface phenotypes, such as TLR-2, TLR-4, MHC-II, CD-80, and CD-86. It also produced many cytokines and chemokines, IL-6, IL-12, TNF- α , CXCL-9, and CXCL-10. On the other hand, in the presence of IL-4/IL-10, the M0 macrophage cell was converted to the M2 macrophage state. In the presence of the GATA-3 transcription factor, Th2 cells recruited M2 macrophage cells and produced IL-4, IL-10, IL-13, IL-33, and TGF- β . It produces many surface phenotypes (TLR-3, CD163, CD206, CD209). It also produced many specific cytokines and chemokines.

1.6 M1 and M2 markers in polarized macrophages

By providing the first line of defence, the phagocytic macrophages are dynamic in nature and play a vital role in innate immunity or natural immunity (Xuan et al., 2015). In the 1960s, Mackaness introduced macrophage polarization or macrophage activation (Martinez & Gordon, 2014). Macrophage polarization has been studied in immunology for several years. M1 phenotype has pro-inflammatory properties and enhances

pathogen-killing activity. On the other hand, the M2 phenotype has anti-inflammatory properties, contains tissue remodelling capacity, and they are tumorigenic and designated as an inflammation pro-resolver (Martinez & Gordon, 2014). Th1-released IFN- γ or bacterial LPS contribute to M1 polarization, whereas parasite or helminth infection or Th2-released anti-inflammatory cytokines like IL-4 and IL-10 contribute to M2 polarization (Malyshev & Malyshev, 2015). Numerous studies disclosed that M1 and M2 phenotypes express unique proteins or specific markers on their surface (Jablonski et al., 2015). CD80, CD68, CD32, CD64, CD86, CCL19 markers are expressed on M1 macrophages, and they can identify these M1 macrophages by those surface markers and M2 macrophages are identified by MRC1, MGL (lectin type receptor), FIZZ1, CD163, CD206, YM-1 (Roszer, 2015). M1 effector functions increased in NO generation and pro-inflammatory cytokines IL-12, IL1 β , IL-6, and TNF- α generation (Figure 1.4). M2 macrophages increased arginase 1 and TGF- β (Jablonski et al., 2015). Also, transcriptomic analysis has delineated unique M1 and M2 markers like CD38 and Fpr2 as M1 markers and c-Myc and Erg as M2 markers (Jablonski et al., 2015). In human beings, metabolism is an essential process. Various essential enzymes regulate this metabolism process and are interconnected by many pathways. When one pathway becomes defective and leads to disease, this interconnectivity becomes obscure (O'Neill et al., 2016). Several signaling pathways, such as-JAK-STAT, JNK-P13/AKT, NOTCH, and TGF β -SMAD recruited in macrophage reprogramming. The status of polarization is indicated by the regulation of these pathways (Malyshev & Malyshev, 2015).

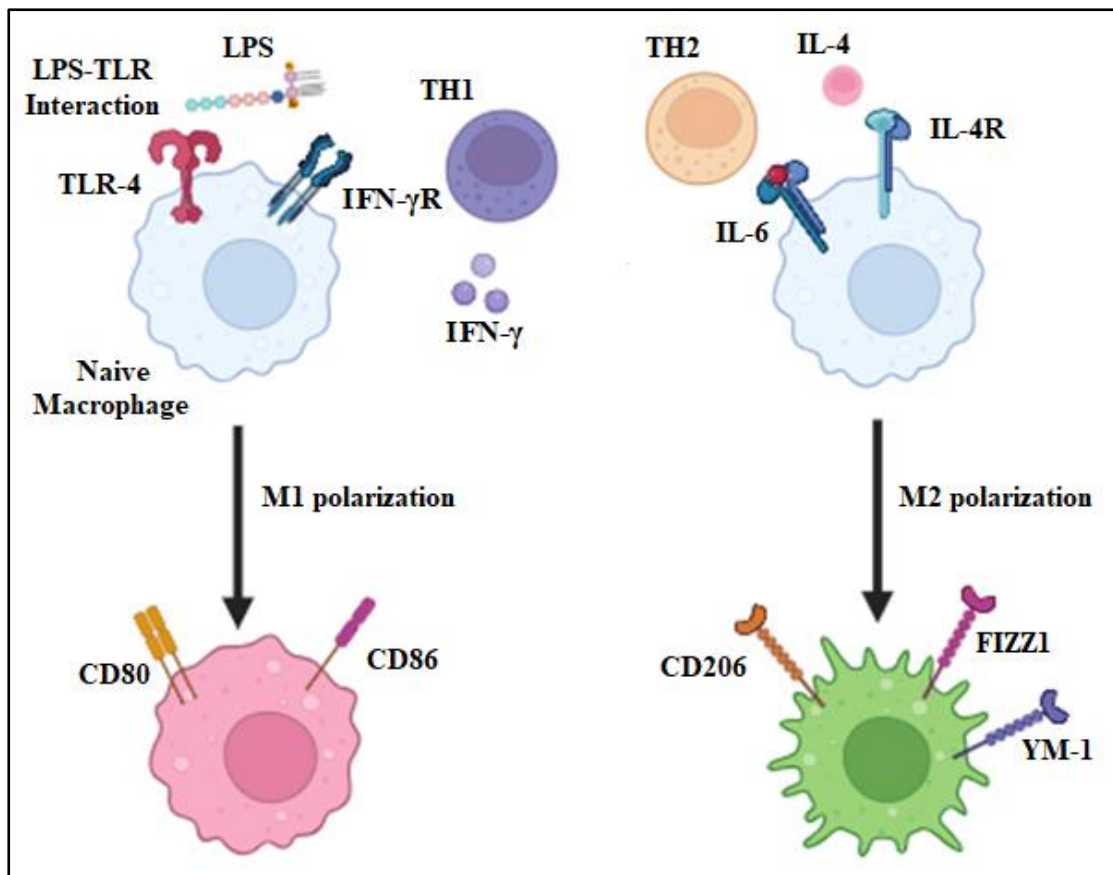


Figure 1.4 Diagram represents the markers of macrophage polarization. When naïve macrophage converted to M1 macrophage polarization state then CD80, CD86 (M1 related) markers were involved. On the other hand, naïve macrophage converted to M2 macrophage polarization state then CD206, FIZZ1, YM1 (M2 related) markers were involved.

1.7 Macrophage polarization in *M. tuberculosis* infection

After the infection with *M. tuberculosis* or with the revival of latent infection in granulomas, the progress of active tuberculosis may occur (Davis & Ramakrishnan, 2009; Volkman et al., 2010; Khabibullina et al., 2022). Within the granuloma, macrophage polarization is decided by type 1 and type 2 inflammatory signals. During M1 polarization, the product of type 1 inflammatory cytokines gives rise to anti-*M. tuberculosis* immune responses, so these cytokines played a major role in resisting *M. tuberculosis* (Quintana-Murci et al., 2007; Diatlova et al., 2023) (Figure 1.5). IFN- γ is described as the driving force behind the M1 program. It has been potentiated to destroy

M. tuberculosis, and it controlled the replication of the remainder (Ehrt et al., 2001; Bhat & Yaseen, 2018). Other studies strongly support that M1 polarization is also an indicator of the initial phase of the anti-*M. tuberculosis* immune response in several animal models, and it is pretty similar to clinical evidence with active TB (Benoit et al., 2008; Ning et al., 2023). In an experiment in a mice model, within the alveolar granuloma compartment, significant amounts of IFN- γ and iNOS signify the M1 polarization during 7 to 30 days post *M. tuberculosis* infection (Redente et al., 2010; Marino et al., 2015). Altogether, the M1 polarization is an integral part of the host immune responses against intracellular pathogenic bacteria and is characterized by the release of proteolytic enzymes, antimicrobial peptides, and pro-inflammatory cytokines and chemokines. It improves phagocytosis and the generation of toxic intracellular granules (Ehrt et al., 2001; Li et al., 2019).

In addition, the type-2 inflammatory milieu is shifted back to type-1 after effective treatment of TB in infected individuals (Raju et al., 2008; Scriba et al., 2017). An earlier report also indicates that M2 signatory IL-4 and IL-13 suppress the autophagy in M1 macrophages and increase *M. tuberculosis* survival (Harris et al., 2007; Khan et al., 2022). Ly et al. (2007) claimed that a higher-level type 2 inflammatory signature is skewing the M1 to M2 having ARG1^{hi} iNOS^{neg} in the alveolar granuloma compartment after day 35 up to day 60 during *M. tuberculosis* infection (Redente et al., 2010). In the later stage, fibrosis in terms of caseous granulomas causes *M. tuberculosis* dissemination, while M2 macrophages obstruct the fibrosis development. It is assumed that the skewing of M2 from M1 shields the immune attack and stand still the pathophysiology caused by *M. tuberculosis* (Dorhoi et al., 2011).

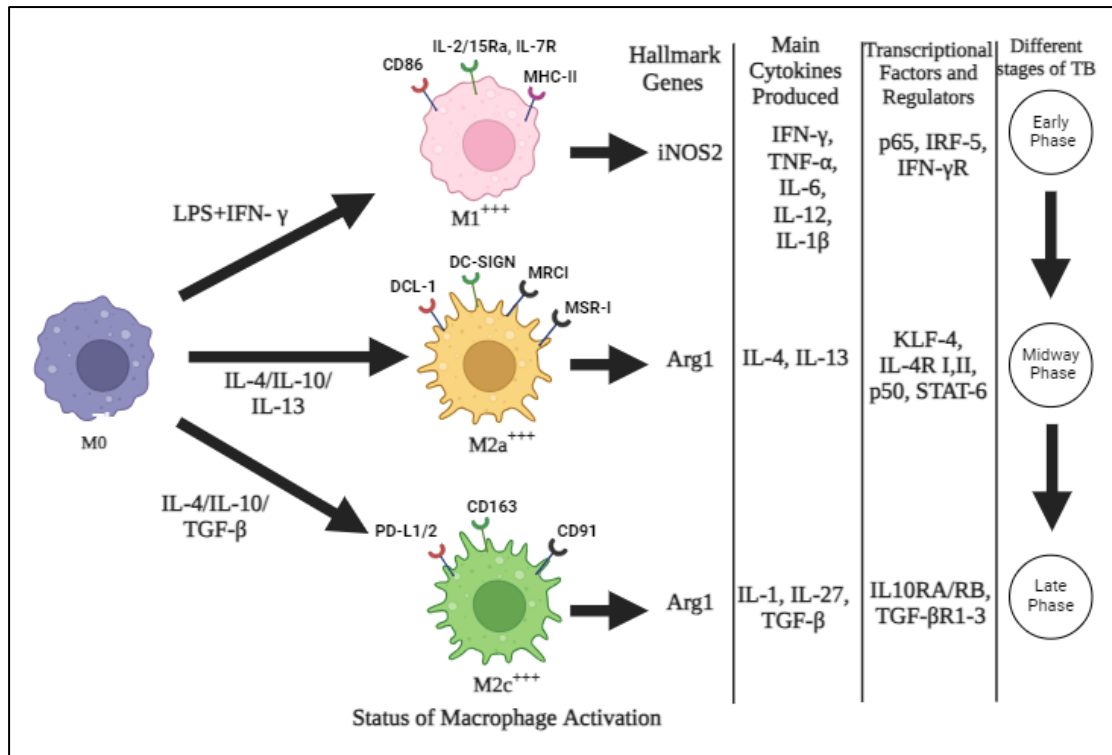


Figure 1.5 Involvement of surface markers, hallmark genes, cytokines, and transcription factors, and the state of macrophage polarization during tuberculosis infection. In the presence of LPS and IFN- γ , M0 macrophage was converted to M1 macrophages, where many surface markers (CD86, MHC-II, etc.) were involved, and iNOS2 hallmark genes were present. In this state, some cytokines were produced, i.e., IFN- γ , TNF- α , IL-6, IL-12, and IL-1 β . Many transcriptions factors, i.e., p65, IRF5, and IFN- γ R recruited. In the presence of IL-4/IL-10/IL-13, M0 macrophage is converted to M2a macrophage state, where many surface markers (DC-SIGN, MRC-I, MSR-I, etc) are involved and Arg1 hallmark gene present. In this state, IL-4, IL-13 cytokines are produced. Here, KLF-4, IL-4R I, II, p50, and STAT6 transcription factors and regulators are recruited. On the other hand, in the presence of IL-4/IL-10/TGF- β , M0 macrophage is converted to M2c macrophage state, where some surface markers (CD163, CD91, etc.) are involved. In this state, IL-1, IL-27 cytokines are produced. Based on the disease progression stage of tuberculosis, in the early phase, M1 state, the middle phase, M2a, and the late phase, M2c state, were observed.

1.8 Immune response during *M. tuberculosis*

1.8.1 Innate immune response during *M. tuberculosis*

In early and chronic infection, macrophages are the main cellular niche for infection of *M. tuberculosis*, so they play a crucial role in *Mycobacterial* pathogenesis (McClean & Tobin, 2016). Macrophages are the chief cells that encounter the pathogen of *M. tuberculosis*, and phagocytosis of *M. tuberculosis* occurs by the alveolar macrophages as well as during the early stage of *M. tuberculosis* infection. It interacts with a variety of different innate immune cells, including neutrophils, natural killer cells, and dendritic cells (Cadena et al., 2016). By numerous pattern recognition receptors (PRRs), TLR-1, TLR-2, TLR-4, TLR-7, TLR-8, and TLR-9 are Toll-like receptors. NOD1, NOD2, NLRP3, and NLRC4 are Nod-like receptors. MR, Dectin-1, Dectin-2, Dectin-3, and DC-SIGN, DCIR, and Mincle are C-type receptors. *M. tuberculosis* is recognized by those innate immune cells (e.g., dendritic cells, NK cells, etc.). To eliminate the *M. tuberculosis* pathogen from the host body, the host receives signaling from pattern recognition receptors or PRR and starts to create various cellular functions, i.e., apoptosis, autophagy, phagocytosis, and inflammasome activation.

Pathogen-associated molecular patterns, or PAMPs, in *M. tuberculosis*, like carbohydrates, glycolipids, and lipoproteins, are recognized by PRRs like Nod-like receptors, Toll-like receptors, and C-type receptors at different stages of infection (early and chronic stages), and they form a vast network of interconnected coordinated signalling pathways that speed up gene expression profiles in host cells (Killick et al., 2013). To demonstrate the significance of inflammatory cytokines, such as IL-1, IFN- γ , IL-12, and macrophage inflammatory protein-1 (MIP1 α /CCL3), gene profiling studies have been conducted proof in the protection against infection of *M. tuberculosis* (Cappelli et al., 2001).

The macrophage polarization spectrum is divided into the M1 and M2 phenotypes based on how macrophages develop and operate (Murray, 2017). Human monocytes develop into M2 macrophages during active tuberculosis, which increases pathogen permissiveness, protease-dependent mortality, and immunomodulation (Lastrucci et al., 2015). This process is characterized by the phenotype $CD16^+CD163^+MerTK^+pSTAT3^+Mycobacterial\ DnaK$ (HSP 70, heat shock protein 70) indexes the M2 macrophages in an IL-10-dependent way (Lopes et al., 2016). Macrophages are also known as myeloid suppressor cells (MSCs), and these macrophages suppress the T-cell response via the secretion and release of TGF- β and IL-10. So, in tuberculosis, these macrophages exhibit both suppressive functions as well as help in the regulation of inflammation and infection (Khan et al., 2016). In a histopathology study, granulomas are the characteristics of tuberculosis that contain various transformed macrophages, i.e., epithelial cells, foam cells, and multinucleated giant cells (Feng et al., 2014). More recently, it was stated that TLR2 signaling promotes polyploidy of the macrophage. It indicates that in the presence of persistent inflammatory stimuli, pathways involved in developing cancer cells surprisingly instruct a polyploid macrophage fate and regulate granulomatous tissue remodeling (Singh et al., 2015).

1.8.1.1 Pattern recognition receptors for identifying *M. tuberculosis* in the innate immune response

Identification of *M. tuberculosis* by pattern recognition receptors such as C-type lectin receptors (CLRs), nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), TLRs, and cyclic GMP-AMP synthase (cGAS)/ stimulator of interferon genes (STING) is the first step in initiating an innate immune response

(Raveslout-Chávez et al., 2021). In mice models, the most necessary TLRs for controlling *M. tuberculosis* are TLR-2 and TLR-9, in which *M. tuberculosis* lipoproteins and lipoglycans are recognized by TLR-2, and unmethylated CpG DNA are identified by TLR-9 (Bafica et al., 2005; Feng et al., 2003; Reiling et al., 2002; Sugawara et al., 2003).

Mice missing both TLR2 and TLR9 are more unprotected or unsafe than TLR2 or TLR9 single knockout mutants. It indicates that each TLR contributes to the immune response in a nonredundant way (Bafica et al., 2005). Numerous CLRs have been asserted to have a functional role in the immunological recognition of *M. tuberculosis* in addition to TLRs. Besides TLRs, CLRs also have a significant role in recognizing *M. tuberculosis*. Moreover, scientists investigated and found that in mutant mice, each individual CLR has a restricted participation (Heitmann et al., 2013; Marakalala et al., 2011), which might be partially clarified by redundancy in function. Eventually, in *in vitro* culture of myeloid cells, the NLR-NOD2 can detect muramyl peptides generated from peptidoglycan of a bacterial cell wall, giving cytokine responses to the *M. tuberculosis* (Coulombe et al., 2009; Juárez et al., 2012; Pandey et al., 2009). At some time, when mice are missing NOD2, they become infection-resistant and show little vulnerability six months after infection (Divangahi et al., 2008).

Three distinct studies claimed that for type-I interferon production, cGAS is very important, and it indicates that DNA is the pathogen-associated molecular pattern (PAMP) that activates STING (Collins et al., 2015; Wassermann et al., 2015; Watson et al., 2015). On the other hand, *M. tuberculosis* has been established to activate type-I interferons by direct STING identification of cyclic-di AMP generated by the bacterium. CLRs, TLRs, and TLRs aid in the immunological response to *M. tuberculosis* by increasing the synthesis of chemokines and pro-inflammatory

cytokines. However, bacterial pathogens exploit an antiviral pathway, which is an example of the cGAS-STING pathway, to promote pathogenesis (Dey et al., 2015).

1.8.2 Adaptive immunity against *M. tuberculosis*

The adaptive immune response defends the host from *M. tuberculosis* and regulates bacterial reproduction. Extreme sensitivity to mycobacterial infections is best seen in lymphopenic HIV patients and gene-deficient animals missing MHC class II or T-cells in general. Direct antimicrobial activities of antigen-specific T cells and cytokine production are the primary characteristics of the adaptive immune response to *M. tuberculosis* infection (Sia & Rengarajan, 2019). Additionally, the longevity of antigen-specific memory T cells provides the foundation for the creation of vaccines that promote protection against mycobacterial infections (Sia & Rengarajan, 2019). B-cells, $\gamma\delta$ T-cells, and CD1-restricted T-cells all have growing roles as well. These offer targeted responses to a wide range of *M. tuberculosis* antigens, which are presented by MHC class I and II in the traditional manner (De Martino et al., 2019). The malignancy of adaptive immune responses is caused by severe inflammation or by being rendered inefficient by repeated antigen exposure. CD4 T-cell responses during *M. tuberculosis* infection. CD8 T-cells protect the host from *M. tuberculosis* infection, and inhibitory receptors play a key role in *M. tuberculosis* infection. In addition, certain cells, such as B-cells, T-cells, $\gamma\delta$ T-cells, CD1-restricted lymphocytes, and mucosal-associated invariant T (MAIT), also protect the host from *M. tuberculosis* infection (Ravesloot-Chávez et al., 2021).

1.9 An agent can cause polarization or repolarization of the macrophage's phenotypes

Refai et al. (2018) claimed that ESAT-6, a plentifully secreted protein of *M. tuberculosis* (Ha et al., 2020), can switch from M1 polarized macrophages to the M2 phenotype. Interestingly, they also claimed that ESAT-6 moderated the manifestation of pro-inflammatory markers (CD80, CD86, IL-12, IL-23, IL-6, CXCL10) of fully activated M1 macrophages (Refai et al., 2018). Wang et al. (2017) claimed that pleural macrophages polarize from M1-like to M2-like phenotype after 3.5 days post-treatment of antibiotics (e.g., rifampicin, isoniazid, ethambutol, pyrazinamide). They also showed that mediators of the proinflammatory markers IFN- γ , TNF- α , and IL-6 in tuberculous pleural effusion (TPE) remained decreased significantly after 2-3 days post-antibiotic treatment (Wang et al., 2017). Pinosylvin, a naturally occurring stilbenoid mostly found in Scots pine trees, alters macrophage polarisation from the pro-inflammatory M1 phenotype to the M2 phenotype, according to Kivimäki et al., (2021). They asserted that pinosylvin has an effect on M2-type activation and inhibits the expression of M1-type markers. They demonstrated that pinosylvin boosted the expression of mannose receptor C type 1 (MRC1) and arginase 1 (Arg1) in murine macrophages (Kivimäki et al., 2021). According to Chatterjee et al., (2021) rocaglates, a natural product derivative from *Aglaia* genus plants (Langlais et al., 2018), can direct polarization of macrophages toward the M1-like phenotype and prepare macrophages at low concentrations of the M1 to induce cytokine IFN- γ and to inhibit their sensitivity to IL-4, an ideal activator of the M2-like phenotype (Chatterjee et al., 2021). By promoting the development of the M1 markers in the cytokine pre-stimulation model, Jayaraman et al. claimed that *Oceanimonas* sp. BPMS22-derived PPI (Protein Protease Inhibitor) transformed into an M1-like phenotype from the resting macrophages (M0) and repolarized them to M1

from the M2 phenotype. Additionally, they said that the PPI incited macrophage M1 repolarization from the M2 macrophage phenotype in the *Leishmania donovani* infection paradigm (Jayaraman et al., 2022). Tian et al. claimed that the CU-CPT22, a TLR2/TLR1 inhibitor (Lin et al., 2021), transformed the M1 macrophages, induced by LPS, to the phenotype of M2 *in vitro*. The M1 marker CD86's expression is significantly repressed by CU-CPT22, and it also promotes the M2 marker CD206's expression, and the inflammatory cytokine production in macrophages is inhibited by CU-CPT22. Their results verified that the M1 phenotype is suppressed by CU-CPT22, and it also promotes the M2 macrophage phenotype (Tian et al., 2021). According to Chen et al, Oligo-Fucoidan extraction from brown seaweed, which is a sulfated polysaccharide (Cheng et al., 2020), induces M1-like macrophages from polarization of monocytes and repolarizes into M1 phenotypes from M2 macrophages. They also claimed that intracellular ROS generated by Oligo-Fucoidan and M1 macrophage polarization is benefited by mitochondrial superoxide, directly or indirectly. In addition, in the *in vitro* process, on M2 macrophage polarization, the chemotherapy drawback is suppressed by Oligo Fucoidan, and in the *in vivo* process, infiltration of M2 macrophages is prevented (Chen et al., 2020). According to Kou et al. (2022) IgG/Bb@BRPL, an opsonized nanoparticle, repolarizes to an anti-inflammatory M2-phenotype from M1 macrophages via ROS rummaging and deactivation of the NF κ B pathway, and for local chondrocytes remodelling, an additional promising microenvironment. They also claimed that with the help of *in vivo* studies, opsonized IgG/Bb@BRPL remarkably enhanced Osteoarthritis (OA) cartilage regeneration and repair. Their study suggests an opsonization-based drug delivery plan for OA treatment (Kou et al., 2022). The combination therapy of ibrutinib and selinexor, according to Jiménez et al., (2020) is capable of increasing mouse survival as well as changing the

innate immune response to an additional inflammatory phenotype, which is precisely defined by downregulation of signal regulatory protein α (SIRP α) and PD-1 in M2 macrophages. Additionally, the proportion of M1 macrophages is increased by this combination therapy, and additional M1 and M2-like properties are modulated with the loss of pro-tumoral M2 characteristics. They also claimed that in malignant cells, this combination treatment had a cytotoxic effect and also preferred an anti-tumoral innate immune response through tumor-infiltrating macrophage polarization shifted to M1 inflammatory and decreased SIRP α and PD-1 expression in the residual tumor-promoting macrophages of M2 (Jiménez et al., 2020). This immunomodulator-based macrophage polarization is summarized in table 1.3.

Table 1.3: Immunomodulator and its polarization.

Immunomodulator Agent	Source/Identity	Polarization or Repolarization	References
ESAT-6	Protein of <i>Mycobacterium tuberculosis</i>	M1 to M2 phenotype	Ha et al., 2020; Refai et al., 2018
Antibiotics	Rifampicin, Isoniazid, Ethambutol, Pyrazinamide	M1 to M2 phenotype	Wang et al., 2017
Pinosylvin	A natural stilbenoid is mainly found in Scots pine plant trees.	M1 to M2 phenotype	Kivimäki et al., 2021
Rocaglates	Natural product derivative from the Aglaia genus plants	Toward M1 phenotype	Langlais et al., 2018; Chatterjee et al., 2021
BPMS22	<i>Oceanimonas</i> sp.	M2 to M1 phenotype	Jayaraman et al., 2022
CU-CPT22	A TLR2/TLR1 inhibitor	M1 to M2 phenotype	Lin et al., 2021; Tian et al., 2021

Oligo-Fucoidan	A sulfated polysaccharide isolated from the brown seaweed.	M2 to M1 phenotype	Cheng et al., 2020; Chen et al., 2020
IgG/Bb@BRPL	An opsonized nanoparticle	M1 to M2 phenotype	Kou et al., 2022
Ibrutinib and selinexor	Ibrutinib is a BTK inhibitor, and Selinexor, a blood–brain barrier (BBB) permeable small molecule.	M2 to M1 phenotype	Jiménez et al., 2020

1.10 Challenges in the characterization of M1-M2 polarization

The lethal intracellular pathogen *M. tuberculosis* can establish a permanent residence inside the host macrophages. *M. tuberculosis* bacilli use several immune-evasion techniques, such as the suppression of pro-inflammatory markers and the host macrophages' release of the immunosuppressive cytokine IL-10 to delay the resolution of its infection and cause severe immune suppression. A few reports indicated that M1 macrophage polarization resulted in granuloma formation, which has an innumerable role in resolving *M. tuberculosis* infection. However, M1-M2 polarization status in macrophages during tuberculosis is still not well characterized during latent, active, and disease-resolving states, which may provide countless efforts to set up the future strategic plan for the sterile cure of tuberculosis, particularly in drug-resistant cases.

1.11 Serine threonine kinase -11 glycoprotein from *Cocos nucifera* L.

C. nucifera L., a prominent member of the Arecaceae family, is frequently called coconut, coconut-of-the-beach, or coco-da-bahia (Fuego et al, 2021). Southeast Asia, which covers the islands between the Pacific Ocean and India, is where the coconut tree first appeared (Esquenazi et al., 2002). This region includes Indonesia, Malaysia, and the Philippines. Their medicinal properties, including nephroprotection, anti-cancer,

hepatoprotection, anti-bacterial (Sinsinwar et al., 2018), antifungal (Khalid Thebo et al., 2016), and anti-helminthic activity, are further enhanced by edible coconut products, agricultural wastes, and coconut shells (Lima et al., 2015; Lima et al., 2016). A serine threonine kinase 11 (STK11) was recently isolated and characterized as a novel glycoprotein derived from the nutshell of *C. nucifera* L., which was found to be encouraged macrophage activation towards pro-inflammation (Srinivasan et al., 2023). The general function of STK11 was to regulate cell activity mediated by members of the AMPK family, including apoptosis (Bhullar et al., 2018), cell polarity, and metabolic processes (Ma et al., 2014; Wilson et al., 2019). Numerous studies had examined the role of the STK (serine-threonine kinase) in defence system of plants (Afzal et al., 2008; Klaus-Heisen et al., 2011; Ho, 2015). STK11 glycoprotein was also reported for its M1 polarization potential from naïve macrophages and could lead to kill the intracellular pathogens (Samanta et al., 2026).

1.12 Aim & Objectives

In this doctoral thesis, at first, we tracked the status of macrophage polarization (M1 or M2) during TB (H37Ra, H37Rv) infected, BCG exposure, and heat killed H37Ra challenged macrophages. After that, we established the activity of novel immunomodulator STK11, isolated from nutshell of *C. nucifera*, alone or in combination with the first line drug rifampicin, for the repolarization of *M. tuberculosis*-induced M2 to M1 macrophages to establish the role of host directed therapy using it to enforce the anti-TB immune responses. Beside the study with active TB, we also examined the potential of STK11 against the latent *M. tuberculosis* infected macrophages also.

1.12.1 Aim

The aim of this study is to establish the state of macrophage polarization during different states of *Mycobacterium tuberculosis* infection and bioprospecting of a novel glycoprotein (STK11) isolated from the nutshell of *C. nucifera* L. on it.

1.12.2 Objectives

- I. To study the macrophage polarization status during *Mycobacterium tuberculosis* infection *in vitro*.
- II. To examine the effect of isolated glycoprotein, STK11, from the nutshell of *Cocos nucifera* L. against the *M. tuberculosis* (H37Ra) bacilli growth through macrophage polarization *in vitro*.
- III. To study the effect of the STK11 in the process of macrophage polarization during challenge with the latent state of *M. tuberculosis* (H37Ra) bacilli.
- IV. To establish the combinatorial effect of STK11 with rifampicin in the treatment of H37Ra-infected macrophages.

Chapter 2: Study the macrophage polarization during *Mycobacterium tuberculosis* infection *in vitro*

2.1 Introduction

Tuberculosis (TB) is a serious communicable disease and the causative agent is *Mycobacterium tuberculosis* (WHO, 2025). The increasing incidence of drug-resistant TB cases (0.39 million in 2024) makes situations more complex (Global TB Report, 2025). TB is among the top 10 causes of death globally and the leading cause of demise from a single infectious agent. WHO Global TB program is giving their best to mitigate this, while, the problem arises due to drug-resistant TB (DR-TB). DR-TB cases show high risk of relapse, treatment failure, prolonged bacilli transmission, and finally death of the patients (Boonsarngsuk et al., 2018). In addition to these, low effectiveness of the Bacillus Calmette-Guerin (BCG) immunization in adulthood are also typically linked to the high incidence of TB in the majority of the suffering countries (Elias et al., 2007; Lawrence, 2024).

Macrophages are found in all major tissues and constitute the first line of defence. These play a vital role in wound healing, tissue integrity, homeostasis, and the regulation of inflammation (Wynn et al., 2013; Gordon et al., 2014). Macrophages are extremely flexible and dynamic innate immune cells that can activate and polarize into either classically (M1) or alternatively (M2) macrophages (Shapouri-Moghaddam et al., 2018). M1-polarized macrophages use arginine to quickly release nitric oxide (NO), while M2 macrophages use arginine to enhance the metabolism of urea and ornithine (Orecchioni et al., 2019; Rath et al., 2014). Additionally, M2 macrophages are categorized by the CD206 expression, IL-10 secretion, macrophage mannose receptor, and contribution in tissue repair (Yunna et al., 2020), while, M1 macrophages are

characterised by their capacity to release pro-inflammatory cytokines i.e., IL-6, IL-1 β , TNF- α , and IL-12, as well as, their anti-microbicidal and tissue-destructive characteristics (Rath et al., 2014).

It is well known that lung resident macrophages, named alveolar macrophages, are the primary host of the *M. tuberculosis* after infection via the aerosol route. Additionally, *M. tuberculosis* effectively exploits the diversity and adaptability of macrophages to promote infection and spread. Besides that, *M. tuberculosis* can also develop and sustain a "metabolically slowed" latent infection phase within macrophages, the key innate immune responders for eradicating the intracellular pathogens, in order to survive within immunological or pharmacological pressure (Marrero et al., 2010; Singh et al., 2012). The macrophage subtype or subtypes that *M. tuberculosis* favours for its latent residency program are primarily unknown. Because it thrives in macrophages, *M. tuberculosis* has developed a variety of defences against the host immunological responses (Queval et al., 2017). Consequently, *M. tuberculosis* is one of the most successful infections because macrophages provide an appropriate environment for its survival (Cambier et al., 2014). In the early phase of tuberculosis infection, M1 or classically activated macrophage polarization is observed, and midway and late phases of tuberculosis, M2a and M2c macrophage polarization stages are observed respectively (Khan et al., 2022). However, the status of macrophage polarization (M1 or M2) during tuberculosis infected macrophages and the disease resolving stages still remains unclear.

In this context, at first, this study has been designed to investigate the time dependent macrophage polarization status during *M. tuberculosis* bacilli infection. Additionally, macrophage polarization status i.e. M1 or M2 during infection and disease resolving

state has been examined with H37Ra, H37Rv, BCG, and heat-killed H37Ra-challenge with macrophages.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Middlebrook 7H9 and 7H10 medium, Middlebrook ADC & OADC growth supplement, Tween 80, glycerol was required for the culture of *M. tuberculosis* bacilli and were purchased from Himedia, India. RPMI-1640 were obtained from Himedia, while FBS and Pen-strep antibiotics were procured from Gibco. Tris buffer, Triton-X, Agarose, Neomycin, Streptomycin, RNA Express reagent was purchased from Himedia. Griess reagent was acquired from SRL. Tubervac BCG vaccine was purchased from Serum Institute of India. H37Ra bacterial strain (MTCC No. 300) was obtained from CSIR Chandigarh. H37Rv bacterial strain (ATCC No. 25618) was obtained from ATCC. A cDNA synthesis kit and RT-PCR kit were procured from Bio-Rad and TAKARA BIO respectively.

2.2.2 *M. tuberculosis* bacilli (H37Rv, H37Ra) culture

The strain of *M. tuberculosis* [H37Ra (MTCC: 300), H37Rv (ATCC: 25618)] was cultured in MB7H9 medium added with glycerol (0.5%), Tween-80 (0.2%), and albumin-dextrose complex enrichment supplement (10%) for 21 days in a shaker incubator at 37 °C. The mid-log growth phase bacilli were harvested after centrifugation at 2500 g for 15 minutes. After two times of centrifugal washing, the bacteria, at a suitable concentration, were suspended in sterile PBS saline (Das et al., 2015; Samanta et al., 2026).

2.2.3 Macrophage cell infection with H37Ra and H37Rv

Macrophages (J774A.1) were cultured in RPMI 1640 media with FBS (10%) in a cell culture flask with regular passage. Cells (J774A.1) were infected with H37Ra, H37Rv at an MOI (1:5= Macrophage: *M. tuberculosis*) (Andreu et al., 2017; Velappan et al., 2017). After 4 h of bacilli challenge, non-ingested bacilli were discarded by washing cells with fresh RPMI 1640 culture media and cultured for the different time points as required for the experiments indicated later (Das et al., 2015; Velappan et al., 2017).

2.2.4 The production of NO in J774A.1 macrophages

The J774A.1 cells (1×10^6) were seeded in 24-well plates for overnight incubation. Then, the cells were infected with H37Ra (MOI=1:5) for different time points (3 – 48 h) initially. Diverse set of experiments were also conducted, where, cells were challenged with heat-killed H37Ra (MOI of 1:5) and BCG vaccine (2×10^6 CFU/ml) beside the infection with H37Rv and H37Ra (MOI=1:5) for 4 h, followed by washing and continued culture in the 5% CO₂ incubator (Zulkipli et al., 2018). After 48 h of incubation, cell culture supernatants (100 µl) from the plate were betrothed for the NO production assessed using the Griess reagent. The absorbance was evaluated at 548 nm in the microplate reader (Jayaraman et al., 2022).

2.2.5 mRNA expressions of target genes for M1 and M2 markers

To examine the mRNA profiles of several M1/M2 markers, and corresponding cytokine markers *in vitro*, total mRNA was collected from experimental groups using Trizol reagents. The reverse transcriptase enzyme was used to obtain the cDNA using 1 µg of total RNA as a template. Arg-1, iNOS2, IFN-γ, YM-1, FIZZ1, CD206, CD68, CD40, IL-6, IL-12, IL-4, and IL-10 mRNA expressions were identified using the specific

forward and reverse primers. Forward and reverse primer sequences of these were listed in Table 2.1 of this chapter. Thermal cycler was run as follows: 35 cycles at 95°C for 5 minutes, 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute, and 72°C for 10 minutes. PCR amplified gene products were processed and run in 1.2% agarose gel electrophoresis, inspected under a UV light, and recorded in the Bio-Rad Gel documentation system. ImageJ software was used to assess the band intensity of each experimental group, and GAPDH was used to normalise the results to determine the relative expression level (Charan Raja et al., 2021).

Table 2.1: Primer sequences of Cellular and cytokines' M1/M2 markers.

Gene	Primer Sequences (5'-3')
iNOS2	F: CAG AGG ACC CAG AGA CAA GC R: AAG ACC AGA GGC AGC ACA TC
Arg 1	F: CAG AAG AAT GGA AGA GTC AG R: CAG ATA TGC AGG GAG TCA CC
YM-1	F: GAA GGA GCC ACT GAG GTC TG R: CAC GGC ACC TCC TAA ATT GT
FIZZ1	F: TGC TGG GAT GAC TGC TAC TG R: CTG GGT TCT CCA CCT CTT CA
CD206	F: ATG CCA AGT GGG AAA ATC TG R: TGT AGC AGT GGC CTG CAT AG
CD68	F: GAA AGG GCT TCC CAT TTC TC R: TCA AGC CAT TGG TTT TCA CA
CD40	F: CCT GGC TTT GGA GTT ATG GA R: CCG GGA CTT TAA ACC ACA GA
IFN- γ	F: CCT GCT TCA CCA CCT TCT TG R: CGA CTC CTT TTC CGC TTC CT
IL-6	F: AGT TGC CTT CTT GGG ACT GA R: TCC ACG ATT TCC CAG AGA AC
IL-12	F: CAC GCC TGA AGA AGA TGA CA R: GAC AGA GAC GCC ATT CCA CA
IL-4	F: GGT GTT CTT CGT TGC TGT GA

	R: TCT CGA ATG TAC CAG GAG CC
IL-10	F: TCA CTC TTC ACC TGC TCC AC R: CTA TGC TGC CTG CTC TTA CTC

2.2.6 Arginase-1 activity

The arginase-1 activity was measured in H37Ra & H37Rv-infected, BCG & heat-killed H37Ra-challenged macrophages. After the experimental time point, cells were lysed using 0.1% Triton X-100 and 25 mM Tris-HCl. 0.5 M L-arginine was used to activate arginase at 56 °C and then hydrolyse arginine at 37 °C for 15–20 minutes. After adding 40 µl of α -isonitrosopropiophenone (liquefied in 100% ethanol) and heating at 95 °C for 30 minutes, the urea content was measured at 540 nm. The quantity of enzyme-catalysed urea production per minute was used to define one unit of arginase activity (Charan Raja et al., 2021).

2.2.7 Cytokine analysis by sandwich ELISA

The cytokine release was estimated from the cell-free supernatant of H37Ra and H37Rv-infected, BCG-exposed, and heat-killed H37Ra-challenged macrophages. As per the manufacturer instruction, sandwich ELISA was carried out using the appropriate cytokine ELISA kits (Bio-legend system) (Jayaraman et al., 2023).

2.2.8 Statistical Analysis

All *in vitro* studies were carried out in triplicate and repeated at least two to three times, and the results were displayed as mean $\pm$ SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test, using GraphPad Prism version 8.0 software, which helps to determine the data at a significant level.

2.3 Results

2.3.1 Arg1 and iNOS2 gene expression, nitrite generation, and arginase activity in macrophages in different time points of H37Ra infection

M1 cellular marker (iNOS2) and M2 cellular marker (Arg1) expression were checked in H37Ra-infected macrophages in the time-dependent assay (3 h, 6 h, 12 h, 24 h, 48 h). Interestingly, after 3 h and 6 h of infection with H37Ra, Arg1 markers were significantly ($p < 0.05$) increased, but Arg1 expression was gradually decreased after 12 h, 24 h, and 48 h of H37Ra infection. On the other hand, iNOS2 expression was decreased after 3 h to 48 h of H37Ra infection (Figure 2.1 A-C). Besides that, arginase activity was significantly ($p < 0.05$) increased after 3 h of H37Ra infection onwards. On the other hand, NO generation was decreased during the time-dependent study but found within the basal level (Figure 2.1 D-E).

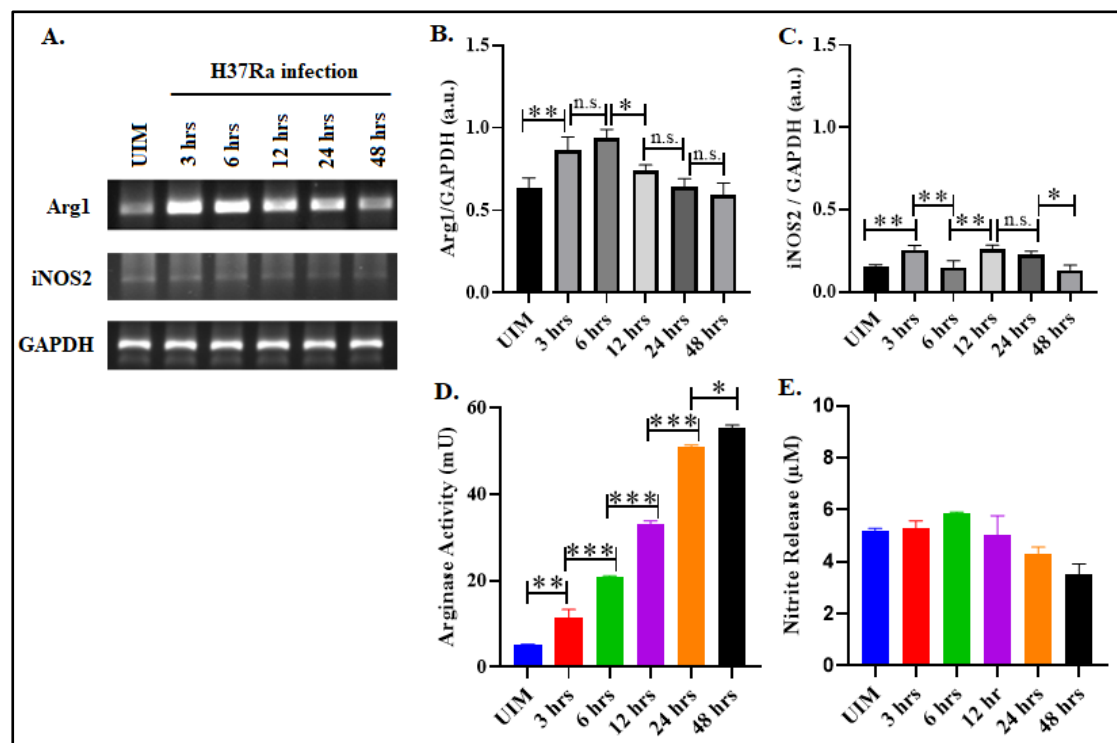


Figure 2.1 Arg1, iNOS2 mRNA expression (A-C), arginase activity (D) and nitrite generation (E), were studied at 3 h, 6 h, 12 h, 24 h, and 48 h of H37Ra-infected

macrophages. The macrophage (1×10^6) cells were infected with H37Ra (OD=0.655) using MOI (1:5= Macrophage: H37Ra) for the indicated times and mRNA expressions of target genes were studied by conventional RT-PCR. A) Expression of the arg-1, iNOS2 and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were presented here. B-C), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. D-E) In a separate set of time dependent experiments, infected cells and cell culture medium were collected separately. Arginase activity was measured from the cell lysate and nitrite release were detected from cell free culture medium. The data presented here were for one of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

2.3.2 M1 and M2 surface markers expression in H37Ra-infected macrophages at different time points

M1 marker (CD68) and M2 markers (CD206, FIZZ1, YM-1) expression were checked during H37Ra-infected macrophages in the time-dependent assay (3 h, 6 h, 12 h, 24 h, 48 h). Interestingly, after 3 h of infection with H37Ra, M1 marker (CD68) expression was decreased but after 6 h to 12 h of H37Ra infection, CD68 expression was slightly increased, and the expression of CD68 was slightly decreased after 24 h, and 48 h of H37Ra infection. On the other hand, the M2 markers (CD206, YM-1, FIZZ1) expression was gradually increased after 6 h of H37Ra infection. For CD206, the expression was significantly ($p < 0.05$) increased during 12 h, 24 h, and 48 h of H37Ra infection. The expression of FIZZ1 was gradually increased during 12 h, 24 h of H37Ra infection, but significantly ($p < 0.05$) decreased after 48 h of infection. The expression of YM-1 was gradually decreased during 12 h and 24 h of H37Ra infection but significantly ($p < 0.05$) increased after 48 h of infection (Figure 2.2 A-E).

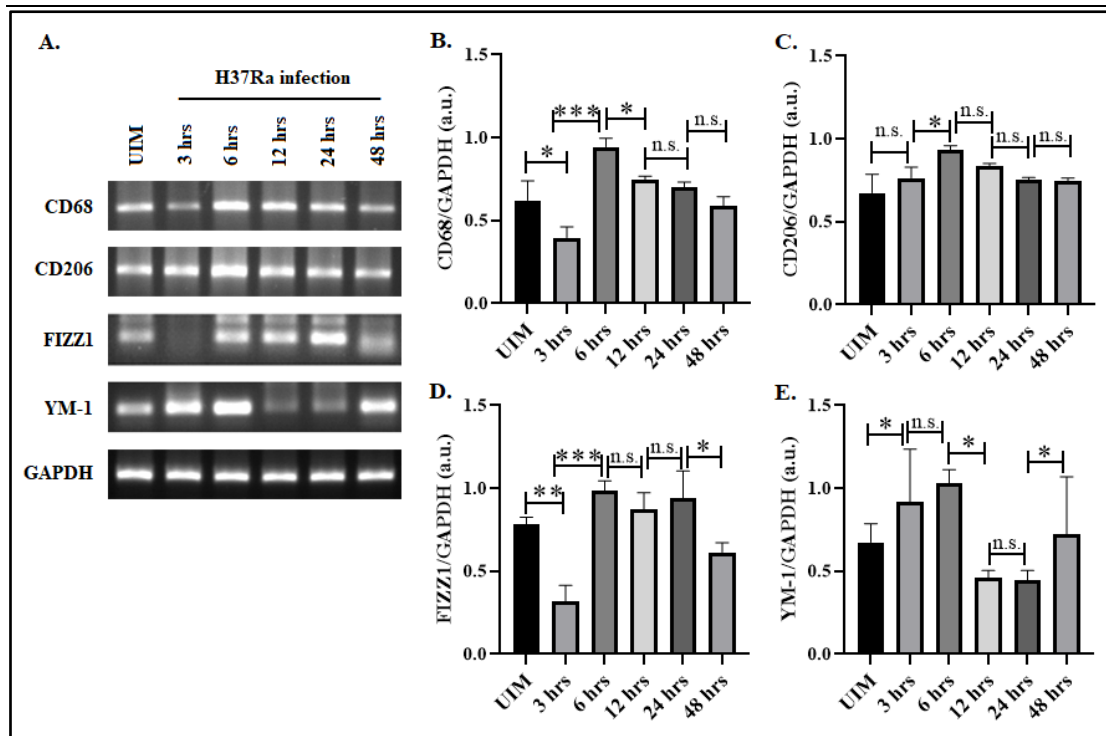


Figure 2.2 Time dependent expressions of M1 and M2 cell surface marker of H37Ra-infected macrophages. The J774A.1 macrophages (1×10^6) were infected with H37Ra as described earlier and mRNA expressions of target genes were studied by conventional RT-PCR. A) Expression of the CD68, CD206, FIZZ1, YM-1 and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were presented here. B-E), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

2.3.3 Cytokines expression in H37Ra-infected macrophages at different time points

To investigate the temporal regulation of cytokine expression in response to H37Ra infection in macrophages, we studied mRNA expression and cytokine release at multiple time points (3 h to 48 h post-infection). Interestingly, after 6 h of infection with H37Ra, anti-inflammatory cytokines (IL-4, IL-10) were significantly ($p < 0.05$) increased, and pro-inflammatory cytokines (IFN- γ , IL-6) expressions were significantly ($p < 0.05$) decreased (Figure 2.3 A-E). We observed that IFN- γ expression was

significantly ($p < 0.05$) increased at 6 h of infection and decreased during different time points of H37Ra infection. Interestingly, a similar result was found in the cytokine release test. After 6 h of H37Ra infection, anti-inflammatory cytokines (IL-4, IL-10) release was significantly ($p < 0.05$) increased, and pro-inflammatory cytokines (IFN- γ , IL-6) release was significantly ($p < 0.05$) decreased (Figure 2.3 F-I).

So, the time dependent experiments were important to know the expected time of macrophage polarization status activation in H37Ra infected macrophages at different time points of 3 h to 48 h. The result showed that after 6 h of H37Ra infection, M2 related cellular marker, and cytokines were expressed. So, in this context, we choose 6 h for H37Ra infection for the future experiments.

2.3.4 Arg1 and iNOS2 expression, nitrite generation and arginase activity during H37Ra, H37Rv-infected, BCG exposed, and heat killed-H37Ra challenged macrophages

We checked Arg1, iNOS2, nitrite generation, and arginase activity during H37Ra, H37Rv-infected, BCG-exposed, and heat-killed H37Ra (HK-H37Ra) challenged macrophages to understand the macrophage polarization and antimicrobial effects in the macrophages. The result showed that the M2 marker (Arg1) was increased during H37Ra and H37Rv-infected macrophages but decreased during BCG-exposed and heat-killed H37Ra-challenged macrophages (Figure 2.4 A-B). On the other hand, the M1 marker (iNOS2) was increased during BCG-exposed and heat-killed H37Ra-challenged macrophages and decreased during H37Ra and H37Rv-infected macrophages significantly ($p < 0.05$). The result of arginase activity was increased during H37Ra and H37Rv-infected macrophages, but decreased during BCG-exposed and heat-killed H37Ra-challenged macrophages. The result of nitrite release claimed

that nitrite generation was decreased during H37Ra and H37Rv-infected macrophages, and increased during BCG-exposed and heat-killed H37Ra-challenged macrophages.

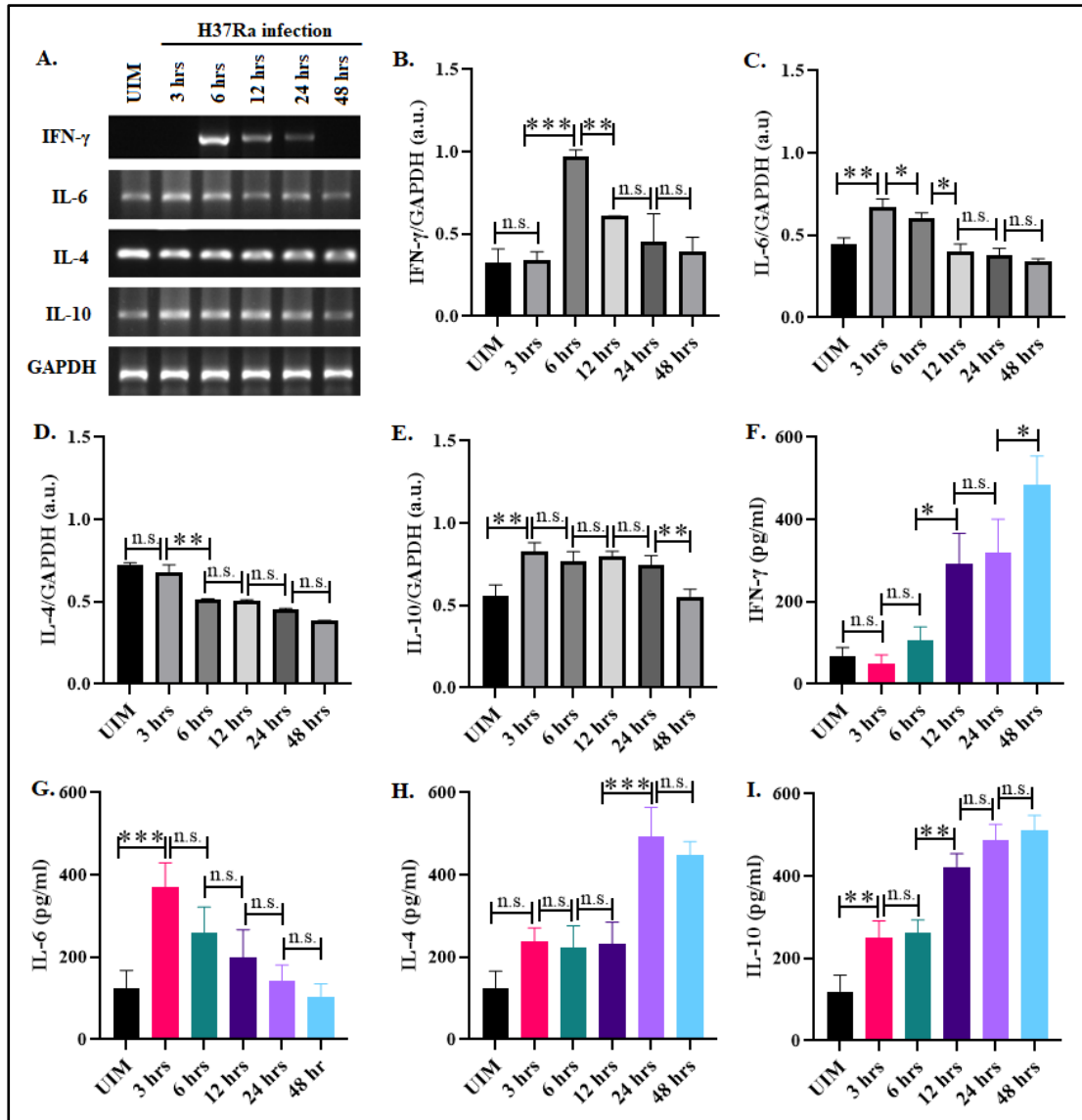


Figure 2.3 Time dependent expressions and release of cytokines in H37Ra-infected macrophages. The J774A.1 macrophages (1×10^6) were infected with H37Ra as described earlier and mRNA expressions of target genes were studied by conventional RT-PCR. A) Expression of the IL-4, IL-6, IL-10, IFN- γ and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were presented here. B-E), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. F-I) In a separate set of time dependent experiments, cells were infected for the indicated time points and cell free culture medium were collected and cytokine release were estimated by sandwich ELISA in it. The data presented here

were for one of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

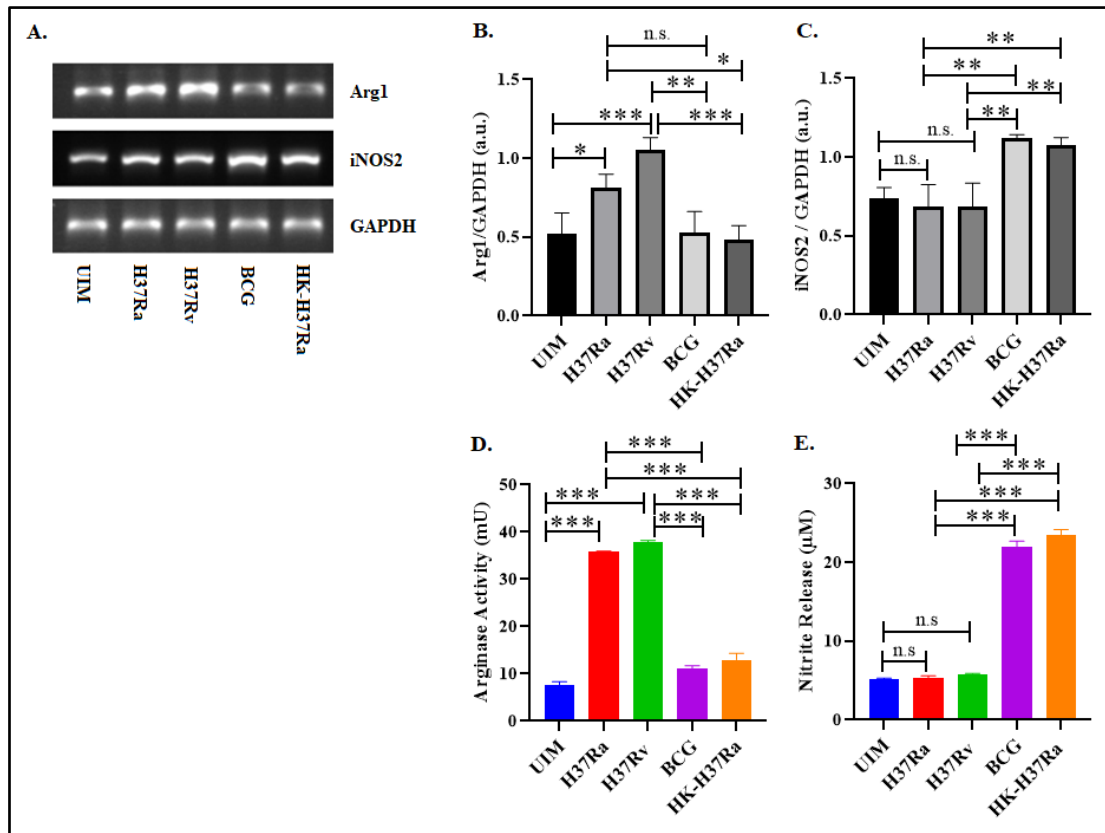


Figure 2.4 mRNA expression of Arg1, iNOS2, arginase activity and nitrite generation during H37Ra, H37Rv-infected, BCG-exposed, and heat-killed H37Ra-challenged macrophages. J774A.1 macrophage (1×10^6) cells were infected with *M. tuberculosis* bacilli (H37Rv and H37Ra) (OD= 0.671 and 0.655, respectively) using MOI (1:5= Macrophage: *M. tuberculosis*) for 6 h. 2×10^6 CFU/ml BCG was exposed (Zulkipli et al., 2018), and heat-killed H37Ra (MOI of 1:5) was challenged for 6 h. After that mRNA expressions of target genes were studied by conventional RT-PCR. A) Expression of the Arg-1, iNOS2 and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were presented here. B-C), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. D-E) In a separate set of experiments for the 48 h, arginase activity was measured from the cell lysate and nitrite release were detected from cell free culture medium. The data presented here were for one of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

2.3.5 M1 and M2 marker's expression during H37Ra, H37Rv-infected and BCG exposed, heat killed H37Ra-challenged macrophages

The expression of M1 and M2 markers were commonly checked during infections with different *M. tuberculosis* strains, like H37Ra, H37Rv-infected, and BCG-exposed, and heat-killed H37Ra challenged macrophages, to understand the type of immune response elicited by each strain. M2 marker (YM-1, FIZZ1, CD206) expressions were upregulated during H37Ra and H37Rv-infected macrophages but downregulated during BCG-exposed, heat-killed H37Ra-challenged macrophages. On the other hand, M1 marker (CD68) expression was upregulated during BCG-exposed and heat-killed H37Ra-challenged macrophages and downregulated during H37Ra and H37Rv-infected macrophages significantly ($p < 0.05$). So, H37Ra and H37Rv-infected macrophages induced M2 polarization in macrophages, but BCG-exposed, heat-killed H37Ra-challenged macrophages induced M1 polarization in macrophages (Figure 2.5 A-E).

2.3.6 Modulation of cytokines in H37Ra, H37Rv-infected, and BCG-exposed, heat killed-H37Ra challenged macrophages

We further checked IFN- γ , IL-12, IL-4, and IL-10 expressions during infections with H37Ra, H37Rv-infected, and BCG-exposed, and heat-killed H37Ra (HK-H37Ra) challenged macrophages, at both mRNA level and protein level for understanding the immune polarization and functional response of macrophages to different *M. tuberculosis* strains. Pro-inflammatory cytokines (IFN- γ , IL-12) expression was upregulated during BCG-exposed, heat-killed H37Ra-challenged macrophages, and downregulated during H37Ra and H37Rv-infected macrophages significantly ($p < 0.05$) (Figure 2.6 A-C). On the other hand, anti-inflammatory cytokines (IL-4, IL-10)

expression was upregulated during H37Ra and H37Rv-infected macrophages but downregulated during BCG-exposed and heat-killed H37Ra-challenged macrophages. A similar result was found in the cytokine release test, i.e., IFN- γ and IL-12 expression were upregulated during BCG-exposed and heat-killed H37Ra-challenged macrophages, and IL-4 and IL-10 expression were upregulated during H37Ra and H37Rv-infected macrophages. So, H37Ra and H37Rv-infected macrophages induced M2 polarization in macrophages, and BCG-exposed and heat-killed H37Ra-challenged macrophages induced M1 polarization in macrophages. So, M1 polarization may be required for the restriction and cure of *M. tuberculosis*.

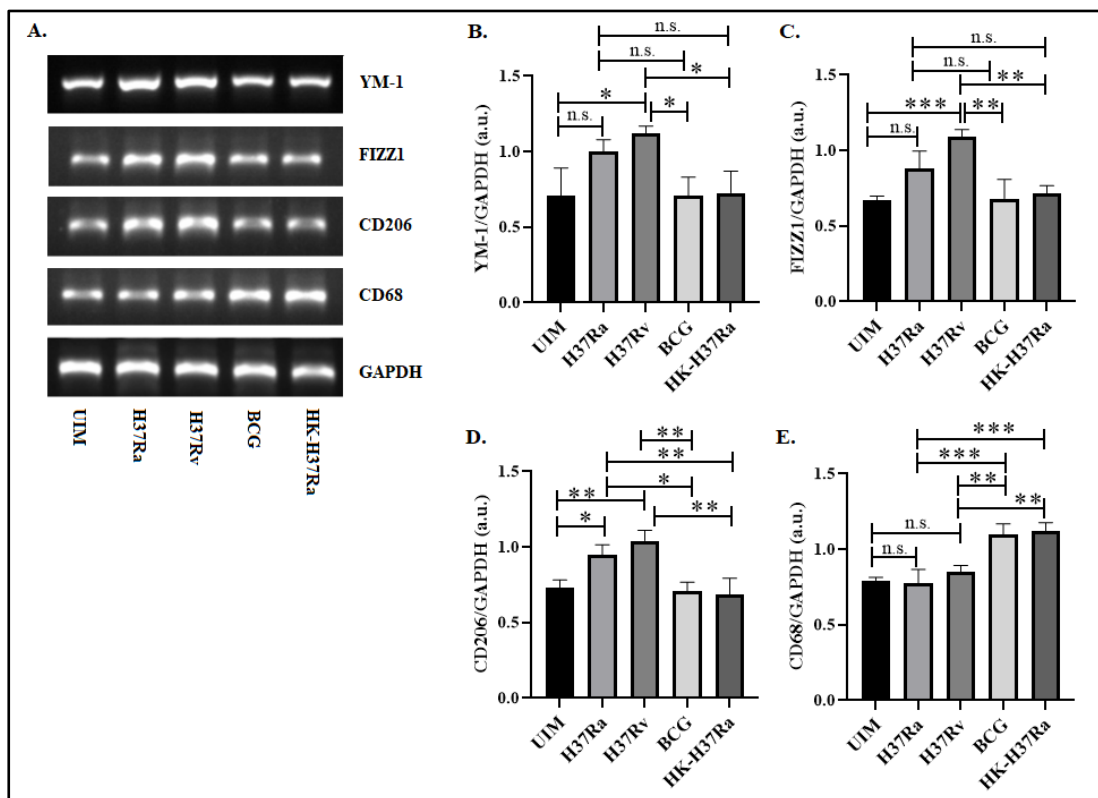


Figure 2.5 mRNA expression of YM-1, FIZZ1, CD206, CD68 marker during H37Ra, H37Rv-infected, BCG-exposed, and heat-killed H37Ra-challenged macrophages. The J774A.1 macrophages (1×10^6) were either infected or challenged with divergent strains as described earlier and mRNA expressions of target genes were studied by conventional RT-PCR. A) Expression of the CD68, CD206, FIZZ1, YM-1 and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were

presented here. B-E), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

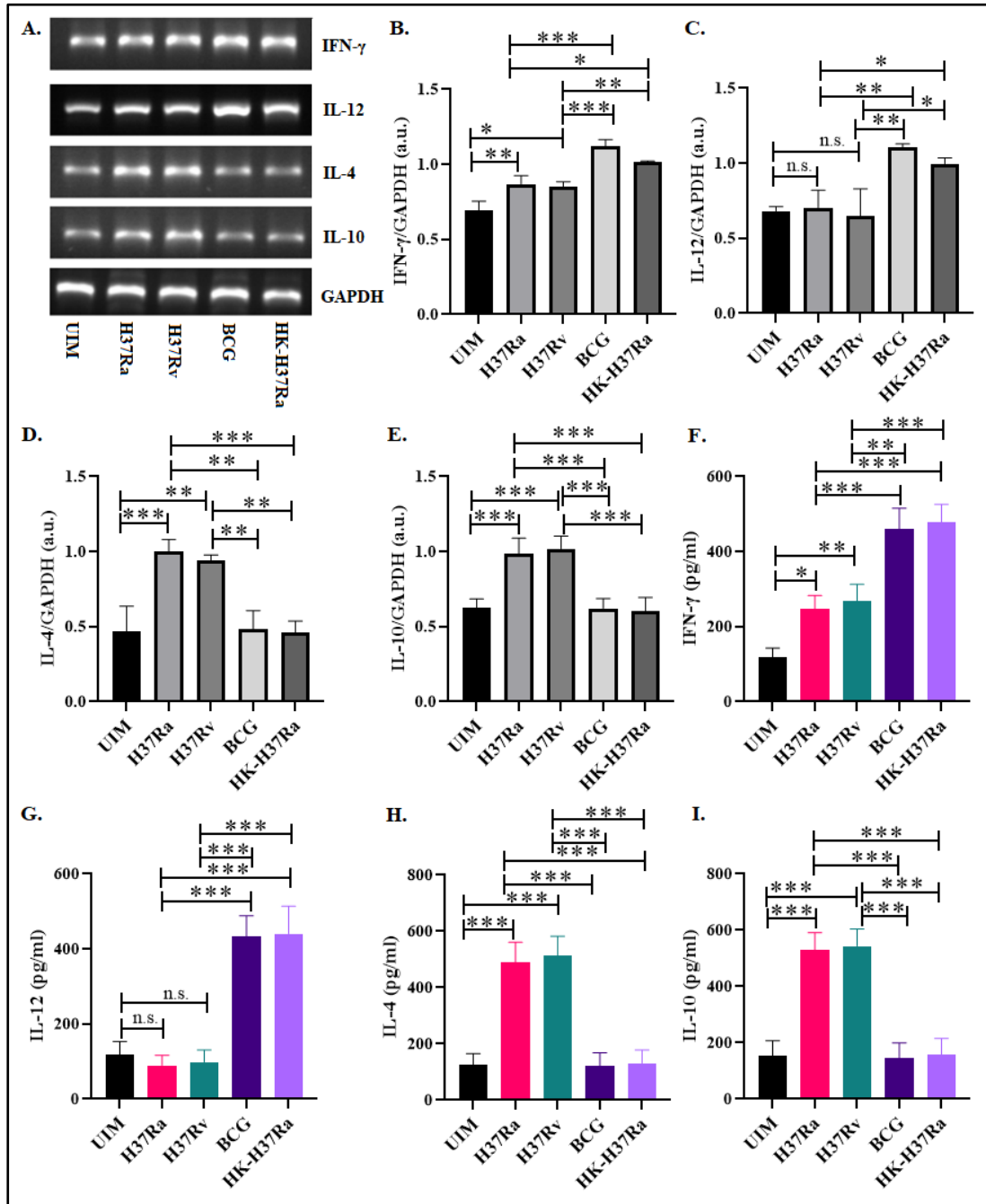


Figure 2.6 mRNA expression and release of different cytokines in H37Ra and H37Rv-infected macrophages and BCG-exposed and heat-killed H37Ra-challenged macrophages. The J774A.1 macrophages (1×10^6) were either infected or challenged with divergent strains as described earlier and mRNA expressions of target genes were studied by conventional RT-PCR, while cytokines release assay was performed by ELISA. A) Expression

of IFN- γ , IL-12, IL-4, IL-6, IL-10, and GAPDH in agarose gel were captured in gel documentation system and one set images from the three independent experiments were presented here. B-E), The corresponding band intensity of three sets experiments were measured in ImageJ and presented as bar diagram. F-I) In a separate set of experiments, cells were challenged for 24 h and cell free culture medium were collected and cytokine release were estimated by sandwich ELISA. The data presented here were for one of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the uninfected control group.

2.4 Discussion

Tuberculosis is a major type of communicable disease that claims millions of lives worldwide (WHO, 2025). In this context, the WHO targeted the sterile cure of TB by 2035, but this goal can be failed due to proper planning and research, and management of drug-resistant cases (Zhuang et al., 2023). This study was designed to establish the time of activation of macrophage polarization at different times of H37Ra-infection (3 to 48 h) and the state of macrophage polarization during the disease progression, and the disease resolving stage (BCG and heat killed H37Ra), which may help to sterile cure of TB by 2035. We all know that macrophage polarization basically has two types- M1 or classically activated and M2 or alternatively activated macrophages (Samanta et al., 2024). But the proper stage of polarization and time of activation of macrophage polarization during disease progression (H37Ra, H37Rv) and disease resolving (BCG exposed, heat-killed H37Ra) state remains unclear.

In this context, we checked various cellular markers (Figure 2.1), M1 and M2 markers (Figure 2.2), pro and anti-inflammatory markers (Figure 2.3), to know the time of activation of macrophage polarization through the time-dependent study. We observed from the results that M2-related markers were significantly ($p < 0.05$) increased after 3-6 h of H37Ra infection, and M1-related markers were significantly ($p < 0.05$) decreased.

Hence, these experiments claimed that the activation of the macrophage polarization may be observed after 3-6 h of H37Ra infection at the mRNA level and protein level. After that, we checked the cellular marker (Figure 2.4), M1 and M2-related markers (Figure 2.5), and pro and anti-inflammatory cytokines (Figure 2.6) to know the status of macrophage polarization during the disease progression and the disease-resolving state of TB infection. These experiments claimed that the M2-related markers were upregulated during H37Ra and H37Rv-infected macrophages, and M1-related markers were upregulated during BCG-exposed and heat-killed H37Ra-challenged macrophages. It may be claimed that M2 polarization was induced by disease progression (H37Ra, H37Rv) state (Mendonca et al., 2023). On the other hand, M1 polarization was associated with disease resolution (BCG and heat-killed H37Ra) state (Putri et al., 2022). M1 polarization may be required for the restriction and cure of tuberculosis. If we can polarize the macrophage cells from M2 to M1 by an agent like an immunomodulator, then TB may be restricted. M1 macrophages are frequently associated with an effective anti-*M. tuberculosis* immunological response, with high expression of iNOS and simultaneous NO generation (Ehrt et al., 2001).

In this regard, it is well known that the Th1 type immune response, which is defined by the production of IL-12, IFN- γ , and TNF- α as well as M1-related markers (Green et al., 2013), is linked to the regression of TB infection. However, the production of anti-inflammatory cytokines and M2-related markers like IL-10 and TGF- β is thought to be associated with a decreased immune response against the disease, which ultimately leads to the inability to control this disease (Jordao & Vieira, 2011). Thus, the outcome of a TB infection is actually controlled by the equilibrium between the protective and inflammatory immune responses.

2.5 Summary & Conclusion

However, several tactics were used to create new treatments to combat it, but the rate of success is limited by the growth of drug-resistant cases. In this context, time-dependent tests revealed that M1 markers were repressed and M2 markers were considerably ($p < 0.05$) elevated following 3-6 h of H37Ra infection. Furthermore, the polarization status of the macrophages was reversed from M2 to M1 by the BCG strain and heat-killed *Mycobacterium*. These findings suggested that M1 polarization, which might be accomplished using immunomodulators, might be necessary for the treatment of *M. tuberculosis*.

Chapter 3: Isolated glycoprotein STK11 from the nutshell of *Cocos nucifera* L. modulated macrophage polarization to prevent the *Mycobacterium tuberculosis* bacilli growth in J774A.1 macrophage

3.1 Introduction

Tuberculosis is one of the greatest threatening communicable diseases in the world. As per the report, tuberculosis claims 1.5 million deaths worldwide, and 10.6 million people fall into this disease annually (WHO, 2025). Present treatment for drug-sensitive TB contains first-line antibiotics, which are managed for approximately 6–9 months (Singhal et al., 2014). Isoniazid and rifampicin are the main drugs with a 95% curing rate in the market (Koul et al., 2011). The treatment duration is very lengthy for drug-susceptible infection, varying from six months to more than two years for multidrug-resistant TB (Pontali et al., 2019). It is also established that *M. tuberculosis* could positively survive within several host environments by altering its metabolic state and avoiding the host's immune defense mechanisms by intracellular growth (Tenland et al., 2019).

In this context, host-directed therapies (HDT) are innovative TB treatment strategies focusing on host characteristics (Guler et al., 2021). HDT aims to treat the disease by moderating the host immune system, but not to target the pathogens. Virulence factors expressed by pathogens for their existence overwhelm host immune responses, which may contribute to disease growth and progression (Hancock et al., 2012). As antimicrobial resistance, HDT is mainly significant in the treatment of infectious diseases. Viruses or bacteria cause infectious diseases, which pose a greater risk to mankind, and antimicrobial resistance enhances this threat (Jeong et al., 2022). By targeting the host rather than the pathogen, emerging novel HDT for TB will avoid

conventional antibiotic resistance (Guler et al., 2021). The new anti-TB drug development is an expensive and lengthy process (van den Boogaard et al., 2009); thus, the HDT study may propose an effective substitute that is readily obtainable. Macrophage cells are one of the main host cells in our immune system (Franken et al., 2016). The results of the previous chapter also claimed macrophage activation status were modulated towards M2 polarization during active TB, while M1 polarization developed during disease resolving state.

Natural products and glycoproteins are known to play a vital role in biological processes (Sitohy & Osman, 2019). Glycoproteins act as antiviral, antibacterial, or antifungal activity (Osman et al., 2016; Fu et al., 2018; Qin et al., 2018). Very few studies have reported that each fraction of a protein induces its nutritional value, physicochemical properties, bioactivity, and use in food or other industries (Angelia et al., 2010). Glycoproteins have been discovered in abundance from natural resources in recent years. Glycoproteins are biological macromolecules that are important for the growth and development of organisms and are receiving enhanced attention globally (Zhang et al., 2023). Furthermore, natural glycoproteins have several remarkable biological properties, such as antioxidant, anti-coagulant, anti-tumor, and anti-microbial properties (Zhang et al., 2023).

In this context, *Cocos nucifera* L., member of the Arecaceae family, derived edible products, agricultural wastes, has been utilized for its therapeutic potential, such as nephroprotection, hepatoprotection, anti-cancer, anti-bacterial, antifungal, and anti-helminthic activity (Lima et al., 2015; 2016; Khalid Thebo et al., 2016; Sinsinwar et al., 2018). The functional role of serine-threonine kinase (STK)-11 in the coconut palm is unidentified. But the general role of the STK11 gene, which is a tumor suppressor gene, controls the activity of cells mediated through the AMPK family members, like

cell polarity, apoptosis, and metabolic functions of cells (Bhullar et al., 2018; Ma et al., 2014; Wilson et al., 2019). The function of the serine-threonine kinase (STK) defence system in plants has been the subject of numerous investigations. According to reports, STKs, a highly studied protein kinase subfamily, aid in various cellular processes, including plant defence (Afzal et al., 2008; Ho, 2015; Klaus-Heisen et al., 2011). On the other hand, the fruity white flesh decoction of the coconut is used to treat fever and malaria in rural Malaysia (Al-Adhroey et al., 2011). HIV infection relieves skin rash, which is treated by the coconut fruit in Kenya (Nagata et al., 2011).

Recently, STK11 from the nutshell of *C. nucifera* L. has been isolated and characterized. The isolated STK11 also proved its potential towards macrophage activation through prompt regulation of cytokine balance (Srinivasan et al., 2023). In this context, the current research aims to investigate the role of the *C. nucifera* L. derived glycoprotein, STK11, in the treatment of tuberculosis, focusing on macrophage polarization through host-directed therapy.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

All the common chemicals were procured in the highest grade available from Himedia, SRL, and Sigma Aldrich. EDTA and rifampicin were procured from SRL. Dialysis membrane, Middlebrook 7H9 medium, Middlebrook ADC, Tween 80, Tris buffer, Triton-X, Agarose, DMEM, FBS, Penicillin, Streptomycin, Neomycin, RNA Express reagent were procured from Himedia. Griess reagent was procured from SRL. H37Ra bacterial strain (MTCC No. 300) was obtained from CSIR Chandigarh. An Amicon centrifugal filter (10 kDa cut off) was procured from Merck-Millipore. cDNA synthesis kit was procured from Bio-Rad. The RT-PCR kit was procured from TAKARA BIO.

3.2.2 Culture of *M. tuberculosis* bacilli strain

M. tuberculosis strain [H37Ra (MTCC-300)] was cultured in Middlebrook 7H9 medium (Himedia) supplemented with 0.5% glycerol, 0.2% Tween 80, and 10% albumin-dextrose complex enrichment in a shaker incubator for 21 days. At 2,500 g, centrifugation for 15 minutes was used to harvest the bacteria during the mid-log growth phase. Following two centrifugal washings, the bacteria were suspended in PBS saline at the appropriate concentration. H37Ra bacteria were sub-cultured after 28 days (Das et al., 2015).

3.2.3 Extraction and purification of nutshell glycoprotein from *C. nucifera* L.

Based on the standard protocol, the coconut shells were washed and cleaned with fresh water. The coconut shells were dried in the sunlight. Then, dried coconut shells were broken into small pieces. Fine powder was prepared after crushing the pieces of coconut shells. Then, 10 g of coconut shell powder was dissolved in 100 ml Tris EDTA buffer with pH 8.2 that contains 0.1% Triton-X and covered with parafilm. Then, it was mixed gently and stirred for 24 h. Then, the coconut shell powder in the TE buffer was centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected from the centrifuge tube. Then the dialysis membrane was activated by EDTA, and the supernatant sample was dialyzed through the dialysis membrane for 48 h, while every 6 h, TE buffer was changed. After dialysis of the sample, the dialysed product of the sample was centrifuged in a cold centrifuge using an amicon centrifugal filter (above 10 kDa). The sample obtained above in the amicon centrifugal filter was dissolved in fresh buffer and stored at -20°C for further use. After that, 20 µl of the dialysed sample was taken in duplicate sets in 96-well plates. 200 µl of Bradford reagent was added to the well plate to assess the total protein content in the sample. Further, absorbance was

measured at 595nm. The molecular weight of the protein was identified by 12% SDS PAGE following standard protocol (Srinivasan et al., 2023).

3.2.4 The effect of STK11 and rifampicin during the growth of H37Ra by resazurin assay

Any compound's effectiveness on the growth of *M. tuberculosis in vitro* can be confirmed in many ways, such as the rapid screening method based on resazurin dye (Jethva et al., 2020), the CFU determination method in broth culture with and without the test compound (Hazan et al., 2012), and the antibiotic dilution method based on agar (Coban et al., 2013). The resazurin test was used to estimate the percentage of killing of the bacteria of STK11 and rifampicin against H37Ra after the treatment using the direct killing assay method. 100 µl of H37Ra bacteria (OD-0.02) were seeded into the 96-well plate. Then, isolated STK11 glycoprotein (0-200 µg/ml) and rifampicin (0-0.12 µg/ml) drugs were added in triplicate. The rest of the volume was filled up to 200 µl with complete MB7H9 media containing 20% Tween 80 and 10X ADC. Additionally, 200 µl of distilled water was added to the wells along the edge of the plate to prevent the media in the plate from being dehydrated. After wrapping the plates with parafilm and aluminium foil, they were placed in a BOD incubator for 7 days at 37°C. Then, 30 µl resazurin (0.1 mg/ml), was added and the plates were incubated for another 24 h. Then, fluorescence intensity was checked at excitation: 530 nm, and emission: 590 nm in a BioTek Synergy H1 microplate reader. The dehydrogenase enzyme of the living cells converted the resazurin to resafurin, oxidizing the deep purple color solution to bright pink. The wells containing antimycobacterial chemicals that prevented the growth of bacteria maintained the deep purple color of the resazurin. The resazurin assay was used to determine the percentage of killing of the bacteria in the wells that

corresponded to the doses of the drugs that prevented bacterial growth (Jyoti et al., 2015).

3.2.5 CFU test from macrophage-mediated killing assay

The colony-forming unit (CFU) tested the efficacy of STK11 glycoprotein on the growth of *M. tuberculosis* in J774A.1 macrophage *in vitro*. J774A.1 cells (1×10^5) were seeded in 24-well plates and incubated overnight. After incubation overnight, macrophages were infected with H37Ra at a multiplicity of infection (MOI) of 1:5 (Macrophage: Mycobacteria) for 4 h. Then STK11 (0-10 $\mu\text{g/ml}$) and rifampicin (0.1 $\mu\text{g/ml}$) were treated in different groups for 48 h in the selected well. After 48 h of treatment, the supernatant was discarded from the plate and washed with PBS. 200 μl RIPA lysis buffer (without protease inhibitor) was added to every cell well plate, the plate was wrapped with parafilm and placed in a rocking condition in the rocker for 30 min. After that, 50 μl of cell lysate was taken from the total lysate sample and placed in another fresh 24-well plate carefully. Then, 450 μl of complete MB7H9 media containing 20% Tween 80 and 10x ADC were added to the cell well plate. The plate was wrapped carefully with parafilm and aluminum foil to avoid dehydration. The plate was placed in a BOD incubator for further culture. After 14 days, 21 days, and 28 days, respectively the bacterial growth was monitored by taking absorbance at 600nm in a BioTek Synergy H1 microplate reader (Elizabeth et al., 2016).

3.2.6 Nitrite production in J774A.1 macrophages

J774A.1 cells (1×10^6) were seeded in 24-well plates. After overnight incubation, macrophages were infected with H37Ra at MOI of 1:5 (Macrophage: *Mycobacteria*) for 4 h. Then, the cells were treated with the *C. nucifera* L. nutshell-derived

glycoprotein, STK11 (0-10 µg/ml), and rifampicin (0.1 µg/ml) carefully and placed in the CO₂ incubator for 48 h. After 48 h of treatment, 100 µl of cell culture supernatants were collected from the plate, and NO generation was measured using the Griess reagent as per the standard protocol. The absorbance was checked at 548 nm in the BioTek Synergy H1 microplate reader (Jayaraman et al., 2022).

3.2.7 mRNA expressions of M1 and M2 markers

Total mRNA was extracted from experimental groups to differentiate the mRNA profiles of several cytokines *in vitro*. 1 µg of total RNA was utilized as a template to synthesize cDNA using reverse transcriptase enzyme, from Takara. Utilizing the particular forward and reverse primers, Arg-1, iNOS2, IFN-γ, YM-1, FIZZ1, CD206, CD68, CD40, IL-6, IL-12, IL-4, IL-10 were determined. The sequences of the forward and reverse primers are listed in table 2.1 and table 3.1. Thermal cycler: 35 cycles for 5 min at 95°C, 30 s at 95°C, 30 s at 62°C, 1 min at 72°C, and 10 min at 72°C. Amplified gene products of PCR were run by 1.2% agarose gel electrophoresis and examined under UV light, and captured in the Bio-Rad Gel documentation system. Band intensity of different experimental group was measured using ImageJ software and normalized with GAPDH to obtain the relative expression level (Charan Raja et al., 2021).

Table 3.1: Ras Genes corresponding primer sequences

Gene	Primer Sequences (5'-3')
N-Ras	F: ATG ACT GAG TAC AAA CTG GTG R: TAC ACA TCA GCA CAC AGG G
K-Ras	F: GTG GTG GTT GGA GCT GGT R: GTC CTT GAC TTC TTC TTC TTC
H-Ras	F: ATG ACA GAA TAC AAG CTT GTG GTG G R: TCA GGA CAG CAC ACA TTT GCA G

GAPDH	F: GAG CCA AAC GGG TCA TCA TC R: CCT GCT TCA CCA CCT TCT TG
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3.2.8 Protein-protein interaction between Ras isoforms and STK11

The built-in 3D model of STK11 was utilized in examining the interaction with various Ras (H-Ras, K-Ras, N-Ras) *in silico*. The sequence of STK11, identified as A0A0N7EZQ1, was taken from a previously reported study (Srinivasan et al., 2023) in the UniProtKB knowledge base (<https://www.uniprot.org/>) and stored in FASTA format for further analysis. Ras receptors were obtained from the PDB (protein data bank) to investigate the activation mechanism through the molecular docking method, performed utilizing Cluspro 2.0, an online server available at <https://cluspro.bu.edu/login.php?redir=/home.php>. The Cluspro v2.0 server follows three main steps: i) Rigid body docking is performed using the Fast Fourier Transform (FFT) correlation by sampling billions of confirmations. ii) The 1000 lowest energy structures are clustered to produce the largest clusters, with Interface Root Mean Square Deviation (IRMSD) characterizing the finest docking complex models. iii) The stability of the docking models (clusters) is validated by removing steric clashes through energy minimization. The interaction between the STK11 protein-protein and the Ras receptors was visualized using PyMOL software (Srinivasan et al., 2023). Further experimentations were performed using K-Ras and H-Ras inhibitors i.e. Sotorasib and Sulindac Sulfide respectively to finally verify the interaction of STK11 with respective Ras. Cells were seeded and infected with H37Ra according to standard protocol followed by addition of Ras inhibitors 2(h) prior treatment with STK11. CFU and nitrite generation was evaluated to check the interaction of STK11 with K-Ras and H-Ras respectively (Canon et al., 2019; Gala et al., 2002).

3.2.9 Measurement of cytokine release by ELISA

Cytokine levels were measured from cell-free supernatants of J774A.1 macrophages of diverse experimental groups using ELISA KIT (BioLegend, San Diego, California) following the manufacturer's instructions (Srinivasan et al., 2023).

3.2.10 Statistical analysis

All *in vitro* experiments were conducted in triplicate, and the data were presented as mean $\pm$ SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test, using GraphPad Prism version 8.0 software, which helps to determine the data at a significant level.

3.3 Results

3.3.1 Isolation of STK11 from the *C. nucifera* L. in TE buffer

STK11 glycoprotein was isolated from the nutshell of *C. nucifera* L., as per a previously reported paper (Srinivasan et al., 2023). Briefly, we isolated the STK11 glycoprotein from the nutshell of *C. nucifera* L. in TE extraction buffer (pH 8.2). STK11 was confirmed as a glycoprotein by 12% SDS-PAGE, which shows a similar result with respect to the previously reported paper (Srinivasan et al., 2023). SDS-PAGE results showed that the molecular weight of STK11 was $\approx$ 46 kDa (Figure 3.1). The yield of the STK11 protein was found to be 194.4 μ g in 10 g of STK11 powder, which was estimated using the Bradford assay method.

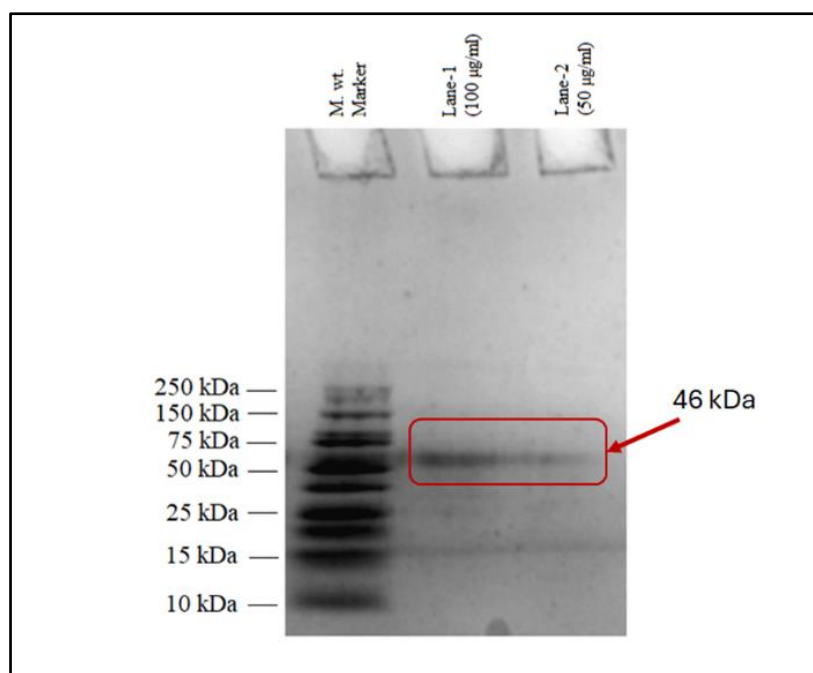


Figure 3.1 12% SDS-PAGE was used to identify the protein molecular weight of the purified *C. nucifera* shell protein. Lane-1: Protein ladder (10-250 kDa), Lane-2: STK11 glycoprotein (100 µg/ml), Lane-3: STK11 glycoprotein (50 µg/ml).

3.3.2 STK11 glycoprotein prevents the H37Ra bacterial growth in resazurin assay

The resazurin assay evaluated cell viability and metabolic activity by measuring resazurin reduction to resafurin by metabolically active cells (Lavogina et al., 2022). Although the resazurin test was used to measure bacterial growth and the effects of treatment. In this context, there was currently no direct evidence indicating that the STK11 glycoprotein can inhibit the growth of *M. tuberculosis* H37Ra. Hence, this study investigated whether isolated STK11 could inhibit the growth of H37Ra bacteria via the resazurin assay. Therefore, this dose-dependent study was performed in triplicate to determine the percentage of killing of the H37Ra bacterial strain treated with STK11 (0-200 µg/ml) and rifampicin (0-0.12 µg/ml). We observed from the results that 3.125 µg/ml of STK11 restricted the H37Ra growth by 13.90% (Figure 3.2A), whereas 0.00375 µg/ml rifampicin restricted it by 3.24% (Figure 3.2B). We also observed that 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml of STK11 can restrict the

H37Ra bacilli growth by 18.91%, 22.49%, 28.49%, 33.06%, and 37.37% respectively. On the other hand, 0.0075 $\mu\text{g/ml}$, 0.015 $\mu\text{g/ml}$, 0.03 $\mu\text{g/ml}$, and 0.06 $\mu\text{g/ml}$ of rifampicin can restrict the H37Ra bacilli growth by 11.83%, 24.51%, 32.32%, and 40.70% respectively. However, we observed that the highest dose of STK11 (200 $\mu\text{g/ml}$) could restrict the bacilli growth of H37Ra, which was 44.83% (Figure 3.2A), whereas 0.12 $\mu\text{g/ml}$ rifampicin could kill the bacteria by 54.98% (Figure 3.2B). Thus, the percentage of H37Ra bacterial killing significantly increased with increasing levels of STK11 (0-200 $\mu\text{g/ml}$) and rifampicin (0-0.12 $\mu\text{g/ml}$).

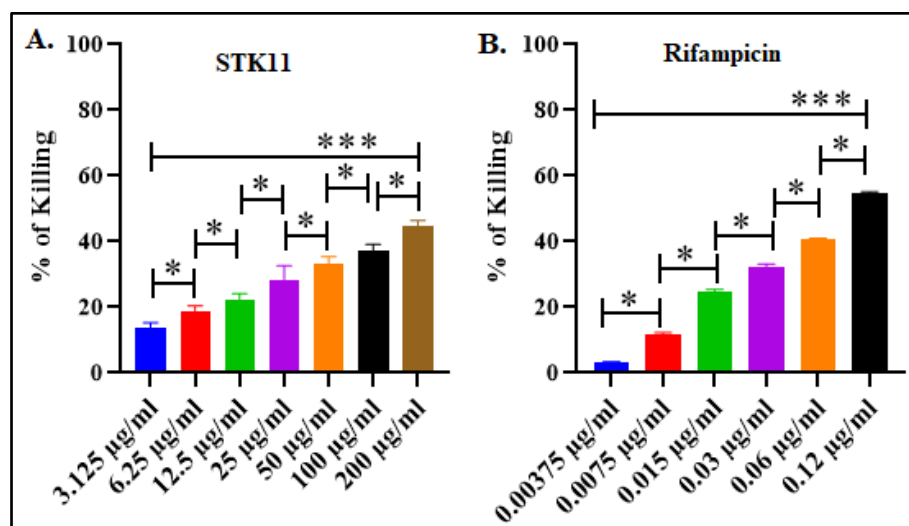


Figure 3.2 Determination of the percentage of killing of H37Ra bacilli after the treatment with (A) STK11 and (B) rifampicin. H37Ra bacteria (OD-0.02) were treated with STK11 (0-200 $\mu\text{g/ml}$) glycoprotein and rifampicin (0-0.12 $\mu\text{g/ml}$) drug. Then the seeded plate was kept in a BOD incubator for 7 Days. Then, resazurin (0.1 mg/ml, 30 μl) was added for 24 h. Then fluorescence was checked in a BioTek Synergy multimode reader at excitation 530 nm, emission 590 nm. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference compared with the untreated control group.

3.3.3 STK11 prevents H37Ra bacterial growth in J774A.1 macrophage cell

The STK11 glycoprotein, encodes a serine/threonine kinase 11 engaged in various cellular processes, including cell metabolism, polarity, and the regulation of the

immune response (Pons-Tostivint et al., 2021). The role of STK11 in bacteria-infected macrophages, particularly with *M. tuberculosis*, was not well-documented. Previously, it was claimed that no cytotoxic effects were observed after treatment below 10 µg/ml of STK11 in murine macrophages (Srinivasan et al., 2023). Hence, we chose the range between 0-10 µg/ml of STK11. Therefore, the growth of H37Ra bacteria in J774A.1 macrophage cell was measured by the colony-forming units (CFU) test at doses of 0-10 µg/ml of STK11. J774A.1 cells were seeded at 1×10^5 per well and then infected with the H37Ra bacteria at an MOI in a 1:5 ratio (Macrophage: H37Ra). Then STK11 and a specific drug (rifampicin) were added for 48 h, and it was incubated for CFU, and the CFU growth was measured after 14 days, 21 days, and 28 days using the multimode reader. We observed that the CFU levels decreased significantly after increasing the dose of STK11 from 0-10 µg/ml to 14 days, 21 days, and 28 days compared to infected macrophages. We observed the highest reduction in CFU levels at 10 µg/ml of STK11 and 0.1 µg/ml of rifampicin after 28 days compared to infected macrophages. STK11 (10 µg/ml) and rifampicin (0.1 µg/ml) could reduce the bacilli growth by 2.74-fold and 2.31-fold, respectively, in H37Ra-infected macrophages (**p<0.001). We also observed that after 14 days, 2.5 µg/ml, 5 µg/ml, 10 µg/ml of STK11 and 0.1 µg/ml of rifampicin can restrict the bacilli growth within the macrophage by 15.79%, 23.25%, 46.12% and 49.4% respectively, compared to 14 days of H37Ra-infected macrophages. We also observed that after 21 days, 2.5 µg/ml, 5 µg/ml, 10 µg/ml of STK11 and 0.1 µg/ml of rifampicin can restrict the bacilli growth within the macrophage by 11.38%, 25.26%, 52.35% and 46.77% respectively, compared to 21 days of H37Ra-infected macrophages. After 28 days, 2.5 µg/ml STK11 inhibited bacilli growth in macrophages by 21.82%, 5 µg/ml STK11 inhibited bacilli growth in macrophages by 50.03%, 10

$\mu\text{g/ml}$ STK11 inhibited bacilli growth in macrophages by 62.86%, and, on the other hand, 0.1 $\mu\text{g/ml}$ rifampicin inhibited bacilli growth by 56.41%. (Figure 3.3).

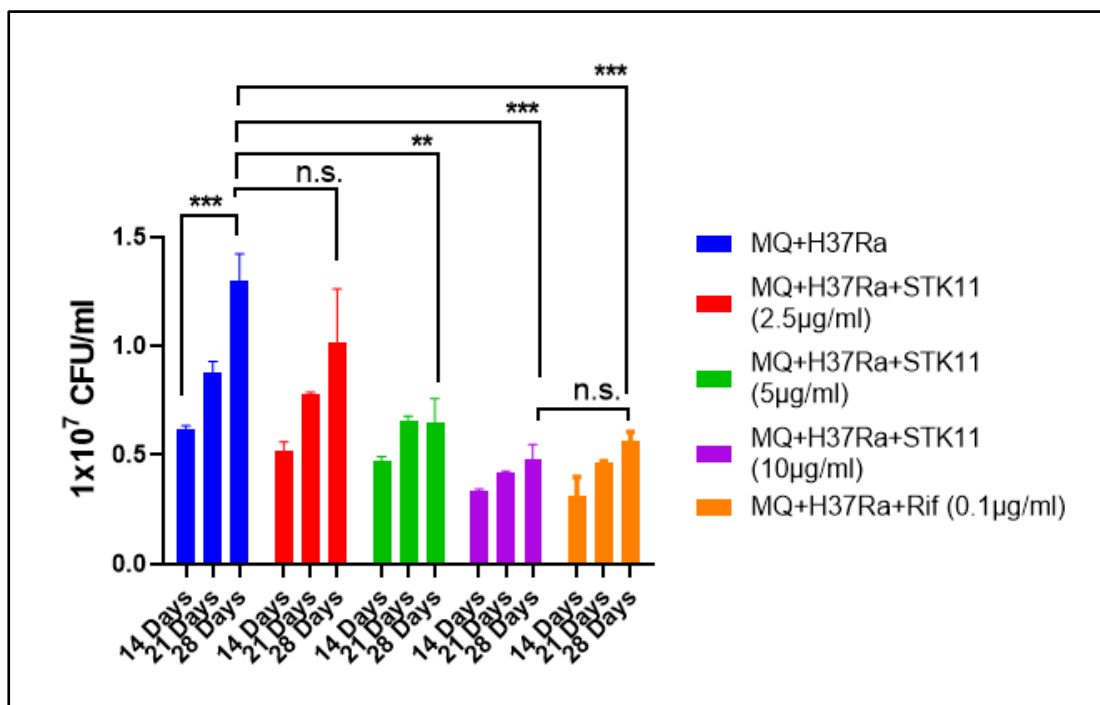


Figure 3.3 CFU level after 14 days, 21 days, and 28 days in the treatment with different doses of STK11. Macrophages (1×10^5) were infected with H37Ra at a 1:5 ratio. After that, macrophages were treated with different concentrations of isolated protein for 48 h. Then, 200 μl RIPA lysis buffer was added. Then, 50 μl of cell lysate with 450 μl of complete MB7H9 media were incubated for CFU count for 14 days, 21 days, and 28 days, and absorbance was checked at 600nm. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicates a significant difference compared with the untreated control group.

3.3.4 STK11 glycoprotein modulated mRNA expression of iNOS2 and Arg-1 and induce nitric oxide generation

Therefore, cellular marker (Arg1, iNOS2) expression during H37Ra-infected macrophages and STK11-treated H37Ra-infected macrophages was examined in triplicate. We observed that the M2 cellular marker, Arg1 expression, was upregulated during H37Ra-infected J774A.1 macrophages and downregulated during STK11-

treated H37Ra-infected macrophages. Besides, we also observed that the M1 cellular marker, iNOS2 expression, was downregulated during H37Ra-infected macrophages but upregulated during STK11-treated H37Ra-infected J774A.1 macrophages (Figure 3.4A, 3.4B & 3.4C). Then the data was statistically analyzed by GraphPad Prism 8.0. Mammalian immune cells generate nitric oxide (NO), which plays an important role in immune responses against pathogens. Naturally, the activation of macrophages needs the nitric oxide generation from L-arginine through a catalyzed reaction mediated by induced nitric oxide synthase (iNOS) (Jayaraman et al., 2022). Treatment of STK11 was done in a dose-dependent manner (0-10 µg/ml) for 48 h to control the NO generation in the H37Ra-infected macrophage cell. Results indicated a 5.11-fold increased NO generation after the treatment with STK11 at a 10 µg/ml dose, while only 2.33-fold was observed after the treatment with rifampicin (0.1 µg/ml) (Figure 3.4D).

3.3.5 STK11 glycoprotein modulated M1 and M2 surface markers in mRNA level

The expression of surface markers (YM-1, FIZZ1, CD206, CD68, CD40) was checked during H37Ra-infected macrophages and STK11-treated H37Ra-infected macrophages. We observed that M2 marker (YM-1, FIZZ1, CD206) expression increased during H37Ra-infected J774A.1 macrophages and slightly decreased during STK11-treated H37Ra-infected macrophages. On the other hand, we observed that M1 markers (CD68, CD40) increased during STK11-treated H37Ra-infected macrophages (Figure 3.5A, 3.5B-F). Therefore, we observed that *C. nucifera L.* derived glycoprotein, STK11, could switch from M2 to M1 polarization, and the results claim that M2 markers were upregulated during H37Ra-infected macrophages but downregulated during STK11-treated H37Ra-infected macrophages.

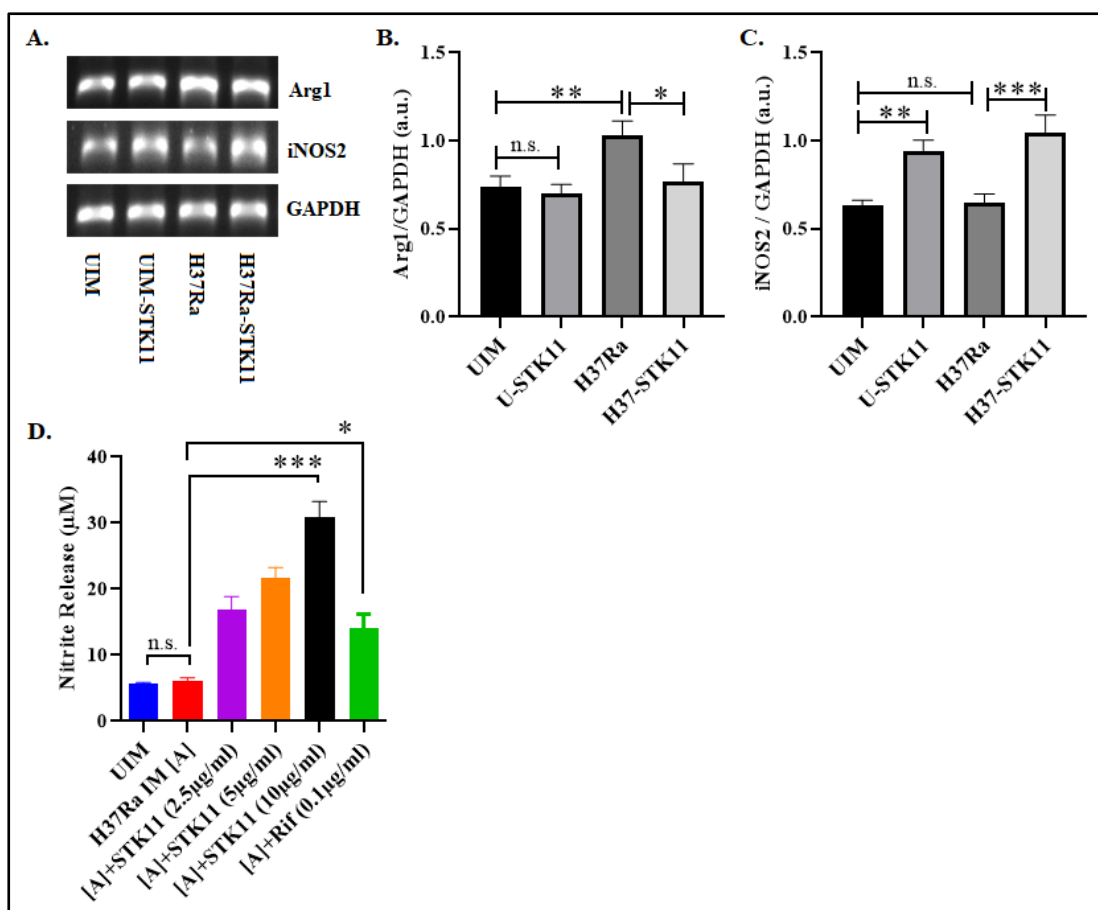


Figure 3.4 Cellular marker expression and nitrite generation were studied during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A, B, C) J774A.1 macrophage (1×10^6) cells were infected with *M. tuberculosis* bacilli (H37Ra) (OD=0.655) using MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. Both the H37Ra-infected and uninfected plates were treated with 10 $\mu\text{g/ml}$ of STK11 glycoprotein for 6 h. Cellular markers (iNOS2, Arg1) gene expression was gained in triplicate using specific primers. (D) Nitrite generation was assessed by Griess reagent from the cell supernatant after 48 h of treatment with the STK11 (0-10 $\mu\text{g/ml}$) and rifampicin (0.1 $\mu\text{g/ml}$). The experiment was conducted three times and produced analogous results, data shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicates a significant difference compared with the untreated control group.

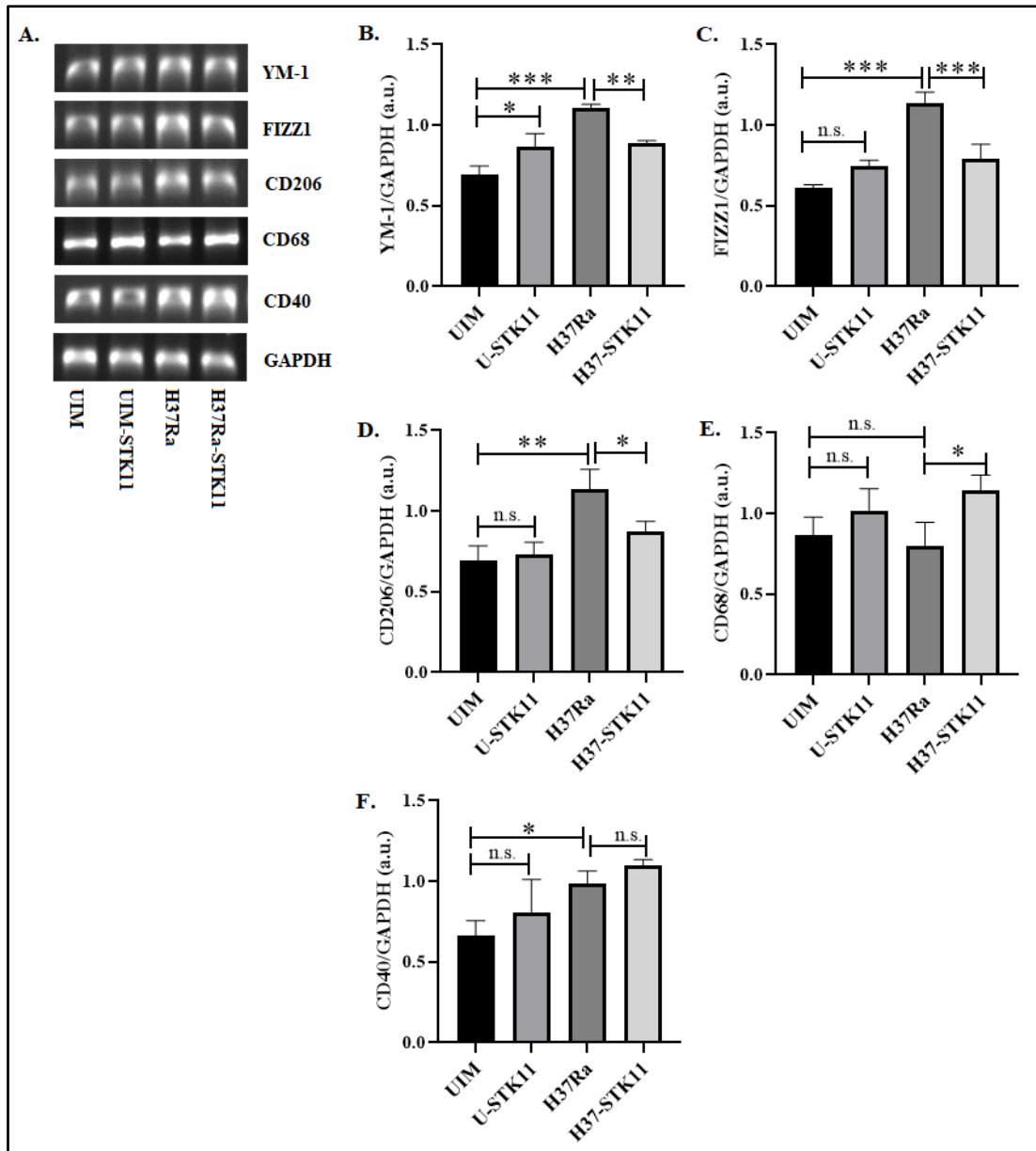


Figure 3.5 M1 and M2 Surface marker expression during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A-F) J774A.1 macrophage (1×10^6) cells were infected with *M. tuberculosis* bacilli (H37Ra) (OD=0.655) at MOI (1:5= Macrophage: *M. tuberculosis*). Following that, treatment with 10 μ g/ml of STK11 glycoprotein was done for 6 h. YM-1, FIZZ1, CD206, CD68, and CD40 gene expression were studied in triplicate form using specific primers. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicates a significant difference compared with the untreated control group.

3.3.6 STK11 glycoprotein modulated the cytokine expression at the mRNA level

A phagocytic cell, such as a macrophage, protects the host from pathogens by regulating pro-inflammatory cytokines, i.e., IFN- γ and IL-12, which stimulate the production of nitric oxide that kills pathogens (Murray, 2017). STK11 modulates cytokine expression at the mRNA level through various signalling pathways, impacting immune responses and inflammation (Koyama et al., 2016). We observed that pro-inflammatory cytokines (IL-12, IL-6) expressions were downregulated during H37Ra-infected macrophages and upregulated during STK11-treated H37Ra-infected macrophages. Along with IFN- γ expression, which was increased during H37Ra-infected macrophages, but slightly decreased during STK11-treated H37Ra-infected macrophages. Besides that, anti-inflammatory cytokines (IL-4, IL-10) expressions were increased during H37Ra-infected macrophages but decreased during STK11-treated H37Ra-infected macrophages (Figure 3.6A, 3.6B-F).

3.3.7 Cytokine release was modulated by STK11 at the protein level

A similar type of result was obtained from cytokine release experiments with the mRNA expression levels. We observed from the ELISA test that pro-inflammatory cytokines (IL-12, IL-6) release was downregulated during H37Ra-infected macrophages and upregulated during STK11-treated H37Ra-infected macrophages. Along with IFN- γ release was upregulated during H37Ra-infected macrophages but slightly decreased during STK11-treated H37Ra-infected macrophages. Besides that, anti-inflammatory cytokines (IL-4, IL-10) release was upregulated during H37Ra-infected macrophages but decreased during STK11-treated H37Ra-infected macrophages (Figure 3.7A-E).

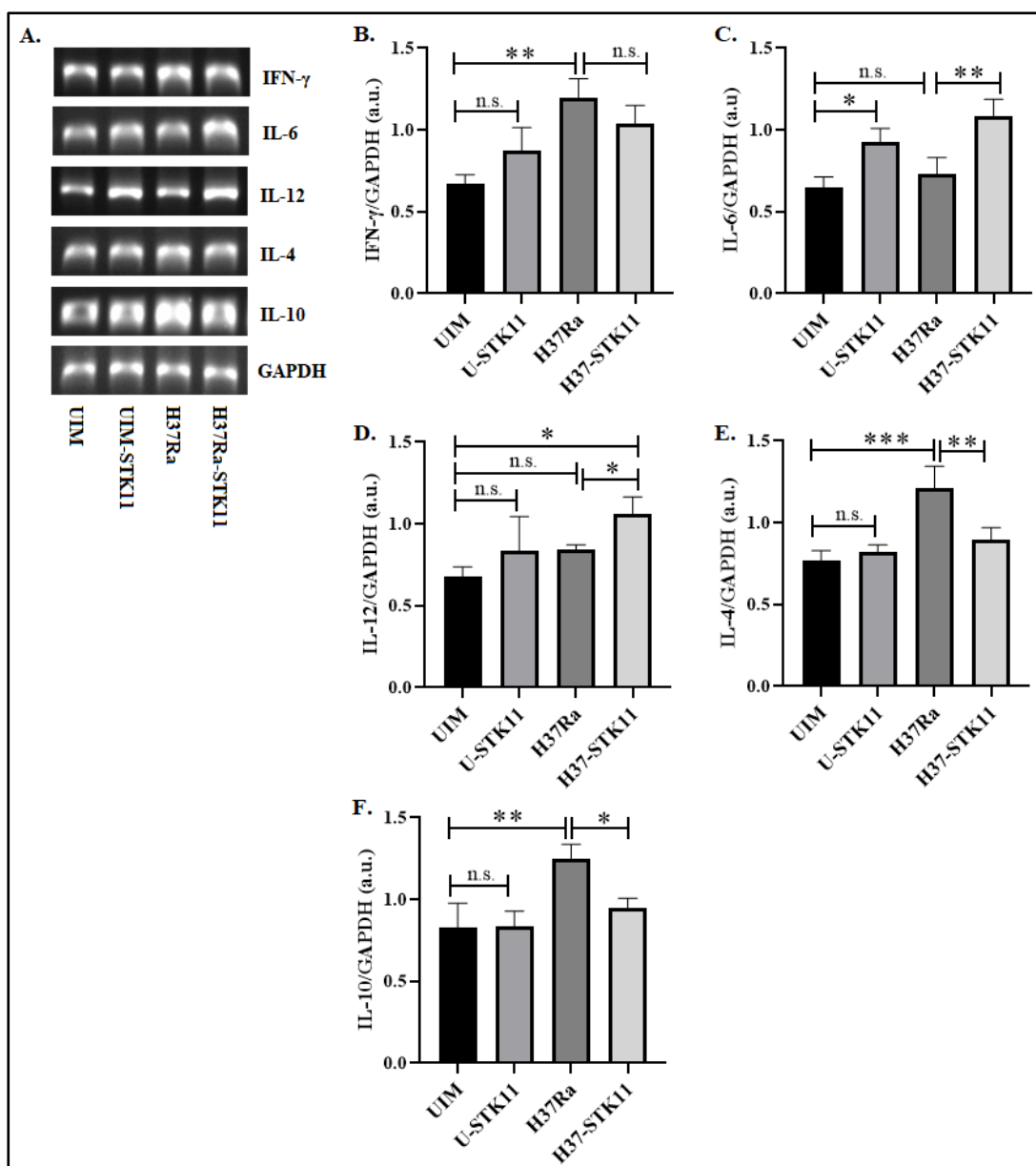


Figure 3.6 Pro and anti-inflammatory cytokine expression was studied during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A-F) J774A.1 macrophage cells (1×10^6) were infected with *M. tuberculosis* bacilli (H37Ra) (OD=0.655) with MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. For 6 h, 10 μ g/ml STK11 was used to treat H37Ra-infected and uninfected macrophages. IFN- γ , IL-6, IL-12, IL-4, and IL-10 cytokine expression were studied in triplicate form using specific primers. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicates a significant difference compared with the untreated control group.

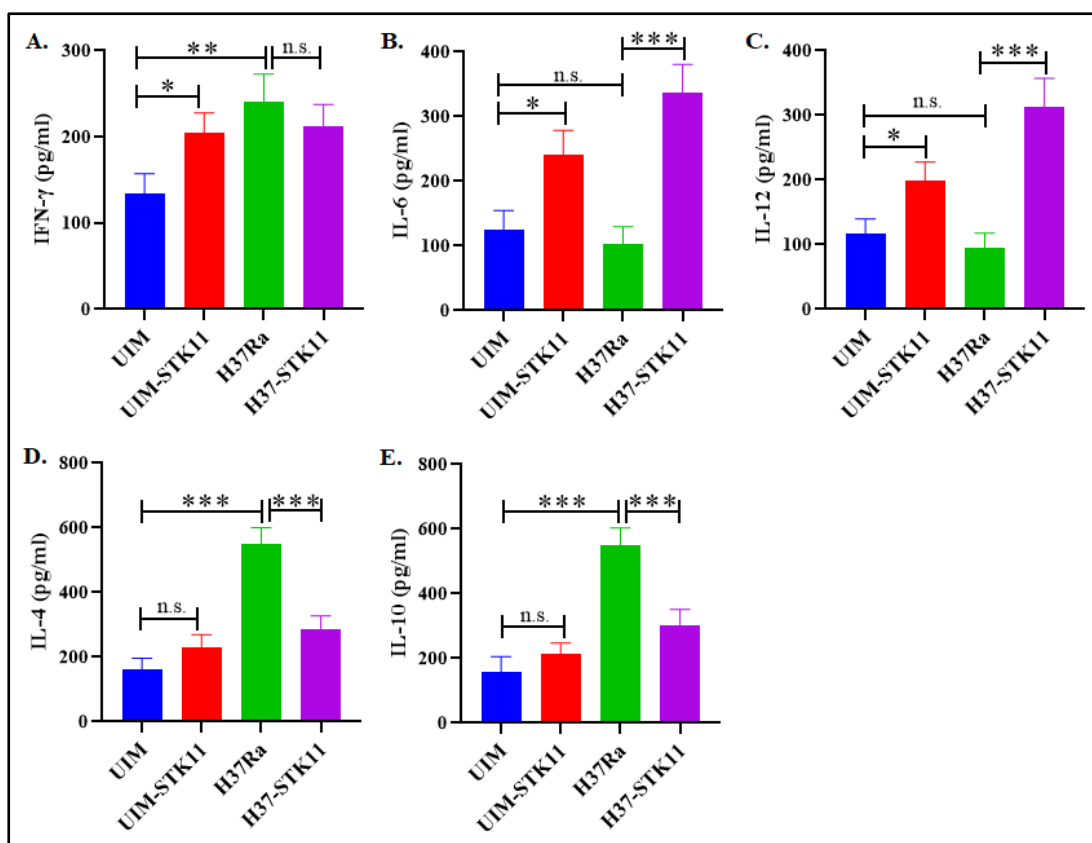


Figure 3.7 Pro and anti-inflammatory cytokine release was studied during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A-E) J774A.1 macrophage cells (1×10^6) were infected with *M. tuberculosis* bacilli (H37Ra) (OD=0.655) with MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. Cytokine release (protein expression) of pro-inflammatory (IFN- γ , IL-12 and IL-6) and anti-inflammatory (IL-10 and IL-4) were determined in triplicate after 24(h) of treatment with STK11 using ELISA. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicates a significant difference compared with the untreated control group.

3.3.8 Protein-Protein docking between the Ras and STK11 glycoprotein and mRNA expression of H-Ras, N-Ras, and K-Ras during H37Ra-infected and STK11-treated H37Ra-infected macrophages

The interaction between the STK11 and Ras proteins (H-Ras, N-Ras, K-Ras) has been of interest in cellular signaling studies (Ihle, 2012). However, detailed direct experimental evidence of protein-protein docking between STK11 and Ras isoforms has been limited. In this context, the *in-silico* interactions at the molecular level between

the STK11 and the Ras were evaluated utilizing the ClusPro 2.0 server, which significantly supported the mRNA expression of Ras in a triplicate study. Interestingly, among all Ras proteins, the lowest binding energy (-905.9 kcal/mol) was observed in the interaction between H-Ras and STK11, which is consistent with the mRNA expression results. However, binding energy -832.0 kcal/mol for K-Ras & -794.5 kcal/mol for N-Ras was found (3.8A-C).

Specific information about the mRNA expression levels of H-Ras, N-Ras, and K-Ras during *M. tuberculosis*-infected macrophages was scarce. In this context, J774A.1 macrophage cells were infected with H37Ra bacteria at a 1:5 ratio for 4 h, and, after being treated with STK11 glycoprotein for 6 h, mRNA expression was studied, followed by cDNA synthesis and PCR for the parameters of N-Ras, K-Ras, and H-Ras. We observed that N-Ras expression was upregulated during H37Ra-infected macrophages but decreased during STK11-treated H37Ra-infected macrophages (Figure 3.8D-E). On the other hand, H-Ras and K-Ras expression were downregulated during H37Ra-infected macrophages but upregulated during STK11-treated H37Ra-infected macrophages. This is illustrated in Figure 3.8D, 3.8F-G.

According to previous reports, Sotorasib is the inhibitor of K-Ras and Sulindac Sulfide is the inhibitor of H-Ras. It has been reported that $0.1\mu\text{M}$ of Sotorasib and $50\mu\text{M}$ of Sulindac Sulfide were standardised concentrations that inhibited the K-Ras and H-Ras (Canon et al., 2019; Gala et al., 2002). In that reference, the infected macrophages were pre-treated with $0.1\mu\text{M}$ Sotorasib and $50\mu\text{M}$ of Sulindac Sulfide respectively 2(h) prior treatment with STK11 to check its interaction with Ras molecules. After 48(h) of treatment, the cell sample were further cultured for 21 days for measuring the CFU. It was observed that the CFU increased by 1.56 and 1.76 folds respectively when H37Ra infected macrophages were treated with K-Ras and H-Ras inhibitors before being

treated with STK11 as compared to treated group (Fig 7H). The nitrite oxide (NO) being the effector molecule in immune responses against pathogens, the NO generation was also examined upon using inhibitors. It was observed that the NO generation decreased by 1.84 and 2.28 folds when H37Ra infected macrophages were treated with K-Ras and H-Ras inhibitors before being treated with STK11 as compared to treated group (Fig 7G). Hence, it can be inferred that the interaction of STK11 with K-Ras and H-Ras is responsible for its activity as a candidate against H37Ra.

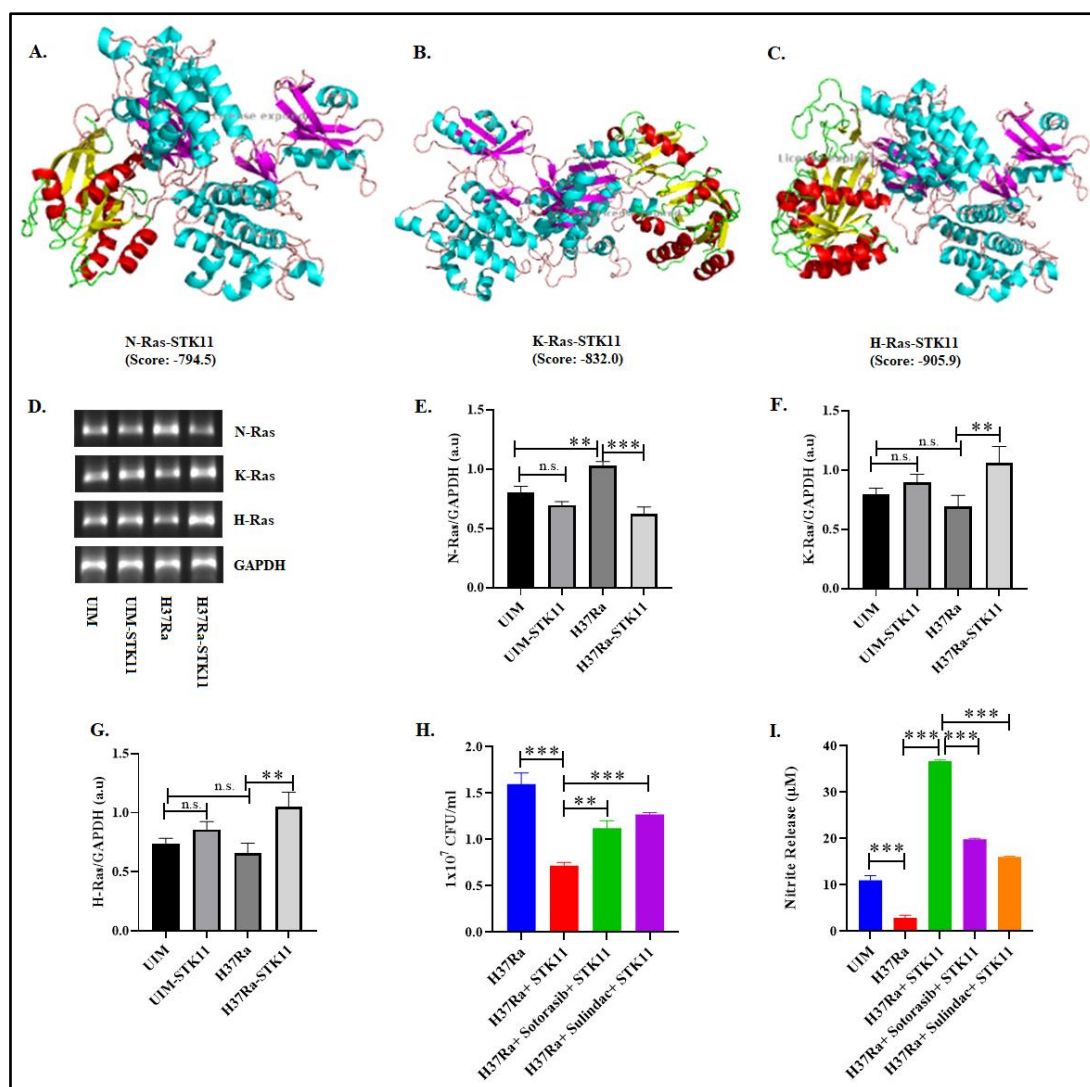


Figure 3.8 Ras-STK11 protein-protein docking (A, B, C), mRNA expressions of N-Ras, K-Ras, and H-Ras (D-G) in infected and uninfected macrophages and effect of K-Ras and H-Ras inhibitors (H, I): The docking results between STK11 and N-Ras, K-Ras, H-Ras

respectively was obtained in Clasp software. J774A.1 macrophage cell (1×10^6) was infected with *M. tuberculosis* Bacilli (H37Ra) (OD=0.655) with MOI (1:5= Macrophage: *M. tuberculosis*). After 4 h, non-ingested *M. tuberculosis* bacilli were removed. After 6(h) of treatment, mRNA expressions of different Ras were observed in uninfected, H37Ra infected as well as STK11 treated H37Ra infected macrophages. CFU and NO generation was checked upon using K-Ras and H-Ras inhibitors. The results were expressed as a mean $\pm$ SD. Asterisks indicated a significant difference compared with the infected control with the treated STK11 group. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

3.4 Discussion

Tuberculosis is responsible for approximately 1.5 million deaths each year (WHO, 2025). HDT drugs that target the host can accelerate and improve the treatment of tuberculosis (Wallis & Hafner, 2015). By altering the pro-(M1) or anti-inflammatory (M2) immune system, host-directed therapies may improve the treatment of tuberculosis (TB) (Krug et al., 2021). Many immunomodulators such as rocaglates, a natural product derivatives from *Aglaia genus* plants, polarized cells toward M1 from M2 (Langlais et al., 2018; Chatterjee et al., 2021), BPMS22 derived from *Oceanimonas* sp. polarized cells from M2 to M1 (Jayaraman et al., 2022), Oligo-Fucoidan, a sulfated polysaccharide isolated from the brown seaweed, polarized cells from M2 to M1 (Cheng et al., 2020; Chen et al., 2020), Ibrutinib, a BTK inhibitor and Selinexor, a blood–brain barrier (BBB) permeable small molecule polarized cells towards M1 from M2 (Jiménez et al., 2020). In this context, we isolated the novel immunomodulator STK11 from *C. nucifera L.* and checked its efficacy for M2 to M1 repolarization based on macrophage polarization in H37Ra-infected macrophages. At a minimum dose, STK11 has been reported to show its immunogenic activity. It is interesting to note that, STK11 stimulates pro-inflammatory cytokine response and NO generation in a TLR-dependent manner (Srinivasan et al., 2023). The SDS-PAGE results showed that STK11 is associated with the bands of 46 kDa (Figure 3.1). According to previous

reports, STKs also assist in safeguarding plants by inducing numerous physiological processes such as secondary metabolite synthesis, stabilization of cell walls, and the prompting of defensive gene expression (Amil-Ruiz et al., 2011; Sekhwal et al., 2015). SnRK2, another plant STK family member, intervene with the ABA signalling and the abiotic response to stress. The negative regulation of plant defences by STK11 was also published (Kulik et al., 2011). An STK in *Arabidopsis*, PBL13, has been shown to negatively control resistance against *Pseudomonas syringae*, a pathogenic bacterium (Lin et al., 2015). Many reports declared that *C. nucifera L.* and its ingredients have an immunomodulatory impact (Narayanankutty et al., 2021; Narayanankutty et al., 2021). In a study using Swiss albino mice and LPS-induced RAW macrophages, it was revealed that the acetone extract of coconut inflorescences possesses antinociceptive and anti-inflammatory properties (Joseph et al., 2021). The liquid shell smoke of *C. nucifera L.* can reconcile the oral diabetic ulcer by raising the responses of the macrophages by dropping the expression level of TNF- α and NF- κ B (Surboyo et al., 2020). From the coconut, the isolated protein fraction has antimicrobial peptides and antioxidants (Li et al., 2018). The anti-mycobacterial activity of rifampicin and isoniazid was previously reported, which exhibited MICs of 0.003 μ g/ml and 0.03 μ g/ml, respectively (Jyoti et al., 2015). This study evaluates the antibacterial effects of STK11 glycoprotein and rifampicin against *M. tuberculosis* H37Ra using the resazurin assay, a well-established method for assessing cell viability. In this present study, we isolated the STK11 immunomodulator and tested its efficacy for the M2 to M1 polarization. Besides, we checked the efficacy of STK11 in the direct killing assay by resazurin assay, which killed the bacteria by increasing the dose of STK11 significantly. The bacterial killing percentage increases as the concentration of STK11 and rifampicin rises (Figure 3.2). On the other hand, the macrophage-mediated killing assay was done

in a triplicate set of *in vitro* experiments, where H37Ra was infected into macrophages at a 1:5 ratio. The observed reduction in CFU levels suggests that STK11 exhibits antimicrobial properties against H37Ra-infected macrophages. Notably, the highest tested concentration (10 µg/ml) of STK11 reduced CFU by 2.74-fold, while rifampicin at 0.1 µg/ml resulted in a 2.31-fold reduction. These results indicate that STK11 may potentiate the antimicrobial effect independently in controlling H37Ra bacterial growth. The statistical significance ($p < 0.001$) of the results further underscores the reliability of our observations. The promising antimicrobial activity of STK11, compared with rifampicin, highlights its potential as an alternative or adjunctive treatment strategy for tuberculosis. These discoveries pave the way for additional research into the therapeutic potential of STK11 in bacterial infections (Figure 3.3). Additionally, *in vivo* studies are required to validate the efficacy of STK11 in animal models of tuberculosis infection.

Utilising certain stimuli, namely LPS, macrophages are driven to two main polarized phenotypes: M2 and M1, which function as anti-inflammation and pro-inflammation, respectively (Murray, 2017; Samanta et al., 2024; Arora et al., 2018; Han et al., 2017; Li et al., 2018). On the contrary, *M. tuberculosis* infection can drive macrophages in the early phase toward M1 phenotypes and move their program in intermediate and late phases into M2 phenotypes (Lugo-Villarino et al., 2011; Benoit et al., 2008). iNOS2 and Arg1 are engaged in two opposite activities, iNOS2 with proinflammatory (M1) and Arg1 with anti-inflammatory (M2) function (Wang et al., 2020; He et al., 2013). The result shows that Arg1 expression in RT-PCR increased significantly during H37Ra-infected macrophages, which induced anti-inflammatory (M2) macrophage polarization. On the other hand, results claimed that STK11-treated H37Ra-infected macrophages can be modulated towards M1 polarization from M2. iNOS2 expression

declared that iNOS2 expression decreased during H37Ra-infected macrophages and increased during STK11-treated macrophages (Figure 3.4A, 3.4B & 3.4C). Nitrite release assay suggested that nitrite release decreased in uninfected and H37Ra-infected macrophages and increased during STK11-treated macrophages as the dose 0-10 µg/ml significantly, which supports the same with the iNOS2 band (Figure 3.4D).

M1 and M2 markers expression supported that M2 markers (YM-1, FIZZ1, CD206) also increases and M1 markers (CD68, CD40) expression decreases in H37Ra-infected macrophages. On the other hand, STK11 treatment can restrict the bacilli's growth within the macrophages, and RT-PCR results supported the same, as M2 markers decrease successfully and M1 markers increase significantly ($p < 0.001$) (Figure 3.5A, 3.5B-F). The RT-PCR result of IL-4, IL-10 cytokines expression increased significantly ($p < 0.001$), and IL-6, IL-12 expression decreased significantly ($p < 0.001$) during H37Ra-infected macrophages (Figure 3.6A, 3.6B-F). However, STK11-treated macrophages restricted the bacilli's growth as M1 cytokines (IL-6, IL-12) expression increased and M2 cytokines (IL-4, IL-10) expression decreased. Similar types of results were also found in cytokine release. So, cytokine release also claimed that STK11 treated H37Ra infected macrophages alter the macrophages polarization from M2 to M1 to restrict the H37Ra infection (Fig. 3.7 A-E). IFN- γ expression typically increased during *M. tuberculosis*-infected macrophages, including with the attenuated H37Ra strain-infected macrophages (He et al., 2013). IFN- γ was a critical cytokine in the immune response against *Mycobacterial* infections, as it activated the macrophages and enhanced their ability to kill intracellular pathogens (Kak et al., 2018).

Small GTPases called Ras proteins (such as H-Ras, K-Ras, and N-Ras) were important in sending signals from cell surface receptors to the nucleus, which affects immunological responses, cell survival, and proliferation (Sadeghi Shaker et al., 2023).

In this context, we established that STK11 was the finest molecule for interacting with Ras by the docking method. STK11 displayed better interaction with H-Ras (docking score -905.9) (Figure 3.8A-C). So, our study revealed that N-Ras expression increased, and H-Ras, K-Ras expression decreased during H37Ra-infected macrophages, but on the other hand, N-Ras expression decreased, and H-Ras, K-Ras expression increased in STK11-treated H37Ra-infected macrophages at the mRNA level, which were very similar to the *in-silico* studies result (Figure 3.8D, 3.8E-G). Another result suggested that N-Ras regulates ERK-1/2 activation by CD40, which results in the anti-inflammatory IL-10 production, whereas H- or K-Ras was required for p38MAPK activation, which results in the creation of pro-inflammatory IL-12 (Nair et al., 2020). Sotorasib (0.1 μ M) and sulindac (50 μ M) have been reported to be the inhibitors of K-Ras and H-Ras respectively and have played a critical role in cancer biology in previous reports (Canon et al., 2019; Gala et al., 2002). So, to clarify the positive interaction of Ras isoform with STK11 to treat H37Ra infected macrophages, the inhibitors for K-Ras and H-Ras were used for experimentation. From the results, it was observed that the CFU increased by significant levels ($p < 0.001$) upon addition of sotorasib and sulindac to the already STK11 treated macrophages as compared to the normal treated group (Fig. 7H). NO generation using the inhibitors also showed decrease in nitrite release as compared to the STK11 treated group where no inhibitors were used (Fig. 7I). Hence, through the inhibition of Ras isoforms and overviewing its effect on STK11 confirms that STK11 has strong interaction with Ras isoforms and STK11 activity is dependent on Ras isoforms.

Consequently, *M. tuberculosis* can go into and reside inside alveolar macrophages and gain protection from some antibiotics (Davenne & McShane, 2016). Additionally, for developing drugs for any intracellular bacteria, one very significant factor is whether

the applicant drug can work inside the cell or not (Jordao & Vieira, 2011). In this context, we assessed the effect of STK11 in *M. tuberculosis* (H37Ra) infected J774A.1 macrophage cells in the macrophage mediated killing assay followed by direct killing assay and the macrophage mediated RT-PCR and protein expression study.

3.5 Summary & Conclusion

Tuberculosis is a very dangerous disease nowadays. So, the WHO targeted how to control this. In this context, this study examines the importance of host-directed therapy against tuberculosis by targeting macrophage polarization with *C. nucifera* L. nutshell-derived STK11. RT-PCR results revealed that M2 markers (Arg1, YM-1, FIZZ1, CD206, IL-4, IL-10) were upregulated, while M1 markers (CD40, CD68, IL-6, IL-12) were downregulated in H37Ra-infected J774A.1 macrophages. Isolated glycoprotein STK11 significantly ($p < 0.05$) restricted the growth of *Mycobacterium tuberculosis* bacilli in the Middlebrook 7H9 medium and inhibited H37Ra bacilli growth within macrophages. STK11 (10 $\mu\text{g/ml}$) was also found to decrease the expression of M2 markers and increase M1 markers in infected macrophages. Additionally, STK11 can interact with Ras isoforms (H-Ras, N-Ras, K-Ras) *in silico*, and the mRNA expression of K-Ras and H-Ras was increased, while N-Ras was decreased in STK11-treated H37Ra-infected macrophages, indicating the involvement of Ras isoforms during STK11 treatment. Hence, STK11 might be associated with the regulation of Ras isoforms, modulating M2 to M1 repolarization in H37Ra-infected macrophages, followed by restricting the *M. tuberculosis* bacilli growth in host macrophages. Through a nitrite-dependent mechanism, the STK11 glycoprotein may restrict the growth of bacilli within macrophages and induce the M1 signature in H37Ra-infected macrophages (Figure 3.9).

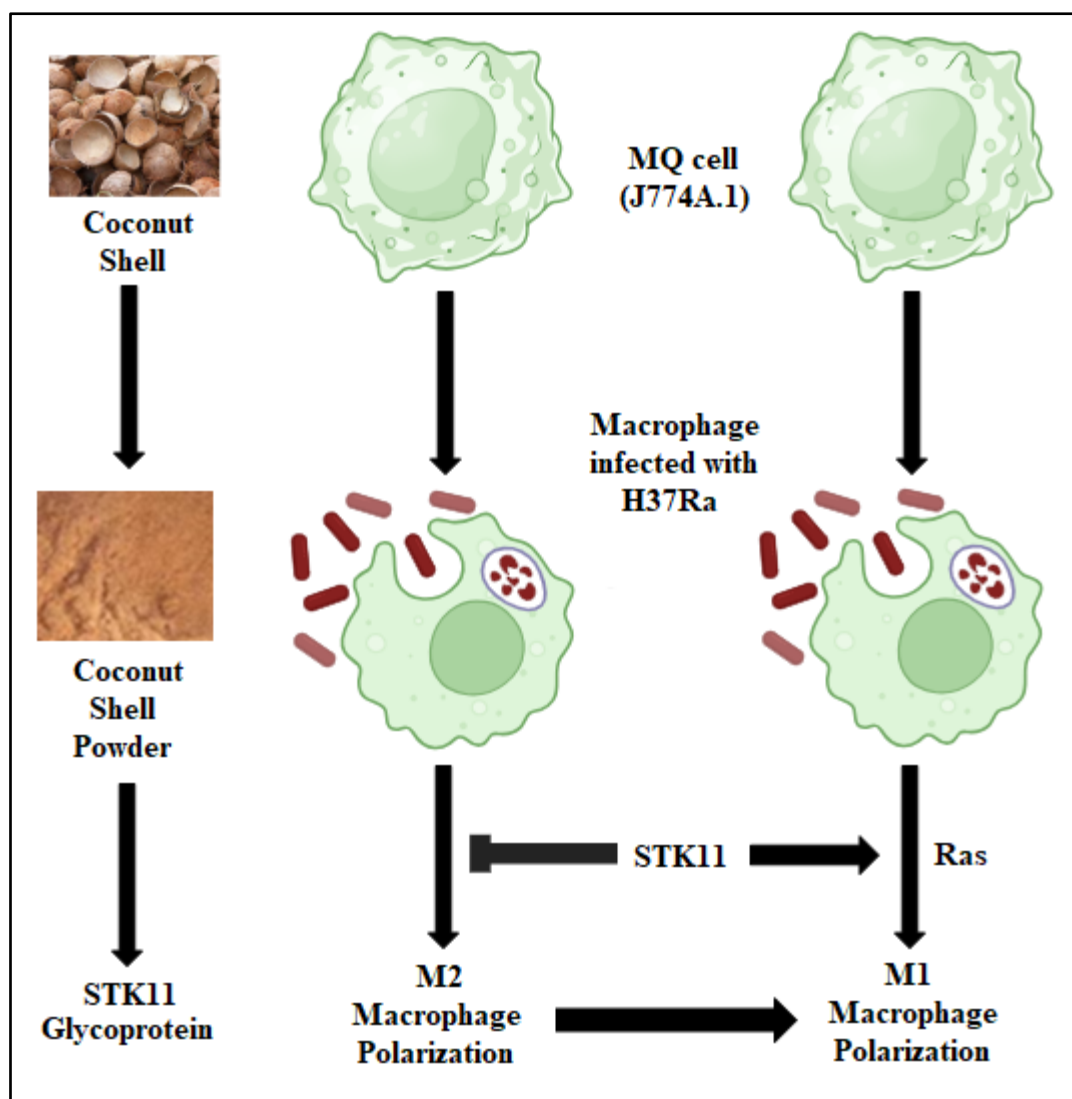


Figure 3.9 STK11 repolarizes the macrophages during tuberculosis. STK11 glycoprotein obtained from the nutshell powder of *C. nucifera* L. On the other hand, H37Ra-infected macrophage cell induces M2 polarization, but when STK11 is used to treat H37Ra it induces M1 polarization through the interaction with Ras. STK11 can repolarize cells from M2 to M1.

Chapter 4: Study of STK11 mediated macrophage polarization to treat the latent *Mycobacterium* bacilli in J774A.1 macrophages

4.1 Introduction

Tuberculosis, one of the greatest life-threatening airborne diseases in the world, is affected by the *Mycobacterium tuberculosis* (WHO, 2025). Based on the mode of tuberculosis infection, there are two types: active and latent TB infection (LTBI) (Gong & Wu, 2021). Recently, WHO predicted that approximate one-third of the global population is infected with latently *M. tuberculosis* bacilli, which is covered 90% of the population infected by it (WHO, 2025). In biology, the term "latent" refers to an organism's dormant state when its surroundings are unsuitable for development and proliferation; in medicine, it indicates a disease stage when its symptoms are not yet clinically recognized (Behr et al., 2018). *M. tuberculosis* remains in a sluggish state for a long time during LTBI (Hasnain et al., 2019; Alzahabi et al., 2020). Most LTBI individuals do not become sick with tuberculosis; however, nearly 5-15% may progress to active tuberculosis disease (Campbell et al., 2020; Getahun et al., 2015). Since people with LTBI are a reservoir for incident TB disease, preventing the progression of LTBI to TB disease is a vital personal and public health objective (Shah & Dorman, 2021). There are no accurate methods for diagnosing LTBI (Huang et al., 2022). By identifying memory T-cell response, which only indicates host sensitization to *M. tuberculosis* antigens, the IGRAs and the tuberculin skin test indirectly assess tuberculosis infection (Getahun et al., 2015). Most people agree that these tests are acceptable but flawed (Pai et al., 2014). Treatment options for latent tuberculosis infection to prevent progression to active clinical disease are limited (Chee et al., 2018). To achieve the goal (sterile cure of TB cases by 2035), it is not enough to identify active TB cases and improve

effective treatment; strategies are also needed to prevent new infections and neutralize existing LTBI (Vasiliu et al., 2024).

In this context, host-directed therapy (HDT) is an innovative therapeutic approach that has been developed in recent years to treat TB infections. Unlike outmoded antimicrobial therapy, HDT usually acts on the host's immune system rather than the pathogens (Kaufmann et al., 2018). Due to antimicrobial resistance (AMR), HDT is especially crucial in cure of infectious diseases. AMR makes infectious diseases that are caused by bacteria or viruses much more dangerous to humankind (Jeong et al., 2022). An estimated 4.95 million fatalities globally were attributed to AMR in 2019 (Ranjbar & Alam., 2023) and in the 21st century, there are difficulties in finding novel antibiotics that are effective against antibiotic-resistant bacteria (Hoffman, 2020). HDT offers enormous potential as a therapeutic approach that can overcome drug resistance, normalize patients' immunological responses, and make up for the deficiencies of existing TB treatments (Jeong et al., 2022).

Several studies proposed that TLRs are vital factors for *M. tuberculosis* infection (Berrington & Hawn, 2007; Hölscher et al., 2008). TLR1, TLR2, TLR4, TLR6, and TLR9 are the main receptors involved in *Mycobacterial* infection (Zihad et al., 2023). Several host cells, such as dendritic cells, macrophages, neutrophils, B-cells, endothelial and epithelial cells of mucosal linings, express TLR family members (Kawasaki & Kawai, 2014; Suzuki et al., 2021). TLR activation upregulates the proinflammatory cytokines transcription, including IL-1 β , IL-6, and TNF- α , which are vital for the recruitment of immune cells to the site of infection and controlling the infection of *M. tuberculosis* (Takeda & Akira, 2004; Das et al., 2016).

The isolated STK11 from nutshell of *C. nucifera* L. was already proved its efficacy in macrophage activation through prompt regulation of cytokine balance and could

interact with TLRs (Srinivasan et al., 2023). Our recent report and described in the previous chapter also proved that STK11 could inhibit the growth of H37Ra bacilli in J774A.1 macrophages through macrophage M1 polarization (Samanta et al., 2026). Hence, we intended to check the effect of STK11 glycoprotein, isolated from the naturally abundant and agricultural waste material, the nutshell of *C. nucifera* L., in latent tuberculosis-challenged macrophages.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

All chemicals, cell culture media, growth supplements, molecular biology grade chemicals were obtained from SRL, Himedia, Sigma Aldrich, Gibco, Bio-Rad, Takara in its highest grade available as stated in the previous chapters (Section: 2.2.1 and 3.2.1).

4.2.2 *M. tuberculosis* (H37Ra) bacilli culture, preparation of the latent state of *M. tuberculosis*

M. tuberculosis (H37Ra) bacilli culture was maintained with regular passage in MB7H9 medium with required growth supplements as stated in previous chapters (Section: 2.2.2) and report (Samanta et al., 2026). The latent state of *M. tuberculosis* was prepared as described by Wayne's hypoxia model (Wayne & Hayes, 1996). The liquid cultures were prepared by filling screw-capped culture tube (30 ml) with the sterile MB7H9 broth with 5% glycerol and 10% ADS. After that mid-log phase *M. tuberculosis* inoculum were carefully added and mixed using pipetting to ensure its' thorough distribution. Methylene blue (1.5 µg/ml) was added to the culture as of colour indicator. Rubber septum with parafilm seal was used to seal the flask and assure air-tightness.

The flasks were then incubated at 37°C in stirring condition. Methylene blue decolorization was noted as the indication of oxygen deprivation within the cultures. Non-replicating persistence (NRP) stage 1 (minimal methylene blue decolorization) and NRP stage 2 (full methylene blue decolorization) were achieved on day 7 and day 12 respectively (Wayne & Hayes, 1996; Jakkala & Ajitkumar., 2019).

4.2.3 The impact of STK11 against the latent H37Ra in the resazurin assay

The resazurin assay was used to assess the percentage of killing of latent H37Ra bacilli after the treatment with STK11 and rifampicin. Briefly, 100 µl of latent H37Ra (OD-0.02) was seeded in 96 well culture plate. Then 0-200 µg/ml of STK11 and 0-0.12 µg/ml of rifampicin was added on the plate in triplicate form. Then, remaining of the volume up to 200 µl was added with MB7H9 medium on that plate. To avoid dehydration on the plate 200 µl of distilled water was added on other well of the plate. Then the plate was carefully wrapped with parafilm and aluminium foil and kept in BOD incubator at 37°C for 7 days. After that, 30 µl of resazurin (0.1 mg/ml) was added for 24 h. Then, fluorescence intensity was measured at excitation: 530 nm and emission: 590 nm in the multimode reader (Samanta et al., 2026; Jyoti et al., 2015).

4.2.4 Intracellular screening of the inhibitory potential of STK11 against the latent *M. tuberculosis* bacilli challenged macrophages

J774A.1 macrophage (1×10^5) cells were incubated in 24-well culture plates for overnight. Then, the cells were challenged with latent H37Ra at the MOI of 1:5 (Macrophage: latent H37Ra) for 4 h (Iona et al., 2012). Next, STK11 (0–10 µg/ml) and rifampicin (0.1 µg/ml) were added for 48 h in the selected well. The supernatant was removed from the plate and washed with PBS after 48 h of treatment. Then, RIPA lysis

buffer (except protease inhibitor) (200 μ l) was added to each well and allowed to rock for 30 minutes. Subsequently, cell lysate sample (50 μ l) was taken and carefully transferred to a fresh, cleaned 24-well plate and complete MB7H9 medium (450 μ l) was added on that plate. Then, the plate was carefully enfolded with aluminum foil and parafilm to evade dehydration. The plate was kept in a BOD incubator for the culture. Then, after 28 days, bacterial growth was checked using the Synergy H1 microplate reader to measure absorbance at 600 nm. GraphPad Prism 8.0 was used to calculate and examine CFU based on the absorbance value (Samanta et al., 2026; Elizabeth et al., 2016).

4.2.5 NO production in J774A.1 macrophage cells

Macrophage cells (1×10^6) were seeded in 24-well culture plates for overnight. Then, the J774A.1 cells were challenged with latent H37Ra at an MOI of 1:5 (Macrophage: latent H37Ra) for 4 h (Iona et al., 2012). The cells were treated with 0-10 μ g/ml of STK11 and 0.1 μ g/ml of rifampicin, and were kept in the CO₂ incubator for 48 h. Then, supernatants (100 μ l) were taken from the plate, and NO production was tested by the Griess reagent at 548 nm as per the proper protocol (Jayaraman et al., 2022).

4.2.6 The expression of mRNA of different pro and anti-inflammatory markers

J774A.1 macrophage (1×10^6) cells were infected or challenged with either H37Ra (OD = 0.646) or latent-H37Ra (OD = 0.218) *M. tuberculosis* bacilli for 4 h followed by treated with STK11 glycoprotein (10 μ g/ml) for another 6 h. Total mRNA extraction was performed from the experimental group *in vitro*. The reverse transcriptase enzyme was used to synthesis cDNA using 1 μ g of total RNA as a template. Using the specific forward and reverse primers of iNOS2, Arg-1, YM-1, CD206, FIZZ1, IFN- γ , CD68,

IL-6, IL-12, CD40, IL-10, IL-4, TLR-4, TLR-2, TLR-9, TLR-6 were assessed. The forward and reverse primer sequences of the target genes were listed in table 2.1 and table 4.1. Thermal cycler conditions were as stated earlier chapters. Using 1.2% agarose gel electrophoresis, amplified PCR gene products were run and observed under UV light and evaluated using the Bio-Rad Gel documentation system. The band intensity was assessed using ImageJ software and standardized with the GAPDH to achieve the relative expression level (Charan Raja et al., 2021).

Table 4.1: TLRs Genes, corresponding primer sequences

Gene	Primer Sequences (5'-3')
TLR-2	F: AAG AGG AAG CCC AAG AAA GC R: CGA TGG AAT CGA TGA TGT TG
TLR-4	F: ACC TGG CTG GTT TAC ACG TC R: CTG CCA GAG ACA TTG CAG AA
TLR-6	F: TTG TTT GCG CCC TGG CCT TAA T R: TGT TCT TGG TGG CAG GTC TTT G
TLR-9	F: ACT GAG CAC CCC TGC TTC TA R: AGA TTA GTC AGC GGC AGG AA
GAPDH	F: GAG CCA AAC GGG TCA TCA TC R: CCT GCT TCA CCA CCT TCT TG

4.2.7 The interaction of protein-protein between STK11 and TLRs

The created 3D model of STK11 was used to examine the interaction with several TLRs (TLR-2, TLR-4, TLR-6, and TLR-9) *in silico*. The STK11 sequence (A0A0N7EZQ1) was occupied from our earlier described study (Srinivasan et al., 2023) in the UniProtKB knowledge base and kept in FASTA format for more analysis. Using Cluspro 2.0, an online server available at <https://cluspro.bu.edu/login.php?redir=/home.php>, TLR receptors were obtained from the protein data bank in order to investigate the mechanism of activation using the

molecular docking approach. Three primary phases are pursued by the Cluspro v2.0 server: a) Rigid body docking is achieved by sampling billions of conformations using the Fast Fourier Transform (FFT) correlation. b) The primary clusters are formed by grouping the 1000 lowest energy structures; the best docking complicated models are described by Interface Root Mean Square Deviation. c) By removing steric conflicts using energy minimisation, the stability of the docking models is verified. The interaction between the STK11 glycoprotein and the TLRs receptors was imagined via PyMOL software (Srinivasan et al., 2023).

4.2.8 Cytokine analysis by sandwich ELISA

The cytokine release were assessed from the cell-free supernatant of latent H37Ra-challenged STK11-treated macrophages. Sandwich ELISA was performed for pro-inflammatory and anti-inflammatory cytokines with respective ELISA kits (Bio-legend system) according to the manufacturer's instructions (Jayaraman et al., 2022).

4.2.9 Statistical analysis

All the *in vitro* experiments were performed in triplicate forms, and the data was introduced here as mean $\pm$ SD. Statistical analysis was achieved utilising two-way ANOVA followed by Tukey's multiple comparison test in Graph Pad Prism version 8.0 software.

4.3 Results

4.3.1 STK11 glycoprotein inhibited the latent H37Ra bacilli

The resazurin assay was employed to examine whether isolated glycoprotein STK11 confined the latent H37Ra. The result of this experiment indicated that STK11 at the lowest dose of 3.125 µg/ml could restrict the latent H37Ra bacilli growth by 12.11%. We also observed that 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml of STK11 killed the latent H37Ra bacilli by 18.53%, 25.42%, 30.84%, 36.51%, and 42.59%, respectively. STK11 at the highest dose of 200 µg/ml employed the killing of the latent H37Ra bacilli by 49.10% (Figure 4.1A). On the other hand, a dose-dependent study of rifampicin was performed. The result indicated that rifampicin at the lowest dose of 0.00375 µg/ml can restrict the latent H37Ra bacilli growth by 15.45%. We also observed that 0.0075 µg/ml, 0.015 µg/ml, 0.03 µg/ml, and 0.06 µg/ml of rifampicin could kill the latent H37Ra bacilli by 27.27%, 37.82% and 48.77%, 59.22% respectively. On the other hand, rifampicin at its highest dose of 0.12 µg/ml might kill the latent H37Ra bacilli by 66.71% (Figure 4.1B). Hence STK11 could be considered to restrict the latent *M. tuberculosis* bacilli like the active bacilli (Figure 3.2 and Figure 4.1).

4.3.2 STK11 prevents the latent H37Ra bacilli in J774A.1 macrophage

Earlier we checked the efficacy of STK11 (10 µg/ml) against the growth of H37Ra bacilli (Samanta et al., 2026) as no cytotoxic impact was seen in the MTT assay after treatment with STK11 below the dose of 10 µg/ml *in vitro* (Srinivasan et al., 2023). Therefore, we selected a dosage of STK11 up to 0–10 µg/ml. After 28 days of culture in triplicate, we observed that CFU levels in latent H37Ra-challenged macrophages were progressively dropped after STK11-treatment at 0–10 µg/ml significantly

($p < 0.001$). The highest level of reduction of CFU was identified at the doses of 10 $\mu\text{g/ml}$ of STK11 and 0.1 $\mu\text{g/ml}$ of rifampicin in latent H37Ra-challenged macrophages. The CFU in latent H37Ra-challenged macrophages were decreased by 3.78-fold and 3.90-fold in 10 $\mu\text{g/ml}$ STK11 and 0.1 $\mu\text{g/ml}$ rifampicin treatment, respectively. In this context, 2.5 $\mu\text{g/ml}$ of STK11 restricted 32.76%, 5 $\mu\text{g/ml}$ of STK11 restricted 57.50%, and 10 $\mu\text{g/ml}$ of STK11 restricted 73.53% of the latent H37Ra bacilli. On the other hand, 0.1 $\mu\text{g/ml}$ of rifampicin restricted the latent H37Ra bacilli by 74.35% (Figure 4.2). Hence, STK11 could restrict the CFU of latent *M. tuberculosis* bacilli within macrophages which was similar type to the effect of rifampicin.

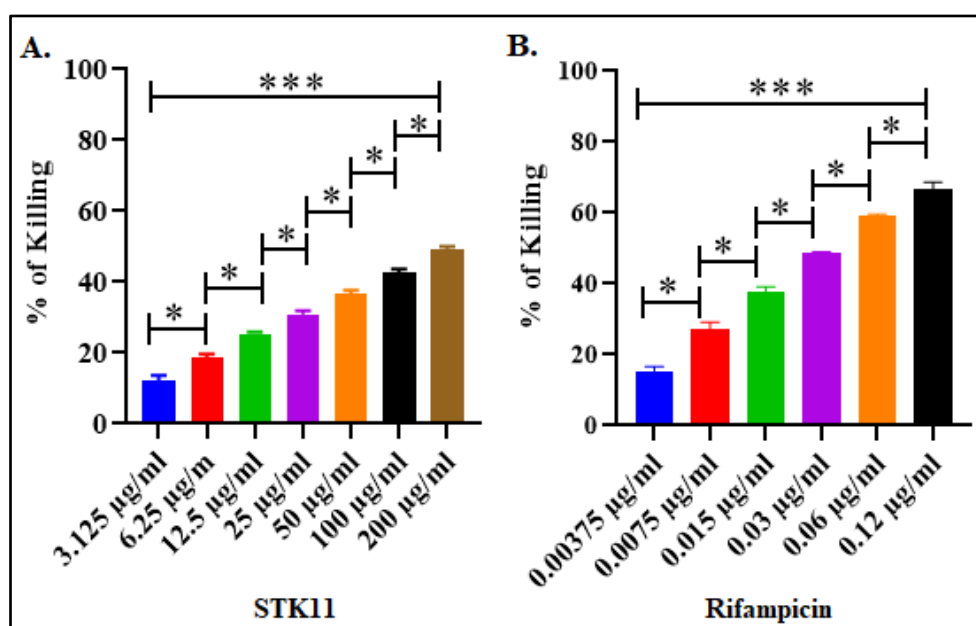


Figure 4.1 Latent H37Ra bacteria killing after treatment with STK11 (A) and rifampicin (B). Briefly, STK11 (0-200 $\mu\text{g/ml}$) and rifampicin (0-0.12 $\mu\text{g/ml}$) were used to treat the latent H37Ra bacilli (OD-0.02). Then, the culture plate was kept in a 37 °C incubator for 7 days. Then, 30 μl of resazurin (0.1 mg/ml) was added for 24 h. Then, multimode reader was used to check the fluorescence at excitation 530 nm and emission 590 nm. Mean $\pm$ SD is used to display the data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ shows the significant difference compared to the untreated control group.

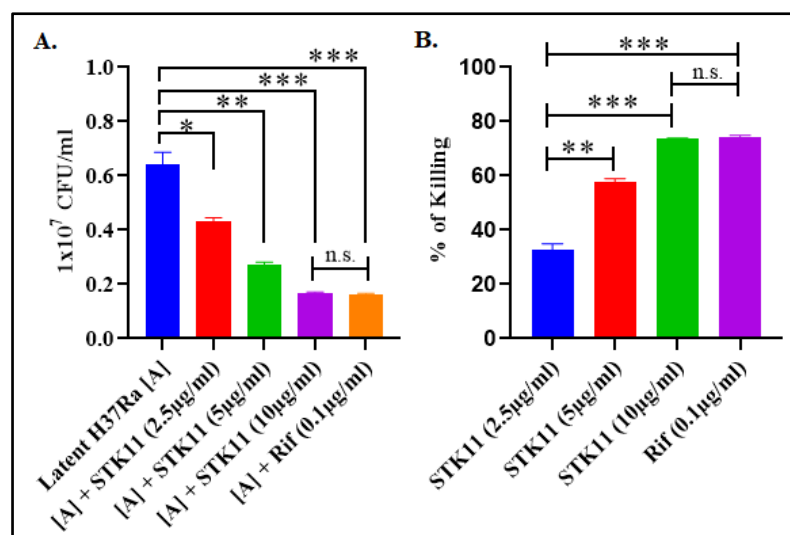


Figure 4.2 CFU levels (A) and percentage of killing (B) of the latent H37Ra in macrophages after the treatment with different doses of STK11. Macrophages (1×10^5) were challenged with latent H37Ra at a 1:5 ratio. Then, macrophages were treated with different dosage (2.5 – 10 μ g/ml) of STK11 for 48 h. Next, RIPA lysis buffer (200 μ l) was added. Then, cell lysate (50 μ l) with MB7H9 medium (450 μ l) were used and kept the plate to further culture in incubator for 28 days. After, 28 days, absorbance was measured at 600 nm to obtain the CFU. The data is displayed as mean $\pm$ SD. A significant difference from the untreated control group is shown by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s-not significant.

4.3.3 mRNA expression of iNOS2 and Arg1 and nitric oxide generation were modulated in STK11 glycoprotein treated latent H37Ra infected macrophages

Cellular markers were checked in the triplicate set during H37Ra-infected, latent H37Ra-challenged, and STK11-treated H37Ra-infected or latent H37Ra-challenged J774A.1 macrophages. The expression of M2 marker (Arg1) was upregulated during H37Ra-infected, latent H37Ra-challenged macrophages and downregulated in STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages. On the other hand, M1 marker (iNOS2) expression was upregulated during STK11-treated H37Ra-infected and latent H37Ra-challenged macrophages and downregulated during H37Ra-infected, latent H37Ra-challenged macrophages (4.3A-C). Hence, STK11 may be used to employ the macrophage activation to restrict the latent TB similar to the active TB.

Nitric oxide (NO), produced by mammalian immune cells, is vital for the immune response to infection. Naturally, macrophage activation necessitates the nitric oxide production from L-arginine through a catalytic reaction mediated by inducible nitric oxide synthase (iNOS) (Jayaraman et al., 2022). For 48 h, STK11 (0–10 $\mu\text{g/ml}$) was treated in a dose-dependent manner to regulate the production of NO in the dormant H37Ra-challenged macrophage cell. According to the results, NO production increased by 1.96-fold after the treatment with STK11 (10 $\mu\text{g/ml}$), but only 1.63-fold and 1.46-fold increased after treatment with STK11 (5 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ respectively), compared to latent H37Ra infection (Figure 4.3D).

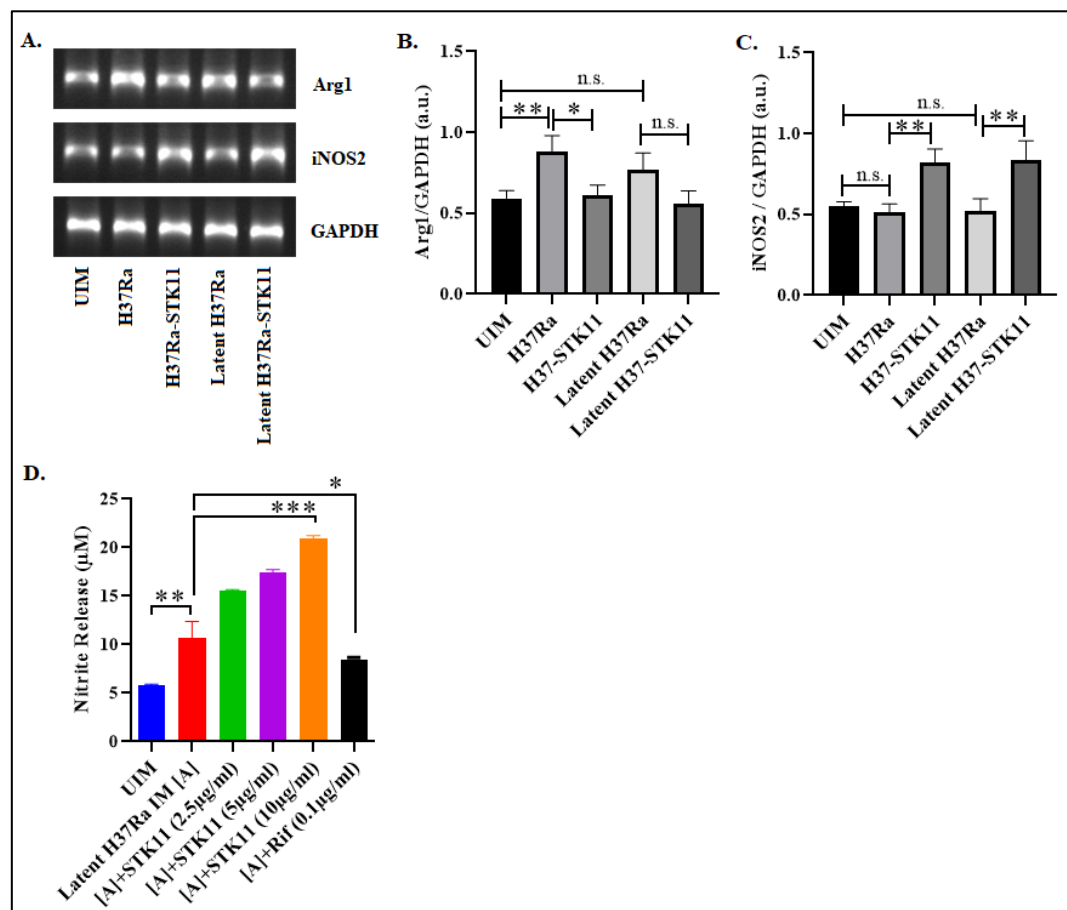


Figure 4.3 mRNA expression of arg-1, iNOS2 and nitrite release during H37Ra-infected, latent H37Ra-challenged, and STK11-treated J774A.1 macrophages. (A, B, C) Using MOI of 1:5 (Macrophage: *M. tuberculosis*), J774A.1 macrophage (1×10^6) cells were infected and challenged with *M. tuberculosis* bacilli (H37Ra and latent-H37Ra) (OD = 0.646 and 0.218,

respectively) for 4 h. 10 µg/ml of STK11 glycoprotein was added to the H37Ra-infected, the latent H37Ra-challenged macrophages for 6 h. The expression of the cellular markers (Arg1, iNOS2) gene was presented from one of the three sets of experiments. (D) After treatment with STK11 (0–10 µg/ml) and rifampicin (0.1 µg/ml) for 48 h, nitrite production was measured using Griess reagent from the cell supernatant. Data is shown as mean ± SD. Three sets of the experiment produced similar type of findings and presented here one of them. Significant differences from the untreated control group and infected group are shown by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant, respectively.

4.3.4 The expressions of M1 and M2 marker during latent H37Ra-challenged and STK11 treated latent H37Ra-challenged macrophages

M1 and M2 surface marker expression was examined in H37Ra-infected, latent H37Ra-challenged, and STK11-treated macrophages. We observed that M2 markers (YM-1, FIZZ1, CD206) were upregulated during H37Ra-infected and latent H37Ra-challenged macrophages but downregulated during STK11-treated H37Ra-infected and latent H37Ra-challenged macrophages (Figure 4.4A, B-D). On the other hand, M1 markers (CD68, CD40) were downregulated during H37Ra-infected and latent H37Ra-challenged macrophages but upregulated during STK11-treated H37Ra-infected and latent H37Ra-challenged macrophages (4.4A, E-F). Hence, it may be claimed that *C. nucifera* L. derived glycoprotein, STK11, modulated the M1 from M2 polarization.

4.3.5 Cytokines expression and release during latent H37Ra-challenged and STK11 treated macrophages

The expression of cytokine was modulated by STK11 at the mRNA level through various signaling pathways (Samanta et al., 2026). The mRNA expression and releases of pro- and anti-inflammatory cytokines were investigated during H37Ra-infected, latent H37Ra-challenged, and STK11-treated H37Ra-infected, latent H37Ra-

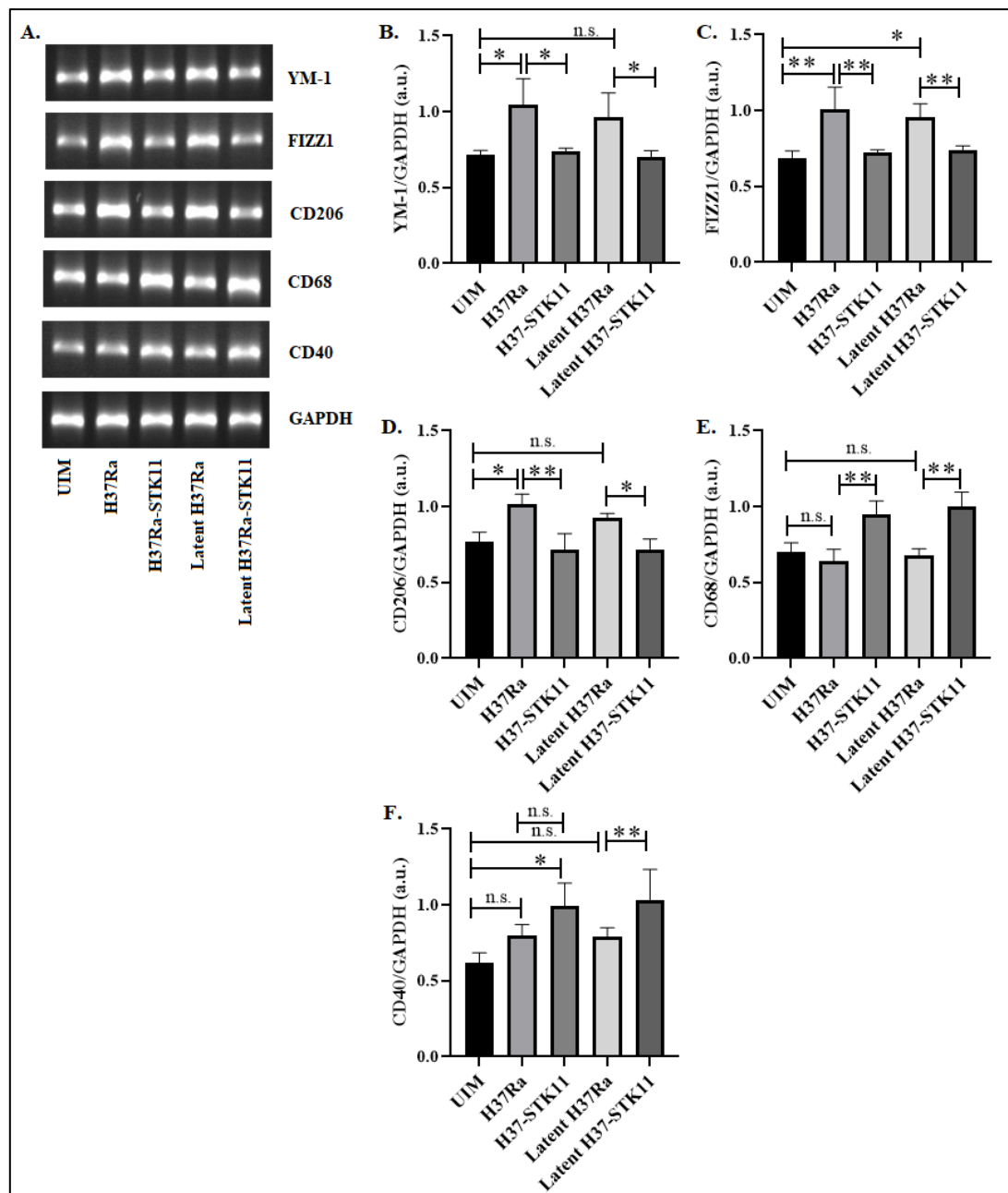


Figure 4.4 The expressions of M1 and M2 markers during STK11 treatment in latent H37Ra-challenged macrophages. (A-F) Macrophage (1×10^6) cells were infected and challenged with *M. tuberculosis* (H37Ra and latent H37Ra) (OD=0.646 and 0.218, respectively) at MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. Later, 10 μ g/ml of STK11 glycoprotein was added for 6 h. Using certain primers, the expression of the genes YM-1, FIZZ1, CD206, CD68, and CD40 was examined in triplicate. The band intensity were measured by ImageJ from the three sets of experiments and the data were shown as mean $\pm$ SD. A significant difference from the untreated control group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

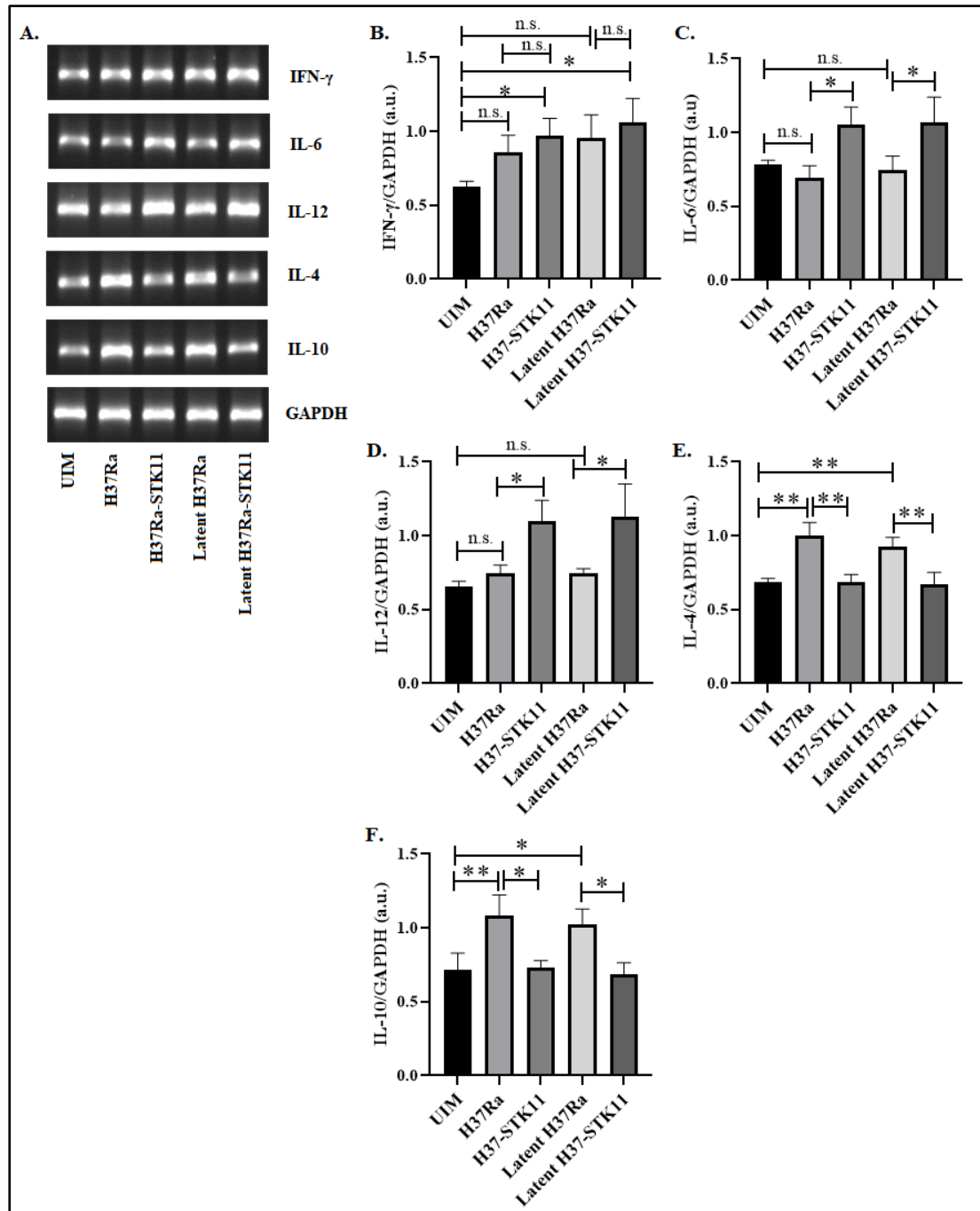


Figure 4.5 The mRNA expression of pro and anti-inflammatory cytokines during H37Ra-infected, latent H37Ra-challenged, and STK11-treated macrophages. (A-F) Macrophage cells (1×10^6) were infected and challenged with *M. tuberculosis* bacilli (H37Ra and latent H37Ra) (OD=0.646 and 0.218, respectively) with multiplicity of infection (1:5= Macrophage: *M. tuberculosis*) for 4 h. The latent H37Ra-challenged macrophages were exposed to 10 μ g/ml STK11 glycoprotein for 6 h. Using certain primers, the expression of IFN- γ , IL-6, IL-12, IL-4, and IL-10 cytokines was examined in triplicate. The band intensity data is displayed as mean $\pm$ SD from three sets of experiments. A significant difference from the untreated control group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

challenged macrophages. We observed that proinflammatory cytokines (IFN- γ , IL-6, IL-12) were downregulated during H37Ra-infected, latent H37Ra-challenged macrophages, and upregulated during STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages in mRNA and protein level (Fig: 4.5A, B-D; 4.6A-C). On the other hand, anti-inflammatory cytokines (IL-4, IL-10) were upregulated during H37Ra-infected, latent H37Ra-challenged macrophages, and downregulated during STK11-treated macrophages in mRNA and protein level (Fig 4.5A, E-F; 4.6D-E).

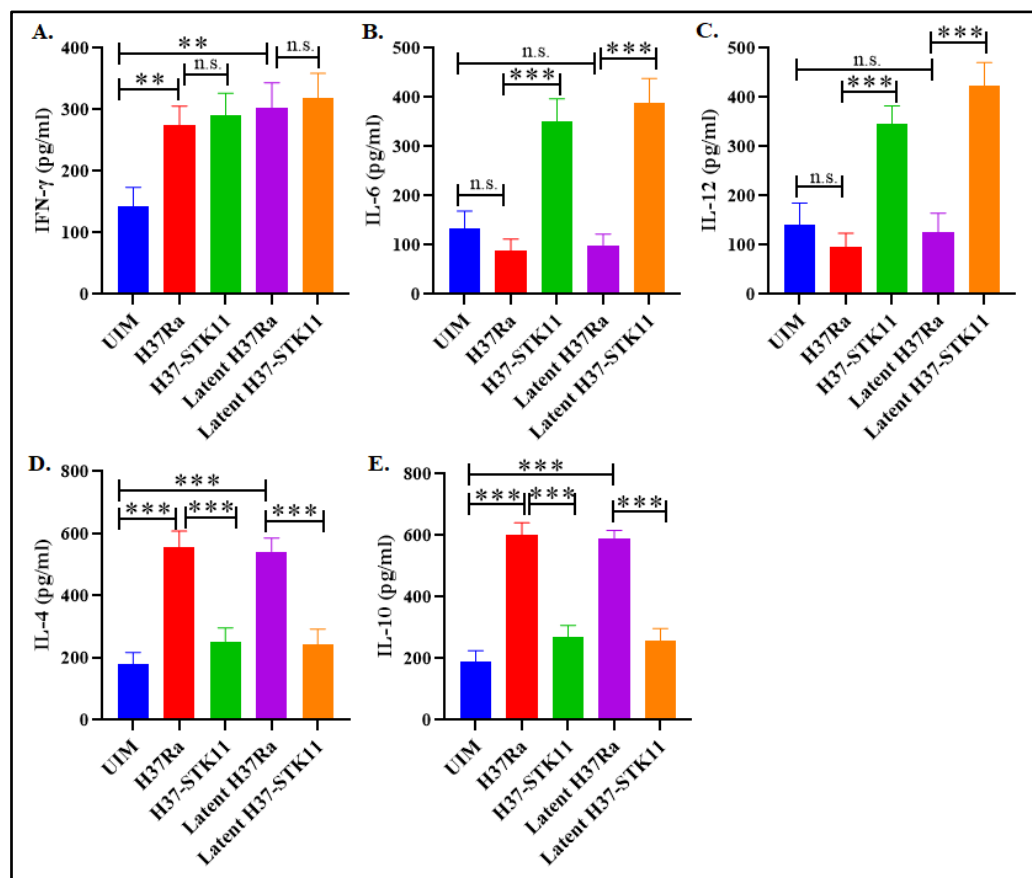


Figure 4.6 The release of pro and anti-inflammatory cytokine was examined during H37Ra-infected, latent H37Ra-challenged, and STK11-treated macrophages at the protein level. (A-E) Macrophage cells (1×10^6) were infected and challenged with *M. tuberculosis* bacilli (H37Ra and latent H37Ra) (OD=0.655 and 0.218, respectively) with MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. After that the cells were treated with STK11 (10 μ g/ml) glycoprotein on the H37Ra-infected plate and the uninfected plate. The level of pro-inflammatory (IL-6, IL-12, IFN- γ) and anti-inflammatory cytokines (IL-4, and IL-10) were measured after 24-h of STK11 treatment. Data is displayed as mean $\pm$ SD. Significant differences from the untreated control group are shown by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

4.3.6 STK11 interacted with TLRs *in silico* and modulated mRNA expression of TLRs during H37Ra-infected, latent H37Ra-challenged J774A.1 macrophages

Toll-like receptors (TLRs) play a significant role in recognizing *M. tuberculosis* by detecting its components, like lipoproteins and DNA, triggering the innate immune response (Kleinnijenhuis et al., 2011). They activate signaling pathways that lead to cytokine production and enhance phagosome maturation, aiding in bacterial killing (Lee et al., 2020). Additionally, TLRs help shape the adaptive immune response by promoting Th1 cell activation, which is essential for controlling TB infection (MacLeod & Wetzler., 2007). In this context, an *in-silico* study conducted that revealed TLRs were docking with the STK11 glycoprotein. Interestingly, among all TLRs, the lowest binding energy was observed when TLR-9 binds with STK11 (-1109.7 kcal/mol). However, the binding energy for TLR-2 (-1057.6 kcal/mol), TLR-4 (-958.9 kcal/mol), and TLR-6 (-885.9) was found (Figure 4.7 A-D).

On the other hand, mRNA expression of TLRs during H37Ra-infected, latent H37Ra-challenged macrophages, and STK11-treated macrophages was studied in triplicate. TLR-2 expression was increased during H37Ra-infected and latent H37Ra-challenged macrophages but significantly decreased with STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages (Figure 4.7E, F). TLR-6 expression was increased during only STK11-treated macrophages, but significantly decreased during H37Ra-infected, latent H37Ra-challenged, and STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages (Figure 4.7E, H). On the other hand, TLR-4 and TLR-9 expression decreased during H37Ra-infected, latent H37Ra-challenged macrophages, but significantly increased during STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages (Figure 4.7E, G, I). Hence, STK11 could interacted with selected TLRs and modulated the expressions in both latent and active *M. tuberculosis*

infection in macrophages, which might be involved in development anti-tubercular immune responses.

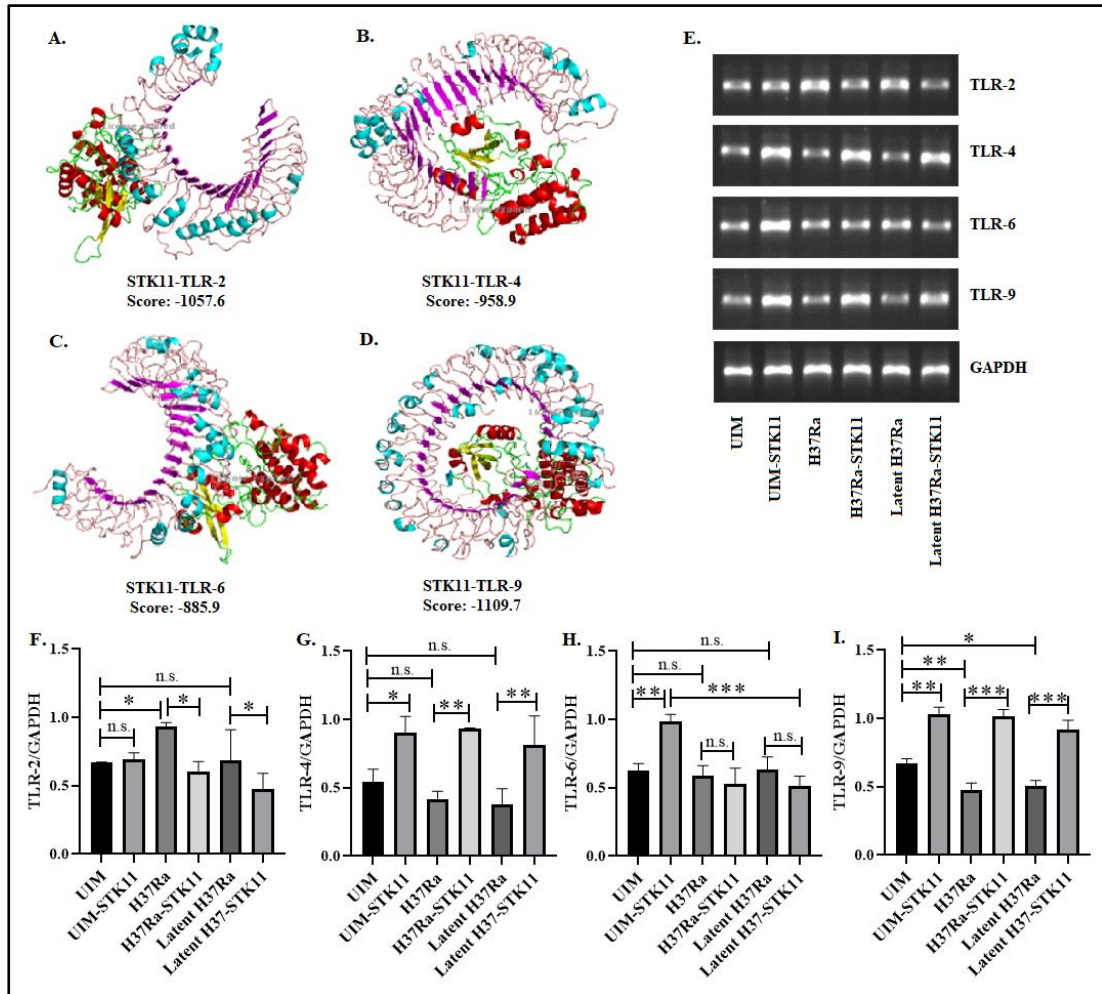


Figure 4.7 STK11-TLRs protein-protein docking and mRNA expression in H37Ra-infected and latent H37Ra-challenged macrophages. (A, B, C, D) The docking result was gained in Clasp software between STK11 with TLR-2, TLR-4, TLR-6, and TLR-9. (E-I) The expression of mRNA of these TLRs was checked during H37Ra-infected, latent H37Ra-challenged macrophages, and STK11-treated macrophages. Macrophage cell (1×10^6) was infected and challenged with *M. tuberculosis* bacilli (H37Ra and latent-H37Ra) (OD=0.646 and 0.218, respectively) with MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. After 4 h, non-ingested *M. tuberculosis* bacilli were discarded. After STK11 (10 μ g/ml) glycoprotein was added to the H37Ra-infected, the latent H37Ra-challenged plate, and the uninfected plate for 6 h. Then, gene expressions of TLR-2, TLR-6, TLR-4, and TLR-9 were studied by the specific primers in triplicate. A significant difference from the untreated control group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

4.4 Discussion

The lungs are primarily affected by TB (WHO, 2025). An earlier study indicated that 23% of the world's population was assessed to be latently infected with *M. tuberculosis*. As 5-15% of LTBI cases might progress to active TB cases, we established the latent state of tuberculosis according to the model of Wayne & Hayes described in 1996 (Wayne & Hayes, 1996). Typically, the latent state was established after 12 days (complete decolorization of methylene blue). This latent state of bacteria was then used for experiments, such as infection and treatment with the immunomodulator as per the previously reported method (Iona et al., 2012). Many reports claimed that *C. nucifera* L. and its ingredients have an immunomodulatory impact (Narayanankutty et al., 2021 & 2022). Reports also indicated that numerous immunomodulators, including rocaglates, a naturally occurring product derivative of plants in the *Aglaia* genus, polarized macrophages from M2 to M1 (Langlais et al., 2018; Chatterjee et al., 2021); BPMS22, produced from *Oceanimonas* sp., towards M1 from M2 (Jayaraman et al., 2022); Oligo-Fucoidan, also known as a sulfated polysaccharide produced from brown seaweed, polarized towards M1 from M2 (Cheng et al., 2020; Chen et al., 2020); Ibrutinib, known as a BTK inhibitor; and Selinexor, also known as a BBB permeable small molecule, polarized from M2 to M1 (Jiménez et al., 2020). STK11 glycoprotein was also reported to polarize from M2 to M1 state in H37Ra-infected macrophages (Samanta et al., 2026). Hence, the role of STK11 against latent H37Ra bacilli growth as host-directed therapy, was studied to target the macrophage polarization. The previous study claimed that STK11 (200 µg/ml) glycoprotein can restrict the H37Ra bacilli growth by 44.98% in the resazurin assay (Samanta et al., 2026). In this context, the latent H37Ra bacilli were also treated with 0-200 µg/ml of STK11. Then, after 7 days, it was added with resazurin for 24 h. The results claimed that STK11 and

rifampicin significantly upregulates the rate of killing percentage of latent H37Ra bacilli with the increasing dose of STK11 and rifampicin (Figure 4.1). Hence, STK11 successfully restricted the latent H37Ra bacilli by 49.10%. After that the latent H37Ra bacilli-challenged macrophages were treated with the STK11 (0-10 µg/ml). Then, after 48 h, treated macrophages were processed for the study of colony-forming units after 28 days. The result was illustrated that STK11 significantly ($p < 0.05$) reduced the colony-forming units of latent H37Ra bacilli (Fig. 4.2). Hence, STK11 acts as a primary drug treatment against latent H37Ra-challenged macrophages, like other first-line drugs, i.e., rifampicin, isoniazid, ethambutol, and pyrazinamide (Prasad et al., 2021). After being activated by LPS and Th1 cytokines, such as IFN- γ , macrophages polarize into M1 macrophages. IL-4 or IL-13 activates the M2 macrophages (Samanta et al., 2024). Numerous reports indicated that macrophage polarization drives into M1 polarization, where the iNOS2 marker with proinflammatory activity is involved in the primary stage of tuberculosis infection (Samanta et al., 2024; Redente et al., 2010; Marino et al., 2015). In the mid and late stages of tuberculosis, macrophage polarization drives into the M2 phenotype, and Arg1 marker with anti-inflammatory activity is involved (Samanta et al., 2024; Ly et al., 2007). The RT-PCR result claimed that the Arg1 marker expression was upregulated and iNOS2 expression was downregulated during H37Ra-infected and latent H37Ra-challenged macrophages, and after STK11-treatment, Arg1 was downregulated and iNOS2 expression was upregulated (Figure 4.3 A-C). NO generation was successfully upregulated during STK11 treatment at the dose of 0-10 µg/ml of STK11 (Figure 4.3D). So, STK11 treatment successfully modifies the host macrophages from M2 to M1 polarization.

The M1 phenotype increases the ability to destroy pathogens and possesses pro-inflammatory properties. On the other hand, the M2 phenotype is tumor-causing, can

remodel tissue, has anti-inflammatory properties, and is classified as an inflammation pro-resolver (Martinez et al., 2014). Hence, in this study, it was claimed that M2 marker expression was upregulated and M1 marker was downregulated during H37Ra-infected and latent H37Ra-challenged macrophages, but STK11-treated macrophages significantly downregulated the M2 markers and upregulated the M1 markers. Hence, latent H37Ra-challenged and H37Ra-infected macrophages induce M2 polarization in macrophages, and STK11-treated macrophages induce M1 polarization within the macrophages and restrict the growth of H37Ra and latent H37Ra bacilli (Figure 4.4 A-F).

M1 effector functions were increased with the NO generation and pro-inflammatory cytokines releases (Jablonski et al., 2015). In this study, anti-inflammatory cytokines (IL-10, IL-4) expression and release were increased, and pro-inflammatory cytokines (IL-12, IL-6) expression and release were decreased during H37Ra-infected and latent H37Ra-challenged macrophages, but STK11 treatment could reverse the expression of the pro-inflammatory and anti-inflammatory markers (Figure 4.5 A-F & Figure 4.6 A-E). So, it also claimed that STK11 is a significant immunomodulator, which is responsible towards M1 from M2 polarization in STK11-treated H37Ra-infected, latent H37Ra-challenged macrophages.

Besides, STK11 was a potent glycoprotein that could interact with the TLRs in an *in-silico* study. We established that STK11 was the best molecule to interact with TLRs by the docking method (Figure 4.7A-D). STK11 exhibits well interaction with TLR-9 (docking score: 1109.7) (Figure 4.7D). TLR-4 and TLR-9 expression were upregulated during STK11-treated macrophages in mRNA expression level, which is very similar to the *in-silico* study, with the lowest docking score. TLR-2 and TLR-6 expression were downregulated during STK11-treated macrophages (Figure 4.7 E-I).

4.5 Summary & Conclusion

LTBI, a serious type of tuberculosis, also needs to be treated by a doctor. But the major problem of LTBI is that it is not symptomatic. But 5-15% LTBI may progress to active tuberculosis. Therefore, we used Wayne's hypoxia model to investigate the effect of *C. nucifera* L. derived STK11 against latent H37Ra *Mycobacteria* through host-directed therapy, focusing on macrophage polarization. We first prepared the latent state of H37Ra as per Wayne's hypoxic model. We then experimented with the latent state of tuberculosis by treating it with STK11. The result of RT-PCR claimed that Arg1, YM-1, FIZZ1, CD206, IL-4, IL-10 (M2 marker) were upregulated and iNOS2, CD40, CD68, IL-6, IL-12 (M1 markers) were downregulated during H37Ra-infected, latent H37Ra-challenged macrophage. On the other hand, M2 markers were significantly downregulated and M1 markers were upregulated during STK11-treated H37Ra-infected and latent H37Ra-challenged macrophages. Hence, the M2 to M1 polarization might play a crucial role to restrict *M. tuberculosis* (H37Ra, latent H37Ra) bacilli growth within the macrophages. STK11 could kill the latent H37Ra bacilli significantly in the resazurin assay. STK11 also restricted the latent H37Ra bacilli within the macrophages in the macrophage-mediated killing assay. Besides, STK11 interacted with the TLRs (TLR-2, TLR-4, TLR-6, TLR-9) in an *in silico* study, and mRNA expression of TLRs during H37Ra-infected and latent H37Ra-challenged and STK11-treated macrophages supported this claim. So, latent H37Ra-challenged macrophages were also restricted by the potent immunomodulator STK11 in a nitrite-dependent manner. Hence, *C. nucifera* L. derived STK11 could be considered as a TLR modulating potent immunomodulator to restrict the *Mycobacterium* load both active and latent within infected macrophages through M2 to M1 repolarization (Figure 4.8).

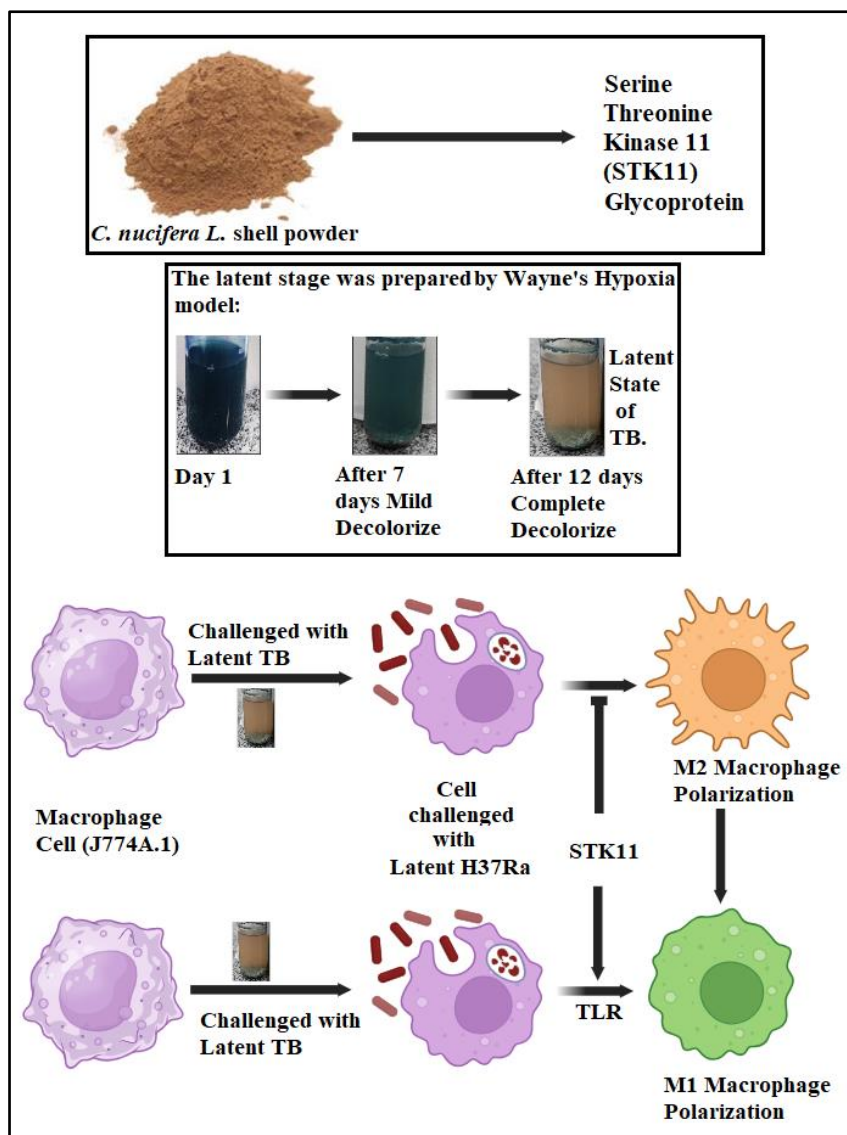


Figure 4.8 The pictorial summary of STK11 restricted latent TB within macrophages. The STK11 glycoprotein obtained from the nutshell powder of *C. nucifera L.* On the other hand, H37Ra-infected and latent H37Ra-challenged macrophage cells induce M2 polarization, but when STK11 is involved, it induces M1 polarization through the interaction with TLR. STK11 can polarize cells from M2 to M1.

Chapter 5: Combinatorial effect of STK11 with rifampicin against *Mycobacterium tuberculosis* infection in J774A.1 macrophage

5.1 Introduction

The typical short-course treatment for tuberculosis patients who have never been treated involves a 4-drug combination of isoniazid, rifampicin, pyrazinamide, and ethambutol for the 2-months of the intensive phase and followed by 2-drug regimens of isoniazid & rifampicin for the next 4-month extensive phase (Van Ingen et al., 2011). However, TB disease, along with MDR-TB, XDR-TB, and RR-TB, presented a serious global public health threat (Liu et al., 2019). Because of less effective and more toxic drugs, TB patients with MDR-TB, RR-TB, or XDR-TB require longer therapy than those with drug-susceptible TB, and the worldwide cure rate drops to 60% (Cohen et al., 2019). Drug-resistant TB currently only be treated with a limited set of drugs, requiring second-line of drugs possessing expensive, less effective, more toxic, and necessitate longer treatment durations (Millard et al., 2015).

In this context, alternative treatments are urgently needed to combat TB patients along with MDR-TB, XDR-TB, or RR-TB and to reduce the duration of existing tuberculosis therapy, even though research on several new drugs is still ongoing (Koul et al., 2011, Motta et al., 2024). According to a study of 68 authorized drugs, the usual time to available a drug to market was 14 years and \$800 million (US dollars) (DiMasi et al., 2003). In this situation, discovering new solutions for current drugs, either alone or in combination therapies, may be a cost-effective option (Boguski et al., 2009; Chong & Sullivan, 2007).

Therefore, host-directed therapies (HDTs) offer an underexplored opportunity to address the issues of treatment failure in complex TB disease, including MDR-

TB/XDR-TB/RR-TB, during the search for new drugs (Rao et al., 2019; Young et al., 2020). Instead of focusing on the pathogens, HDT aims to treat the disease by partially regulating the host immune system (Hancock et al., 2012). HDT repurposes existing immune-modulation and has become a compelling adjuvant therapeutic option. By modifying host factors, HDTs aim to augment favourable responses and reduce unfavourable responses for the host, which may either lead to tissue damage or continuation of *Mycobacterial* growth (Kaufmann et al., 2018). As a therapeutic strategy, HDT has great promise for overcoming drug resistance, restoring patients' immunological responses, and addressing the drawbacks of current TB treatments (Jeong et al., 2022).

In this context, STK11 glycoprotein, as shown promising effect through HDT on macrophage activation against both active and latent stage of *M. tuberculosis*, we intended to study its combinatorial efficacy with rifampicin against infected macrophages. We have chosen rifampicin as it is a first-line drug available on the market (Maug et al., 2020). Beside the CFU within host cell, herein we have studied the macrophage polarization status of *M. tuberculosis* infection.

5.2 Materials & Methods

5.2.1 Chemicals and reagents

All chemicals were obtained from SRL, Himedia, and Sigma Aldrich. EDTA, Griess reagent, and rifampicin were purchased from SRL. MB7H9 medium, Dialysis membrane, Middlebrook ADC, Tris buffer, Tween 80, Agarose, Triton-X, FBS, DMEM, Penicillin, Neomycin, Streptomycin, RNA Express reagents were purchased from Himedia. A 10 kDa cut-off Amicon centrifugal filter was purchased from Merck-

Millipore. A cDNA synthesis kit was procured from Bio-Rad. The RT-PCR kit was purchased from TAKARA BIO.

5.2.2 Culture of *M. tuberculosis* (H37Ra) bacilli strain

The H37Ra strain was cultured in the MB7H9 medium with glycerol (0.5%), tween 80 (0.2%), and albumin-dextrose complex enrichment (10%) in a shaker incubator (37°C for 21 days). The mid-log growth phase bacteria were extracted by centrifugation at 2,500×g for 15 minutes. Following two centrifugal washes, the bacteria were washed and diluted in PBS saline at the appropriate concentrations (Samanta et al., 2026).

5.2.3 Extraction and purification of STK11 glycoprotein from *C. nucifera* L. nutshells

The previously described procedure was followed to isolate STK11 (Srinivasan et al., 2023; Samanta et al., 2026). The coconut shells were cleaned and washed with fresh water. The shells were broken into small pieces and ground into a powder after being sun-dried. In 100 ml of Tris EDTA buffer (pH 8.2) containing 0.1% Triton-X, 10 grams of powdered coconut shell were dissolved. The samples were centrifuged for 10 minutes at 3000 rpm following a 24-h stirring period. The supernatant was dialyzed in TE buffer for 48 h, changing the buffer every 6 h. To extract STK11, the dialyzed material was centrifuged using an Amicon centrifugal filter (above 10 kDa) (Srinivasan et al., 2023; Samanta et al., 2026).

5.2.4 Examine the combined effects of STK11 and rifampicin on H37Ra growth

The microtiter resazurin test was used to assess the percentage of killing of H37Ra bacilli after STK11 and rifampicin combinatorial treatment using the direct killing

assay method. The 96-well plate was seeded with 100 µl of H37Ra bacteria (OD-0.02). Rifampicin (0.12 µg/ml) and STK11 (0 to 200 µg/ml) were added together in triplicate. The remaining volume was made up to 200 µl using full MB7H9 media with 10X ADC and 20% Tween 80. To keep the media in the plate from becoming dehydrated, 200 µl of distilled water was also supplied to the wells around the plate's edge. The plates were wrapped in aluminium foil and parafilm and then incubated at 37 °C for seven days. The plates were incubated for an additional 24 h after adding 30 µl of resazurin (0.1 mg/ml) after 7 days. Next, using a BioTek Synergy H1 microplate reader, fluorescence intensity was measured at excitation: 530 nm and emission: 590 nm (Jyoti et al., 2015; Samanta et al., 2026).

5.2.5 Screening of intracellular inhibitory potential of STK11 with rifampicin against the growth of *M. tuberculosis* inside the macrophages

The combinatorial efficacy of STK11 and rifampicin was examined on the growth of *M. tuberculosis* in J774A.1 macrophage *in vitro* and CFU was determined. In 24-well plates, J774A.1 cells (1×10^5) were cultured for overnight. After that H37Ra bacilli were used for the infection to macrophages as described in chapter 3. H37Ra infected macrophages were treated with STK11 (2.5 µg/ml) and rifampicin (0.1 µg/ml) either alone or combination for 48 h. After that, the supernatant was removed from the plate and washed with PBS. The cells were lysed with RIPA lysis buffer (without protease inhibitor). Subsequently, 50 µl of cell lysate was taken from the entire lysate sample and carefully transferred to a fresh, cleaned 24-well plate and 450 µl of complete MB7H9 media with 10x ADC and 20% Tween 80 was added to that cell well plate. To prevent dehydration, the plate was carefully covered in aluminium foil and parafilm. Then the plate was put in a BOD incubator to cultivate the culture for 28 days and

bacterial growth was monitored using the BioTek Synergy H1 microplate reader by reading the absorbance at 600 nm. GraphPad Prism 8.0 was used to calculate and analyze CFU based on the absorbance value (Elizabeth et al., 2016; Samanta et al., 2026).

5.2.6 Nitrite production after the combination treatment in J774A.1 macrophages

In 24-well plates, cells (1×10^6) were seeded and incubated overnight. Macrophages were infected with H37Ra at a MOI of 1:5 (Macrophage: H37Ra). The cells were carefully put in the CO2 incubator for 48 h after being treated with STK11 (2.5 µg/ml) and rifampicin (0.1 µg/ml) alone or in combination. After 48-h, 100 µl of cell culture supernatants were collected from the plate, and NO production was assessed using the Griess reagent according with the proper protocol. The BioTek Synergy H1 microplate reader was used to measure the absorbance at 548 nm (Jayaraman et al., 2022; Samanta et al., 2026).

5.2.7 Study of mRNA expression of M1 and M2 markers after the combined treatment of STK11 with rifampicin

Macrophages were seeded and infected with H37Ra followed by treated with STK11 and rifampicin alone or in combination for 6 hrs. After that, total mRNA of the experimental group was extracted *in vitro*. 1 µg of total RNA used as a template for the cDNA synthesis. mRNA expressions of iNOS2, Arg-1, YM-1, CD206, FIZZ1, IFN-γ, CD68, IL-6, IL-12, CD40, IL-10, and IL-4 were evaluated by conventional RT-PCR using the particular forward and reverse primers. The forward and reverse primer sequences are shown in table 2.1. Amplified PCR gene products were run on 1.2% agarose gel electrophoresis, examined under UV light, and assessed using the Bio-Rad

Gel documentation system. The band intensity was measured using Image J software and standardized with the GAPDH to achieve the relative expression level (Charan Raja et al., 2021; Samanta et al., 2026).

5.2.8 Cytokine release assay by sandwich ELISA after the combination treatment of STK11 with rifampicin

The changes in cytokine release were estimated from the cell-free supernatant of H37Ra-infected followed by STK11 with rifampicin in combination-treated macrophages. Sandwich ELISA was performed for pro-inflammatory and anti-inflammatory cytokines (IL-6, IL-12, IFN- γ , IL-4, and IL-10) with respective cytokines ELISA kits (Bio-legend system) according to the manufacturer's instructions (Jayaraman et al., 2022).

5.2.9 Statistical analysis

All the experiment was carried out in triplicate, and the results were shown as mean $\pm$ SD. Using GraphPad Prism version 8.0 software, two-way ANOVA and Tukey's multiple comparison test were used for statistical analysis, which aids in determining the results at a significant level.

5.3 Results

5.3.1 STK11 with rifampicin in combination inhibit the H37Ra bacilli growth

The experimental results indicated that STK11 (200 µg/ml) could inhibit the growth of H37Ra bacilli by 42.78%, and rifampicin (0.12 µg/ml) could inhibit the growth of H37Ra bacilli by 50.05%. On the other hand, rifampicin (0.12 µg/ml) with STK11 (6.25 µg/ml) restricted the growth of H37Ra bacilli by 51.08%. The restriction of the bacilli growth was increased with the increasing doses of STK11 with rifampicin (0.12 µg/ml). Interestingly, 63.19% maximum growth inhibition was observed when treated with combination of rifampicin (0.12 µg/ml) with STK11 (200 µg/ml) (Figure 5.1).

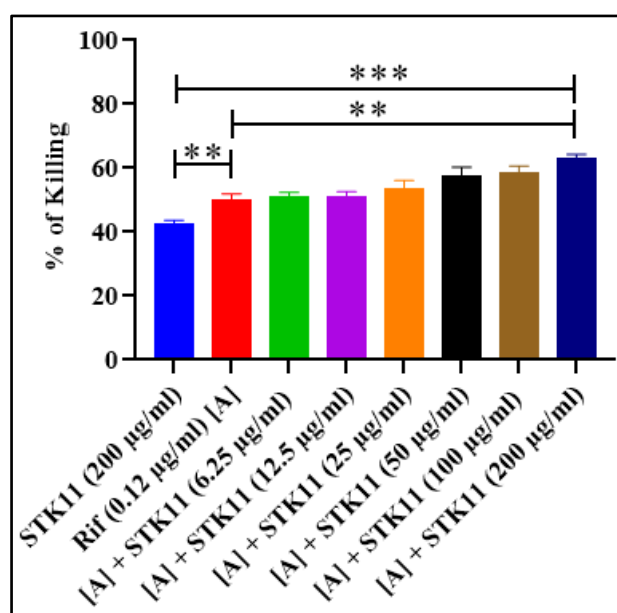


Figure 5.1 Determination of the percentage of killing of H37Ra bacilli after the combination treatment of rifampicin with STK11. Rifampicin (0.12 µg/ml) and STK11 (0-200 µg/ml) glycoprotein were used to treat H37Ra bacteria (OD-0.02). Later, the seeded plate was kept for 7 days at 37°C in a BOD incubator. Resazurin (0.1 mg/ml, 30 µl) was then added and allowed for a 24 h. A BioTek Synergy multimode reader was then used to measure fluorescence at excitation 530 nm and emission 590 nm. The data is presented as mean ± SD. A significant difference from the untreated control group is shown by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

5.3.2 STK11 with rifampicin restricted the H37Ra bacilli growth in J774A.1 macrophage

The combined effect of STK11 with rifampicin against tuberculosis-infected macrophages has not been studied, especially those caused by *M. tuberculosis*. The results of the earlier chapters indicated that 10 µg/ml of STK11 showed maximum effect (62.86% of killing). Therefore, we had selected a suboptimal dose of STK11 for the combination therapy and tested its efficacy in presence with 0.1 µg/ml of rifampicin, as it exerted maximum effect (56.41% of killing). The important purpose of this study was to establish whether STK11 could enhance the efficacy of rifampicin or not. We observed that CFU levels were decreased significantly ($p < 0.001$) during treatment with STK11 (2.5 µg/ml) and rifampicin (0.1 µg/ml) compared to H37Ra-infected macrophages, as well as relative to individual treatments with STK11 or rifampicin. After 28 days, the data showed a 3.59-fold, 2.06-fold, and 1.32-fold reduction in CFU after the combined doses of 2.5 µg/ml of STK11 with 0.1 µg/ml of rifampicin, 0.1 µg/ml of rifampicin alone, and 2.5 µg/ml of STK11 alone, respectively, compared to H37Ra-infected macrophages (Figure 5.2A). In this context, 2.5 µg/ml of STK11 inhibited H37Ra bacilli growth by 24.71%, 0.1 µg/ml of rifampicin by 51.49%, and their combination by 72.20% (Figure 5.2B). Hence, 2.5 µg/ml of STK11 could enhance responsiveness of rifampicin from 51.49% to 72.20%. Therefore, combined treatment with STK11 and rifampicin might be significantly more effective than individual treatments in limiting the growth of H37Ra within host cell macrophages.

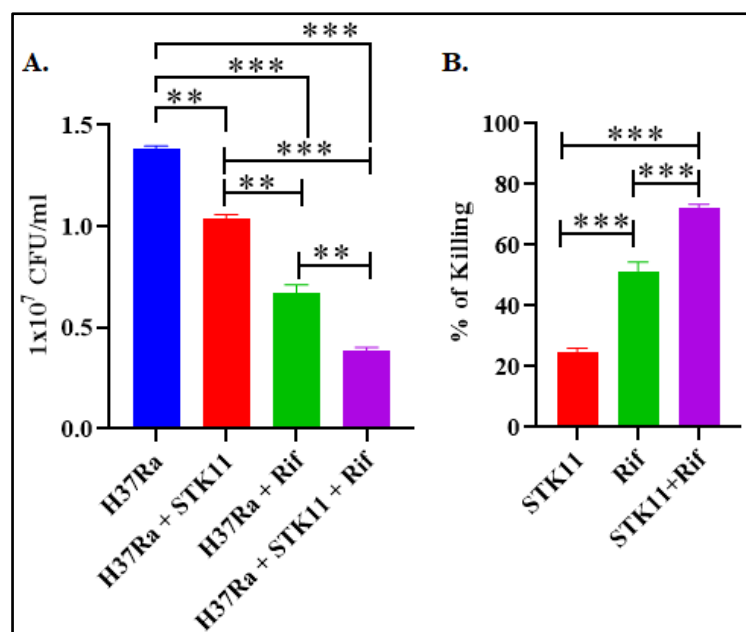


Figure 5.2 Combinatorial effect of STK11 and rifampicin on CFU level and percentage of killing in *M. tuberculosis*-infected macrophages. Macrophages (1×10^5) were infected with H37Ra as described in previous chapters. Then, macrophages were treated with STK11 (2.5 $\mu\text{g/ml}$) and rifampicin (0.1 $\mu\text{g/ml}$) in combination or alone for 48 h. Then, 200 μl RIPA lysis buffer was added. Then, 50 μl of cell lysate with 450 μl of complete MB7H9 media were incubated for CFU count for 28 days, and absorbance was checked at 600nm. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference compared with the untreated control group.

5.3.3 STK11 with rifampicin in combination modulated Arg1 and iNOS2 expression and nitric oxide generation in H37Ra infected J774A.1 cell

Cellular markers were checked during the combined treatment of H37Ra-infected macrophages. M2 marker, Arg1, expression was increased during H37Ra-infected J774A.1 macrophages but effectively decreased during the combined treatment of STK11 with rifampicin, compared with individual drugs-treated macrophages. On the other hand, the M1 marker, iNOS2 expression, was effectively increased during the combined treatment of STK11 with rifampicin, compared to individually treated macrophages (Figure 5.3 A-C). Nitric oxide (NO) produced by mammalian immune cells is essential for the immune response to intracellular infection. Naturally,

macrophage activation required the production of nitric oxide from L-arginine through a catalytic reaction mediated by inducible nitric oxide synthase (iNOS) (Jayaraman et al., 2022). Results indicated that 6.82-fold, 3.46-fold, and 2.78-fold NO generation were increased after the treatment with STK11 (2.5 $\mu\text{g/ml}$) with rifampicin (0.1 $\mu\text{g/ml}$) in combination, 2.5 $\mu\text{g/ml}$ of STK11 and 0.1 $\mu\text{g/ml}$ of rifampicin, respectively (Figure 5.3D).

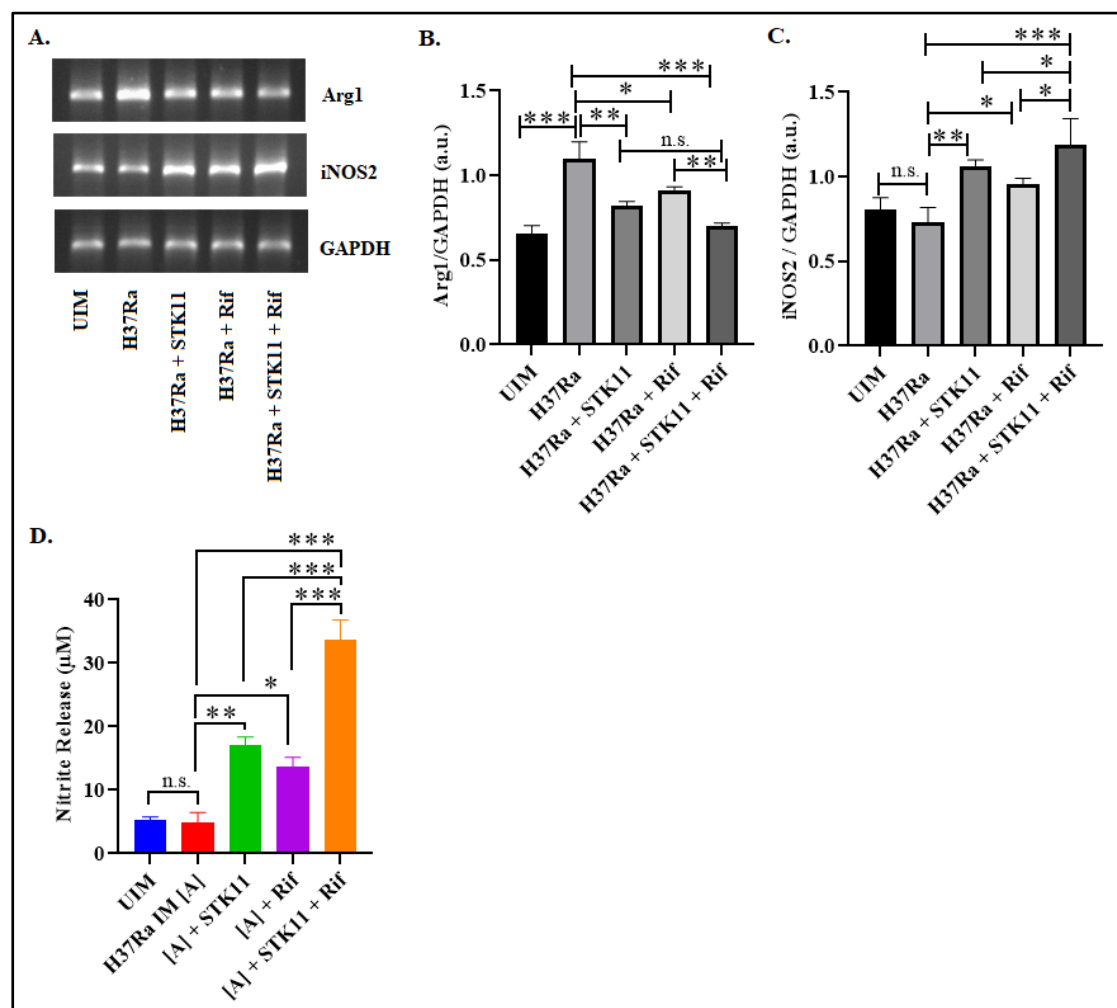


Figure 5.3 mRNA expression of Arg-1, iNOS2 and nitrite generation after treatment with STK11 and rifampicin in combination in H37Ra-infected macrophages. (A, B, C) J774A.1 (1×10^6) cells were infected with H37Ra (OD=0.655) using MOI of 1:5 (Macrophage: H37Ra) for 4 h. H37Ra-infected plates were treated with 2.5 $\mu\text{g/ml}$ of STK11, 0.1 $\mu\text{g/ml}$ of rifampicin, and 2.5 $\mu\text{g/ml}$ of STK11 with 0.1 $\mu\text{g/ml}$ of rifampicin as a combination treatment for 6 h. Using certain primers, the gene expression of cellular markers (iNOS2, Arg1) was obtained in

triplicate. (D) Following 48 h of treatment after infection, nitrite production was measured using Griess reagent from the cell supernatant. Three rounds of the experiment had similar type of results, and the data indicated one of them. Significant changes from the untreated control group are shown by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant, respectively.

5.3.4 Alterations in M1 and M2 markers during combined treatment of STK11 and rifampicin in H37Ra-infected macrophage

M1 and M2 marker expressions were checked during H37Ra-infected, STK11-treated, rifampicin-treated, and STK11 with rifampicin in combination-treated H37Ra-infected macrophages. M2 markers (YM1, FIZZ1, CD206) expressions were increased during H37Ra-infected J774A.1 macrophages and effectively decreased during STK11 with rifampicin in combination-treated macrophages than in individual treated macrophages (Figure 5.4 A-D).

M1 markers (CD68 and CD40) expressions were decreased during H37Ra-infected macrophages but effectively increased during STK11 with rifampicin in combination treatment compared to H37Ra-infected macrophages, but the maximum level of increased M1 markers were observed during STK11 with rifampicin as combination-treated macrophages compared to STK11 or rifampicin-alone treated (Figure 5.4A, E-F). So, combined treatment of STK11 with rifampicin is much more effective than STK11 or rifampicin treatment alone through host-directed therapy, depending upon macrophage polarization.

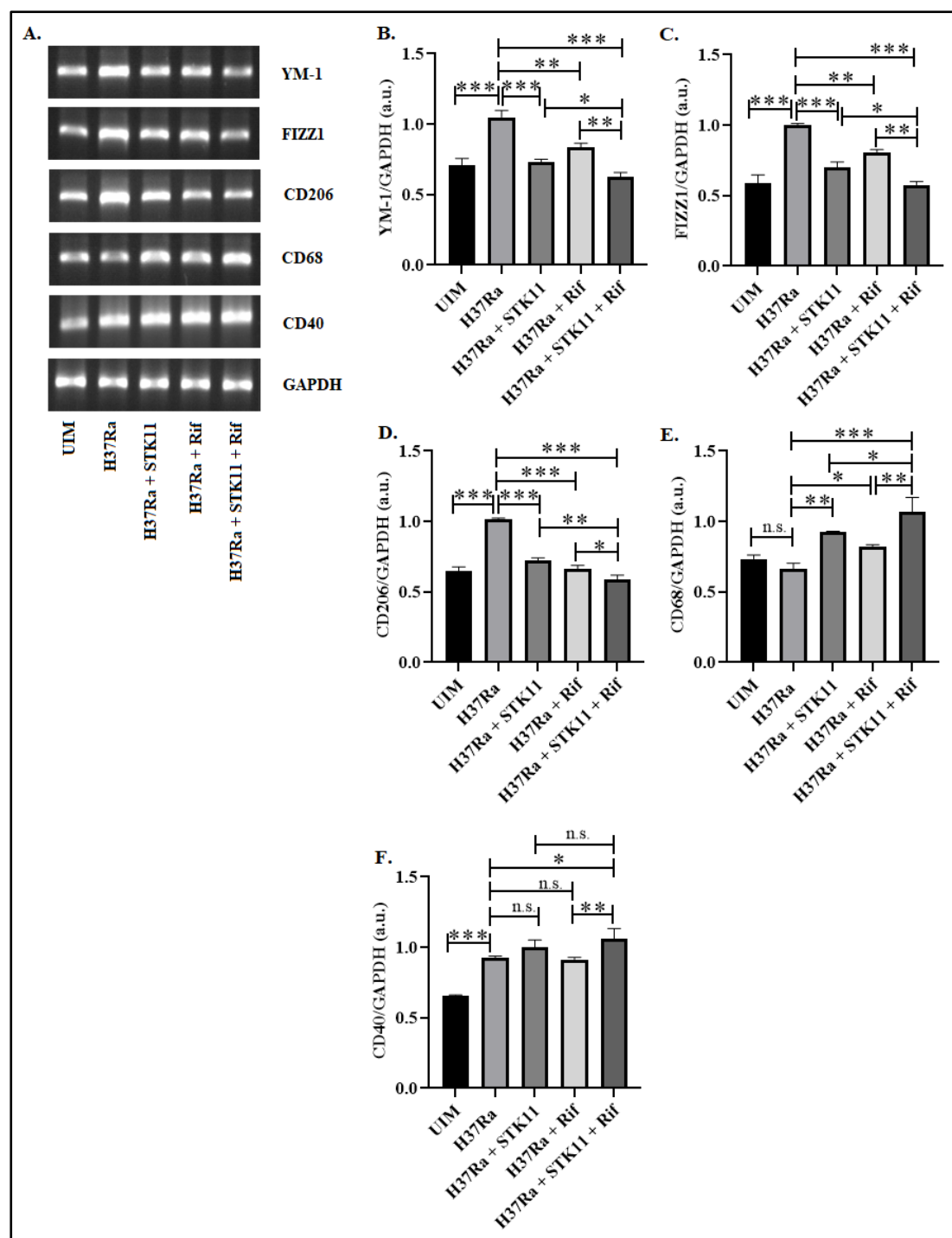


Figure 5.4 mRNA expression of different M1 and M2 markers were studied during STK11, rifampicin, and STK11 with rifampicin-treated macrophages. Macrophage cells (1×10^6) were infected with H37Ra bacilli (OD=0.655) for 4 h and were treated for 6 h. M1 and M2 markers (YM-1, FIZZ1, CD206, CD68, CD40) gene expression was gained in triplicate using specific primers. The band intensity was measured by ImageJ from the three sets of experiments and the data were shown as mean $\pm$ SD. A significant difference from the untreated group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

5.3.5 Cytokine expression during the combined treatment of STK11 with Rifampicin in H37Ra-infected macrophages

By regulating pro-inflammatory cytokines like IFN- γ , IL-6, IL-12, which promote the synthesis of nitric oxide that kills the pathogens by macrophages and protect the host from infections (Murray, 2017). Through various signalling pathways, STK11 with rifampicin influenced cytokine expressions at the mRNA level, influencing inflammation and immunological responses (Koyama et al., 2016). In this context, pro-inflammatory cytokines (IFN- γ , IL-6, IL-12) and anti-inflammatory cytokines (IL-4 and IL-10) were checked during H37Ra-infected, STK11 with rifampicin-combination- and individual drug treated macrophages. Anti-inflammatory cytokines (IL-4, IL-10) expressions were upregulated during H37Ra-infected J774A.1 macrophages but were effectively downregulated during the combination of STK11 with rifampicin-treated macrophages than individual treated macrophages (Figure 5.5A, E-F). On the other hand, proinflammatory cytokines (IFN- γ , IL-6, IL-12) expressions were effectively increased during the combination of STK11 with rifampicin-treated macrophages (Figure 5.5A-D). We observed that the combinatorial treatment of STK11 with rifampicin could effectively alter the macrophage polarization than individual treatment from M2 to M1. Thus, depending on macrophage polarization, combined treatment of rifampicin with STK11 was found much more effective than rifampicin and STK11 individual treatment through host-directed therapy.

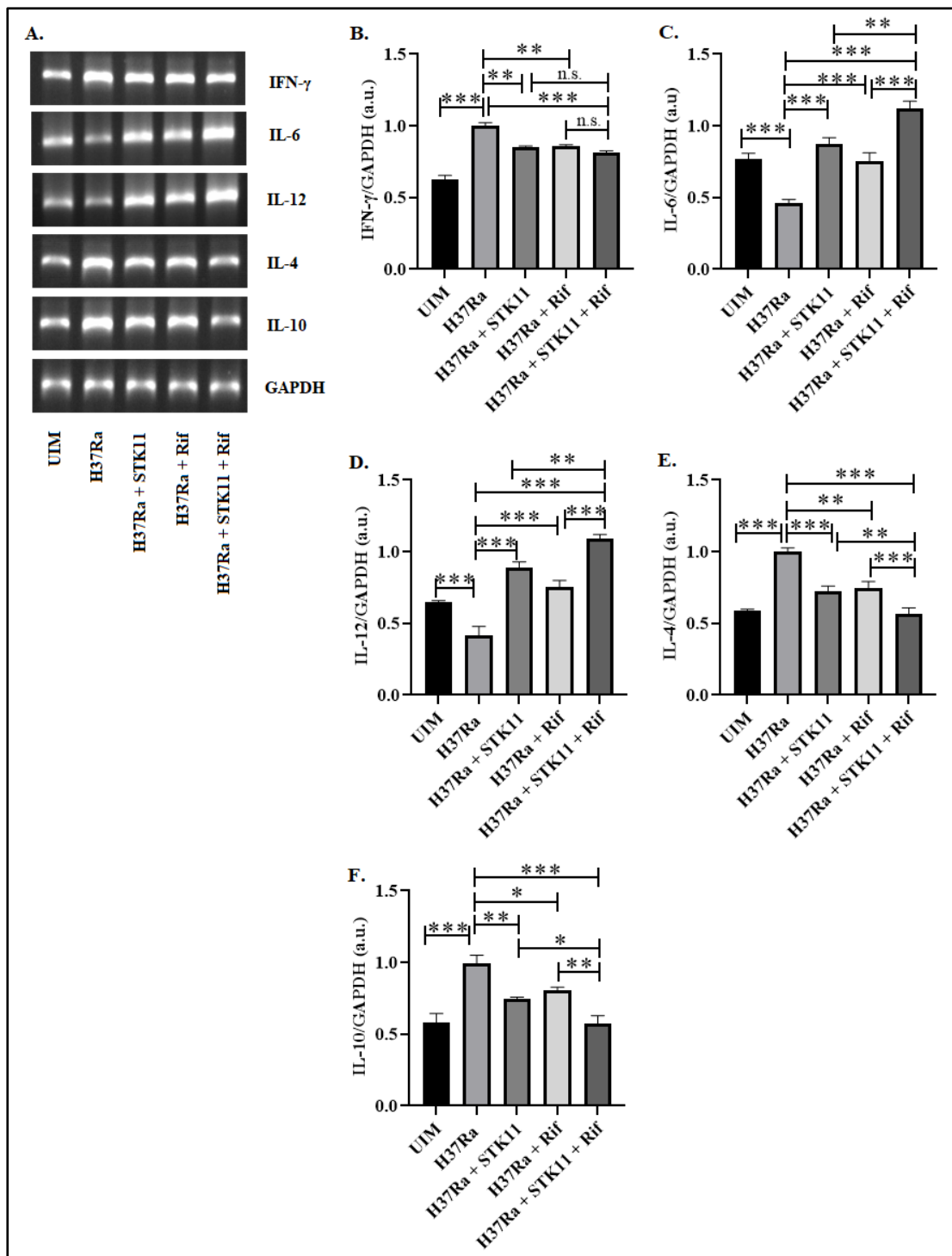


Figure 5.5 mRNA expression of different pro and anti-inflammatory cytokines during H37Ra-infected and STK11, rifampicin, and STK11 with rifampicin-treated H37Ra-infected macrophages. Macrophage cells (1×10^6) were infected with H37Ra bacilli (OD=0.655) using MOI of 1:5 (Macrophage: *M. tuberculosis*) for 4 h. H37Ra-infected plates were treated with 2.5 μ g/ml of STK11 glycoprotein, 0.1 μ g/ml of rifampicin, and 2.5 μ g/ml of STK11 with 0.1 μ g/ml of rifampicin as a combination treatment for 6 h. Pro and anti-inflammatory cytokines (IFN- γ , IL-6, IL-12, IL-4, IL-10) gene expression was measured in

triplicate using specific primers by conventional RT-PCR. The band intensity data is displayed as mean $\pm$ SD from three sets of experiments. A significant difference from the untreated control group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s.-not significant.

5.3.6 Cytokine release during the combined treatment of STK11 with rifampicin in H37Ra-infected macrophages

Similar to the mRNA expression, M1-M2-related cytokines were also modulated at the protein level, except for IFN- γ . Pro-inflammatory cytokines (IL-6, IL-12) releases were decreased, and anti-inflammatory cytokines (IL-4, IL-10) releases were effectively increased during H37Ra-infected macrophages. On the other hand, IL-6, IL-12 release increased, and IL-4, IL-10 release were effectively decreased during the STK11 with rifampicin in combination-treated H37Ra-infected macrophages than the individual treated macrophages (Figure 5.6 B-E). On the other hand, IFN- γ played the dual role during tuberculosis, hence we didn't get a significant difference in it among the effects of combination therapy (Figure 5.6A).

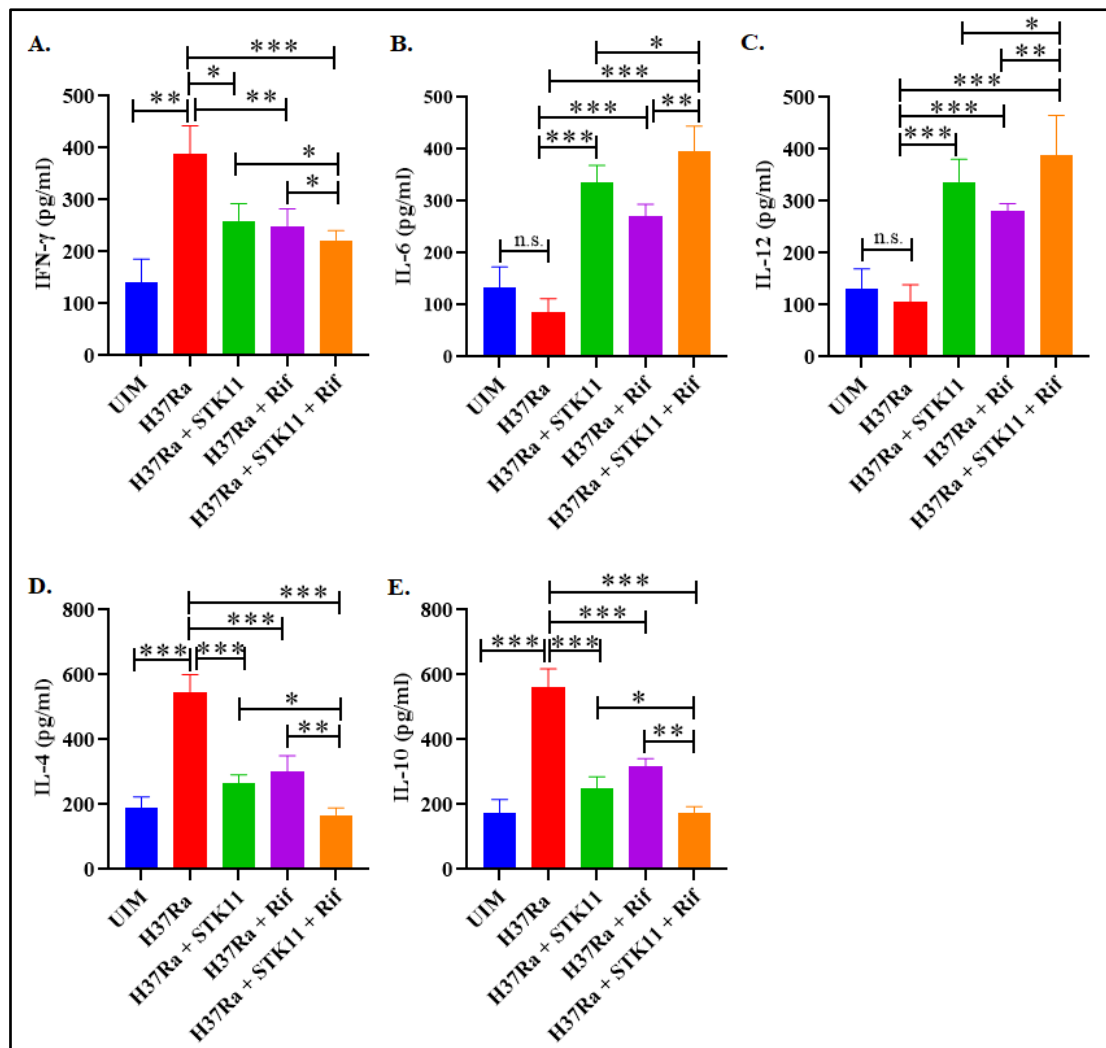


Figure 5.6 Different cytokines released after combination treatment between STK11 and rifampicin in H37Ra-infected macrophages. (A-E) Macrophage cells (1×10^6) were infected with H37Ra bacilli (OD=0.655) with MOI (1:5= Macrophage: *M. tuberculosis*) for 4 h. After 4 h, the cells were treated with 2.5 $\mu\text{g/ml}$ of STK11 glycoprotein, 0.1 $\mu\text{g/ml}$ of rifampicin, and 2.5 $\mu\text{g/ml}$ of STK11 with 0.1 $\mu\text{g/ml}$ of rifampicin in combination. Then, after 24 h, the cell supernatant was subjected to ELISA to check the level of pro-inflammatory and anti-inflammatory cytokines. IFN- γ , IL-6, IL-12, IL-4, and IL-10 cytokine release were studied in triplicate form in an ELISA. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.-not significant, indicate a significant difference compared with the untreated control group.

5.4 Discussion

Tuberculosis is a serious type of deadly infectious disease in the world nowadays (WHO, 2025). In addition to significant side effects, current treatment regimens for TB disease, including MDR-TB, XDR-TB, and RR-TB, were characterized by long durations (18-24 months) and the used of second-line drugs with toxicity, which contributed to less effectiveness, as well as, high rates of treatment dropout. In this context, there was an unmet need for shorter, safer, and effective treatments (Quan et al., 2025). In the treatment of tuberculosis infections, HDT was found as an innovative supplementary therapeutic approach that had been developed recently (Kaufmann et al., 2018). HDT protected lungs, increases long-term survival, and shortens treatment period by reducing lung inflammation, enhancing focused drug penetration, and inducing antimicrobial activity of phagocytes (Zhao et al., 2024). As a result, HDT helped address the current problems of complex tuberculosis treatment option and the possibility of drug-resistant (Jeong et al., 2022). HDT might progress the tuberculosis treatment by shifting the pro-(M1) or anti-inflammatory (M2) immune system (TB) (Krug et al., 2021). In this context, we isolated the novel immunomodulator STK11 from *C. nucifera* L. (chapter 3) and checked its efficacy in combination with rifampicin drug for M2 to M1 repolarization based on macrophage polarization in H37Ra-infected macrophages. It had been shown that STK11 exhibited immunogenic activity at a low dose. Interestingly, STK11 was TLR-dependent and promoted a strong pro-inflammatory cytokine response and NO production (Srinivasan et al, 2023), which also proved in case of *M. tuberculosis* infection as observed in the chapter 4.

Besides, we checked the efficacy of STK11 in combination with rifampicin of different doses against tuberculosis by resazurin assay, which significantly killed the bacteria by increasing the dose of STK11 with rifampicin. The bacterial killing percentage was

effectively increased in the combination treatment of STK11 with rifampicin compared to individual treatment. The results showed that the highest dose of STK11 with rifampicin can restrict H37Ra bacterial growth more effectively than individual treatment (Figure 5.1).

We also investigated the combinatorial effect of STK11 with rifampicin in H37Ra-infected macrophages. In the macrophage infection model, rifampicin (0.1 µg/ml) alone restricted the intracellular bacilli by 51.49%. Interestingly, in the presence of STK11 (2.5 µg/ml), it could reach up to 72.66%. Hence, 2.5 µg/ml of STK11 glycoprotein in combination with 0.1 µg/ml of rifampicin could restrict the H37Ra bacterial growth within the macrophage more effectively than the individual treatment (Figure 5.2).

Arg1 and iNOS2 showed distinct and counter regulating functions; where, Arg1 had an anti-inflammatory (M2) role, whereas iNOS2 showed a proinflammatory (M1) function (Wang et al., 2020; He et al., 2013). On the other hand, *M. tuberculosis* infection could cause macrophages to change into M2 phenotypes in the intermediate and late phases of tuberculosis and driven them towards M1 phenotypes in the early phases (Lugo-Villarino et al., 2011; Benoit et al., 2008). The result showed that the Arg1 marker was increased during H37Ra-infected macrophages, but on the other hand, iNOS2 expression was effectively increased during the combination-treated H37Ra-infected macrophages than the individual treatment (Figure 5.3A-C). So, the combinatorial treatment of STK11 with rifampicin could modify the macrophage polarization from M2 to M1 more effectively than the individual treatment. On the other hand, the results of nitrite release also claimed that it was effectively increased during the combination-treatment of STK11 with rifampicin than in the individual-drug-treated macrophages (5.3D).

Various M1 (CD40, CD68) and M2 (YM-1, FIZZ1, CD206) markers were also checked during the combination-treated macrophages (Figure 5.4). Our previous study exhibited that the mRNA expressions of M2 markers were increased and M1 markers were decreased during H37Ra-infected macrophages (Samanta et al., 2026). In this context, M2 markers were effectively decreased, and M1 markers were effectively increased during the STK11 with rifampicin combination-treated macrophages than the individual treatment. On the other hand, pro-inflammatory (IFN- γ , IL-6, IL-12) and anti-inflammatory (IL-4, IL-10) cytokines expression and release were checked during the combination-treated macrophages to know the effect of STK11 with rifampicin, especially to know the polarization or repolarization effect from M2 to M1. The result claimed that IL-6, IL-12 expressions were effectively increased, and IL-4, IL-10 expressions were effectively decreased during the combination-treated macrophages compared to the individual treatment (Figure 5.5-5.6). These cytokines balance towards pro-inflammation might promoted the iNOS2 expression, which was the cause of induction of nitrite generation to kill the H37Ra bacilli within macrophages.

So, all the results claimed that the STK11 in combination with rifampicin induced the HDT through M2 to M1 macrophage polarization in proficient way that resulted restricting H37Ra bacilli growth intracellularly.

5.5 Summary & Conclusion

This study evaluated the significance of host-directed therapy against tuberculosis-infected macrophages, focusing on macrophage polarization using *C. nucifera* L. derived STK11 in combination with rifampicin. The results indicated that STK11 in different doses in combination with rifampicin can restrict the growth of H37Ra more effectively than the individual in the resazurin assay. STK11 with rifampicin also

restricted the bacilli's growth more effectively than individual treatment within the macrophages in the macrophage-mediated killing assay. In the macrophage TB infection model, rifampicin (0.1 $\mu\text{g/ml}$) alone reduced the intracellular bacilli by 51.49%. Remarkably, this was increased up to 72.66% when STK11 (2.5 $\mu\text{g/ml}$) was present. The results also claimed that Arg1, YM-1, FIZZ1, CD206, IL-4, IL-10 (M2 marker) were upregulated and iNOS2, CD40, CD68, IL-6, IL-12 (M1 markers) were downregulated during H37Ra infected macrophage. On the other hand, M2 markers were effectively downregulated, and M1 markers were effectively upregulated during the combination treatment of STK11 with rifampicin than the individual treatment. Therefore, STK11 improved rifampicin's effectiveness. Clubbing all together, STK11, either alone or in combination with rifampicin, was identified as a strong immunomodulator that could soon be used in host-directed therapy to treat tuberculosis.

Chapter 6: Conclusion

6.1 Conclusion

Tuberculosis is a deadly disease caused by the bacterium *M. tuberculosis*, which claims 1.5 million deaths and 10 million infections worldwide. In this context, WHO targeted the goal of sterile cure of Tuberculosis by 2035. Hence, several strategies were adopted to develop novel therapeutics against it, although the emergence of drug resistance is restricting the rate of success. In this context, host-directed therapy can be an effective opportunity to combat this deadly disease. A study of macrophage polarization status during disease progression and disease-resolving state might help us to design better host-directed therapeutics against tuberculosis. Hence, this study aimed to establish the state of macrophage polarization during disease progression (H37Ra, H37Rv) and disease resolving (BCG, Heat killed H37Ra) states of *M. tuberculosis* infection and bioprospecting of a novel glycoprotein (STK11) isolated from the nutshell of *Cocos nucifera L.* as a possibility of host-directed therapeutics.

The results of time-dependent experiments showed that after 6 hrs of H37Ra infection, M2 markers (Arg-1, YM-1, FIZZ-1, CD206, IL-4, IL-10) were significantly ($p < 0.05$) increased, while M1 markers (iNOS2, CD68, IL-6) were suppressed (Figure 2.1–2.3). Additionally, M2 markers were also upregulated during H37Rv-infected macrophages, but, M2 markers were downregulated and as well as M1 markers were upregulated during BCG and heat-killed H37Ra-challenged macrophages (Figure 2.4–2.6). Hence, the BCG strain and heat-killed *Mycobacterium* reversed the macrophages polarization status from M2 to M1. These results indicated that M1 polarization might be required for the cure of *M. tuberculosis*, which could be achieved using modest immunomodulators. In this context, we had taken STK11 (46 kDa glycoprotein)

isolated from the nutshell of *C. nucifera* L. that earlier proved its efficacy towards the macrophage activation.

Here in, at first, STK11 was isolated and treated against H37Ra *Mycobacterium* and examined its efficacy. Results strongly claimed that higher concentrations of STK11 (200 µg/ml) could restrict 44.83% bacilli growth in MB7H9 Middlebrook culture medium; while, the standard drug rifampicin (0.12 µg/ml) could restrict 54.98% bacilli growth (Figure 3.2). Being the intracellular nature of *M. tuberculosis* within host macrophages to establish the TB disease, we intended to study the efficacy of STK11 within infection model *in vitro*. Our results clearly claimed that almost 20-fold less concentration of direct killing assay i.e. 10 µg/ml of STK11 significantly ($p < 0.001$) decreased by 62.86% of intracellular bacilli growth within macrophages indicating the possibilities of host-directed therapy using it (Figure 3.3). It was also noteworthy that, rifampicin (0.1 µg/ml) could restrict the bacilli growth within macrophages by 56.41% (Figure 3.3). Here, it was also needed to mention that effect of STK11 was noticeably higher than the effect of rifampicin, irrespective of doses required. Additionally, M2 markers (Arg-1, YM-1, FIZZ-1, CD206, IL-4, IL-10) were increased significantly ($p < 0.05$) during H37Ra-infected macrophages, while, M1 markers (iNOS2, CD68, CD40, IFN- γ , IL-6, IL-12) were significantly ($p < 0.05$) upregulated in STK11-treated H37Ra-infected macrophages (Figure 3.4–3.7). Hence, STK11 might enforce the M1 polarization in H37Ra-infected macrophages to restrict the bacilli growth within the host cells. Further, *in silico* study and mRNA expression of Ras isoforms indicated that STK11 interacted with H-Ras and K-Ras and promoted nitrite dependent killing of this intracellular H37Ra bacilli (Figure 3.8). Hence, STK11 modulated Ras isoforms which might be involved in M2 to M1 polarization during H37Ra infection.

It is well established that sterile cure of tuberculosis is not possible without addressing the latent TB. In this context, further experiments were conducted to establish the efficacy of STK11 against latent H37Ra bacilli using Wayne's hypoxia model. Interestingly, STK11 (200 µg/ml) could kill the latent bacilli by 49.10%, which was comparable to the effect against active bacilli (Figure 4.1). Here in, similar to the previous experimental results, STK11 (10 µg/ml) was found more effective in infection model, where, decrease of 73.53% latent bacterial load were observed with in macrophages (Figure 4.2). Rifampicin (0.1 µg/ml) also lessened the latent bacterial load by 74.35%. Hence, STK11 could be considered as host directed therapeutics against both active and latent stage of *M. tuberculosis* infection. Corroborating to the results of previous experiments, STK11 also imposed the M1 polarization from the M2 polarization in latent bacilli-carrying macrophages (Figure 4.3 – 4.6). Further experiments claimed that STK11 interacted with TLR-2, TLR-4, TLR-6 and TLR-9 *in silico* and modulated their expressions (Figure 4.7), which might lead to the development of anti-TB immune responses to overcome the latent infection significantly.

Finally, we examined the combinatorial effect of STK11 with rifampicin against *M. tuberculosis* (H37Ra). STK11 (200 µg/ml) in combination with rifampicin (0.12 µg/ml) could inhibit the growth of H37Ra bacilli by 63.19%, which were significantly ($p < 0.01$) higher than the effect of individual components. In addition to these, rifampicin (0.1 µg/ml) alone restricted the intracellular bacilli by 51.49% in the infection model of macrophages. Interestingly, in the presence of STK11 (2.5 µg/ml), it could reach up to 72.20%. Hence, STK11 could enhance the efficacy of the rifampicin during the treatment of the infected macrophages. Combinatorial treatment with rifampicin and STK11 also endorsed the M1 markers and suppressed the M2 markers in H37Ra-

infected macrophages with prompt nitrite generation. These might be involved in killing of *Mycobacterium* bacilli within the infected macrophages. It was also observed that STK11 with rifampicin was more effective treatment option than the alone components.

Altogether, STK11 alone or with rifampicin, could be recognised as a potent immunomodulator interacting with TLRs and Ras isoforms that might be appraised for the treatment of tuberculosis through host-directed therapy in the near future (Figure 6.1). However, further studies are required to enlighten this work.

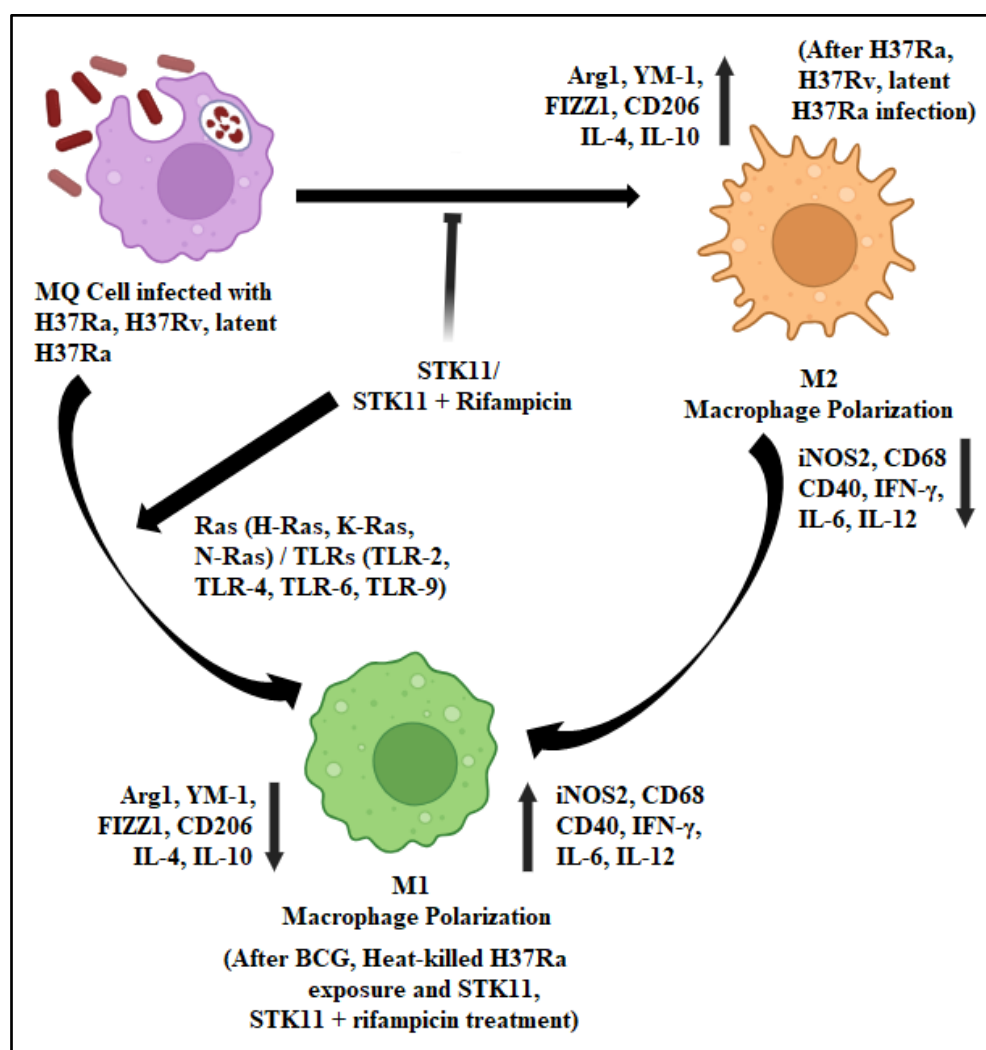


Figure 6.1 Effect of STK11 towards macrophage polarization to treat tuberculosis. H37Ra, H37Rv, or latent H37Ra *Mycobacterium* levied the M2 polarization, while BCG and

heat-killed strain developed the M1 active state. STK11 alone and in combination with rifampicin repolarised the infected macrophages from M2 to M1 and restricted the *M. tuberculosis* bacilli growth within host macrophages.

6.2 Future scope of this work

- I. The effect of the STK11 glycoprotein can be studied in *in vivo* animal model of tuberculosis.
- II. The details signaling pathway involved in STK11 induced macrophage polarization can be established
- III. Effect of STK11 in combination with standard drugs other than rifampicin can be validated in vitro and in vivo.
- IV. Effect of STK11 on drug resistant TB can be examined.

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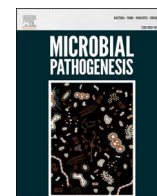
Appendix 1: List of publications and conferences

List of publications related to this thesis:

1. **Samanta, A.**, Maity, S., Hazra, R., Jayaraman, A., & Mahapatra, S. K. (2024). Macrophage polarization or repolarization in tuberculosis. *Asian Pacific Journal of Tropical Medicine*, 17(10), 435-444. https://doi.org/10.4103/apjtm_116_24. **SCI-E indexed, Impact Factor: 3.1**
2. **Samanta, A.**, Datta, N., Pramanik, M., Srinivasan, S., & Mahapatra, S. K. (2026). Isolated glycoprotein STK11 from the nutshell of *Cocos nucifera* L. modulated macrophage polarization to prevent the *Mycobacterium tuberculosis* bacilli growth in J774A.1 macrophage. *Microbial pathogenesis*, 215, 108456. Advance online publication. <https://doi.org/10.1016/j.micpath.2026.108456>. **SCI-E indexed, Impact Factor: 3.5**
3. **Samanta, A.**, Datta, N., Pramanik, M., Dasmahapatra, S., & Mahapatra, S. K. The glycoprotein STK11 derived from the nutshell of *Cocos nucifera* L. alters macrophage polarization to inhibit latent *Mycobacterium* bacilli in J774A.1 macrophages **(Communicated)**
4. **Samanta, A.**, Pramanik, M., Datta, N., Dasmahapatra, S., & Mahapatra, S. K. Combinatorial effect of STK11 with rifampicin prevents the growth of *Mycobacterium tuberculosis* bacilli more effectively through the M2 to M1 repolarization in J774A.1 macrophage **(Communicated to DMID)**

List of paper presented at conference:

1. Isolated glycoprotein from *Cocos nucifera* L. nutshell modulated macrophage polarization to prevent the Tuberculosis bacilli growth. **Arijeet Samanta**, Nayanika Datta, Madhusri Pramanik, Sajal Dasmahapatra, Sujatha Srinivasan, Santanu Kar Mahapatra. 2024, Science Beyond Boundary: Invention, Discovery, Innovation and Society "RASAYAN 20". **(International Symposium), (Received the Best Poster Presentation Award)**.
2. Isolated glycoprotein from *Cocos nucifera* L. nutshell prevents the tuberculosis bacilli growth in macrophages. **Arijeet Samanta**, Santanu Kar Mahapatra. 7th Regional Science & Technology Congress: 2024-25, Region-4 abstract volume 1. **(National level Conference)**.
3. The nutshell of *Cocos nucifera* L. derived glycoprotein STK11 alters macrophage polarization to inhibit latent *Mycobacterium tuberculosis* bacilli in J774A.1 macrophage. **Arijeet Samanta**, Santanu Kar Mahapatra. 7th Annual National Conference "MCCCON-2026" on "Trends in Synthetic Biology and Sustainable Development" from 4th to 6th February, 2026. **(National level Conference)**.



Isolated glycoprotein STK11 from the nutshell of *Cocos nucifera* L. modulated macrophage polarization to prevent the *Mycobacterium tuberculosis* bacilli growth in J774A.1 macrophage

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ABSTRACT

This study examines the importance of host-directed therapy against tuberculosis by targeting macrophage polarization with *Cocos nucifera* L. nutshell-derived STK11. RT-PCR results revealed that M2 markers (Arg1, YM-1, FIZZ1, CD206, IL-4, IL-10) were upregulated, while M1 markers (CD40, CD68, IL-6, IL-12) were downregulated in H37Ra-infected J774A.1 macrophages. Isolated glycoprotein STK11 significantly ($p < 0.05$) restricted the growth of *Mycobacterium tuberculosis* bacilli in the Middlebrook 7H9 medium and inhibited H37Ra bacilli growth within macrophages. STK11 (10 μ g/ml) was also found to decrease the expression of M2 markers and increase M1 markers in infected macrophages. Simultaneously, cytokine release results claimed to upregulate the M1 related cytokines (IL-6, IL-12) and to downregulate the M2 related cytokines (IL-4, IL-10) during STK11 treated H37Ra infected macrophages. Additionally, STK11 can interact with Ras isoforms (H-Ras, N-Ras, K-Ras) *in silico*, the mRNA expression of K-Ras and H-Ras were found to increased, and, N-Ras was decreased in STK11-treated H37Ra-infected macrophages indicating the involvement of Ras isoforms in STK-11 treatment. Hence STK-11 might be associated with the regulation of Ras isoforms, modulating M2 to M1 repolarization in H37Ra-infected macrophages followed by restricting the *M. tuberculosis* bacilli growth in host macrophages.

1. Introduction

Tuberculosis is one of the greatest threatening communicable diseases in the world. It is caused by the bacterium *Mycobacterium tuberculosis*. As per the report, tuberculosis claims 1.5 million deaths worldwide, and 10.6 million people fall into this disease annually [1]. Through aerosol particle inhalation, *M. tuberculosis* is transmitted to the lungs, where it contaminates alveolar macrophages and avoids destruction by interfering with the mechanisms of innate antimicrobials [2]. Present treatment for drug-sensitive TB contains first-line antibiotics, which are managed for approximately 6–9 months [3]. Isoniazid and rifampicin are the main drugs with a 95% curing rate in the market [4]. *M. tuberculosis* could positively survive within several host environments by altering its metabolic state and avoiding the host's immune defense mechanisms by intracellular growth [5]. Consequently, it is very challenging to treat TB, and extensive chemotherapy with a multi-drug regimen is required for its treatment. The treatment duration is very

lengthy for drug-susceptible infection, varying from six months to more than two years for multidrug-resistant TB [5].

In this context, host-directed therapies (HDT) are innovative TB treatment strategies focusing on host characteristics [6]. HDT aims to treat the disease by moderating the host immune system, but not targeting the pathogens directly. Virulence factors expressed by pathogens for their existence overwhelm host immune responses, which may contribute to disease growth and progression [7]. As antimicrobial resistance persists, HDT is mainly significant in the treatment of infectious diseases. Viruses or bacteria cause infectious diseases, which pose a greater risk to mankind, and antimicrobial resistance enhances this threat [8]. By targeting the host rather than the pathogen, emerging novel HDT for TB may avoid conventional antibiotic resistance [6]. The new anti-TB drugs development is an expensive and lengthy process [9], thus, the HDT study may propose an effective substitute that is readily obtainable. Macrophage cells are one of the main host cells in our immune system [10]. Macrophage polarization is often defined as ‘the

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macrophage's activation at a given point in time" [11]. Macrophage polarization is basically of two types: classically activated (M1) and alternatively activated (M2) macrophages [12].

Natural products and glycoproteins are known to play a vital role in biological processes [13–16]. Glycoproteins act as antiviral, antibacterial, or antifungal activity [17–19]. Very few studies have reported that each fraction of a protein induces its nutritional value, physicochemical properties, bioactivity, and use in food or other industries [20]. Glycoproteins have been discovered in abundance from natural resources in recent years. Glycoproteins are biological macromolecules that are important for the growth and development of organisms and are receiving enhanced attention globally [21]. Furthermore, natural glycoproteins have several remarkable biological properties, such as anti-oxidant, anti-coagulant, anti-tumor, and anti-microbial properties [21].

In this context, a significant member of the Arecaceae family, *Cocos nucifera* (L.), is referred to as coco, coconut, coconut-of-the-beach, or coco-da-bahia [22]. The coconut tree originated in Southeast Asia (Indonesia, Malaysia, and the Philippines), which includes the islands between the Pacific Ocean and India [23]. Edible products of coconut, agricultural wastes, and shells of coconut also enhance their therapeutic potential, like nephroprotection, hepatoprotection, anti-cancer, antibacterial, antifungal, and anti-helminthic activity [24–27]. The functional role of serine-threonine kinase (STK)-11 in the coconut palm is unidentified. But, the general role of the STK11 gene, which is a tumor suppressor gene, is that it controls the activity of cells, mediated through the AMPK family members, like cell polarity, apoptosis, and metabolic functions of cells [28–30]. The function of the serine-threonine kinase (STK) defence system in plants has been the subject of numerous investigations. According to reports, STKs, a highly studied protein kinase subfamily, aid in various cellular processes, including plant defence [31–33]. On the other hand, the fruity white flesh decoction of the coconut is used to treat fever and malaria in rural Malaysia [34]. HIV infection relieves skin rash, which is treated by the coconut fruit in Kenya [35].

Recently, CNP-F2 fragment which has core similarity with STK-11 have been isolated and characterized from the nut shell of *C. nucifera* L. The isolated STK11 (CNP-F2) also proved its' potential towards macrophage activation through prompt regulation of cytokines balance [36]. In this context, the current research aims to investigate the role of the *C. nucifera* L. derived glycoprotein, STK11, in the treatment of tuberculosis, emphasizing on macrophage polarization through host-directed therapy.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were procured from Himedia, SRL, and Sigma Aldrich. EDTA was procured from SRL. Dialysis membrane, Middlebrook 7H9 medium, Middlebrook ADC, Tween 80, Tris buffer, Triton-X, Agarose, DMEM, FBS, Penicillin, Streptomycin, Neomycin, RNA Express reagent were procured from Himedia. Griess reagent was procured from SRL. H37Ra bacterial strain (MTCC No. 300) was obtained from CSIR Chandigarh. An amicon centrifugal filter (10 kDa cut off) and rifampicin were procured from Merck-Millipore. cDNA synthesis kit was procured from Bio-Rad. The RT-PCR kit was procured from TAKARA BIO.

2.2. Culture of *M. tuberculosis* bacilli strain

M. tuberculosis strain [H37Ra (MTCC-300)] was cultured in Middlebrook 7H9 medium (Himedia) supplemented with 0.5% glycerol, 0.2% Tween 80, and 10% albumin-dextrose complex enrichment in a shaker incubator for 21 days. At 2500 g, centrifugation for 15 min was used to harvest the bacteria during the mid-log growth phase. Following two centrifugal washings, the bacteria were suspended in PBS saline at

the appropriate concentration. H37Ra bacteria were subcultured after 28 days [37].

2.3. Extraction and purification of nutshell glycoprotein from *C. nucifera* L. in Tris-EDTA (TE) buffer

Based on the standard protocol, the coconut shells were washed and cleaned with fresh water. The coconut shells were dried in the sunlight. Then, dried coconut shells were broken into small pieces. Fine powder was prepared after crushing the pieces of coconut shells. Then, 10 g of coconut shell powder was dissolved in 100 ml Tris EDTA buffer with pH 8.2 that contains 0.1% Triton-X and covered with parafilm. Then, it was mixed gently and stirred for 24 h. Then, the coconut shell powder in the TE buffer was centrifuged at 3000 rpm for 10 min, and the supernatant was collected from the centrifuge tube. Then the dialysis membrane was activated by EDTA, and the supernatant sample was dialyzed through the dialysis membrane for 48 h, while every 6 h, TE buffer was changed. After dialysis of the sample, the dialysed product of the sample was centrifuged in a cold centrifuge using an amicon centrifugal filter (above 10 kDa). The sample obtained above in the amicon centrifugal filter was dissolved in fresh buffer and stored at -20°C for further use. After that, 20 μl of the dialysed sample was taken in duplicate sets in 96-well plates. 200 μl of Bradford reagent was added to the well plate to assess the total protein content in the sample. Further, absorbance was measured at 595 nm. The molecular weight of the protein was identified by 12% SDS PAGE following standard protocol [36].

2.4. The effect of STK11 and rifampicin during the growth of H37Ra by resazurin assay

Any compound's effectiveness on the growth of *M. tuberculosis* *in vitro* can be confirmed in many ways, such as the rapid screening method based on resazurin dye [38], the CFU determination method in broth culture with and without the test compound [39], and the antibiotic dilution method based on agar [40]. The resazurin test was used to estimate the percentage of killing of the bacteria of STK11 and rifampicin against H37Ra after the treatment using the direct killing assay method. 100 μl of H37Ra bacteria (OD-0.02) were seeded into the 96-well plate. Then, isolated STK11 glycoprotein (0–200 $\mu\text{g}/\text{ml}$) and rifampicin (0–0.12 $\mu\text{g}/\text{ml}$) drugs were added in triplicate. The rest of the volume was filled up to 200 μl with complete MB7H9 media containing 20% Tween 80 and 10X ADC. Additionally, 200 μl of distilled water was added to the wells along the edge of the plate to prevent the media in the plate from being dehydrated. After wrapping the plates with parafilm and aluminium foil, they were placed in a BOD incubator for 7 days at 37°C . Then, 30 μl resazurin (0.1 mg/ml), was added and the plates were incubated for another 24 h. Then, fluorescence intensity was checked at excitation: 530 nm, and emission: 590 nm in a BioTek Synergy H1 microplate reader. The dehydrogenase enzyme of the living cells converted the resazurin to resafurin, oxidizing the deep purple color solution to bright pink. The wells containing antimycobacterial chemicals that prevented the growth of bacteria maintained the deep purple color of the resazurin. The resazurin assay was used to determine the percentage of killing of the bacteria in the wells that corresponded to the doses of the drugs that prevented bacterial growth [41].

2.5. CFU test from macrophage-mediated killing assay

The colony-forming unit (CFU) tested the efficacy of STK11 glycoprotein on the growth of *M. tuberculosis* in J774A.1 macrophage *in vitro*. J774A.1 cells (1×10^5) were seeded in 24-well plates and incubated overnight. After incubation overnight, macrophages were infected with H37Ra at a multiplicity of infection (MOI) of 1:5 (Macrophage: Mycobacteria) for 4 h. Then STK11 (0–10 $\mu\text{g}/\text{ml}$) and rifampicin (0.1 $\mu\text{g}/\text{ml}$) were treated in different groups for 48 h in the selected well. After 48 h of treatment, the supernatant was discarded from the plate and washed

with PBS. 200 µl RIPA lysis buffer (without protease inhibitor) was added to every cell well plate, the plate was wrapped with parafilm and placed in a rocking condition in the rocker for 30 min. After that, 50 µl of cell lysate was taken from the total lysate sample and placed in another fresh 24-well plate carefully. Then, 450 µl of complete MB7H9 media containing 20% Tween 80 and 10x ADC were added to the cell well plate. The plate was wrapped carefully with parafilm and aluminum foil to avoid dehydration. The plate was placed in a BOD incubator for further culture. After 14 days, 21 days, and 28 days, respectively the bacterial growth was monitored by taking absorbance at 600 nm in a BioTek Synergy H1 microplate reader [42].

2.6. Nitrite production in J774A.1 macrophages

J774A.1 cells (1×10^5) were seeded in 24-well plates. After overnight incubation, macrophages were infected with H37Ra at MOI of 1:5 (Macrophage: *Mycobacteria*) for 4 h. Then, the cells were treated with the *C. nucifera* (L.) nutshell-derived glycoprotein, STK11 (0-10 µg/ml), and rifampicin (0.1 µg/ml) carefully and placed in the CO2 incubator for 48 h. After 48 h of treatment, 100 µl of cell culture supernatants were collected from the plate, and NO generation was measured using the Griess reagent as per the standard protocol. The absorbance was checked at 548 nm in the BioTek Synergy H1 microplate reader [43].

2.7. mRNA expressions of M1 and M2 markers

Total mRNA was extracted from experimental groups to differentiate the mRNA profiles of several cytokines *in vitro*. 1 µg of total RNA was utilized as a template to synthesize cDNA using reverse transcriptase enzyme, from Takara. Utilizing the particular forward and reverse primers, Arg-1, iNOS2, IFN-γ, YM-1, FIZZ1, CD206, CD68, CD40, IL-6, IL-12, IL-4, IL-10 were determined. The sequences of the forward and

Table 1

Cellular marker, M1/M2, cytokines, and Ras Genes, corresponding primer sequences, and melting temperature.

Gene	Primer Sequences (5'-3')	Melting temperature (T _m)
iNOS2	F: CAG AGG ACC CAG AGA CAA GC R: AAG ACC AGA GGC AGC ACA TC	61.0 °C
Arg 1	F: CAG AAG AAT GGA AGA GTC AG R: CAG ATA TGC AGG GAG TCA CC	52.0 °C
YM-1	F: GAA GGA GCC ACT GAG GTC TG R: CAC GGC ACC TCC TAA ATT GT	55.0 °C
FIZZ1	F: TGC TGG GAT GAC TGC TAC TG R: CTG GGT TCT CCA CCT CTT CA	55.0 °C
CD206	F: ATG CCA AGT GGG AAA ATC TG R: TGT AGC AGT GGC CTG CAT AG	54.0 °C
CD68	F: GAA AGG GCT TCC CAT TTC TC R: TCA AGC CAT TGG TTT TCA CA	51.0 °C
CD40	F: CCT GGC TTT GGA GTT ATG GA R: CCG GGA CTT TAA ACC ACA GA	59.0 °C
IFN-γ	F: CCT GCT TCA CCA CCT TCT TG R: CGA CTC CTT TTC CGC TTC CT	59.0 °C
IL-6	F: AGT TGC CTT CTT GGG ACT GA R: TCC ACG ATT TCC CAG AGA AC	59.0 °C
IL-12	F: CAC GCC TGA AGA AGA TGA CA R: GAC AGA GAC GCC ATT CCA CA	60.0 °C
IL-4	F: GGT GTT CTT CGT TGC TGT GA R: TCT CGA ATG TAC CAG GAG CC	59.1 °C
IL-10	F: TCA CTC TTC ACC TGC TCC AC R: CTA TGC TGC CTG CTC TTA CTC	55.0 °C
N-Ras	F: ATG ACT GAG TAC AAA CTG GTG R: TAC ACA TGA GCA CAC AGG G	54.0 °C
K-Ras	F: GTG GTG GTT GGA GCT GGT R: GTC CTT GAC TTC TTC TTC TTC	55.0 °C
H-Ras	F: ATG ACA GAA TAC AAG CTT GTG GTG G R: TCA GGA CAG CAC ACA TTT GCA G	63.0 °C
GAPDH	F: GAG CCA AAC GGG TCA TCA TC R: CCT GCT TCA CCA CCT TCT TG	59.0 °C

reverse primers are listed in Table 1. Thermal cyclor: 35 cycles for 5 min at 95 °C, 30 s at 95 °C, 30 s at 62 °C, 1 min at 72 °C, and 10 min at 72 °C. Amplified gene products of PCR were run by 1.2% agarose gel electrophoresis and examined under UV light, and captured in the Bio-Rad Gel documentation system. Band intensity of different experimental group was measured using ImageJ software and normalized with GAPDH to obtain the relative expression level [44].

2.8. Protein-protein interaction between Ras isoforms and STK11

The built-in 3D model of STK11 was utilized in examining the interaction with various Ras (H-Ras, K-Ras, N-Ras) *in silico*. The sequence of STK11, identified as A0A0N7EZQ1, was taken from a previously reported study [36] in the UniProtKB knowledge base (<https://www.uniprot.org/>) and stored in FASTA format for further analysis. Ras receptors were obtained from the PDB (protein data bank) to investigate the activation mechanism through the molecular docking method, performed utilizing Cluspro 2.0, an online server available at <https://cluspro.bu.edu/login.php?redir=/home.php>. The Cluspro v2.0 server follows three main steps: i) Rigid body docking is performed using the Fast Fourier Transform (FFT) correlation by sampling billions of conformations. ii) The 1000 lowest energy structures are clustered to produce the largest clusters, with Interface Root Mean Square Deviation (IRMSD) characterizing the finest docking complex models. iii) The stability of the docking models (clusters) is validated by removing steric clashes through energy minimization. The interaction between the STK11 protein-protein and the Ras receptors was visualized using PyMOL software [36]. Further experimentations were performed using K-Ras and H-Ras inhibitors i.e. Sotorasib and Sulindac Sulfide respectively to finally verify the interaction of STK11 with respective Ras. Cells were seeded and infected with H37Ra according to standard protocol followed by addition of Ras inhibitors 2(h) prior treatment with STK11. CFU and nitrite generation was evaluated to check the interaction of STK11 with K-Ras and H-Ras respectively [45,46].

2.9. Measurement of cytokine release by ELISA

Cytokine levels were measured from cell-free supernatants of J774A.1 macrophages of diverse experimental groups using ELISA KIT (BioLegend, San Diego, California) following the manufacturer's instructions [36].

2.10. Statistical analysis

All *in vitro* experiments were conducted in triplicate, and the data were presented as mean ± SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test, using GraphPad Prism version 8.0 software, which helps to determine the data at a significant level.

3. Results

3.1. Isolation of STK11 from the *C. nucifera* L. in TE buffer

C. nucifera L. derived glycoprotein, STK11, was isolated, as described in a previously established protocol [36]. Briefly, we isolated the glycoprotein STK11 from the *C. nucifera* L. nutshell in a TE buffer (pH 8.2). The purity of the purified STK11 was verified as a glycoprotein by 12% SDS-PAGE, which shows a similar kind of result with respect to the previous report [36]. SDS-PAGE results showed that the molecular weight of STK11 was ≈46 kDa (Fig. 1). The yield of the STK11 protein was found to be 194.4 µg in 10 g of STK11 powder which was estimated using the Bradford assay method.

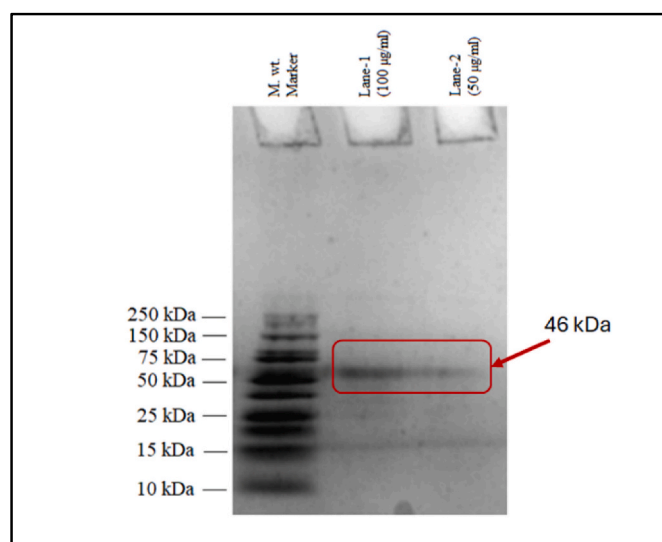


Fig. 1. 12% SDS-PAGE was used to identify the protein molecular weight of the purified *C. nucifera* shell protein. Lane-1: Protein ladder (10-250 kDa), Lane-2: STK11 glycoprotein (100 µg/ml), Lane-3: STK11 glycoprotein (50 µg/ml).

3.2. STK11 glycoprotein can prevent the H37Ra bacterial growth in resazurin assay

The resazurin assay evaluated cell viability and metabolic activity by measuring resazurin reduction to resorufin by metabolically active cells [47]. Although the resazurin test was used to measure bacterial growth and the effects of treatment. In this context, there was currently no direct evidence indicating that the STK11 glycoprotein can inhibit the growth of *M. tuberculosis* H37Ra. Hence, this study investigated whether isolated STK11 could inhibit the growth of H37Ra bacteria via the resazurin

assay. Therefore, this dose-dependent study was performed in triplicate to determine the percentage of killing of the H37Ra bacterial strain treated with STK11 (0-200 µg/ml) and rifampicin (0-0.12 µg/ml). We observed from the results that 3.125 µg/ml of STK11 restricted the H37Ra growth by 13.90% (Fig. 2A), whereas 0.00375 µg/ml rifampicin restricted by 3.24% (Fig. 2B). However, we observed that at the highest dose of STK11 (200 µg/ml) can restrict the bacilli growth of H37Ra was 44.83% (Fig. 2A), whereas 0.12 µg/ml rifampicin could kill 54.98% of the bacteria (Fig. 2B). Thus, the percentage of H37Ra bacterial killing significantly increased with increasing levels of STK11 (0-200 µg/ml) and rifampicin (0-0.12 µg/ml).

3.3. *C. nucifera* L. shell derived glycoprotein, STK11 can prevent H37Ra bacterial growth in J774A.1 macrophage cell

The STK11 glycoprotein, encodes a serine/threonine kinase 11 engaged in various cellular processes, including cell metabolism, polarity, and the regulation of the immune response [48]. The role of STK11 in bacteria-infected macrophages, particularly with *M. tuberculosis*, was not well-documented. Previously, it was claimed that no cytotoxic effects were observed after treatment below 10 µg/ml of STK11 in murine macrophages [36]. Hence, we chose the range between 0 and 10 µg/ml of STK11. Therefore, the growth of H37Ra bacteria in J774A.1 macrophage cell was measured by colony-forming units (CFU) test at doses of 0-10 µg/ml of STK11. J774A.1 cells were seeded at 1×10^5 per well and then infected with the H37Ra bacteria at an MOI in a 1:5 ratio (Macrophage: H37Ra). Then STK11 and a specific drug (rifampicin) were added for 48 h, and it was cultured for CFU, and the CFU growth was measured after 14 days, 21 days, and 28 days using the multimode reader. We observed that the CFU levels decreased significantly after increasing the dose of STK11 from 0 to 10 µg/ml to 28 days compared to infected macrophages. We observed the highest reduction in CFU levels at 10 µg/ml of STK11 and 0.1 µg/ml of rifampicin after 28 days compared to infected macrophages. STK11 (10 µg/ml) and

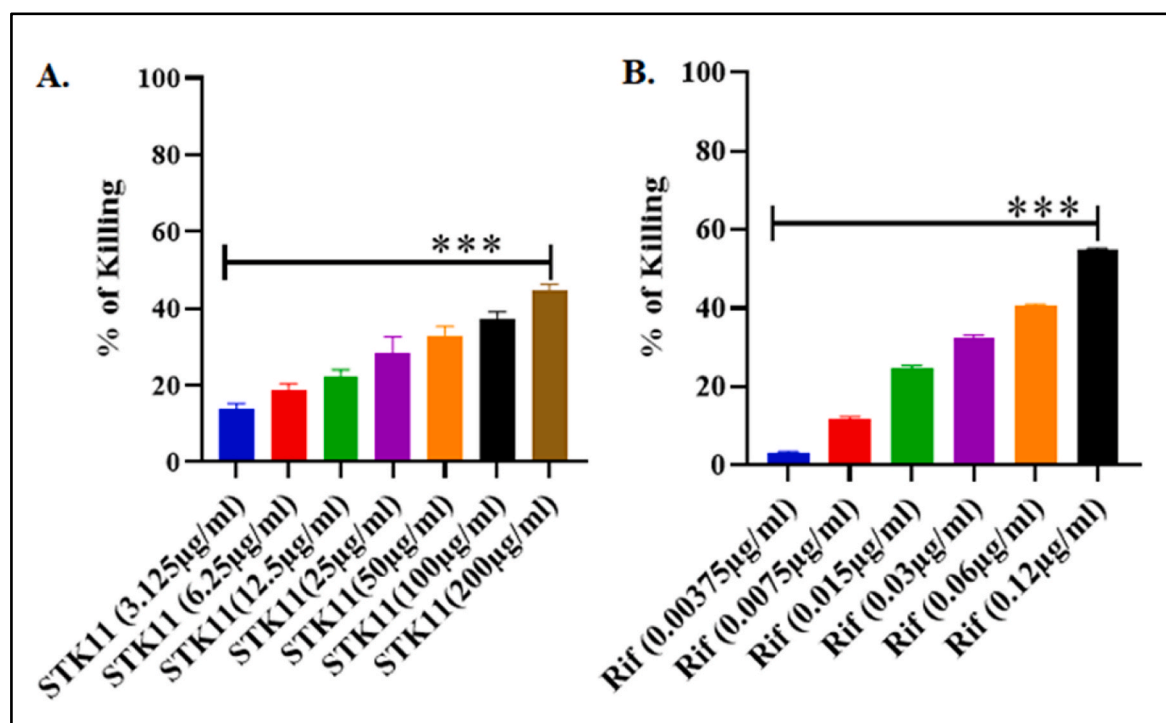


Fig. 2. Determination of the percentage of killing of H37Ra bacilli after the treatment with (A) STK11 and (B) rifampicin. H37Ra bacteria (OD-0.02) were treated with STK11 (0-200 µg/ml) glycoprotein and rifampicin (0-0.12 µg/ml) drug. Then the seeded plate was kept in a BOD incubator for 7 Days. Then, resazurin (0.1 mg/ml, 30 µl) was added for 24 h. Then fluorescence was checked in a BioTek synergy multimode reader at excitation 530 nm, emission 590 nm. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference compared with the untreated control group.

rifampicin (0.1 µg/ml) could reduce the bacilli growth by 2.74-fold and 2.31-fold, respectively, in H37Ra-infected STK11 and rifampicin-treated macrophages (***p* < 0.001). After 28 days, 10 µg/ml STK11 inhibited bacilli growth in macrophages by 62.86%, 5 µg/ml STK11 inhibited bacilli growth in macrophages by 50.03%, 2.5 µg/ml STK11 inhibited bacilli growth in macrophages by 21.82%, and on the other hand, 0.12 µg/ml rifampicin inhibited bacilli growth by 56.41%. (Fig. 3).

3.4. STK11 glycoprotein modulated mRNA expression of iNOS2 and Arg-1 and induce nitric oxide generation

The STK11 glycoprotein acts as an important modulator of macrophage function by regulating iNOS2 and Arg-1 mRNA expression [49]. Therefore, cellular marker (Arg1, iNOS2) expression during H37Ra-infected macrophages and STK11-treated H37Ra-infected macrophages was examined in triplicate. We observed that the M2 cellular marker, Arg-1 expression, was upregulated during H37Ra-infected J774A.1 macrophages and downregulated upon STK11 treatment. Besides, we also observed that the M1 cellular marker, iNOS2 expression, was downregulated in H37Ra-infected macrophages but upregulated in STK11-treated H37Ra-infected J774A.1 macrophages (Fig. 4A, B & 4C).

Mammalian immune cells generate nitric oxide (NO), which plays an important role in immune responses against pathogens. Naturally, the activation of macrophages needs the nitric oxide generation from L-arginine through a catalyzed reaction mediated by induced nitric oxide synthase (iNOS) [43]. Treatment with STK11 was done in a dose-dependent manner (0-10 µg/ml) for 48 h in the H37Ra-infected macrophage cells and NO generation was checked. Results indicated 5.11-fold increase in NO generation after the treatment with STK11 at a 10 µg/ml dose, while only 2.33-fold was observed after the treatment with rifampicin (0.1 µg/ml) (Fig. 4D).

3.5. STK11 glycoprotein modulated M1 and M2 surface markers in mRNA level

STK11 regulates M1 and M2 surface markers at the mRNA level, influencing macrophage polarization [50]. The expression of surface markers (YM-1, FIZZ1, CD206, CD68, CD40) was checked in H37Ra-infected macrophages and STK11-treated H37Ra-infected macrophages. We observed that M2 marker (YM-1, FIZZ1, CD206)

expression increased in H37Ra-infected J774A.1 macrophages and slightly decreased in STK11-treated H37Ra-infected macrophages. On the other hand, we observed that M1 markers (CD68, CD40) increased in STK11-treated H37Ra-infected macrophages (Fig. 5A, 5B-5F). Therefore, we observed that *C. nificera* L. derived glycoprotein, STK11, could switch polarization from M2 to M1, and the results claim that M2 markers were upregulated in H37Ra-infected macrophages but down-regulated in STK11-treated H37Ra-infected macrophages.

3.6. STK11 glycoprotein modulated the cytokines expression and cytokine release

A phagocytic cell, such as a macrophage, protects the host from pathogens by regulating pro-inflammatory cytokines, i.e., IFN-γ and IL-12, which stimulate the production of nitric oxide that kills pathogens [11]. STK11 modulates cytokine expression at the mRNA level through various signalling pathways, impacting immune responses and inflammation [51]. We observed that pro-inflammatory cytokines (IL-12, IL-6) expressions were downregulated in H37Ra-infected macrophages and upregulated in STK11-treated H37Ra-infected macrophages. IFN-γ expression was increased in H37Ra-infected macrophages but slightly decreased in STK11-treated H37Ra-infected macrophages. Besides that, anti-inflammatory cytokines (IL-4, IL-10) expressions were increased in H37Ra-infected macrophages but decreased in STK11-treated H37Ra-infected macrophages (Fig. 6A, 6B-6F).

A similar type of result was obtained in cytokine release experiments as that of the mRNA expression levels. We observed from the experiments that pro-inflammatory cytokine (IL-12, IL-6) release was down-regulated in H37Ra-infected macrophages and upregulated in STK11-treated H37Ra-infected macrophages. In contrast the IFN-γ release was upregulated in H37Ra-infected macrophages but slightly decreased in STK11-treated H37Ra-infected macrophages. Besides that, anti-inflammatory cytokines (IL-4, IL-10) release was upregulated in H37Ra-infected macrophages while decreased in STK11-treated H37Ra-infected macrophages (Fig. 6G-K).

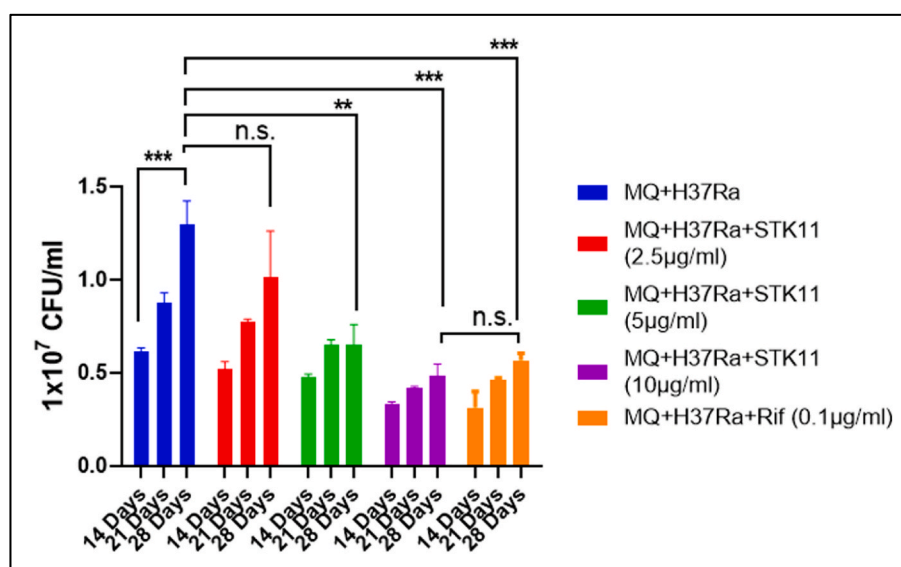


Fig. 3. CFU level after 14 days, 21 days, and 28 days in the treatment with different doses of STK11. Macrophages (1×10^5) were infected with H37Ra at a 1:5 ratio. After that, macrophages were treated with different concentrations of STK11 for 48 h. Then, 200 µl RIPA lysis buffer was added. Then, 50 µl of cell lysate with 450 µl of complete MB7H9 media were incubated for CFU count for 14 days, 21 days, and 28 days, respectively and absorbance was checked at 600 nm. Data is shown as mean $\pm$ SD. **p* < 0.05, ***p* < 0.01; ****p* < 0.001 indicate a significant difference compared with the untreated control group.

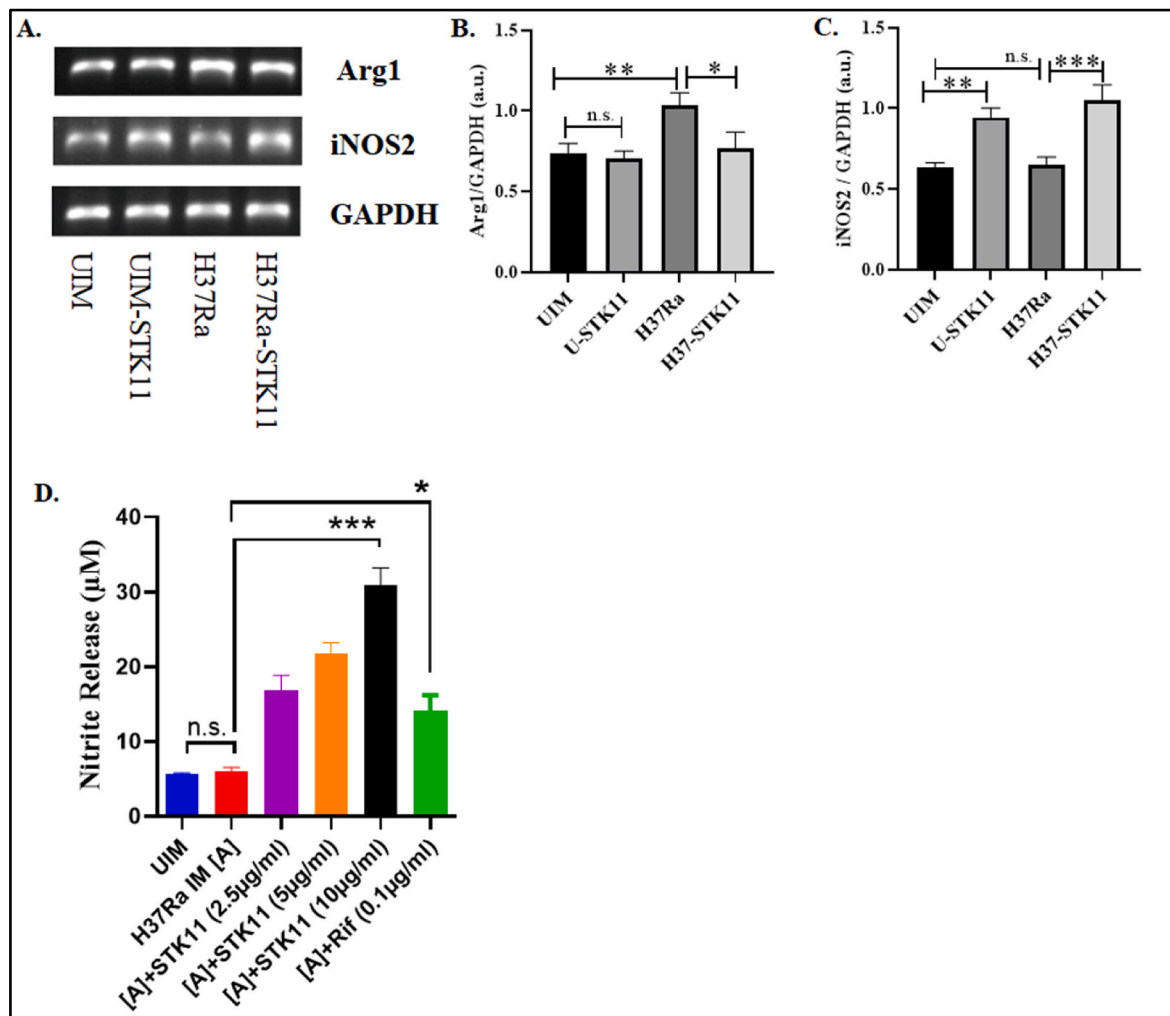


Fig. 4. Cellular marker expression (A, B, C) and nitrite generation (D) were studied during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A, B, C) J774A.1 macrophage (1×10^6) cells were infected with *M. tuberculosis* bacilli (H37Ra) (OD = 0.655) using MOI (1:5 = Macrophage: *M. tuberculosis*) for 4 h. Both the H37Ra-infected and uninfected plates were treated with 10 μ g/ml of STK11 glycoprotein for 6 h. Cellular markers (iNOS2, Arg1) gene expression was gained in triplicate using specific primers. (D) Nitrite generation was assessed by Griess reagent from the cell supernatant after 48 h of treatment with the STK11 (0–10 μ g/ml) and rifampicin (0.1 μ g/ml). The experiment was conducted three times and produced analogous results, data shown. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ show significant differences compared with the untreated control group, respectively.

3.7. Protein-protein docking between the Ras and STK11 glycoprotein and mRNA expression of H-Ras, N-Ras, and K-Ras during H37Ra-infected and STK11-treated H37Ra-infected macrophages

After the macrophage repolarization from M2 to M1 in STK11 treated H37Ra infected macrophages, we preliminary checked Ras-STK11 interaction in *in silico*. As well as mRNA expression of Ras isoforms upon treating the uninfected and infected macrophages with STK11. The interaction between the STK11 and Ras proteins (H-Ras, N-Ras, K-Ras) has been of interest in cellular signalling studies [52]. However, detailed direct experimental evidence of protein-protein docking between STK11 and Ras isoforms has been limited. In this context, the *in-silico* interactions at the molecular level between the STK11 and the Ras were evaluated utilizing the ClusPro 2.0 server, which significantly supported the mRNA expression of Ras in a triplicate study. Interestingly, among all Ras proteins, the lowest binding energy (−905.9 kcal/mol) was observed in the interaction between H-Ras and STK11, which is consistent with the mRNA expression results. However, binding energy −832.0 kcal/mol for K-Ras & −794.5 kcal/mol for N-Ras was found (7A, 7B & 7C).

Specific information about the mRNA expression levels of H-Ras, N-Ras, and K-Ras during *M. tuberculosis*-infected macrophages was scarce.

In this context, uninfected and infected J774A.1 macrophages had been treated with 10 μ g/ml concentration of STK11 and mRNA expressions of Ras molecules were checked. We observed that N-Ras expression was upregulated significantly ($p < 0.001$) in H37Ra-infected macrophages but was decreased during STK11-treated H37Ra-infected macrophages (Fig. 7D and E). On the other hand, H-Ras and K-Ras expressions were downregulated significantly in H37Ra-infected macrophages but were upregulated in STK11-treated H37Ra-infected macrophages concluding that upon treatment of H37Ra infected macrophages with STK11 mRNA expression of K-Ras and H-Ras enhanced preliminarily indicating the interaction of STK11 with K-Ras and H-Ras (Fig. 7D, 7F– 7G).

According to previous reports, Sotorasib is the inhibitor of K-Ras and Sulindac Sulfide is the inhibitor of H-Ras. It has been reported that 0.1 μ M of Sotorasib and 50 μ M of Sulindac Sulfide were standardised concentrations that inhibited the K-Ras and H-Ras [45,46]. In that reference, the infected macrophages were pre-treated with 0.1 μ M Sotorasib and 50 μ M of Sulindac Sulfide respectively 2(h) prior treatment with STK11 to check its interaction with Ras molecules. After 48 (h) of treatment, the cell sample were further cultured for 21 days for measuring the CFU. It was observed that the CFU increased by 1.56 and 1.76 folds respectively when H37Ra infected macrophages were treated with K-Ras and H-Ras inhibitors before being treated with STK11 as

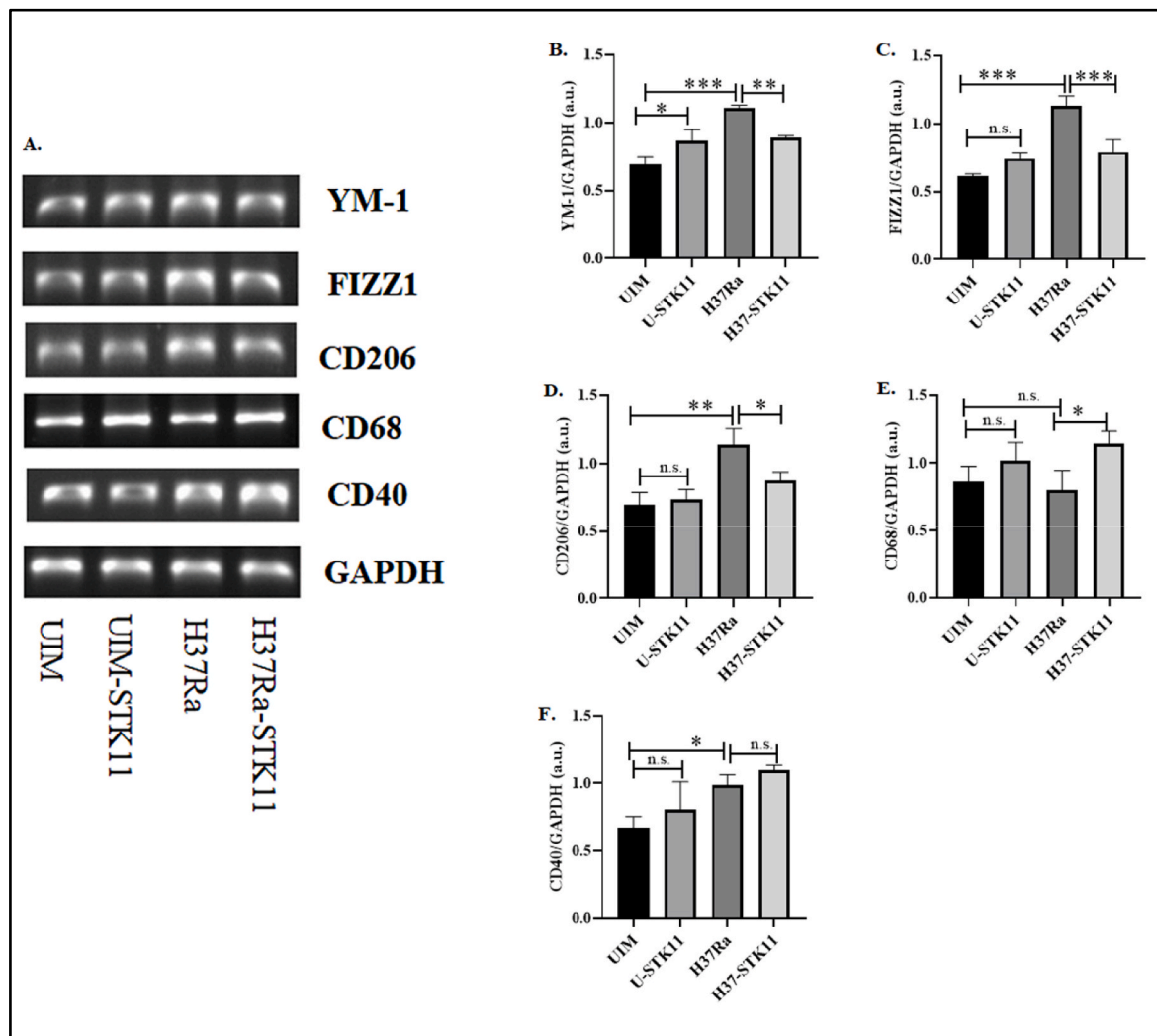


Fig. 5. M1 and M2 Surface marker expression during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A-F) J774A.1 macrophage (1×10^6) cells were infected with *M. tuberculosis* bacilli (H37Ra) (OD = 0.655) at MOI (1:5 = Macrophage: *M. tuberculosis*). Following that, treatment with 10 μ g/ml of STK11 glycoprotein was done for 6 h. YM-1, FIZZ1, CD206, CD68, and CD40 gene expression were studied in triplicate form using specific primers. Data is shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ indicate a significant difference compared with the untreated control group.

compared to treated group (Fig. 7H). The nitrite oxide (NO) being the effector molecule in immune responses against pathogens, the NO generation was also examined upon using inhibitors. It was observed that the NO generation decreased by 1.84 and 2.28 folds when H37Ra infected macrophages were treated with K-Ras and H-Ras inhibitors before being treated with STK11 as compared to treated group (Fig. 7G). Hence, it can be inferred that the interaction of STK11 with K-Ras and H-Ras is responsible for its activity as a candidate against H37Ra.

4. Discussion

Tuberculosis is responsible for approximately 1.5 million deaths each year [1]. HDT drugs that target the host can accelerate and improve the treatment of tuberculosis [53]. By altering the pro-(M1) or anti-inflammatory (M2) immune system, host-directed therapies may improve the treatment of tuberculosis (TB) [54]. Many immunomodulators such as rocaglates, a natural product derivatives from *Aglaia* genus plants, polarized macrophages toward M1 from M2 [55,56], BPMS22 derived from *Oceanimonas* sp. polarized macrophages from M2 to M1 [43], Oligo-Fuoidan, a sulfated polysaccharide isolated from the brown seaweed, polarized cells from M2 to M1 [57,58], Ibrutinib, a BTK inhibitor and Selinexor, a blood-brain barrier (BBB) permeable small

molecule polarized macrophages towards M1 from M2 [59]. In this context, we isolated the novel immunomodulator STK11 from *C. nucifera* L. and checked its efficacy in M2 to M1 repolarization in H37Ra-infected macrophages. At a minimum dose, STK11 has been reported to show its immunogenic activity. It is interesting to note that, STK11 stimulates pro-inflammatory cytokine response and NO generation in a TLR-dependent manner [36]. The SDS-PAGE results showed that STK11 is associated with the bands of 46 kDa (Fig. 1). According to previous reports, STKs also assist in safeguarding plants by inducing numerous physiological processes such as secondary metabolite synthesis, stabilization of cell walls, and the prompting of defensive gene expression [60, 61]. SnRK2, the plant STK family members, revealed that they intervene in the ABA signalling and the abiotic response to stress. Another report on negative regulation of plant defences was published [62]. An STK in *Arabidopsis*, PBL13, has been shown to negatively control resistance against *Pseudomonas syringae*, a pathogenic bacterium [63]. Many reports declared that *C. nucifera* L. and its ingredients have an immunomodulatory impact [64,65]. In a study using Swiss albino mice and LPS-induced RAW macrophages, it was revealed that the acetone extract of coconut inflorescences possesses antinociceptive and anti-inflammatory properties [66]. The liquid shell smoke of *C. nucifera* L. can reconcile the oral diabetic ulcer by raising the responses of the

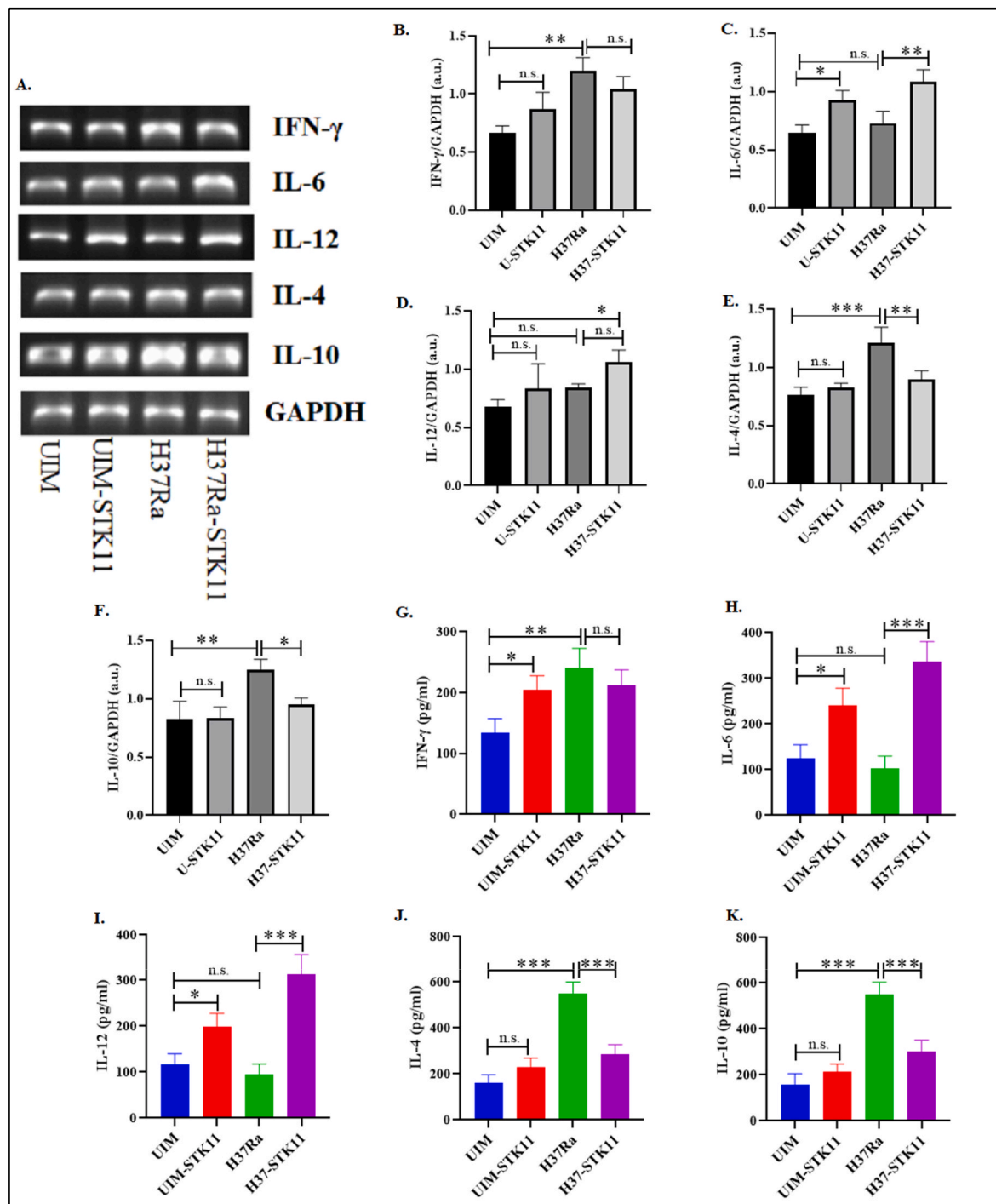


Fig. 6. Pro and anti-inflammatory cytokine expression was studied during H37Ra-infected and STK11-treated H37Ra-infected macrophages. (A-F) J774A.1 macrophage cells (1×10^6) were infected with *M. tuberculosis* bacilli (H37Ra) (OD = 0.655) with MOI (1:5 = Macrophage: *M. tuberculosis*) for 4 h. For 6 h, 10 μ g/ml STK11 was used to treat H37Ra-infected and uninfected macrophages. IFN- γ , IL-6, IL-12, IL-4, and IL-10 cytokines expression were studied in triplicate using specific primers. Cytokine release (protein expression) of pro-inflammatory (IFN- γ , IL-12 and IL-6) and anti-inflammatory (IL-10 and IL-4) were determined in triplicate after 24(h) of treatment with STK11 using ELISA(G-K). All the results are expressed in mean $\pm$ SD. The asterisks indicate the significance level for untreated uninfected controls *p < 0.05, **p < 0.01, ***p < 0.001.

macrophages by dropping the expression level of TNF- α and NF- κ B [67]. From the coconut, the isolated protein fraction has antimicrobial peptides and antioxidants [68]. The anti-mycobacterial activity of rifampicin and isoniazid was previously reported, which exhibited MICs of 0.003 μ g/ml and 0.03 μ g/ml, respectively [41]. This study evaluates the antibacterial effects of STK11 glycoprotein and rifampicin against *M. tuberculosis* H37Ra using the resazurin assay, a well-established method

for assessing cell viability. Besides, we checked the efficacy of STK11 in the direct killing assay by resazurin assay, which killed the bacteria by increasing the dose of STK11 significantly. The bacterial killing percentage increases as the concentration of STK11 and rifampicin rises (Fig. 2). On the other hand, the macrophage-mediated killing assay was done in a triplicate set of *in vitro* experiments, where H37Ra was infected into macrophages at a 1:5 ratio. The observed reduction in CFU levels

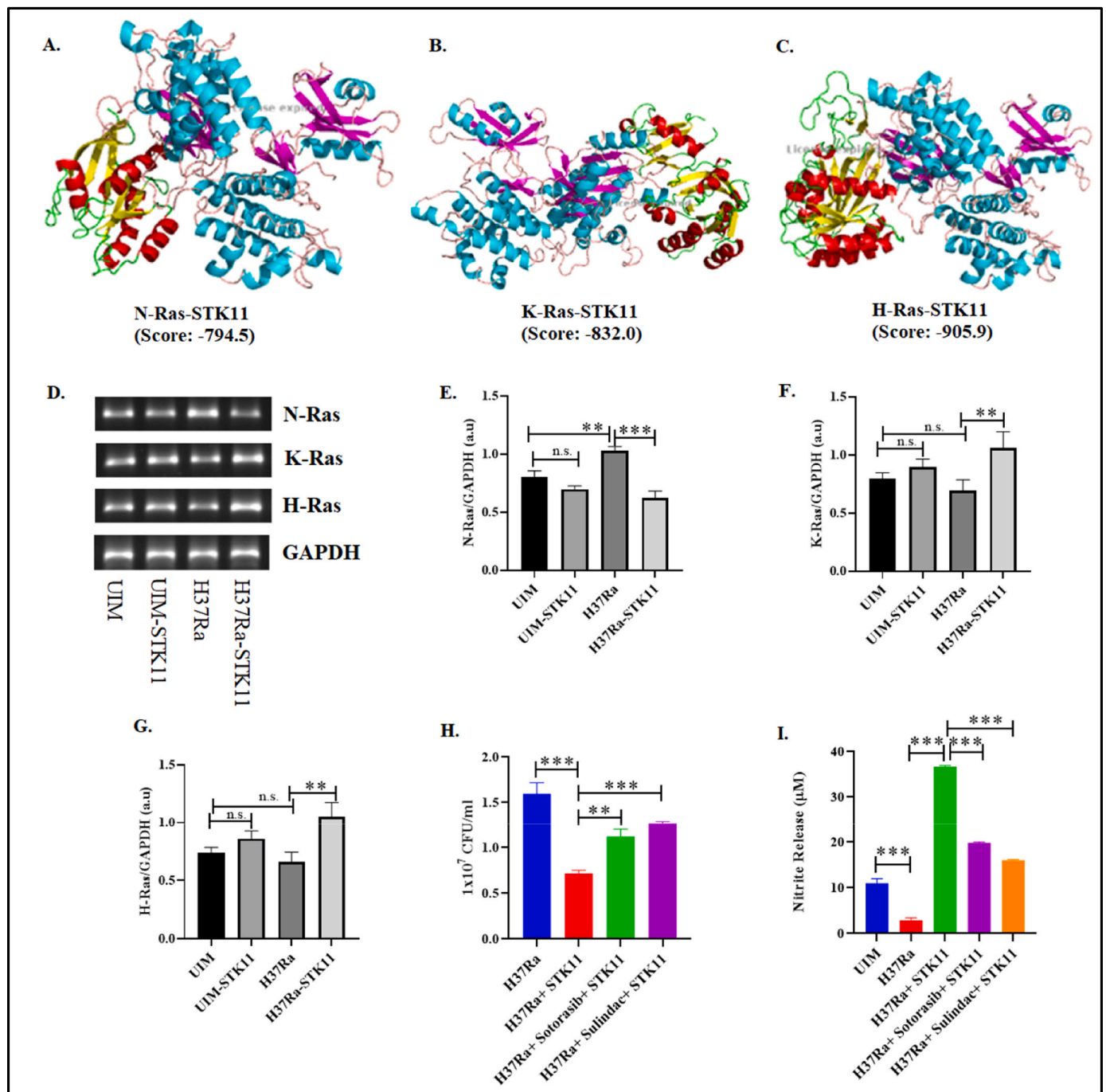


Fig. 7. Ras-STK11 protein-protein docking (A, B, C), mRNA expressions of N-Ras, K-Ras, and H-Ras (D-G) in infected and uninfected macrophages and effect of K-Ras and H-Ras inhibitors (H, I): The docking results between STK11 and N-Ras, K-Ras, H-Ras respectively was obtained in Clasp software. J774A.1 macrophage cell (1×10^6) was infected with *M. tuberculosis* Bacilli (H37Ra) (OD = 0.655) with MOI (1:5 = Macrophage: *M. tuberculosis*). After 4 h, non-ingested *M. tuberculosis* bacilli were removed. After 6(h) of treatment, mRNA expressions of different Ras were observed in uninfected, H37Ra infected as well as STK11 treated H37Ra infected macrophages. CFU and NO generation was checked upon using K-Ras and H-Ras inhibitors. The results were expressed as a mean $\pm$ SD. Asterisks indicated a significant difference compared with the infected control with the STK11 treated group. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

suggests that STK11 exhibits antimicrobial properties against H37Ra-infected macrophages. Notably, the highest tested concentration (10 μ g/ml) of STK11 reduced CFU by 2.74-fold, while rifampicin at 0.1 μ g/ml resulted in a 2.31-fold reduction. These results indicate that STK11 may potentiate the antimicrobial effect independently in controlling H37Ra bacterial growth. The statistical significance ($p < 0.001$) of the results further underscores the reliability of our observations. The promising antimicrobial activity of STK11, compared with rifampicin, highlights its potential as an alternative or adjunctive treatment strategy

for tuberculosis. These discoveries pave the way for additional research into the therapeutic potential of STK11 in bacterial infections (Fig. 3). Additionally, *in vivo* studies are required to validate the efficacy of STK11 in animal models of tuberculosis infection.

Utilizing certain stimuli, namely LPS, macrophages are driven to two main polarized phenotypes: M2 and M1, which function as anti-inflammatory and pro-inflammatory respectively [11,12]; [69–71]. On the contrary, *M. tuberculosis* infection can drive macrophages in the early phase toward M1 phenotypes and change in intermediate and late

phases into M2 phenotypes [72,73]. iNOS2 and Arg1 are engaged in two opposite activities, iNOS2 with proinflammatory (M1) and Arg1 with anti-inflammatory (M2) function [74,75]. The result shows that Arg1 expression in RT-PCR increased significantly during H37Ra-infected macrophages, which induced anti-inflammatory (M2) macrophage polarization. On the other hand, results claimed that STK11-treated H37Ra-infected macrophages can be modulated towards M1 polarization from M2. iNOS2 expression declared that iNOS2 expression decreased during H37Ra-infected macrophages and increased in STK11-treated macrophages (Fig. 4A, B & 4C). Nitrite release assay suggested that nitrite release decreased in uninfected and H37Ra-infected macrophages and increased during STK11-treated macrophages as the dose 0-10 µg/ml significantly, which supports the same with the iNOS2 (Fig. 4D).

M1 and M2 markers expression supported that M2 markers (YM-1, FIZZ1, CD206) also increases and M1 markers (CD68, CD40) expression decreases in H37Ra-infected macrophages. On the other hand, STK11 treatment can restrict the bacilli's growth within the macrophages, and RT-PCR results supported the same, as M2 markers decrease successfully and M1 markers increase significantly ($p < 0.001$) (Fig. 5A, 5B-5F). The RT-PCR result of IL-4, IL-10 cytokines expression increased significantly ($p < 0.001$), and IL-6, IL-12 expression decreased significantly ($p < 0.001$) during H37Ra-infected macrophages (Fig. 6A, 6B-6F). However, STK11-treated macrophages restricted the bacilli's growth as M1 cytokines (IL-6, IL-12) expression increased and M2 cytokines (IL-4, IL-10) expression decreased. Similar types of results were also found in cytokine release. So, cytokine release also claimed that STK11 treated H37Ra infected macrophages alter the macrophages polarization from M2 to M1 to restrict the H37Ra infection (Fig. 6G-K). IFN- γ expression typically increased during *M. tuberculosis*-infected macrophages, including with the attenuated H37Ra strain-infected macrophages [75]. IFN- γ was a critical cytokine in the immune response against *Mycobacterium* infections, as it activated the macrophages and enhanced their ability to kill intracellular pathogens [76].

Small GTPases called Ras proteins (such as H-Ras, K-Ras, and N-Ras) were important in sending signals from cell surface receptors to the nucleus, which affects immunological responses, cell survival, and proliferation [77]. In this context, we established that STK11 was the finest molecule for interacting with Ras by the docking method. STK11 displayed better interaction with H-Ras (docking score -905.9) (Fig. 7A, B & 7C). So, our study revealed that N-Ras expression increased, and H-Ras, K-Ras expression decreased during H37Ra-infected macrophages, but on the other hand, N-Ras expression decreased, and H-Ras, K-Ras expression increased in STK11-treated H37Ra-infected macrophages at the mRNA level, which were very similar to the *in-silico* studies result (Fig. 7D, 7E-7G). Another result suggested that N-Ras regulates ERK-1/2 activation by CD40, which results in the anti-inflammatory IL-10 production, whereas H- or K-Ras was required for p38MAPK activation, which results in the creation of pro-inflammatory IL-12 [78]. Sotorasib (0.1 µM) and sulindac (50 µM) have been reported to be the inhibitors of K-Ras and H-Ras respectively and have played a critical role in cancer biology in previous reports [45,46]. So, to clarify the positive interaction of Ras isoform with STK11 to treat H37Ra infected macrophages, the inhibitors for K-Ras and H-Ras were used for experimentation. From the results, it was observed that the CFU increased by significant levels ($p < 0.001$) upon addition of sotorasib and sulindac to the already STK11 treated macrophages as compared to the normal treated group (Fig. 7H). NO generation using the inhibitors also showed decrease in nitrite release as compared to the STK11 treated group where no inhibitors were used (Fig. 7I). Hence, through the inhibition of Ras isoforms and overviewing its effect on STK11 confirms that STK11 has strong interaction with Ras isoforms and STK11 activity is dependent on Ras isoforms.

Therefore, it is well known and established that immune response of Th1 type which is characterized by IFN- γ , IL-12 and TNF- α secretion as well as M1 related markers [79], is associated with the tuberculosis

infection's regression. However, the anti-inflammatory cytokine production as well as M2 related markers such as IL-10, TGF- β is supposed to be related with the reduced immune response against the disease which eventually results in the failure to regulate this terrible disease [80]. Therefore, the balance between the protective and the inflammatory immune response truly regulates the fate of the infection of tuberculosis.

Consequently, *M. tuberculosis* can go into and reside inside alveolar macrophages and gain protection from some antibiotics [81]. Additionally, for developing drugs for any intracellular bacteria, one very significant factor is whether the applicant drug can work inside the cell or not [80]. In this context, we assessed the effect of STK11 in *M. tuberculosis* (H37Ra) infected J774A.1 macrophage cells in the macrophage mediated killing assay followed by direct killing assay and the macrophage mediated RT-PCR and protein expression study.

5. Conclusion

Tuberculosis is a very dangerous disease nowadays. So, the WHO targeted how to control this. In this context, nutshell of *Cocos nucifera L.* plant-derived isolated glycoprotein, STK11, can restricted the bacilli growth significantly in the resazurin assay at 0-200 µg/ml dose of STK11 and also restricted the bacilli growth within the macrophage in macrophage mediated killing assay and CFU results also claimed that bacilli growth significantly decreased at 28 days after the increased treatment of STK11 (0-10 µg/ml) significantly. An *in-silico* study stated that H-Ras and K-Ras can interact most with STK11, and RT-PCR results supported the same, as H-Ras, K-Ras expression increased during STK11-treated H37Ra-infected macrophages. The effectiveness of STK11 for M2 to M1 polarization was examined, and the results from the expression of cytokines, M1 and M2 indicators, and cellular markers validated this claim. Through a nitrite-dependent mechanism, the STK11 glycoprotein may regulate the development of bacilli within macrophages and induce the M1 signature in H37Ra-infected macrophages.

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There is nothing to declare.

CRediT authorship contribution statement

Arijeet Samanta: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Nayanika Datta:** Methodology, Writing – review & editing. **Madhusri Pramanik:** Investigation, Methodology. **Sujatha Srinivasan:** Data curation, Methodology. **Santanu Kar Mahapatra:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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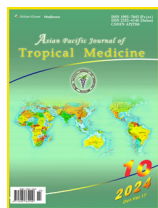
Data availability

Data will be made available on request.

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Review Article

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Macrophage polarization or repolarization in tuberculosis

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ABSTRACT

Tuberculosis caused by *Mycobacterium (M.) tuberculosis* remains a global public health threat. Over the last few decades, anti-tubercular research mainly focused on mechanisms of identifying by which activated macrophages can slaughter or the proliferation of *M. tuberculosis* bacilli prevented in a cell-dependent manner. In this regard, for disease resolution, inflammatory cytokines are very crucial. Here, we demonstrate how macrophages act as the first line of defense against the *M. tuberculosis*. Studies have revealed a dual role in *M. tuberculosis* infection played by macrophages. It is worth mentioning that the macrophages are the crucial immune effector and antigen-presenting cells that play the anti-tubercular response, which is the habitat of *M. tuberculosis*, hence, followed by progressing the disease protecting *M. tuberculosis*. This dual role can be correlated with the different macrophage polarization statuses, namely, M1 and M2. Herein, we have stated how the several polarization conditions of macrophages are directly linked to the immune responses during host and *M. tuberculosis* pathogen interactions. We have proposed that macrophage polarization and repolarization are of paramount significance for the anti-tubercular immune response that may involve a sterile cure of the disease. This article summarizes the immune response to *M. tuberculosis*, the polarization states of macrophages during *M. tuberculosis* and the repolarization of macrophages by some agents during some diseases including *M. tuberculosis*, which may be an important factor in the World Health Organization's target to cure tuberculosis by 2035.

KEYWORDS: Tuberculosis; Macrophage polarization; Effector responses; Antitubercular immune response

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium (M.) tuberculosis* poses a serious public health threat in many countries[1–3]. The effects of tuberculosis continue to be a major source of illness and mortality on a global scale. The World Health Organization (WHO) estimates that in 2024, TB will be responsible for nearly 1.5 million annual deaths. Once alveolar macrophages phagocytose *M. tuberculosis* enters the body through inhalation into the lungs, they then travel to the pulmonary interstitial space, where they draw in immune cells such as mononuclear derived macrophages and create tuberculous granuloma by surrounding themselves with germs[4]. Based on the mode of infection, *M. tuberculosis* infection is of two types-latent TB and active TB[5]. In latent TB, the tuberculous granuloma's crucial function is to restrict the infecting bacteria and prevent the infection's spreading (active TB)[6].

Macrophages are the initial line of defense against specific bacteria through phagocytosis, cytokine secretion, and antigen presentation which differentiate from monocytes and bone marrow hematopoietic stem cells[7,8]. Macrophages showed enormous functional plasticity and in response to the microenvironment, they polarize into a definite phenotype. As per their useful features and morphology, macrophages are basically two types: M1 and M2 stand for classically and alternatively activated macrophages, respectively.

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IFN- γ and lipopolysaccharide (LPS) cause macrophages to polarize into M1 cells. M1 macrophages boost the expression of inflammatory cytokines as IL-12, TNF- α , IL-6, iNOS, and CD86. These have the ability to eradicate intracellular infections and tumour cells, hence enhancing the Th-1 immune response[9,10].

In contrast, anti-inflammatory activity is associated with M2 macrophages[4]. As per different stimuli, M2a, M2b, and M2c are the designations for the three subpopulations of M2 phenotypic macrophages, which differ according to various stimuli. Scavenger receptor, mannose receptor, and arginase-1 (Arg-1) expression are induced in M2a macrophages by IL-4 and IL-13, whereas IL-1R ligands and immune complexes activate M2b macrophages, raising IL-10 production and promoting the response of Th2. Following the released pentraxin 3 and chitinase 3 which play a role in wound healing, IL-10 predominantly activates M2c macrophages[11].

Progressive research claimed that macrophages are the primary host cells against *M. tuberculosis* infection, which are crucial immune effector cells capable of tubercular antigen-presentation followed by inducing an anti-TB immune response. On the contrary, *M. tuberculosis*' residence is macrophages, and it defends *M. tuberculosis*[12]. Hence, these host cell macrophages play a significant dual role during the infection of *M. tuberculosis*[4]. It implies that *M. tuberculosis* has progressed some approaches to survive, although it is capable to replicate within the macrophages. *M. tuberculosis* within the macrophages also acts to prevent the fusion of phagosome-lysosome, inhibits the maturation of phagolysosome, and induces M2 macrophage polarization[6]. M2 phenotype macrophages create an oppressive microenvironment. This can promote latent infection and intracellular growth of *M. tuberculosis*. In order to effectively treat tuberculosis, a greater understanding of macrophage polarization during *M. tuberculosis* infection may be helpful[4].

This mini-review summarizes the concept of macrophage polarization, polarization states of macrophages during *M. tuberculosis*, the concept of immune response during *M. tuberculosis*, and macrophage polarization and repolarization by some agents during some diseases including *M. tuberculosis*, which may be directly associated with the disease progression to anti-tubercular immune response and these could be an important factor in the WHO's goal of curing TB by 2035.

2. Subtypes and activation of macrophage polarization and its function

The human system is highly intricate in its functioning due to the association of many different systems. The immune system is complex, with a network of various fighter immune cells in action. An encounter or challenge with any non-native substance triggers a shot of host immune response, provoking innate and adaptive responses against it. Dendritic cells, macrophages, and natural killer cells are the immune cells connected to innate immune response.

Among the other immune cells, monocyte-derived macrophages are recognized as very versatile. Earlier considered only to be associated with the host defense and apoptotic cells clearance, but now the plethora of literature indicates that macrophages are more than just phagocytic cells and play a pivotal role in many facets due to their dynamicity and their property to orient to the environment they are defined with[13].

Polarization is often defined as "the activation of the macrophages at a given point in time"[14]. Macrophages can be polarized into two different states of activity, known as M1 (classically activated) and M2 (alternatively activated). These two macrophage subsets have been identified to evoke a wide range of functionalities[15–18]. Secreted cytokines, cell surface indicators, and biological roles of these macrophages vary depending upon the polarization state[19]. The final result of arginine metabolism is M1/M2 polarity that occurs through two opposite pathways. One of them is the iNOS pathway, which generates nitric oxide and citrulline from arginine, resulting in macrophages that resemble M1 cells. The other is the arginase route, which converts the M2-like macrophage-producing enzyme arginase into urea and ornithine[20].

Lipopolysaccharide (LPS) and Th1 cytokines including IFN- γ and TNF- α activate macrophages, which then polarize into M1 macrophages. Nonetheless, the transcription factor, T-bet, plays the crucial role in the Th1 cytokines release[11]. TLR-2, TLR-4, CD80, CD86, and MHC-II are several surface characteristics that are present on M1 macrophages. These cells secrete cytokines and chemokines including TNF- α , IL-1, IL-6, IL-12, CXCL 9, and CXCL 10, which have a beneficial effect on normal macrophages, M0 or unpolarized macrophages, and cause them to change into the M1 state. Transcription factors such as NF- κ B, STAT1, STAT5, IRF3 and IRF5 are essential for regulating M1 genes. Additionally, in the case of M1 macrophages, two transcription factors named NF- κ B and STAT1 play a vital role in the M1 macrophages' microbicidal and tumoricidal activities[14,21–23]. Pro-inflammatory cytokines produce against bacterial or viral encounters by macrophages leading to M1 polarization. Neutrophils, natural killer cells (NK cells) and T helper cell type 1 or Th1 are engaged by the cytokines, and these compounds increase pro-inflammatory cytokines and kill external pathogens[24]. Moreover, the activity of killing the M1 phenotype is increased by nitrite production. M1 macrophages possess antitumoral (inhibiting the development of tumours) activities and anti-angiogenic (reducing the new blood vessel's growth that supports tumour growth rather than blocking the growth of tumour cells themselves) activities[24]. Unpolarized macrophages enter either M1 (classical activation) or M2 (alternative activation) when the microenvironment changes. Macrophage reprogramming is the term for this trend[24].

On the other hand, parasite infection and anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF- β) drive M2 macrophages to become polarized as opposed to unpolarized macrophages[21,22]. The M2 macrophages are only directly activated by IL-4 and IL-

13. Other cytokines like IL-33, IL-25 help to produce T-helper type 2 (Th2) cytokines due to activation of GATA-3 in helper T-cells, which increases the activation of M2 macrophages[25]. In addition, M2 is classified into 3 subgroups, *i.e.*, M2a, M2b and M2c. M2 macrophages are known to be "biomedically important" because of their tissue remodelling properties, explained in many inflammatory disease studies and as inflammation pro-resolvers. M2 macrophages are an integral part of tumour microenvironmental as tumour associated macrophages (TAM) that promote blood vessel formation (angiogenesis), thereby ensuring tumour survival[13,26]. M2 macrophages are identified by the expression of some surface markers such as CD 206, CD 163, CD 209, mannitol receptor, FIZZ 1, YM 1/2. Chemokines and cytokines *i.e.*, TGF- β , IL-10, CCL1, CCL17, CCL18, CCL22 and CCL24[22,27] alter unpolarized macrophage or M0 to M2 polarization state[28]. Transcription factors *i.e.*, STAT6, JMJD3, IRF4, and PPAR control the expression of the M2 genes. Till now, the STAT pathway is the one which activates the M2 macrophages[14]. The M1/M2 polarization signals and their effector functions are summarized in Figure 1.

3. M1 and M2 markers in polarized macrophages

By providing the first line of defense, the phagocytic macrophages are dynamic in nature and play a vital role in innate immunity or natural immunity[29]. Depending upon the microenvironment, macrophages orchestrate between two states, "M1/classical activation" or "M2/alternative activation"[30]. In the 1960s, Mackaness introduced macrophage polarization or macrophage activation[30]. Macrophage polarization has been studied in immunology for several years. M1 phenotype has pro-inflammatory properties and enhances pathogen-killing activity. On the other hand, the M2 phenotype has anti-inflammatory properties, contains tissue

remodeling capacity, and they are tumorigenic and designated as an inflammation pro-resolvers[30]. Th1-released IFN- γ or bacterial LPS contribute to M1 polarization, whereas parasite or helminth infection or Th2-released anti-inflammatory cytokines like IL-4 and IL-10 contribute to M2 polarization[24]. Numerous studies disclosed that M1 and M2 phenotypes express unique proteins or specific markers on their surface[31]. CD80, CD68, CD32, CD64, CD86, CCL19 markers are expressed on M1 macrophages, and they can identify these M1 macrophages by those surface markers and M2 macrophages are identified by MRC1, MGL (lectin type receptor), FIZZ1, CD163, CD206, YM-1[13]. M1 effector functions increased in NO generation and pro-inflammatory cytokines IL-12, IL1 β , IL-6, and TNF- α generation (Figure 2). M2 macrophages increased arginase 1 and TGF- β [31]. Also, transcriptomic analysis has delineated unique M1 and M2 markers like CD38 and Fpr2 as M1 markers and c-Myc and Erg as M2 markers[31]. In human beings, metabolism is an essential process. Various essential enzymes regulate this metabolism process and are interconnected by many pathways. When one pathway becomes defective and leads to disease, this interconnectivity becomes obscure[32]. Several signaling pathways such as-JAK-STAT, JNK-P13/AKT, NOTCH and TGF- β -SMAD recruit in macrophage reprogramming. The status of polarization is indicated by the regulation of these pathways[24].

4. Macrophage polarization in *M. tuberculosis* infection

After the infection with *M. tuberculosis* or with the revival of latent infection in granulomas, the progress of active tuberculosis may occur[33–35]. Within granuloma, macrophage polarization is decided by type 1 and type 2 inflammatory signals. During M1 polarization, the product of type 1 inflammatory cytokines gives rise to anti-*M. tuberculosis* immune responses, so these cytokines played a major

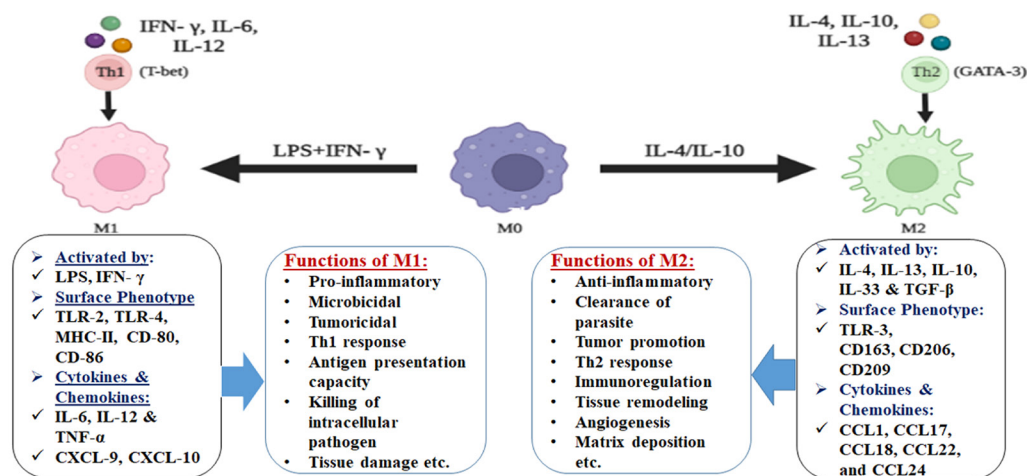


Figure 1. Macrophage polarization and specific function of M1 and M2. In presence of LPS and IFN- γ , M0 macrophage converted to M1 macrophage state. In presence of T-bet transcription factor, Th1 cell recruited M1 macrophage and produced IFN- γ , IL-6, IL-12 cytokines. It produced many surface phenotypes, such as TLR-2, TLR-4, MHC-II, CD-80 and CD-86. It also produced many cytokines and chemokines, IL-6, IL-12, TNF- α , CXCL-9 and CXCL-10. On the other hand, in presence of IL-4/IL-10, M0 macrophage cell was converted to M2 macrophage state. In presence of GATA-3 transcription factor, Th2 cell recruited M2 macrophage cell and produced IL-4, IL-10, IL-13, IL-33 and TGF- β . It produce many surface phenotypes (TLR-3, CD163, CD206, CD209). It also produced many specific cytokines and chemokines.

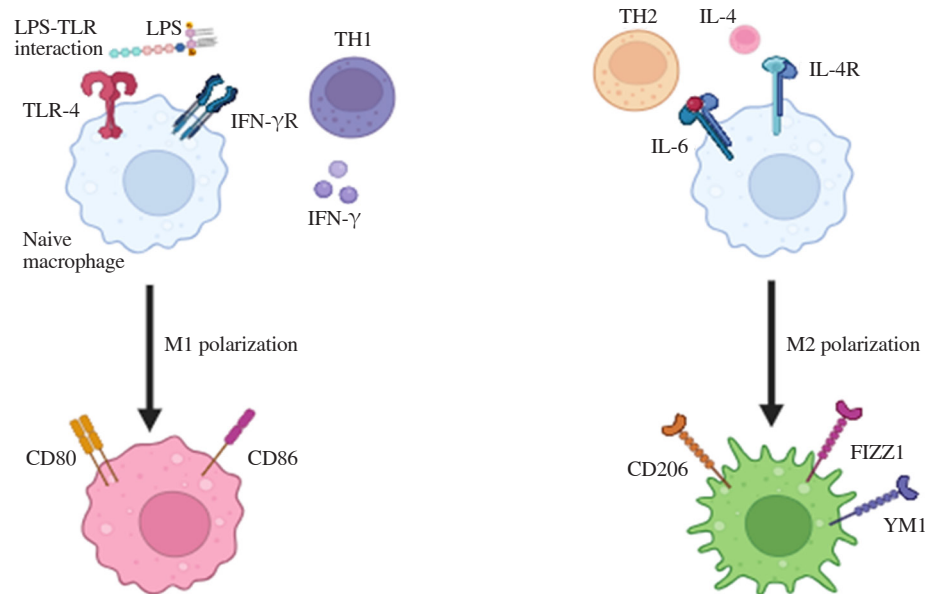


Figure 2. Diagram represents the markers of macrophage polarization.

role in resisting *M. tuberculosis*[36,37] (Figure 3). IFN- γ is described as the driving force behind the M1 program. It has potentiated to destroy *M. tuberculosis*, and it controlled the replication of remainder[38,39]. Other studies strongly support that M1 polarization is also an indicator of the initial phase of the anti-*M. tuberculosis* immune response in several animal models, and it is pretty similar to clinical evidence with active TB[40,41]. In an experiment in a mice model, within the alveolar granuloma compartment, significant amounts of IFN- γ and iNOS signify the M1 polarization during 7 to 30 days post *M. tuberculosis* infection[42,43]. Altogether, the M1 polarization is an integral part of the host immune responses against intracellular pathogenic bacteria and is characterized by the release of proteolytic enzymes, antimicrobial peptides, and pro-inflammatory cytokines and chemokines. It improves phagocytosis and the generation of toxic intracellular granules[38,44].

In addition, the type-2 inflammatory milieu is shifted back to type-1 after effective treatment of TB in infected individuals[45,46]. An earlier report also indicates that M2 signatory IL-4 and IL-13 suppress the autophagy in M1 macrophages and increase *M. tuberculosis* survival[47,48]. Ly *et al.* claimed that higher level type-2 inflammatory signature is skewing the M1 to M2 having ARG1hi iNOSneg in the alveolar granuloma compartment after day 35 up to day 60 during *M. tuberculosis* infection[42,49]. In the later stage, fibrosis in terms of caseous granulomas causes *M. tuberculosis* dissemination, while M2 macrophages obstruct the fibrosis development. It is assumed that the skewing of M2 from M1 shields the immune attack and standstill the pathophysiology caused by *M. tuberculosis*[50].

5. Immune response during *M. tuberculosis*

5.1. Innate immune response during *M. tuberculosis*

In early and chronic infection, macrophages are the main cellular niche for infection of *M. tuberculosis*, so they play a crucial role in Mycobacterial pathogenesis[51]. Macrophages are the chief cells that encounter the pathogen of *M. tuberculosis*, and phagocytosis of *M. tuberculosis* occurs by the alveolar macrophages as well as during the early stage of *M. tuberculosis* infection. It interacts with a variety of different innate immune cells, including neutrophils, natural killer cells, and dendritic cells[52]. By numerous pattern recognition receptors (PRRs), TLR1, TLR2, TLR4, TLR7, TLR8, and TLR9 are Toll-like receptors. NOD1, NOD2, NLRP3, and NLRC4 are Nod-like receptors. MR, Dectin-1, Dectin-2, Dectin-3 and DC-SIGN, CL-LK, DCIR, Mincle are C-type receptors. *M. tuberculosis* is recognized by those innate immune cells (*e.g.*, dendritic cells, NK cells, *etc.*). To eliminate *M. tuberculosis* pathogen from the host body, the host receives signaling from pattern recognition receptors or PRR and starts to create various cellular functions *i.e.*, apoptosis, autophagy, phagocytosis, and inflammasome activation.

Pathogen-associated molecular patterns, or PAMPs, in *M. tuberculosis* like carbohydrates, glycolipids, and lipoproteins are recognized by PRRs like Nod-like receptors, Toll-like receptors, and C-type receptors at different stages of infection (early and chronic stages), and they form a vast network of interconnected coordinated signalling pathways that speed up gene expression profiles in host cells[53]. In order to demonstrate the significance of inflammatory cytokines, such as IL-1, IFN- γ , IL-12, and

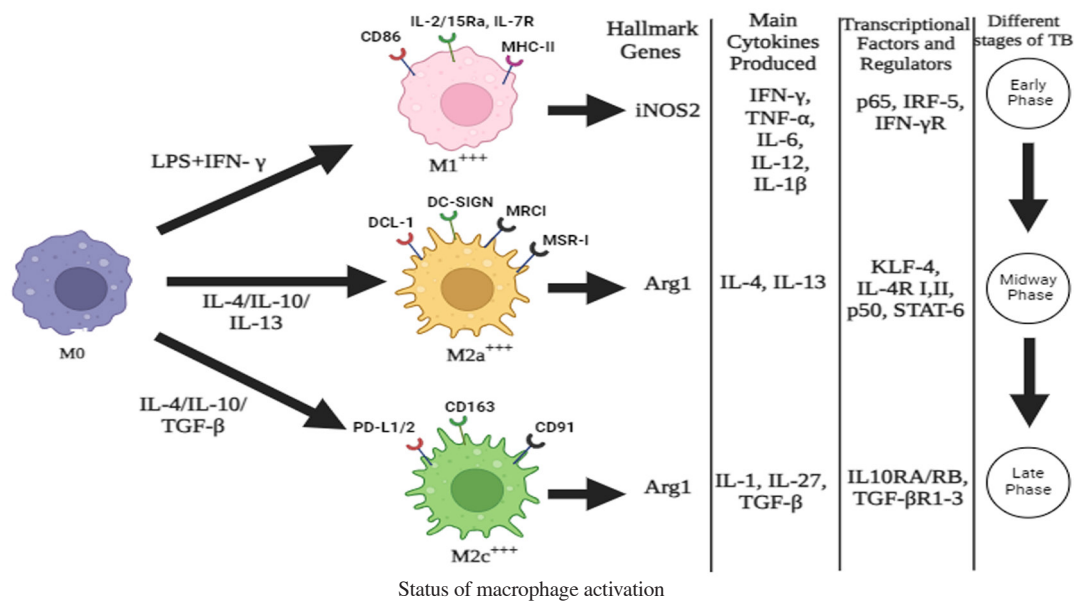


Figure 3. Involvement of surface markers, hallmark genes, cytokines, and transcription factors and the state of macrophage polarization during tuberculosis infection. In presence of LPS and IFN-γ, M0 macrophage was converted to M1 macrophage where many surface markers (CD86, MHC-II, etc.) involved and iNOS2 hallmark genes present. In this state, some cytokines were produced, i.e., IFN-γ, TNF-α, IL-6, IL-12, IL-1β. Many transcriptions factor, i.e., p65, IRF-5, IFN-γR recruited. In presence of IL-4/IL-10/IL-13, M0 macrophage is converted to M2a macrophage state where many surface markers (DC-SIGN, MRC-I, MSR-I, etc) involved and Arg1 hallmark gene present. In this state, IL-4, IL-13 cytokines produced. Here, KLF-4, IL-4R I,II, p50, STAT-6 transcription factors and regulators recruited. On the other hand, in presence of IL-4/IL-10/TGF-β, M0 macrophage is converted to M2c macrophage state, where some surface markers (CD163, CD91, etc.) involved. In this state, IL-1, IL-27 cytokines produced. Based on the disease progression stage of tuberculosis, in early phase M1 state, middle phase M2a, and late phase M2c state was observed.

macrophage inflammatory protein-1 (MIP1α/CCL3), gene profiling studies have been conducted proof in the protection against infection of *M. tuberculosis*[54].

The macrophage polarization spectrum is divided into the M1 and M2 phenotypes based on how macrophages develop and operate[14]. Human monocytes develop into M2 macrophages during active tuberculosis, which increases pathogen permissiveness, protease-dependent mortality, and immunomodulation[55]. This process is characterized by the phenotype CD16+CD163+MerTK+pSTAT3+. Mycobacterial DnaK (HSP 70, heat shock protein 70) indexes the M2 macrophages in an IL-10-dependent way[56]. Macrophages are also known as myeloid suppressor cells (MSCs), and these macrophages suppress the T-cell response *via* the secretion and release of TGF-β and IL-10. So, in tuberculosis, these macrophages exhibit both suppressive functions as well as help in the regulation of inflammation and infection[57]. In a histopathology study, granulomas are the characteristics of tuberculosis that contain various transformed macrophages, i.e., epithelial cells, foam cells, and multinucleated giant cells[58]. More recently, it was stated that TLR2 signaling promotes polyploidy of the macrophage, and TLR2 inhibits genomic instability by regulating Myc and ATR expression. It indicates that in presence of persistent inflammatory stimuli, pathways involved in developing cancer cells surprisingly instruct a polyploid macrophage fate and regulate granulomatous tissue remodeling[59].

5.1.1. Pattern recognition receptors for identifying *M. tuberculosis* in innate immune response

Identification of *M. tuberculosis* by pattern recognition receptors such as C-type lectin receptors (CLRs), nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), Toll-like receptors (TLRs), and Cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) is the first step in initiating an innate immune response[60]. In mice models, the most necessary TLRs for controlling *M. tuberculosis* are TLR2 and TLR9, in which *M. tuberculosis* lipoproteins and lipoglycans are recognized by TLR2 and unmethylated CpG DNA are identified by TLR9[61–64].

Mice missing both TLR2 and TLR9 are more unprotected or unsafe than TLR2 or TLR9 single knockout mutants. It indicates that each TLR contributes to the immune response in a nonredundant way[61]. Numerous CLRs have been asserted to have a functional role in the immunological recognition of *M. tuberculosis* in addition to TLRs. Besides TLRs, CLRs also have a significant role in recognizing *M. tuberculosis*. Moreover, scientists investigated and found that in mutant mice, each individual CLRs have a restricted participation[65,66], which might be partially clarified by redundancy in function. Eventually, in *in vitro* culture of myeloid cells, the NLR NOD2 can detect muramyl peptides generated from peptidoglycan of a bacterial cell wall, giving cytokine responses to the *M. tuberculosis*[67–69]. At some time, when mice are missing NOD2, they become infection resistant and show little vulnerability six months after infection[70].

Three distinct studies claimed that for type I interferon production, cGAS is very important, and it indicates that DNA is the pathogen-associated molecular pattern (PAMP) that activates STING[71–73]. On the other hand, *M. tuberculosis* has been established to activate type I interferons by direct STING identification of cyclic-di-AMP generated by the bacterium. CLRs, TLRs, and TLRs aid in the immunological response to *M. tuberculosis* by increasing the synthesis of chemokines and pro-inflammatory cytokines. However, bacterial pathogens exploit an antiviral pathway, which is an example of the cGAS-STING pathway, to promote pathogenesis[74].

5.2. Adaptive immunity against *M. tuberculosis*

The adaptive immune response defends the host from *M. tuberculosis* and regulates bacterial reproduction. Extreme sensitivity to mycobacterial infections is best seen in lymphopenic HIV patients and gene-deficient animals missing MHC class II or T-cells in general. Direct antimicrobial activities of antigen-specific T cells and cytokine production are the primary characteristics of the adaptive immune response to *M. tuberculosis* infection[75]. Additionally, the longevity of antigen-specific memory T cells provides the foundation for the creation of vaccines that promote protection against mycobacterial infections[75]. B-cells, $\gamma\delta$ T-cells, and CD1-restricted T-cells all have growing roles as well. These offer targeted responses to a wide range of *M. tuberculosis* antigens, which are presented by MHC class I and II in the traditional manner[76]. The malignancy of adaptive immune responses is caused by severe inflammation or by being rendered inefficient by repeated antigen exposure. CD4 T-cell responses during *M. tuberculosis* infection. CD8 T-cells protect the host from *M. tuberculosis* infection, and inhibitory receptors play a key role in *M. tuberculosis* infection. In addition, certain cells, such as B-cells, T-cells, and $\gamma\delta$ T-cells, CD1-restricted lymphocytes, and mucosal-associated invariant T (MAIT), also protect the host from *M. tuberculosis* infection[60].

6. An agent can cause polarization or repolarization of the macrophage's phenotypes

Refai *et al.* claimed that ESAT-6, a plentifully secreted protein of *M. tuberculosis*[77], can switch from M1 polarized macrophages to the M2 phenotype. Interestingly, they also claimed that ESAT-6 moderated the manifestation of pro-inflammatory markers (CD80, CD86, IL-12, IL-23, IL-6, CXCL-10) of fully activated M1 macrophages[78]. Wang *et al.* claimed that pleural macrophages polarize from M1-like to M2-like phenotype after 3.5 days post-treatment of antibiotics (*e.g.* rifampicin, isoniazid, ethambutol, pyrazinamide). They also showed that mediators of the pro-

inflammatory markers IFN- γ , TNF- α and IL-6 in tuberculous pleural effusion (TPE), remained decreased significantly after 2-3 days post antibiotics treatment[79]. Pinosylvin, a naturally occurring stilbenoid mostly found in Scots pine trees, alters macrophage polarisation from the pro-inflammatory M1 phenotype to the M2 phenotype, according to Kivimäki *et al.*[80]. They asserted that pinosylvin has an effect on M2-type activation and inhibits the expression of M1-type markers. They demonstrated that pinosylvin boosted the expression of mannose receptor C type 1 (MRC1) and arginase-1 (Arg-1) in murine macrophage[80]. According to Chatterjee *et al.* rocaglates, a natural product derivative from *Aglaia* genus plants[81], can direct polarization of macrophage toward the M1-like phenotype and prepare macrophages at little concentrations of the M1 to induce cytokine IFN- γ and to inhibit their sensibility to IL-4, an ideal activator of the M2-like phenotype[82]. By promoting the development of the M1 markers in the cytokine pre-stimulation model, Jayaraman *et al.* claimed that *Oceanimonas* sp. BPMS22-derived PPI (Protein Protease Inhibitor) transformed into an M1-like phenotype from the resting macrophages (M0) and repolarized to M1 from the M2 phenotype. Additionally, they said that the PPI-incited macrophage M1 repolarization from the M2 macrophage phenotype in the *Leishmania donovani* infection paradigm[83]. Tian *et al.* claimed that the CU-CPT22, a TLR2/TLR1 inhibitor[84], transformed the M1 macrophages, induced by LPS, to the phenotype of M2 *in vitro*. The M1 marker CD86's expression is significantly repressed by CU-CPT22 and it also promotes to the M2 marker CD206's expression, and the inflammatory cytokine production in macrophages is inhibited by CU-CPT22. Their results verified that the M1 phenotype is suppressed by CU-CPT22 and it also promotes the M2 macrophage phenotype[85].

According to Chen *et al.* Oligo-Fucoidan extraction from brown seaweed, which is a sulfated polysaccharide[86], induces to M1-like macrophages from polarization of monocyte and repolarized into M1 phenotypes from M2 macrophages. They also claimed that intracellular ROS slaked by Oligo-Fucoidan and M1 macrophage polarization is benefited by mitochondrial superoxide, directly or indirectly. In addition, in *in vitro* process, on M2 macrophage polarization, the chemotherapy drawback is suppressed by Oligo-Fucoidan and in *in vivo* process, infiltration of M2 macrophage is prevented[87]. According to Kou *et al.* IgG/Bb@BRPL, an opsonized nanoparticle, repolarizes to anti-inflammatory M2-phenotype from M1 macrophages *via* ROS rummaging and deactivation of NF- κ B pathway, and for local chondrocytes remodeling an additional promising microenvironment. They also claimed that with the help of *in vivo* studies, opsonized IgG/Bb@BRPL remarkably indorsed Osteoarthritis (OA) cartilage regeneration and repair. Their study suggests an opsonization-based drug delivery plan for OA treatment[88]. The combination therapy of ibrutinib and

Table 1. Immunomodulators and macrophage polarization.

Immunomodulators	Source/Identity	Polarization or repolarization	References
ESAT-6	Protein of <i>M. tuberculosis</i>	M1 to M2 phenotype	[77,78]
Antibiotics	Rifampicin, isoniazid, ethambutol, pyrazinamide	M1 to M2 phenotype	[79]
Pinosylvin	A natural stilbenoid mainly found in Scots pine plant trees	M1 to M2 phenotype	[80]
Rocaglates	Natural product derivative from <i>Aglaia</i> genus plants	Toward M1 phenotype	[81,82]
BPMS22	<i>Oceanimonas</i> sp.	M2 to M1 phenotype	[83]
CU-CPT22	A TLR2/TLR1 inhibitor	M1 to M2 phenotype	[84,85]
Oligo-Fucoidan	A sulfated polysaccharide isolated from the brown seaweed	M2 to M1 phenotype	[86,87]
IgG/Bb@BRPL	An opsonized nanoparticle	M1 to M2 phenotype	[88]
Ibrutinib and selinexor	Ibrutinib is a BTK inhibitor, Selinexor, a blood–brain barrier (BBB) permeable small molecule	M2 to M1 phenotype	[89]

selinexor, according to Jiménez *et al*, is capable of increasing mouse survival as well as changing the innate immune response to an additional inflammatory phenotype, which is precisely defined by downregulation of signal regulatory protein α (SIRP α) and PD-1 in M2 macrophages[89]. Additionally, the proportion of M1 macrophages is increased by this combination therapy, and additional M1 and M2-like properties are modulated with the loss of pro-tumoral M2 characteristics. They also claimed that in malignant cells, this combination treatment had a cytotoxic effect and also preferred an anti-tumoral innate immune response through tumor-infiltrating macrophage polarization shifted to M1 inflammatory and decreased SIRP and PD-1 expression in the residual tumor-promoting macrophages of M2[89]. These immunomodulator based macrophage polarization is summarized in Table 1.

7. Challenges in the characterization of M1–M2 polarization

The lethal intracellular pathogen *M. tuberculosis* is able to establish a permanent residence inside the host macrophages. *M. tuberculosis* bacilli uses a number of immune-evasion techniques, such as the suppression of pro-inflammatory markers and the host macrophages' release of the immunosuppressive cytokine IL-10 to delay the resolution of its infection and cause severe immune suppression. A few reports indicated that M1 macrophage polarization resulted in granuloma formation, which has an innumerable role in resolving *M. tuberculosis* infection. However, M1-M2 polarization status in macrophages during tuberculosis is still not well characterized during latent, active, and disease-resolute states that may provide countless efforts to set up the future strategic plan for the sterile cure of tuberculosis, particularly in drug-resistant cases.

8. Conclusions and perspectives

Tissue resident macrophage is the most significant immune effector and antigen-presenting cell. It is in charge of the anti-tubercular

immune responses after activation of it. Unfortunately, the host macrophage is also the habitat of *M. tuberculosis* disease and protects *M. tuberculosis*. However, this dual role is not well characterized even through studying different innate and adaptive immune responses against TB. Several reports indicated that macrophage polarization/repolarization status is directly correlated with disease progression and resolved state, which is not yet thoroughly studied during TB. Very few reports indicated that M1 type macrophage polarization can be modulated into the M2 type macrophage polarization and vice-versa by natural, semi-synthetic, and chemical agents as host-directed therapies, antitubercular peptide, first-line drugs, and so many. Hence, the future study on macrophage polarization and repolarization is emerging during *M. tuberculosis* infection mediated tuberculosis. This may contribute to the WHO's mission to eradicate tuberculosis by 2035.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Both AS and AJ wrote the original draft. AS, SM, RH reviewed, edited and revised the manuscript. SKM conceptualized, supervised, visualized and performed as project administration

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