Modulation of the activation of dendritic cells and macrophages by the proteins of *Mycobacterium tuberculosis*

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Certificate

The project work entitled "**Modulation of the activation of dendritic cells and macrophages by the proteins of *Mycobacterium tuberculosis***" submitted by **Sanpreet Singh** for the degree of **Doctor of Philosophy**, has been carried out under the supervision of **Dr. Vijayender Bhalla** and **Dr. Javed Naim Agrewala** at the CSIR-Institute of Microbial Technology, Chandigarh.

The research work is novel and has not been either partially or completely submitted for any other degree or diploma from any other institute or university.

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Dedicated to

My Family

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CSIR-IMTECH

ABBREVIATIONS

Ab	Antibody
Acr1	Alpha-crystallin1
ACK	Ammonium chloride lysis buffer
AMs	Alveolar Macrophages
ANOVA	Analysis of variance
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
BMCs	Bone marrow cells
BMDC	Bone marrow derived dendritic cell
Bregs	B regulatory cells
BSA	Bovine serum albumin
CCR7	C-C Motif Chemokine Receptor 7
CFA	Complete Freund's adjuvant
CFUs	Colony forming units
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CL-LK	Collectin LK
CLRs	C-type lectin receptors
CMPs	Common myeloid progenitor cells
CLPs	Common lymphoid progenitorcells
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DCs	Dendritic cells
DC^{MPT64}	DCs + MPT64
DCIR	Dendritic cell immunoreceptor
DC-SIGN	DC-specific intercellular adhesion molecule-3 grabbing nonintegrin
ECs	Epithelioid cells

ESAT-6	Early Secreted Antigenic Target 6 kDa
FASII	Fatty acid synthase type II
FBS	Foetal bovine serum
FCS	Foetal calf serum
Fc	Fragment crystallizable
Fig	Figure
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMPs	Granulocyte-macrophage progenitors
H_2DCFDA	2,7-dichlorofluorescin diacetate
HIV/AIDS	Human Immunodeficiency virus/Acquired Immune Deficiency Syndrome
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic stem cells
iMFI	Integrated mean/median fluorescence intensity
iNOS	Inducible nitric oxide synthase
i.v.	Intravenous
IFNs	Interferons
IFN-γ	Interferon gamma
IL-4	Interleukin 4
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LAL	Limulus amebocyte lysate
LCs	Langerhans cells
RD2	Region of deletions 2
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
ManLAM	Mannose-capped lipoarabinomannan
M-CSF	Macrophage colony stimulating factor
MDPs	MΦ/DC progenitor cells
MDSCs	Myeloid derived suppressor cells

MFI	Mean fluorescent intensity
MGCs	Multinucleated giant cells
MGO	Methylglyoxal
MHC I/II	Major histocompatibility complex I/II
MMP-1	Matrix metalloproteases 1
MOTT	Mycobacteria other than *Mtb*
MPT64	*Mycobacterium tuberculosis* protein
MR	Mannose receptor
MSCs	Mesenchymal stem cells
Mtb	*Mycobacterium tuberculosis*
MΦ	Macrophage
NEDD	Neural precursor cell expressed developmentally downregulated 8
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cells
NLRs	Nucleotide binding oligomerization domain binding like receptor
NO	Nitric oxide
NOD	Nucleotide binding oligomerization domain
NOX2	NADPH oxidase
OADC	Oleic Acid albumin dextrose catalase
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBST	1X PBS + 0.1% Tween-20
PGE2	Prostaglandin E2
pH	Negative logarithm of hydrogen ion concentration
PknE	Protein kinaseE
PMNs	Polymorphonuclear neutrophils
PPARγ	Peroxisome proliferator-activated receptor gamma
PPD	Purified protein derivative
PRRs	Pattern recognition receptors

PTAT	Patch Test for Active Tuberculosis
PtpA	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
r	Recombinant
rpm	rotations per minute
RLRs	Retinoic acid inducible gene-I like receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SENP8	SUMO/sentrin-specific peptidase family member 8
s.c.	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error mean
SNs	Supernatants
T1R	Toll interleukin 1 receptor
TB	Tuberculosis
TBA	Tetra-butyl alcohol
TCR	T cell receptor
Tfh	T follicular cell
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TLRs	Toll like receptors
TNF-α	Tumour Necrosis Factor alpha
Tregs	T regulatory cells
WHO	World Health Organization

Symbols

α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
±	Plus-minus
µ	Micro
≤	Less than or equal to
≥	More than or equal to

Units of measurement

%	Percentage
×g	Centrifugal force equal to gravitational force
µg	Microgram
µl	Microlitre
°C	Degrees Celsius
d	Day(s)
h	Hour(s)
kDa	kilo Dalton
kHz	kilo Hertz
M	Molar
mg	Milligrams
min	Minutes
ml	Milliliter
mM	Millimolar
mV	Millivolts
ng	Nanogram
nM	Nanomolar
nm	Nanometer
OD	Optical density
V	Volts

Techniques

ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence assisted cell sorting
SEM	Scanning electron microscopy
CLSM	Confocal laser scanning microscopy
WB	Western Blotting
qRT-PCR	Quantitative reverse transcription PCR

Chemicals

$CaCl_2$	Calcium chloride
C_2H_3N	Acetonitrile
$C_3H_4N_2$	Imidazole
DMSO	Dimethylsulfoxide
H_2SO_4	Sulphuric acid
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HRP	Horse radish peroxidase
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Potassium bicarbonate
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride

NH_4HCO_3	Ammonium bicarbonate
OPD	Ortho phenylenediamine
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulphate
Tris-HCl	Tris-Hydrochloric acid

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Chapter-1

Introduction

1. INTRODUCTION

Mycobacterium tuberculosis (*Mtb)* persists skillfully by deceiving the host immune system. It adopts multiple contrivances *viz* disrupts the fusion of phagosome-lysosome (Mehra et al., 2013), neutralizes the function of reactive nitrogen and oxygen free radicals (Lerner et al., 2015), induces necrosis of macrophages (Queval et al., 2017; Wang et al., 2012), impairs the antigen processing and presentation (Cambier et al., 2014; Harding and Boom, 2010), hampers apoptosis of infected cells (Velmurugan et al., 2007), inhibits autophagy and lysosomal functions (Ouimet et al., 2016) and impedes the production of interferons (Mayer-Barber et al., 2011). Suggesting that the pathogen is not only utilized by the host to generate protective immunity, but the bacterium too successfully subjugates the host immune system (Ernst, 2018; Zhai et al., 2019).

The *Mtb* uses its proteins like Acr1 to successfully inhibit the maturation of DCs (Amir et al., 2017; Siddiqui et al., 2014), DCs exposed to ManLAM failed to fully mature and showed a decline in the level of MHC I and MHC II, CD83, CD86, and CCR7 (Dulphy et al., 2007), and LprA, LprG, and LpgH too downregulates the MHC II expression (Popov and Schultze, 2008; Wolf et al., 2007). Further, an encounter of DCs with *Mtb* secretory Ag (MTSA) during differentiation promotes the survival of the intracellular bacterium by inhibiting the production of ROS (Sinha et al., 2006). Moreover, ESAT-6 has been reported to inhibit the phagolysosome fusion (Tian et al., 2005). Similarly, PtpA and SapM proteins inhibit phagosome maturation. PtpA suppresses the acidification of the phagosomes (Wong et al., 2011) and Ndk interacts with the Rac1 protein of host cells and prevents the assembly of the NOX2 (Miller et al., 2010). Many proteins such as PknE (protein kinaseE), Ndk, nuoG, SecA2, and ptpA have been reported to induce necrosis and apoptosis of infected host cells (Jayakumar et al., 2008; Sun et al., 2013; Velmurugan et al., 2007). Furthermore, *Mtb* elicits the generation of MDSCs that fail to activate T cells (Ribechini et

al., 2019). Along with Tregs, myeloid-derived suppressor cells (MDSCs) presence is reported in TB granulomas to hinder the functionality of effector T cell and therefore encourages the disease severity (Banchereau and Steinman, 1998). MDSCs utilize nitric oxide (NO), arginase 1, and methylglyoxal (MGO) with other constituents that cause nitrosylation of T cell receptors, programmed cell death of T cells, and generation of T regulatory cells (Baumann et al., 2020; El Daker et al., 2015; Knaul et al., 2014). The vast detrimental effect of MDSCs is mediated by the strong suppression of Th1 and Th17 immunity along with a decline in the secretion of innate cytokines (Yang et al., 2014). Consequently, it becomes crucial to decode the function of unexplored proteins exploited by *Mtb* to support the generation of MDSCs and thereby elude the immune system.

MPT64 is a secretory protein (m.wt. ~24 kDa) and is less studied for its immunomodulatory role (Harboe et al., 1986; Wang et al., 2007). It associates with the beta grasp proteins family (Sehnal et al., 2021). The level of MPT64 is high in the serum and sputum of active TB patients (Mehaffy et al., 2017). Moreover, MPT64 enhances the secretion of IL-6, TNF-α, IL-1β and IL-10 by the macrophages, and therefore may be responsible for suppressing the immune system (Fan et al., 2013). Recently, it has been shown that MPT64 inhibits the apoptosis of macrophages through up-regulation of bcl-2 and promotes the survival of *Mtb* (Wang et al., 2014). Further, the MPT64 mutant strain (Mtb∆*mpt64*) generates less bacterial burden in mice, as compared to wildtype *Mtb* (Stamm et al., 2019).

Keeping in view the aforesaid facts, we have tried to delineate here the immunomodulatory role of MPT64 on DCs activation and differentiation. Interestingly, we noted that the early encounter of DCs with MPT64 (DC^{MPT64}) during their differentiation, skewed them towards a myeloid suppressor cell phenotype. The DC^{MPT64} showed accumulation of methylglyoxal, an advanced glycation end product, which along with other soluble mediators is implicated in suppressing

the host immune system. DC^{MPT64} preferably generated Tregs and suppressed Th1, Th2 and Th17 cells activation. Moreover, DC^{MPT64} demonstrated decreased tendency to phagocytose and kill *Mtb*. Consequently, it may be concluded from the study that *Mtb* utilizes MPT64 to make DC^{MPT64} a safe shelter for its persistence, and this may be one of the mechanisms adopted by the mycobacterium to escape the host immune system.

Chapter-2

Aims and Objectives

2. AIMS AND OBJECTIVES

Mycobacterium tuberculosis is a causative agent of TB. It persists for thousands of years and is the major cause of death worldwide. *Mtb* has emerged as a smart and successful pathogen, which can survive in the hostile milieu of the host. It utilizes a myriad of strategies to circumvent the host immune response. Macrophages (MΦs) and dendritic cells (DCs) are the primary innate cells infected by *Mtb*. Within these cells, *Mtb* survives for a long time and modulates the immune response mounted against it (Wayne, 2001). DCs are the prime cells that bridge innate and adaptive immunity. Various reports have elucidated that DCs functionality can be modulated by the *Mtb* antigens (Chatterjee et al., 2011; Latchumanan et al., 2002). During infection, *Mtb* secretes numerous proteins that implicate a modulatory effect on the APCs (Gehring et al., 2004; Sherman et al., 2001). Within the infected host cells, *Mtb* inhibits phagolysosome fusion for its survival and persistence by secreting an array of proteins (Sturgill-Koszycki et al., 1994). The ESAT-6 inhibits phagolysosome fusion (Simeone et al., 2012), PtpA and SapM restrict the phagosome maturation and acidification (Wong et al., 2011), Ndk interacts with Rac1 protein of host cells and prevents the assembly of NOX2 (Miller et al., 2010). Moreover, *Mtb* has devised various approaches to control the surface exhibition of MHC II and costimulatory molecules on the APCs (Cooper, 2009). Proteins like LprA, LprG and LpgH inhibit the MHC II expression (Gehring et al., 2004; Noss et al., 2001; Pecora et al., 2006). Acr1 has diametric role on DCs. Pre-exposure of Acr1 during DC maturation results in impairment in its function. In contrast, an encounter of Acr1 after DC maturation promotes the bolster of their stimulatory function (Amir et al., 2017; Siddiqui et al., 2014). The depletion of Acr1 leads to the loss of dormant stage by *Mtb* (Hu et al., 2015).

Apoptosis is the key pathway by which infected APCs control *Mtb* infection. *Mtb* employs cascade of molecules that instigates necrosis and inhibits the apoptosis of infected host cells, resulting in the spread

of *Mtb* to nearby cells and organs. Various proteins of *Mtb* viz PknE (Protein kinaseE), Ndk, nuoG, SecA2 and ptpA have been associated with the killing of the infected cells (Jayakumar et al., 2008; Sun et al., 2013; Velmurugan et al., 2007). MPT64 is an important protein of *Mtb*, which is chiefly secreted during the active phase of the infection, and remains least explored for its immunomodulatory role on DCs. Recently, it has been shown that MPT64 inhibits the apoptosis of macrophages and other immune cells (Wang et al., 2014). Further, MPT64 contributes to *Mtb* pathogenesis, as evidenced by its enhanced expression and pro-apoptotic markers and induction of the phenomenon in epithelioid and multinucleated giant cells (Mustafa et al., 2007, 2008).

The above-mentioned studies illustrate that *Mtb* release numerous molecules to tame and tune the host immunity. DC imparts an imperative role in limiting the progression of *Mtb* by linking the innate and adaptive immune systems (Banchereau and Steinman, 1998). The role of MPT64 in modulating the generation and differentiation of DCs remains a novel area to be yet explored. Thereupon, in the present study we examined the influence of MPT64 on the differentiation and activation of DCs to achieve the following objectives:

1. Cloning and expression of *Mtb* protein MPT64 in *E. coli*.
2. Purification of MPT64.
3. Impact of MPT64 on the activation and differentiation of dendritic cells (DCs) and macrophages (MΦs).
4. Examining the skewing of the bone marrow cells differentiation towards myeloid cells from DCs by MPT64.
5. Studying the role of MPT64 on phagocytosis of *Mtb* and controlling the intracellular survival of the bacterium in the DCs.
6. Elucidation of the mechanism responsible for skewing of DCs to myeloid cells.

Chapter-3

Review of Literature

3. REVIEW OF LITERATURE

3.1. Pathogenesis of *Mtb*. The potential of evading host immune response by various mechanisms makes *Mtb* one of the smartest and most successful human pathogens (Korb et al., 2016). TB is not a new disease to mankind; rather, it is infecting the human population for thousands of years (Morse et al., 1964). Because of its high rate of infection and increased drug resistance cases, the WHO in 1993 disclosed TB as global health emergency. According to the Global TB report, 2021, there occurred nearly 1.3 million deaths among people negative for HIV with additional 2,14,000 deaths among people positive for HIV . India accounted for 34% of the total number of TB deaths in 2020. Geographically, most of the TB cases have been reported in 2020 and are from South-East Asia, Africa and Western Pacific regions with some other countries also. India accounts for 26% of cases of TB as a global load in 2020 and is among the top countries with high incidences of TB (WHO, 2020). The structure of *Mtb* makes it more resistant to immune response and antibiotics. The presence of mycolic acid, lipoarabinomannan, phosphatidylinositol mannosides and arabinogalactan in the cell wall of *Mtb* confers them a smooth and waxy appearance along with resistance against the acidic and basic environment of cell, antibiotics and lysosomal enzymes.

Mtb was discovered by Robert Koch (1882) as a Gram-positive, acid-fast bacillus (Sakula, 1982). This pathogen has the potential to reside within host cells in latent form and has the tendency to reactivate when an individual becomes immunocompromised (Lin and Flynn, 2010; Parrish et al., 1998). The pathogenesis of TB starts with the inhalation of *Mtb* as a small droplet (1-2 μm). Further, it has been seen that 1-10 bacilli are enough to cause disease in a healthy individual (Russell, 2007). Within the host, the primary targets of infection are alveolar macrophages and dendritic cells. These alveolar macrophages phagocytose the *Mtb* and initiate a cascade of immune events. Inside the macrophage, these bacilli inhibit the phagolysosome fusion for its

survival and progression (Sturgill-Koszycki et al., 1994). Further, these bacilli then translocate to the cytoplasm of the cell and initiate replicating over there. This leads to the initiation of pro-inflammatory response, and because of that, there occurs an accumulation of mononuclear cells from the nearby circulation around the infected host cell, leading to a granuloma formation (Russell et al., 2009). Granuloma is a compact structure with an infected alveolar macrophage as its core surrounded by layers of foamy macrophages, neutrophils, mononuclear cells and lymphocytes. Further, the recruitment of *Mtb*-specific T cells occurs around three weeks post-infection (Russell et al., 2009). *Mtb* phagocytosed DCs migrate to the local lymph nodes and prime T cells; further, these lymphocytes migrate to the infection site and become the part of granuloma structure. On the other side, *Mtb* starts growing extracellularly in the lipid-rich necrotic granuloma, which becomes firm with reduced blood vessels. This caseous structure of granuloma becomes hypoxic and limits the proliferation of *Mtb*. Upon reactivation of this disease, there is increased secretion of matrix metalloprotease 1 (MMP-1) by macrophages in the granuloma, leading to the softening of this hard granuloma structure with the release of all these bacilli into the airway, which upon coughing and sneezing comes into the environment and infects the nearby person (Kaplan et al., 2003; Ong et al., 2014). Granuloma is a hallmark of identification of TB in the host and is a complex structure needed to be explored more. As it has been evidenced that granuloma formation has a protective role in terms of induction of T cell immune response in animals (Chackerian et al., 2002); on the other side it has a detrimental role as it promotes proliferation and survival of *Mtb* (Volkman et al., 2010). So, the role of this structure in terms of protection against *Mtb* remains to be delineated. Most importantly, this granulomatous structure provides protection to *Mtb* from IFN-γ secreting T cells.

3.2. Active and Latent TB. Active TB is a condition, which is characterized as clinically symptomatic i.e., an individual affected with *Mtb* having TB symptoms. In active TB there is induction of Th1 immune response by the APCs having *Mtb* antigen presentation potential. In general terms, when an infected person sneezes or coughs, will generate aerosols having the *Mtb* bacilli. These aerosols taken up by nearby individuals and make them diseased, as *Mtb* subsequently infect the macrophages and DCs, leading to granuloma formation followed by proliferation and spread of *Mtb* bacilli from the necrotic granuloma to other locations and organs. There are various medicines available for the treatment of active TB. In contrast, there is another form of TB, i.e., latent TB. Generally, latency, dormancy and persistence are the terms used to define latent *Mtb* infection and its pathogenesis (Gomez and McKinney, 2004). Basically, latency is a tuberculous lesion without any symptoms. Latency is a well-established condition between the host and pathogen. During latency, the host will not be able to generate an immune response against the pathogen and vice-versa. This happens in the case of *Mtb*, by well-controlled growth within the host cell (Orme and Roberts, 2001). Latent TB is a clinically asymptomatic and non-contagious (Soper and Amberson, 1938). An interesting fact about latent TB is that it cannot be cured with any anti-tuberculous drugs, as *Mtb* during this stage is non-responsive to drugs. *Mtb* in latent condition can survive for a longer time and upon getting favourable conditions, it will relapse into an active form (Comstock et al., 1974; Wayne, 1994). Hence, the complete elimination of this pathogen is very difficult and is a slow process. Another interesting fact about latent *Mtb* is that it is also known as dormant i.e., metabolically inactive (Soper and Amberson, 1938). During dormancy, *Mtb* is viable but is not culturable using standard methods (Barer and Harwood, 1999). Further, to study the hypoxia-induced persistence of non-replicating *Mtb*, Wayne and colleagues developed an *in vitro* persistence model of *Mtb*, known as the "Wayne model". At the cellular level, *Mtb* resides in a very hostile condition, where they

modulate the host cell (macrophages or DCs) for its persistence and growth. Most importantly, *Mtb* is able to evade the immune response generated against it through immunomodulatory mechanisms (Gomez and McKinney, 2004). Further, *Mtb* has been seen to inhibit the apoptosis of macrophages. And upon infection, there is induction of heat shock proteins (HSPs). Through proteomic studies, it was established that during stress, starvation or hypoxia conditions, there is induction of heat shock protein specifically the alpha-crystallin chaperone protein homologue (16 kDa, hspX, Acr1) and Acr2 (Wayne and Hayes, 1996; Yuan et al., 1996). Various studies have shown the relation between the Acr1 and immune-regulation of immune cells (Wayne and Lin, 1982).

3.3. Immune response and *Mtb* infection. The entrance of aerosolized *Mtb* in host lungs instigates enormous immune cells such as alveolar MΦs, neutrophils, DCs followed by migration of various other immune cells to the infection site. Initially, monocytes are recruited at the site of infections and then further, these cells differentiate into alveolar MΦs and DCs (Hmama et al., 2015). In various other diseases, the innate immune system is very efficient in mopping off the infection; but in some cases, an adaptive immune response has to come into action. In the case of *Mtb* infection, this bacilli successfully survives and persists in the macrophages and dendritic cells within the lungs with more efficient survival in the alveolar macrophages. Activation of *Mtb* infected macrophages by IFN-γ of Th1 cells is one of the main cell-cell interactions needed to clear off this infection (Flynn et al., 1993). Hence, the immune cells of the host play an indispensable role in limiting the *Mtb* infection.

Mtb infected alveolar MΦs move to the lung interstitium and on the other side *Mtb* infected DCs or monocytic-derived dendritic cells localize to the draining lymph nodes; so that they can present the internalized and processed bacterial peptides to T cells and generates an efficient adaptive immune response (Cohen et al., 2018). The adaptive immune

system is widely characterized and understood in the case of *Mtb* infection, but with the identification of MDSCs, it seems like more studies are needed to delineate the innate immune system. MDSCs have suppressive potential and are localized in the granuloma (Dorhoi and Du Plessis, 2017). In the case of TB infection, a very strong and effective innate and adaptive immune response is needed to curtail this infection. But, MDSCs suppress both these immune responses through suppression or inhibition of lymphocytes and NK cells functions. MDSCs mediate all these inhibitory effects through secretion of nitric oxide, upregulation of arginase 1 activity, nitrosylation of T cell receptor (TCR), apoptosis of lymphocytes and induction of Tregs (du Plessis et al., 2013; El Daker et al., 2015; Yang et al., 2014). The vast detrimental effect of MDSCs is mediated by strong suppression of Th1 immune response along with a decline in the secretion of innate cytokines against TB infection.

3.3.1. Innate immune response. The primary line of the host defense system is the innate immune response. Upon initial encounter with invading pathogen, this branch of the immune system came into action through various components such as soluble mediators (cytokines, complements, ROS, RNS, NO, etc.), cellular components (dendritic cells, macrophages, neutrophils etc.) neutralize the invading pathogen. Antigen-presenting cells (DCs and MΦs) impart an imperative part in the activation of the adaptive immune system through the turning on of T cells through peptide-MHC complex and cytokines. The pathogen is recognized through PRRs located over the surface of immune cells (Mogensen, 2009). These PRRs recognize the PAMPs of the pathogen and initiate cascades of the immune response. These PAMPs are exclusive to each pathogen and help in the distinguishing of self from non-self molecules. Along with identification role of microbes, these PAMPs have various physiological roles in the microbes. PAMPs are composed of proteins, nucleic acid components, peptidoglycan, lipopolysaccharide (LPS), lipoproteins and lipoteichoic acid in the cell

wall of microbes (Dempsey et al., 2003). Similarly, PRRs also have many roles such as phagocytosis, opsonization, induction/inhibition of signaling pathways, inflammasome activation, apoptosis and chemotaxis (Khan et al., 2016; Lerner et al., 2015). PRRs are constituted by CLRs, TLRs, RLRs and NLRs and having a role in identification and uptake of *Mtb* (Akira et al., 2001; Killick et al., 2013).

3.3.1.1. TLRs. TLRs are conserved PRRs identified in all organisms i.e., from insects to humans (Dempsey et al., 2003). Based on the cellular location, TLRs are classified into plasma membrane-anchored (TLR1, 2, 4, 5, 6) and endosomal (TLR7, 8, 9). Endosomal TLRs recognize microbial nucleic acids (Kawai and Akira, 2010) and plasma membrane-anchored TLRs recognize components of Gram-negative, and Gram-positive microbes (LPS, flagellin, lipoproteins etc). Upon infection, recruitment of different adaptor molecules to TLRs turn on NF-κB, MAPK and PI3/Akt signaling pathways; hence, prompting the secretion of protective cytokines and interferons (IFNs) type I (Kawai and Akira, 2010). *Mtb* is primarily recognized through lipoarabinomannan present on its surface by TLRs (TLR-2) (van Crevel et al., 2002). This TLR-2 recognizes LAM, 38 kDa, 19 kDa, triacylated and diacylated lipoproteins of *Mtb* by forming homo or heterodimers with TLR-1 and TLR-6, which leads to instigation of NF-κB and MAPK pathways (Texereau et al., 2005). Further, it has been seen that mice deficient in MYD88 are more susceptible to TB infection (Sugawara et al., 2003). Similarly, TLR-2 deficient mice have impaired granuloma formation potential and are more susceptible to *Mtb* infection (Drennan et al., 2004). Also, TLR-4 knockout strain has been observed to die, after *Mtb* challenges within 15 weeks. TLR-2 polymorphism in humans makes them more prone to pulmonary TB (Drennan et al., 2004). TLR-2 recognizes 1pqH lipoprotein of *Mtb* (Pecora et al., 2006). The induction of the TLR-4 pathway is important for elicitation of the effective immune response against *Mtb*. TLR-9 recognizes the unmethylated CpG motifs of microbes. In the case of *Mtb,* TLR-9 knockout mice are

immensely susceptible to *Mtb* infection in comparison to normal mice (Bafica et al., 2005). Interestingly, it has been noticed that uptake of bacteria without TLRs signaling impaired the immune response against that pathogen (van Crevel et al., 2002).

3.3.1.2. NLRs. The NLR family consists of NOD1, NOD2, NLRP3 and NLRC4, which recognizes the component of microbes and induces an inflammatory immune response (Kim et al., 2016b). In general, NOD1 and NOD2 recognize the bacterial peptidoglycan and induces an inflammatory immune response by releasing cytokines that cause the localization of neutrophils, MΦs and DCs at the site of infection. In the case of *Mtb* infection, *Mtb* escapes the phagolysosome fusion in the cytosol of MΦs through secretion of ESAT-6 secretion system-1 (Simeone et al., 2012). So, it shows that PRRs, like NLRs, have an essential role in the detection of *Mtb*. Initiation of NOD2 through its ligand (muramyl dipeptide) in *Mtb* infected macrophages have shown better activation and control of *Mtb* growth through the autophagy induction (Juarez et al., 2012). Also, NOD2 knockout mice show less protection against the *Mtb* (Divangahi et al., 2008).

3.3.1.3. CLRs and RLRs. These PRRs are CLRs containing, collectins, selectins, proteoglycans and phagocytic receptors. Generally, CLRs contain one or more carbohydrate-binding domains that can recognize carbohydrates, lipids, and proteins in a calcium-dependent and independent manner. In *Mtb* infection, mannose receptor (MR), Mincle, DC-SIGN, Dectin-1 and 2, CL-LK and dendritic cell immunoreceptor (DCIR) are well known. MR binds to the ManLAM and causes the phagocytosis of mycobacteria by human macrophages (Kang et al., 2005). Upon mycobacterial infection Dectin-1 along with TLR-2 activates the macrophages (Yadav and Schorey, 2006). Similarly, Dectin-2 has an important role against *Mtb* (Yonekawa et al., 2014). Mincle also has an imperative role in providing protection against the *Mtb*, as it recognizes the trehalose 6, 6′-dimycolate of *Mtb* (Matsunaga and Moody, 2009). Like MR, CL-LK and DC-SIGN binds to ManLAM

(Geurtsen et al., 2009). Similarly, RIG-1, MDA-5 and LGP2 constitute the RLRs family. These induce the Type I IFN upon recognizing the RNA released in the cytosol (Akira et al., 2006). So, it shows that all PRRs (TLRs, CLRs, RLRs and NLRs) have a very precise and noticeable role *Mtb* infection. Still, a lot of work or research is needed to be carried out to decipher the unidentified PRRs and their association with specific ligands.

3.3.1.4. *Mtb* infection and the production of cytokines. Cytokines are low molecular weight glycoproteins, secreted mainly by the immune cells. As a primary line of defense, innate immune cells secrete soluble components or mediators such as cytokines, chemokines, ROS, RNS, NO, etc. There are two subtypes of cytokines based on the functionality, that is, one which induces a protective immune response, known as pro-inflammatory and another one that hinders or inhibits the induction of immune response known as anti-inflammatory cytokines. The most important pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-12) are secreted by MΦs and DCs during *Mtb* infection. Also, the secretion of these cytokines is induced by the activation of PRRs on recognizing the PAMPs. Upon uptake of *Mtb* by alveolar MΦs, leads to the activation of these cells in TLR-2 and 4 dependent manners; which ultimately causes the release of pro-inflammatory cytokines (Brightbill et al., 1999). *Mtb* prompts the release of IL-12 that causes differentiation of T cells into Th1, and is finally required for constraining or limiting the *Mtb* growth within the host cell (Zhang et al., 1994). IL-12 not only activates or generates the Th1 cells, but rather a generation of Th1 cells leads to the activation of macrophages through secretion of IFN-γ, which helps in the dissemination of *Mtb*. Further, it was shown that individuals with IL-12 receptor mutations were more susceptible to *Mtb* infection (Altare et al., 1998). Another very potent cytokine is the TNF-α, secreted by the MΦs, DCs and neutrophils in response to *Mtb* infection. In the case of infected MΦs, this cytokine induces apoptosis and along with that it also induces many reactive oxygen intermediates and reactive nitrogen intermediates

production, which ultimately destroys the infected host cell. Intriguingly, being the protective role played by TNF-α, there are some chronic diseases that it is being used to cure. But the problem is that usage of TNF-α for curing some chronic health issues, leads to the relapse of *Mtb* hidden inside the MΦs from its dormant stage (Flynn et al., 1995). TNF-α induces granuloma formation in a much-regulated manner, by inducing the recruitment of leukocytes. IL-6, another pro-inflammatory cytokine also limits or hampers the *Mtb* growth in infected MΦs (Flesch and Kaufmann, 1990). It has been observed in IL-6 knockout mice, that upon giving *Mtb* infection, they died very early compared to wild-type mice even at a dose of bacilli which can be well tolerated by wild-type mice (Ladel et al., 1997). Upon *Mtb* infection, to IL-6 deficient or mutant mice, there is induction of Th2 immune response as evidenced by a huge exhibition of IL-4 and IFN-γ. The most prominent reason for the death of knockout (IL-6) mice is non-induction of protective Th1 immune response. Anti-inflammatory cytokines are well known to impede the generation of the immune response against *Mtb*. There is another cytokine, IL-4 which drives the generation of Th2 immune response, which is an antagonist to Th1 immune response against *Mtb* infection. The generation of the Th2 immune response hinders the Th1 immune response, and because of that, there is an almost negligible release of IFN-γ. Moreover, IL-4, IL-10 and TGF-β play a very deteriorating role against the *Mtb* infection. Also, it was seen that helminth co-infection in TB-infected individuals has more Th2 immune response as compared to Th1 immune response (Hernandez-Pando et al., 1997). Various reasons can be behind this, but one of many reasons can be the non-even efficacy of the BCG vaccine.

3.3.1.5. Immune cells and *Mtb* infection. There are various cells involved in mycobacterial infections such as DCs, MΦs, neutrophils, NK cells, MDSCs and lymphocytes. These cells play different roles in response to *Mtb* infection. Most of these cells have an overlapping and similar role against *Mtb* (Fig. A).

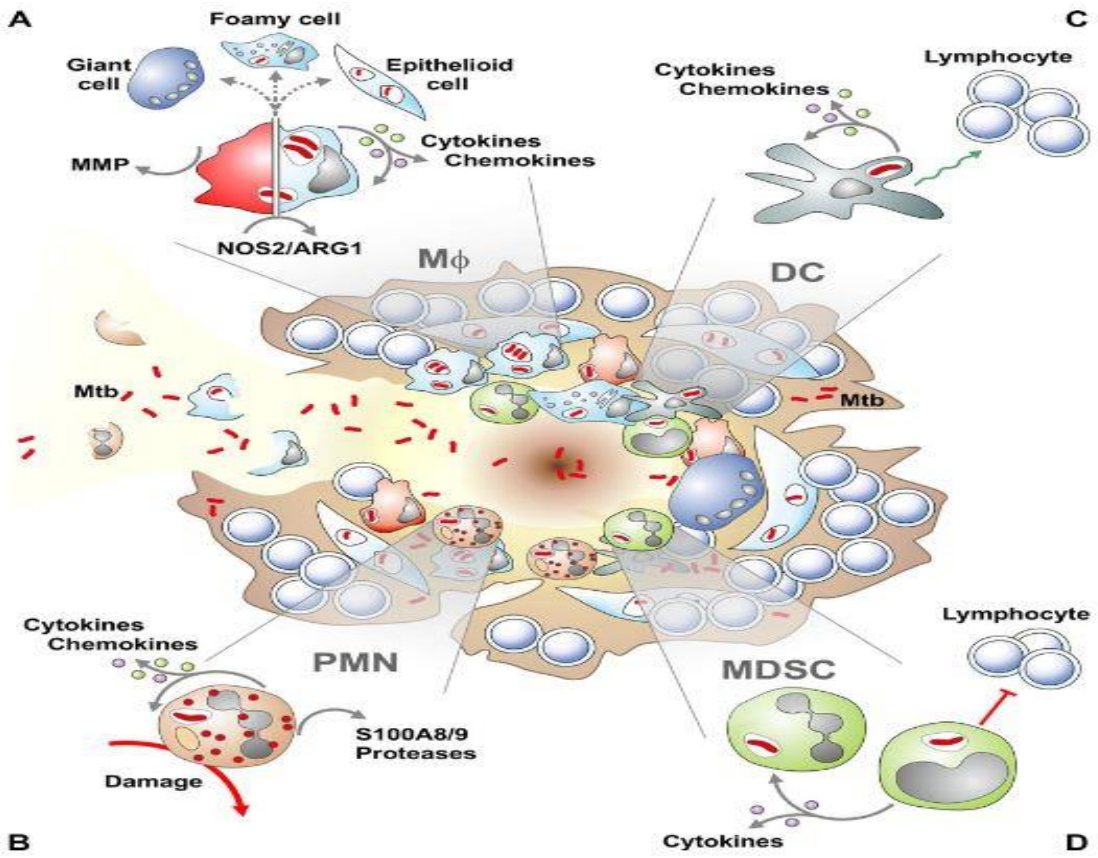


Figure A. Immune cells in TB granuloma. Granuloma is a hard caseous structure formed after *Mtb* infection to constrain its spread to nearby cells or tissues. MΦs, DCs, polymorphonuclear neutrophils (PMNs), MDSCs and lymphocytes mostly constitute the granuloma. (A) MΦs upon *Mtb* infection secretes cytokines, chemokines, arginase 1 and nitric oxide synthase 2 (NOS2). Matrix metalloproteases (MMP) secreted by the macrophages induce cavitation in granulomas followed by tissue remodeling. (B) PMNs secrete various soluble components such as cytokines and S100A8/9 proteases that induce cavitation in the granulomas which leads to an enhanced progression of TB. (C) DCs link innate immunity with adaptive immunity in response to *Mtb* Infection by activating T cells. (D) MDSCs through various secretory components impose immunosuppression on other immune cells. Hence, promotes the severity of the disease (Dorhoi and Kaufmann, 2015).

3.3.1.6. Dendritic cells. DCs links the innate and adaptive immune systems and hence have an imperative role (Prendergast and Kirman, 2013). Among APCs, only DCs can activate the naïve T cells since they constitutively express the optimum level of costimulatory molecules and MHC I/II. Adversely, B cells and MΦs are other professional APCs, which only act on effector cells (Austyn et al., 1983; Klinkert, 1984; McCormack et al., 1992; Steinman and Witmer, 1978). The presence of PRRs on DC recognizes PAMPs of bacteria, fungi, viruses and protozoa followed by its activation and maturation. Upon activation, DC translocates the MHC complex I/II on its surface, enhances costimulatory molecules expression and secretes various cytokines and chemokines (van Vliet et al., 2007). Activated DCs translocate to secondary lymphoid organs to turn on the naïve T cells (CD4 and CD8). Generally, to turn on naïve T cells, three important signals are delivered by DC. The first signal is the MHC peptide-TCR complex, the second is the costimulatory molecules and the third signal is pro-inflammatory cytokines. Therefore, DCs have an imperative role in the elicitation of the T cell response (Lenschow et al., 1996) (Fig. B).

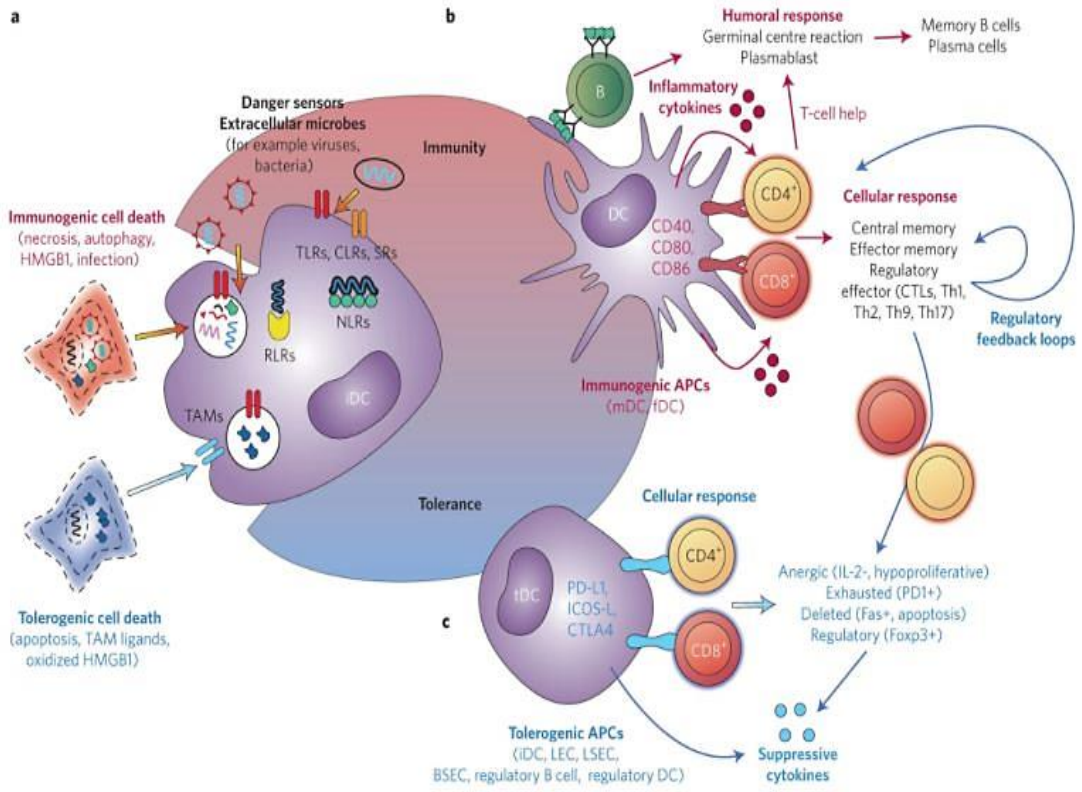


Figure B. Dendritic cell bridges innate and adaptive immunity. Through PRRs, DCs sense pathogens' PAMPs and initiate cascades of the immune response. (a) Immature DCs recognize immunogenic signals or tolerogenic signals of infected or dying cells in a steady-state through various PRRs (TLRs, CLRs, NLRs, RLRs, and SRs). (b) Upon sensing immunogenic signals, DCs activate effector B cells and T cells. (c) Peripheral tolerance is maintained through signals from apoptotic cells during homeostatic turnover that inhibits the activation and maturation of DCs (Irvine et al., 2013).

A lot of work has been carried out on CD11c expressing cells, known as conventional DCs (cDCs). These cells have the tendency to turn on naïve T cells against *Mtb* (Banchereau and Steinman, 1998; Khader et al., 2006). Pulmonary cDCs express high levels of CD11c, CD11b and varied CD103 upon *Mtb* infection (Mayer-Barber et al., 2011). Expression of CD103 marks cDCs as lung resident DCs having migratory potential (Anderson et al., 2014). Along with CD103, lung cDCs express CD80, CD86, and MHC II and secrete high amounts of

IL-12 (Mayer-Barber et al., 2011). In the case of TB, the generation of T cell response is very slow because of the late reaching of CD4 T cells in the lungs of infected mice after several weeks. This delay leads to the proliferation and enhancement in *Mtb* bacilli numbers, which also makes the *Mtb* too well established after infection before the adaptive immune cells can reach it. During infection, infected bone marrow-derived DCs not MΦs have the migratory capacity to draining lymph nodes and activates CD4 T cells (Bhatt et al., 2004). In case of *Mtb* infection, ManLAM ligation with DC-SIGN leads to impairment in the maturation and activation of DCs. Further, it has been seen that ManLAM induces the secretions of IL-10, impairing the maturation of DCs. Also, it modulates the DCs in such a manner that DCs inhibits the IL-12 secretion and finally impair in the activation of the adaptive immune response (Nigou et al., 2001). Another component of *Mtb* is PE27, it induces the Th1 immune response and mediates this induction by binding to TLR-4 followed by activation of MAP kinase and NF-κB pathways (Kim et al., 2016a). Similarly, RpfE, a latent phase component of *Mtb*, prompts the release of protective cytokines and inhibits the secretion of anti-inflammatory cytokines (Choi et al., 2015). Moreover, Rv2463 and Rv3416 of *Mtb* restricts the apoptosis and autophagy in DCs through modulating the expression of NEDD8 and SENP8 (Chadha et al., 2015). Another population of DCs residing in the lung's parenchyma is highly inflammatory in nature, these are monocytic derived and known as mDCs (CD11c, CD13, Ly6C, TLR-2, MHC II, CD80, CD86) (Mayer-Barber et al., 2011). They exhibit immense antigen presentation capacity and secrete a high amount of pro-inflammatory cytokines and iNOS *in vivo*.

3.3.1.7. Generation and differentiation of DCs. The mononuclear phagocyte system (monocytes, MΦs and DCs) constitutes a large portion of the innate immune system. This system has a very wide mode of action against pathogens. In case of *Mtb* infection, MΦs, DCs and monocytic-derived cells have been seen to have a very unique role

in terms of neutralizing and eliminating this bacillus. Generation of these cells occurs in bone marrow followed by their maturation and differentiation in other lymphoid or non-lymphoid organs. Immature DCs are continuously produced in bone marrow from hematopoietic stem cells (HSCs). Initially, HSCs differentiate into common myeloid and lymphoid progenitor cells (CMPs and CLPs). Further, CMPs differentiate into granulocyte-macrophage progenitors (GMPs) followed by MΦ/DC progenitor cells (MDPs). These MDPs then differentiate into monocytes and common DC precursor cells, and then further these common DC precursor cells differentiate into DCs or MΦs. Further, these DCs and MΦs can be classified into different subtypes depending upon their region of localization as a resident, circulatory and myeloid. DCs are well-known APCs with the potential for activation of naïve T cells. Along with this various other functions are being carried out by the DCs. DCs can very efficiently discriminate between self and non-self-antigens/pathogens. Being multifunctional in nature, DCs can impart protective immunity and can become tolerant. So, the microenvironment created around the DCs will decide its further ontogeny. DCs are the heterogeneous cell population that can be further classified based on their anatomical positions such as interstitial DCs (tissues), Langerhans cells (LCs)(epidermis), and thymic DCs in lymphoid organs. Based upon the maturation stage DCs can be classified as immature DCs, semi-mature DCs and mature DCs. Myeloid DCs can be differentiated from precursor cells of bone marrow and monocytes isolated from peripheral blood using growth factors such as GM-CSF (Granulocyte-macrophage colony-stimulating factor) and IL-4. In the case of MΦs differentiation, M-CSF (macrophage colony-stimulating factor) as a growth factor is being used *in vitro* for the generation from bone marrow precursor cells and from monocytes isolated from peripheral blood.

DCs exhibit a wide array of PRRs on their surface to efficiently recognize the wide spectrum of pathogens through their PAMPs. One of the interesting facts is that DCs express TLR-3, which normally other cells

don't express. The most important aspect of DCs functionality is its stages of maturation. A fully matured DC is efficient enough to fight back and eliminate any pathogen, on the other side immature DCs tend to become tolerogenic if their surrounding environment is highly anti-inflammatory. Various components of *Mtb* are known that can modulate the maturation of DCs such as ESAT-6, 19 kDa, ManLAM and Acr1 in a TLRs (TLR-2 and TLR-4) dependent manner (Amir et al., 2017; Henderson et al., 1997; Hertz et al., 2001; Satchidanandam et al., 2014; Tsuji et al., 2000). 19 kDa is a mycobacterial lipoprotein that can induce Th2 immune response; similarly, glycosylated Rv1860 hampers the generation of Th1 and Th17 immune responses (Satchidanandam et al., 2014). On immunizing a MyD88$^{-/-}$ (adaptor protein in TLR signaling) mice with ovalbumin in complete Freund's adjuvant, there is impaired Th1 immune response generation. Moreover, MyD88$^{-/-}$ mice show a high bacterial load with diminished adaptive and innate immune response (Abel et al., 2002; Fremond et al., 2004).

3.3.1.8. Macrophages. The first cell to encounter *Mtb* is MΦs. These cells become the primary site of infection, as well as a shelter during the acute and chronic stages of mycobacterial infection (McClean and Tobin, 2016). Various protective arms are being employed by these cells to curtail the *Mtb* infection such as phagosome acidification, secretion of cytokines and RNI/ROI along with some other processes. Alveolar MΦs are the first cell type that came into contact with *Mtb*. These cells have been observed to secrete nitric oxide and ROS in murine as well as in humans, but still, it is not able to eliminate the *Mtb* before it gets established (Nicholson et al., 1996). These infected alveolar MΦs then recruit other cells such as monocyte-derived macrophages, neutrophils, and lymphocytes at the site of infection (Cadena et al., 2016). PRRs located on macrophages identify and recognize various PAMPs of *Mtb* and induce the immune response accordingly. Already, it has been observed that IL-1β, IL-12 and IL-6 are being secreted in response to *Mtb* infection and modulate various host cellular functions (phagocytosis, apoptosis, autophagy, inflammasome formation). In

response to LPS, GM-CSF, IFN-γ, TNF-α or similar microbial stimuli macrophages are polarized towards classical type (M1), having an effector function against external insult. On the other side, M2 MΦs are anti-inflammatory in nature and poor antigen-presenting cells generated upon induced through IL-4, IL-13, IL-10 and TGF-β (Ginhoux et al., 2016; Sica et al., 2015). Most importantly M2 MΦs suppresses the Th1 immune response, which is very important for inhibiting the *Mtb* infection. Arginase1 (Arg1) is the key marker of M2 MΦs, mice deficient in Arg1 show better protection against *Mtb* (Duque-Correa et al., 2014). But, it has been observed that some components of *Mtb* induce M2 MΦs generation, like Hsp70 (DnaK) (Lopes et al., 2016). Granuloma is the key identification mark of TB through histopathology, and various macrophage populations constitute this structure. Foamy MΦs form concentric layers around the infected alveolar macrophages along with epithelioid cells and multinucleated giant cells (Feng et al., 2014; Peyron et al., 2008). ESAT-6 and mycolic acid induce the formation of foamy cells (Peyron et al., 2008; Singh et al., 2015). This happens because ESAT-6 has been observed to induce changes in metabolic machinery so that foam cells can become. These foamy MΦs are poor antigen-presenting cells with a tendency to secrete TGF-β. Now, a lot of reports are coming to that show that the pathogenicity of *Mtb* is regulated by the host cell's metabolic machinery (Mehrotra et al., 2014). It has been observed that virulent *Mtb* within the alveolar MΦs modulates the cell in such a way that it evades immune response through various strategies such as obstruction of phagolysosome fusion, and detoxification of oxygen and nitrogen radicals and dormancy (Gengenbacher and Kaufmann, 2012). In order to burst the cells, *Mtb* bacilli are needed to come to at least a threshold of 25 bacilli/cell (Repasy et al., 2013).

3.3.1.9. Neutrophils. These are the first innate cells, which migrate at the site of infection or injury. There is a huge population of neutrophils located in the airway of infected individuals. But these cells are poorly

studied till now in *Mtb* experimental models, because of their very short life span. These cells are also known as polymorphonuclear (PMN) cells. The identification of these cells is a little difficult, through staining with Gr-1^+ antibody. As this antibody identifies or stains both Ly6C and Ly6G, markers are present on monocytes, macrophages and DCs. But now staining with Ly6G makes it more specific for neutrophil identification *in vivo*. It has been observed that a decline in neutrophil population after infection of *Mtb* in airways, enhances the bacterial load in the hosts (Blomgran and Ernst, 2011; Pedrosa et al., 2000). Also, depletion of neutrophils from whole blood *in vitro* leads to increased *Mtb* bacilli growth (Martineau et al., 2007). Granules from neutrophils taken up by macrophages have been shown to have an inhibitory effect on bacterial proliferation (Tan et al., 2006). Neutrophils, through ROS, neutrophil extracellular traps (NETs), TNF-α, elastase, myeloperoxidase, collagenase and antimicrobial peptides show their antimycobacterial efficacy (Blomgran and Ernst, 2011). Also, these cells have the tendency to boost the priming of T cells through DCs. It has been evidenced that with disease progression there are pathological changes occurring in neutrophils as observed in humans (Berry et al., 2010). There can be many reasons for these pathological changes initiated by neutrophils such as induction of necrosis in the lungs, and liquefaction of granuloma followed by the collapse of lung functions. Further, it has been seen that *Mtb* initiates necrosis in neutrophils and inhibits apoptosis through factors encoded by its RD-1 region (Corleis et al., 2012). Recently, ESAT-6 has been observed for the induction of necrosis and secretion of NET and MPO from neutrophils in a calcium-dependent manner (Francis et al., 2014). More studies are needed to study the relationship of *Mtb* and neutrophils in terms of disease progression or inhibition.

3.3.1.10. NK cells. NK cells or natural killer cells have a strong cytolytic activity and act very early in response to *Mtb* infection and are not MHC restricted (Allen et al., 2015). These cells can hamper the

growth of mycobacteria through the release of granzymes, perforin and granulolysin; and on the other side by activation of macrophages. Reduced frequency of NK cells has been noticed in patients with pulmonary mycobacterial infection. Further, many components of *Mtb*, like mycolic acid act as ligands for NKp44 on NK cells (Esin et al., 2013). NK cells enhance the phagolysosomal fusion by secreting IFN-γ and IL-22. Also, it boosts the proliferation of T cells (γ/δ) through CD54, TNF-α, IL-12 and GM-CSF (Allen et al., 2015).

3.3.1.11. Discovery of MDSCs. Till the early '70s, the cells imparting immunosuppressive roles were very less known. But it was the late '70s when the information started appearing on suppressive cells, and later these cells are defined as MDSCs (Holda et al., 1985; Strober, 1984). Initially, these cells were known as suppressive cells and were not of great importance to the scientific community. The first general report on the isolation of MDSCs came when it was isolated from bone marrow and spleen of mice having a tumour, and MDSCs have the tendency to restrict the adaptive immune response (*in vivo* and *in vitro*) against the tumour cells (Roder et al., 1978; Subiza et al., 1989). Later, these cells were known as natural suppressive cells, regulatory myeloid cells, and immature myeloid cells. But, finally, in 2007, Gabrilovich and his colleagues named these cells myeloid-derived suppressor cells, based on the basis of their origin, as well as function (Gabrilovich et al., 2007). It took nearly 37 years to name these cells as MDSCs. Since then, many studies have been conducted to decipher their role, as well as function in various diseases. Expansion of the MDSCs population has been observed in the lungs during *Mtb* infection and can become a shelter home for the progression and growth of *Mtb* (Knaul et al., 2014).

3.3.1.12. MDSCs and TB. *Mtb* is a complex pathogen and is not fully understood in terms of its infective potential. Various mechanisms are being used by this pathogen to sustain within the host. One of the major factors impeding TB elimination is not sufficient understanding of its pathogenesis mechanism. Recently, many reports have shown

that TB is a multifaceted disease rather than bifacial i.e., asymptomatic latent infection and active infection (Barry et al., 2009; Delogu and Goletti, 2014; Drain et al., 2018). In the case of TB, Th1 and $CD8^+$ cells are very important for imparting protection. In the case of latent tuberculosis, patients show a protective T cell response as compared to the active tuberculosis (Masungi et al., 2002; Temmerman et al., 2004).

A myeloid cell is one of the innate immune system cells, initially identified in the cancer (Gabrilovich and Nagaraj, 2009). Myeloid cells along with monocytes and neutrophils, are the first effector cells in response to *Mtb* infection in the host and help in restricting the *Mtb* load and disease progression. They do so by activating pro-inflammatory signaling pathways, enhanced bactericidal mechanisms, increased bacilli ingestion and induction of pathogen-specific adaptive immune response (Dorhoi and Kaufmann, 2015; Liu et al., 2018; Srivastava et al., 2014). Interestingly, myeloid cells do not always give a protective immune response, on the other side they facilitate pathological processes, such as the progression of TB through immunosuppression and malfunctioned inflammation (Dorhoi and Kaufmann, 2015). Chronic *Mtb* infections elicit the generation of MDSCs (Bennett et al., 1978; Gabrilovich and Nagaraj, 2009; Kato and Yamamoto, 1982; Wang et al., 2010). The role of MDSCs is well investigated and is being investigated in the field of cancer, but its role in the field of bacterial infections is in its infancy stage. MDSCs can be further classified on the basis of morphology; monocyte-like (M-MDSC) and neutrophil/granulocyte-like (PMN-MDSC). Further, on the basis of surface markers profiles M-MDSC are HLA-$DR^{-/low}CD11b^+CD33^{+/high}CD14^+CD15^-$ (in humans) and Gr-$1^{dim/+}CD11b^+Ly6C^+Ly6G^{-/low}$ (in mice); and PMN-MDSC are HLA-$DR^{-/low}CD11b^+CD33^{dim}CD14^-CD15^+$ (in humans) and Gr-$1^{dim/+}CD11b^+Ly6C^{low}Ly6G^+$ (in mice) (Bronte et al., 2016; Dolcetti et al., 2010; Gabrilovich, 2017; Movahedi et al., 2008). The first report regarding generation of regulatory myeloid cells during mycobacterial infection came from studies with *Mycobacterium bovis* BCG (Dorhoi and

Kaufmann, 2015; Kato and Yamamoto, 1982; Mellow and Sabbadini, 1985). Moreover, reports have shown that BCG promotes the expansion of splenic and bone marrow-derived suppressor cells having the potential to inhibit cell-mediated immune response and proliferation of T cells (Bennett and Marsh, 1980; Kato and Yamamoto, 1982; Kendall and Sabbadini, 1981; Mellow and Sabbadini, 1985). Further, it was seen that these natural suppressor cells upon exposure to mycobacterial products in complete Freund's adjuvant (CFA) in mice, resembles morphologically and functionally to MDSCs (Wang et al., 2010) and are characterized on the basis of expression of Gr-1, CD11b; constraining of T cell proliferation and release of IFN-γ. Later on, many studies came upon the generation of MDSCs during BCG infection; and presence in active TB patients (du Plessis et al., 2013; Martino et al., 2010). There are reports which have shown that these regulatory myeloid cells accumulate in the lungs, bone marrow, blood and spleen during mycobacterial infection and later on, differentiated and represent the heterogeneous population (Tsiganov et al., 2014). Also, MDSCs have been reported in the blood of murine models during BCG vaccination. From these studies, it was established that mycobacterial products have the potential of generating MDSCs. These MDSCs play a bifacial role in tuberculosis, on one side they provide cellular shelter for their survival and growth and on the other side abate the T cell responses.

The frequency of MDSCs in children and adults suffering from TB is almost the same as that found in cancer patients (du Plessis et al., 2013). In the case of TB patient's lung compartmentalization; PMN-MDSC is more localized in bronchoalveolar lavage (BAL), whereas M-MDSC in pleural effusions (du Plessis et al., 2013; El Daker et al., 2015). It has been observed that MDSCs compartmentalization is associated with disease stage and site-specificity. In naïve mice, MDSCs frequency is low in bone marrow, but upon TB infection, MDSCs population increases in lungs and other organs (Tsiganov et al., 2014). With the progression of TB, MDSCs accumulation occurs in the lung

parenchyma of susceptible mice (Knaul et al., 2014; Obregon-Henao et al., 2013). It has been observed that *ex vivo* developed human M-MDSC augments replication of mycobacteria in *in vitro* formed granuloma (Agrawal et al., 2018). It is well known that the frequency of MDSCs is directly linked with disease progression, but in the case of TB-resistant mice, those who don't have necrotic granulomas have very low levels of MDSCs. On the other side necrotic granuloma mice strains such as C3HeB/FeJ, 129S2 (immunocompetent) and $NOS2^{-/-}$ (knock out) show high frequencies of MDSCs (Knaul et al., 2014; Tsiganov et al., 2014). Further, it has been observed that MDSCs frequency declines in recovered TB patients and is at par level to that of healthy persons (du Plessis et al., 2013).

MDSCs directly interact with mycobacterium and it is well known that lung residing M-MDSCs retains *Mtb* and enhances *Mtb* growth through IL-4/IL-4Rα signaling (Knaul et al., 2014). MDSCs secrete nitric oxide (NO), but are inefficient in controlling mycobacterial growth (Martino et al., 2010). Most importantly, it has been observed that tumour-infiltrating MDSCs utilizes fatty acid-β-oxidation as source of energy (Hossain et al., 2015). Similarly, in the case of mycobacterial infection, MDSCs phagocytose the mycobacterium but have poor microbicidal potential against it. *Mtb* utilizes the host cholesterol and fatty acids as a nutrient source (Daniel et al., 2011; Guirado et al., 2015) for its survival and progression. But the exact metabolic status of *Mtb* and its localization within MDSCs is further needed to be decoded.

Despite being many factors responsible for the low efficacy of BCG vaccine (route of administration, helminth co-infection, mycobacterial strains and geographical location), MDSCs have been identified as one of the factors for reducing the potential of BCG vaccine (Jayashankar and Hafner, 2016; Moliva et al., 2017). Many studies have now reported the presence of MDSC in TB. MDSCs get activated and start proliferation in response to chronic TB insult (Ribechini et al., 2017). Signaling behind MDSCs activation and expansion is still needed to be

delineated and is a difficult task. In case of TB infection, GM-CSF, prostaglandins, IL-6 and S100A8/9 induce myelopoiesis by hampering the final maturation of myeloid progenitor cells (Gabrilovich, 2017; Veglia et al., 2018). All these factors induce the generation of MDSCs followed by their activation through an array of cytokines, PAMPs, DAMPs stimulation (Gabrilovich, 2017; Ribechini et al., 2017; Veglia et al., 2018). Along with these various other factors such as MMP9, prokineticin 2 enhances MDSCs proliferation (Knaul et al., 2014). M-MDSC have been observed to be induced during *Mtb*, *M. smegmatis* and BCG infection (Martino et al., 2010). Also, CFA contains mycobacterial glycolipids observed for the expansion of MDSCs (Wang et al., 2010).

3.3.1.13. MDSCs immunosuppressive mechanism during mycobacterial infection. Generally, MDSCs implicate its immunosuppressive action through various ways, such as secretion of soluble factors, through cell surface receptors, fine-tuning of metabolites and other biomolecules (Bronte et al., 2016; Dorhoi and Du Plessis, 2017). It has been observed that MDSCs interacts with DCs, MΦs and induces the generation of Tregs and Bregs (Lei et al., 2015; Park et al., 2016).

MDSCs suppressive activity has been observed to be associated with L-arginine metabolism. In case of MDSCs it is well known that L-arginine acts as a substrate for two enzymes, iNOS and arginase. L-arginine is converted into urea and L-ornithine by arginase and NO generation occurs through iNOS action. Generation of NO through upregulation of iNOS is well known in TB infection in human and mice (El Daker et al., 2015; Yang et al., 2014). MDSCs impart its suppressive action on T cell function through these two enzymes and is well documented (Bronte and Zanovello, 2005; Rodriguez and Ochoa, 2008). Nitric oxide is a lipophilic gas having many biological functions, like inflammation, immunomodulation, microbicidal and tumour eradication. It's a very dynamic molecule having diametric role, i.e., in low concentration it has beneficial and protective role along with role in regulating

physiological processes; In higher concentration it has deleterious effect not only against microbes, tumour cells but also against host cells. But still, its exact role in immune-regulation is not clear. In case of MDSCs, upregulation in activity of both iNOS and arginase causes depletion of L-arginine (Knaul et al., 2014), which further causes superoxide production in low amount by iNOS. This superoxide then reacts with NO and forms $ONOO^-$ and other reactive nitrogen species (RNS) that cause apoptosis in T cells (Bronte et al., 2003; du Plessis et al., 2013). On the other way arginase high activity in MDSCs leads to faster catabolism of L-arginine followed by its depletion from the microenvironment or cell. Shortage of L-arginine restricts T cell proliferation and functions through various mechanisms (Bingisser et al., 1998; Rivoltini et al., 2002; Rodriguez et al., 2005). On the other side it is also well known that MDSCs inhibits T cell response, when present in close proximity through release of nitric oxide (Knaul et al., 2014; Obregon-Henao et al., 2013). All these mediators or interactions by which MDSCs suppresses immune response are well known in murine TB models and humans. In BCG vaccinated mice, it has been seen that there was hampering of T cell proliferation and IFN-γ release by MDSCs in iNOS dependent way (Martino et al., 2010). In humans increased PD-L1 expression has been observed in response to *in vitro* mycobacterial infection (Agrawal et al., 2018) further leading to inhibition of T cell proliferation. In TB, role of MDSCs mediated immunosuppression is further needed to be deciphered in terms of Tregs, Bregs induction, alteration in activity of NK cell and interaction of MDSCs with other myeloid cells. The most important aspect in curing TB through immunotherapy is usage of anti-TNF agents; surprisingly, MDSCs have a very crucial role in TB reactivation in absence of TNF-α (Clay et al., 2008; Miller and Ernst, 2009).

Further, in-depth characterization of these cells both morphologically and functionally through mass cytometry, histo-cytometry, RNAseq, proteomics, etc., will help in understanding the role played by these cells in various other diseases and in immunomodulation.

3.3.1.14. Metabolic reprogramming of MDSC/Role of Metabolic shift in *Mtb* survival in MDSCs. MDSCs have very less or poor antigenic presentation potential, besides this they substantially uptakes *Mtb* and restricts generation of Th1 immune response (Kolahian et al., 2016). *Mtb* prefers to be phagocytosed and live within foamy macrophage. As there is large depot of lipids present over there. Upon phagocytosed in foamy macrophage this bacilli induce metabolic and immune-modulatory changes for its survival and persistence (Daniel et al., 2011; Peyron et al., 2008). Not only these lipids act as energy source for *Mtb*, but rather also involved in cell wall development and have an important role in its revival from latent state (Bhatt et al., 2007; Daniel et al., 2011). Also, through fatty acid elongation by fatty acid synthase II complex (FASII), components of mycolic acid are produced (Bhatt et al., 2007). Along with this fatty acid is involved in the generation of sulfolipids, polyacytrehaloses and phthiocerol-dimycoceroic acid as virulence factors of *Mtb* (Lovewell et al., 2016; Ouellet et al., 2011). On the other side when there is shortage of carbon source, *Mtb* shifts the metabolic changes towards glyoxylate shunt for its survival and metabolism of lipid (Russell et al., 2009). This metabolic shift is occurred through up-regulation of isocitratelyase. It has been reported that in macrophages of murine TB models there is upregulation in expression of PGE_2 and COX-2, and is because of lipids associated with macrophages. This increase in expression of PGE_2 and COX-2 leads to hampering of Th1 immune response. And it is well known that PGE_2 and COX-2 are inducers of MDSCs. So, it might be these MDSCs which hinders the immune response against *Mtb* and promotes their survival (Agard et al., 2013; D'Avila et al., 2006; Knight et al., 2018). Most important is that *Mtb* catabolizes carbohydrates, lipids and amino acids within host to survive and that is one of the reason of presence of heterogeneous population of *Mtb* depending upon site of infection and stages (Lovewell et al., 2016). This shows that host lipid sources are not merely act as one of nutrient source for *Mtb* survival and pathogenesis but also modulates the host immune response.

In myeloid cells metabolic shifts are dynamic phenomenon. These metabolic shift i.e., shift from glycolytic metabolism to lipid metabolism is needed for the proper functionality of these cells (Sica et al., 2017). In case of MDSCs there is a very transparent shift from glycolysis to β-fatty acid oxidation (Al-Khami et al., 2016). Recently, a lot of reports are hypothesizing that MDSCs promotes *Mtb* survival and progression through modulation of metabolic shift. This metabolic shift in MDSCs from glycolysis to fatty acid oxidation is well characterized in cancer field. Along with these metabolic shifts there is increased lipid accumulation has been observed in these MDSCs. The central player of lipid metabolism in MDSCs is peroxisome proliferator-activated receptor gamma (PPARγ); and in TB, TLR-2 and TLR-4 upregulates it, leading to the formation of lipid droplets (Chinetti et al., 2000; Necela et al., 2008). Upregulation in expression of CD36 (LDL receptor) can be correlated with uptake of lipid and accumulation of *Mtb* within cell (Mahajan et al., 2012). In case of TB granuloma, there is huge amount of lipid, because of presence of foamy macrophages. *Mtb* very smartly hijack the host cell metabolic machinery to fulfill its growth requirement within the cell (Caceres et al., 2009; Korb et al., 2016; Russell et al., 2009). Interestingly, it has been observed that *Mtb* prefers to be up-taken or phagocytosed by foamy macrophages, as it is rich source of lipid where *Mtb* bacilli can easily survive and have an immune-modulatory microenvironment (Daniel et al., 2011; Peyron et al., 2008). In TB granuloma, these lipid laden foamy macrophages are derived from normal macrophages as a result of misbalance in influx and efflux of lipids (Kruth et al., 2002). Triglycerides, phospholipids and cholesterol are these lipid components and specifically the low density lipoprotein (LDL). In case of TB granuloma one of the reason for the presence of foamy macrophages are, providing niche for the survival and progression of *Mtb* in terms nutrient source (Peyron et al., 2008). In a similar way, MDSCs may provide a lipid rich sheltering niche for the *Mtb* survival and progression (Hossain et al., 2015). It is well known that there is high number of MDSCs in pleural effusion fluid of TB

patients with less T cell response against *Mtb* (du Plessis et al., 2013). Ultimately, lipid might act as one of the important carbon sources in MDSCs present in TB granuloma for the survival of *Mtb* during the infection (Peyron et al., 2008; Russell et al., 2009).

3.3.2. T cells and adaptive immune response in TB. Another immunity, which limits the spread of TB infection in the host is adaptive immunity. Initially, upon infection with *Mtb*, alveolar macrophages and DCs came into the limelight to curtail the infection. But as the MΦs and DCs get infected, they on one side initiate the immune response against this pathogen and the other side becomes a niche for the growth and survival of these bacilli. The innate immune system is not highly specific in nature. But in the case of TB infection, specificity is needed to stop this infection and the adaptive immune system has acquired this specificity with time and is highly conserved. It is well known that in the case of *Mtb* infection protection or immunity and pathogenesis of disease both are regulated and mediated by T cells (Cooper, 2009). The absence of an adaptive immune response against *Mtb* boosts the bacterial burden within the host and also makes it more susceptible to infection (Lefford, 1975). On the other side, adaptive immune system (T cells) have crucial role in controlling the infection, but along with this function it induces lesion and necrosis; which ultimately leads to the transmission of *Mtb* bacilli. So, it shows that the adaptive immune system has a very decisive part during *Mtb* infection.

Mtb upon phagocytosed by alveolar MΦs induces arrays of the immune response against this pathogen. With time there occurs granuloma formation, a structure consisting of infected MΦs along with other immune cells (MΦs, neutrophils, T cells, DCs). But, DCs have a separate mode of action, upon encountering *Mtb*; DCs having *Mtb* moves to the draining lymph and turn on the naïve T cells through presentation of antigens. It's only DCs which have the potential to activate the naïve T cells. As DCs constitutively exhibits both MHC I/II complexes along with costimulatory molecules and cytokines. But the

major loophole in stopping TB infection is the delay in initiation of T cell response. After infection, T cells get activated and reach the site of infection after 2-3 weeks. In granuloma structure, T cells interact with other mononuclear cells (DCs, MΦs, monocytes) and soluble mediators to inhibit the spread of *Mtb* infection, but this complex interplay between these cells is not able to successfully eradicate the *Mtb* bacilli (Cooper, 2009). CD4 lymphocytes have a major role during *Mtb* infection. Further, it has been confirmed from the increased number of tuberculosis reports in HIV patients (Kaufmann and Andersen, 1998). Turning on CD4 lymphocytes by DCs leads to pro-inflammatory cytokines secretion. These cytokines further prompt the instigation of other immune cells and skewing of immune response more towards Th1 and Th17 types. Moreover, CD8 T cells also have an imperative role in *Mtb* infection. On one side these soluble mediators secreted by CD8 T cells activate other immune cells and on the other side kill *Mtb* infected macrophages and *Mtb* also. Another important T cell subset is γ/δ T cells, as these cells do have not CD4 and CD8 marker but can kill the *Mtb*, or induces an immune response against it after they recognize the lipid antigens of *Mtb* through the CD1 receptor (Moody et al., 1996). The Th1 and Th17 immune responses are well known as anti-TB immune responses (Chackerian et al., 2001). IFN-γ, TNF-α and IL-2 secreting T cells are more effective against the *Mtb* and are better than other lymphocytes (Seder et al., 2008). DCs along with activation, prompts T cell differentiation more towards Th1 and Th17 types. Overall, a lot of strategies are being now adopted where activation of T cells is being targeted, so that the delay in translocating of these lymphocytes at the site of *Mtb* infection can be shortened or removed.

In host T cells have a central part in neutralization and elimination of pathogens. Lymphocyte development occurs in the thymus, and TCR on their surface along with specialized glycoprotein classifies them to different sub-types. T cells having CD4 and CD8 glycoproteins on their surface are known as CD4 T cells and CD8 T cells. Differentiation of

naïve T cells is instigated through various cytokines present in their surrounding microenvironment. The presence or stimulation of cytokines induces the CD4 lymphocytes differentiation. IL-12 and IFN-γ prompt Th1 differentiation from naïve T cells. Similarly, IL-4 instigates Th2 cells, and IL-6 and TGF-β augments differentiation of Th17 cell. Additionally, IL-4 and TGF-β prompt Th9, IL-6 and TNF prompt Th22, IL-6 and IL-21 to form Tfh (T follicular cell) and finally, IL-2 and TGF-β instigate differentiation into Tregs (Raphael et al., 2015). All these CD4 T cell subsets secrets specific cytokines as their signature mark; such as Th1 (interferon-γ and TNF), Th2 (interleukin 4, 5 and 13), Th9 (interleukin 9), Th17 (interleukin 17, 21, 22, 25 and 26), Th22 (interleukin 22), Tfh (interleukin 21) and Tregs (interleukin 10 and TGF-β) (Murai et al., 2009; Raphael et al., 2015). Literature shows that different types of microbial/antigens stimulate DCs to behave and secrete cytokines distinctly. DCs and macrophages have the flexibility of inducing T cells in terms of microbial insult encountered by them. LPS or IFN-γ induces DCs to secrete IL-12, which ultimately induces a Th1 immune response. On the other side IL-10 and prostaglandin E2 (PGE2) induce DCs to release IL-4 and reduce secretion of IL-12, which leads to generation of Th2 immune response. Similarly, there are various factors which induce the DCs to secrete different types of cytokines and finally lead to generation of different subtypes of T cell. In tuberculosis, induction of Th1 and Th17 immune responses is very imperative for restricting this infection (Lyadova and Panteleev, 2015).

3.4. *Mtb* and its components. *Mtb*, the causative agent of TB, is responsible for causing high morbidity and mortality worldwide. *Mtb* (H37Rv) genome was completely sequenced in 1998 followed by genome sequenced of clinical isolates of *Mtb* (CDC1551) and *Mycobacterium bovis* AF2122/97 (Cole et al., 1998; Garnier et al., 2003). *Mtb* H37Rv is a virulent strain, whereas, *Mtb* H37Ra is avirulent strain derived from H37 and was isolated (1905) from pulmonary tuberculosis 19 year old male patient (Steenken and Gardner, 1946). The derivation of H37Ra

occurs from virulent strain H37Rv with aging i.e., H37Rv culture was incubated for a period of 3-4 months and after that there is emergence of a secondary growth with no potential of causing disease. After that the alphabets 'a' (avirulent) and 'v' (virulent) was designated to H37 strain (Steenken Jr, 1935). Comparing to H37Rv genome (4,411,532), H37Ra genome is contains additional 8,445 bp. The increase in size of H37Ra genome is because of presence of 53 insertions and 21 deletions and moreover there are 198 single nucleotide variations (SNVs) (Zheng et al., 2008).

It has reported that major secretory proteins in the culture filtrate of *Mtb* are Rv3804c (Ag85a), Rv1886c (Ag85b), Rv0129c (Ag85c), Rv3803c (Ag85d), MPT63 (Rv926c), MPT64 (Rv1980c), ESAT-6 family, Rv0040c and lipoproteins as observed through SDS-PAGE. In case of ESAT-6 family, Rv0287 (EsxG), Rv1198 (EsxL), Rv1793 (EsxN), Rv2346c (EsxO), Rv3874 (EsxB) and Rv3875 (EsxA) constitutes a major portion of secretory proteins of *Mtb*. Using various molecular techniques such as MALDI-TOF MS, LC-MS, 2D-PAGE, SDS-PAGE; a total 257 proteins has been identified as secretory proteins (Malen et al., 2007).

MPT64 is an actively secretory protein with most of unknown function and specific to *Mtb* complex (Harboe et al., 1986; Wang et al., 2007). Moreover, it has been considered that mycobacterial secretory proteins are immunodominant and provides protection to host (Andersen, 1997). Interestingly region encoding MPT64 gene is deleted in many Bacillus Calmette Guèrin strains (Behr et al., 1999). MPT64 also known as Rv1980c is 687 bp long protein with 228 amino acids. MPT64 (2HHI) NMR solution structure (Sehnal et al., 2021; Wang et al., 2007) depicts it as a protein from new family of beta grasp proteins (Fig. C). MPT64 has a molecular weight of approx. 24 kDa and isoelectric point is 4.6. This protein is located outside the cell membrane and mostly located in cell wall and extracellular space (Wolfe et al., 2010). Moreover, MPB64

from *M. bovis* is identical to MPT64 from H37Rv. In case of *M. bovis* BCG strains, MPT64 is being encoded by one of the gene of region of deletion 2. Most importantly, MPT64 from *M. bovis* BCG, has β-grasp like domains consisting of α-helix and β-sheets at its N terminal region (Wang et al., 2007). Another, important aspect of secretory proteins is induction of pathogenesis. MPT64 (Rv1980c) protein isolated from the non-virulent Mtb strain H37Ra has been characterized. The reason for choosing H37Ra strain for its isolation, expression and purification was to reduce the concern of toxicity associated with virulent (H37Rv) strain, while exploring its pathogenicity mechanism (Bange et al., 1999; Chu and Yuann, 2011). As, it has been seen that *Mtb* has evolved many strategies for its survival, one among many strategies is inhibition of phagosome maturation and acidification. But it's very surprising that there is no phagosome modification upon phagocytosis of dead *Mtb* bacilli (Beatty and Russell, 2000; Stewart et al., 2005). The expression level of MPT64 protein in active TB individuals is high and has been observed in their serum and sputum (Mehaffy et al., 2017). As this protein along with other proteins is secreted by actively dividing *Mtb* (Gaillard et al., 2011). The major function of this protein is to promote the growth and persistence of bacilli in host cell.

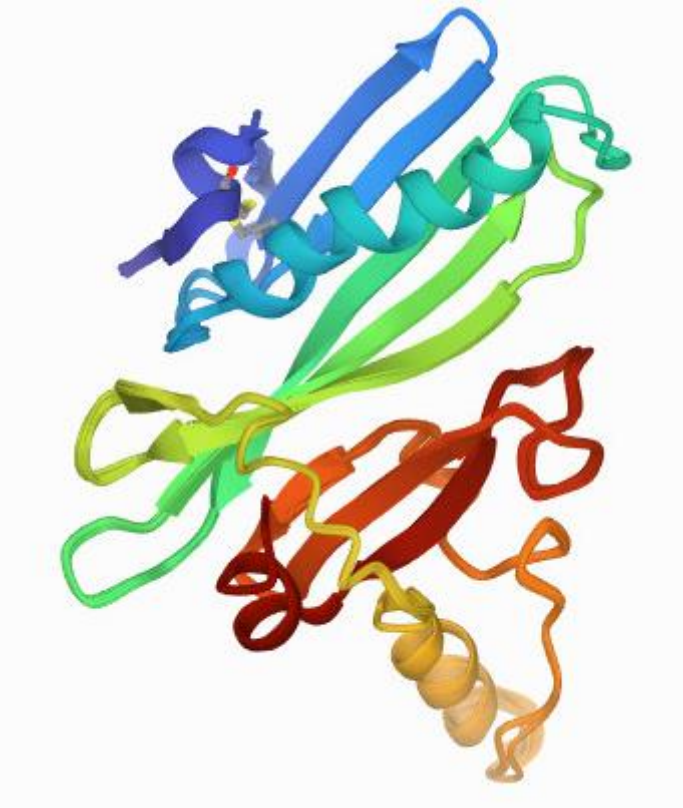


Figure C. Structure of MPT64 protein. MPT64 also known as Rv1980c is 687 bp long protein with 228 amino acids. MPT64 (2HHI) NMR solution structure depicts it as a protein from a new family of beta grasp proteins (Sehnal et al., 2021; Wang et al., 2007).

It has been hypothesized that the secretory proteins of *Mtb* are the first ones that interact with the immune system of the host. It has been seen that MPT64 is recognized by human T helper 1 cells and hence can be a potential tool for TB diagnostics and as a vaccine candidate (Mustafa, 2010). But a lot of variations have been seen in the diagnostic accuracy of MPT64, and might be owing to changes in MPT64 structure or sequence. In MPT64 antigen, 23 T cell epitopes have been recognized through IEDB (Immune Epitopes Database) (Ernst et al., 2008). Many variations in these epitopes have been observed in terms of nucleotide alterations. It has been reported that, MPT64 gene from MTBC shows polymorphisms, and this lead to a change in the antigenic structure of this protein. Ultimately, this change leads to the immune evasion (Jiang et al., 2013). And hence allow the survival and persistence of *Mtb* within the host.

MPT64 has been observed to boost the secretion of pro-inflammatory and IL-10 cytokines from MΦs (RAW274.7) cells and might be involved in inhibiting the apoptosis of MΦs (Fan et al., 2013). In granuloma, TNF-α and IFN-γ have been noticed to activate MΦs with augmented bactericidal activity (Flynn et al., 1995). Interestingly, IL-10 and TGF-β are also observed in the granuloma. This shows that there is very dynamic correlation between these cytokines in the granuloma (Kindler et al., 1989). But anti-inflammatory cytokines augments growth and the survival of *Mtb* by inhibiting the activation of MΦs and immune response (Murray et al., 1997). Further, it has been reported that virulent *Mtb* inhibits the apoptosis by modulating the secretion of Fas, FasL, Bax and Bcl2 (Keane et al., 2000; Mogga et al., 2002). This ultimately leads to the progression in the growth of *Mtb*. In granuloma, MΦs differentiate into epithelioid cells (ECs) and combine to form multinucleated giant cells (MGCs) (Takashima et al., 1993). Increased expression of MPT64 and Fas, FasL, TGF-β was observed in the MGCs and it shows that MPT64 might inhibits the apoptosis of infected MGCs and promotes the survival and growth of *Mtb* in them (Mustafa et al., 2008).

A lot of work on MPT64 has been focused in terms of using it as a diagnostic tool. MPT64 has been used as an aptasensor for detection of *Mtb* (Sypabekova et al., 2019). Usage of MPT64 in the Patch Test for Active Tuberculosis (PTAT) has an advantage over the use of PPD (purified protein derivative) in terms of less cross-reactivity with mycobacteria other than *Mtb* (MOTT) (Harboe et al., 1986; Nakamura et al., 2001). As secretory proteins are mostly immunogenic, so MPT64 has been delineated as a vaccine candidate. MPT64 alone or in combination with other secretory proteins such as Ag85 and ESAT-6 has been checked for their efficacy against *Mtb*. Intriguingly, MPT64 alone or in combination with other antigens have not shown better protection efficacy than that of BCG vaccine (Bai et al., 2008; Tian et al., 2004). Furthermore, it has been observed in *Mtb* H37Rv that

deletion of RD2 locus declines the pathogen load in lungs and spleen of mice after 3 months of infection (Sonnenberg and Belisle, 1997). Intriguingly, it has been observed that infection with MPT64 mutant strain (Mtb∆*mpt64*) as compared to wild-type *Mtb* strain generates less bacterial burden in mice, 3 weeks post-infection (Stamm et al., 2019).

3.5. Immune response to *Mtb* components. A better understanding of the mechanism concerning host-pathogen interaction is the clue for innovation of new vaccines, drug targets and diagnostics. Within the host the *Mtb* infects alveolar MΦs and DCs; this alveolar MΦs uptake the *Mtb* and initiates a cascade of immune events. Most importantly, *Mtb*, inside the MΦs inhibits the phagolysosome fusion for its survival and persistence (Sturgill-Koszycki et al., 1994). Because with host, *Mtb* has also evolved itself and generated multiple immune response evasion strategies. Along with causing infection, *Mtb* and its components have many immunomodulatory and modulatory roles in host cellular processes (Fig. D). Within cells, phagosome fusion with lysosome is needed for the maturation of phagosome after infection; and this phagolysosome fusion results in acidification and accumulation of proteolytic enzymes in it. *Mtb* has evolved many strategies to survive within the MΦs one is the inhibition of the phagolysosome fusion (Russell, 2001). But *Mtb* evades the phagolysosome fusion in the cytosol of MΦs (Simeone et al., 2012). Also, proteins like PtpA and SapM hinder the maturation of phagosomes. PtpA (protein tyrosine phosphatase) is a substrate for VPS33B (Vesicular protein sorting 33B). Moreover this protein of *Mtb* inhibits the acidification of the phagosomes (Wong et al., 2011). Another modulatory role of *Mtb* protein Ndk (nucleoside diphosphate kinase) is that it interacts with Rac1 protein of host cell and stops the assembly of NOX2 (Miller et al., 2010). NOX2 (NADPH oxidase) produce reactive oxygen species (ROS)(Gabig and Babior, 1981) and has various cellular effects. One of the important roles of ROS is antimicrobial efficacy against various intracellular pathogens.

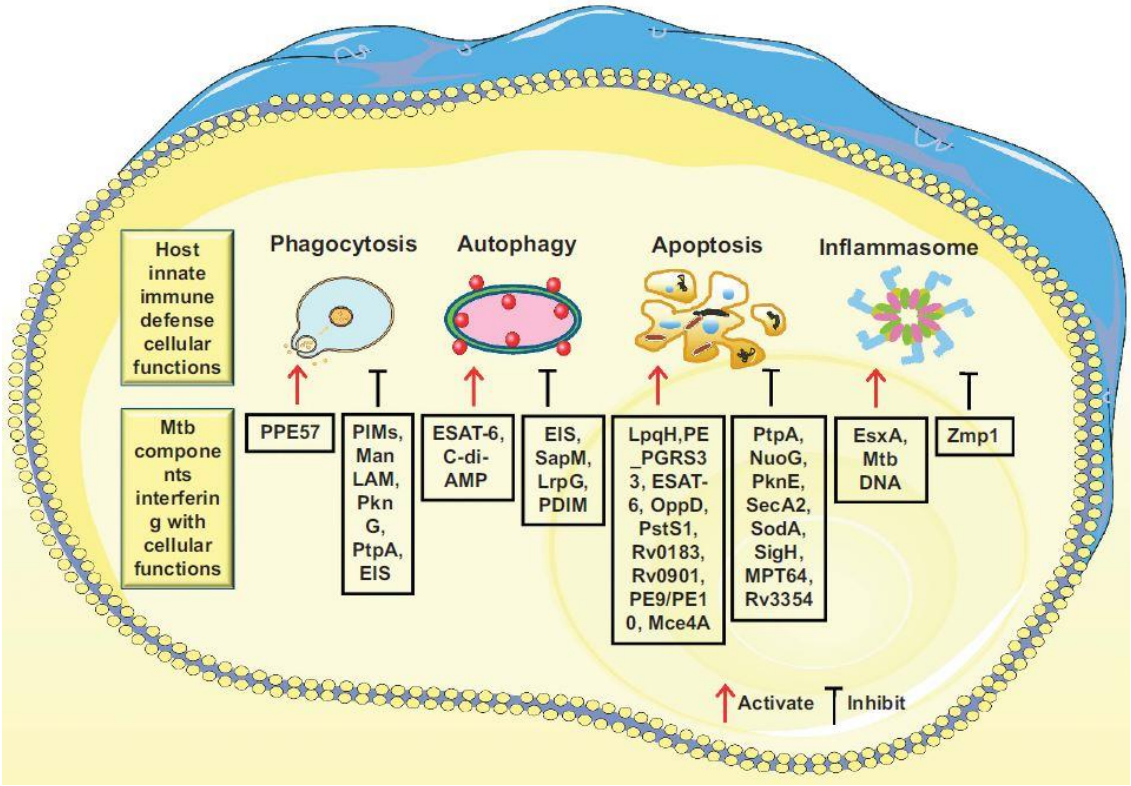


Figure D. Modulation of host cellular functions by *Mtb* components. Myeloid cells (MΦs, DCs, neutrophils) and NK cells perform a cardinal function during *Mtb* infection. Upon recognition of *Mtb* components by the myeloid cells through pattern recognition receptors initiates phagocytosis, apoptosis, autophagy and inflammasome activation. Various components of *Mtb* either stimulates (PPE57, ESAT-6, C-di-AMP, Rv0183, Rv0901, PE9/PE10, Mce4A, PstS1, OppD, PE_PGRS33, LpqH, EsxA, DNA) or inhibits (Zmp1, MPT64, Rv3354, SodA, SecA2, PknE, NuoG, PtpA, SigH, EIS, SapM, LrpG, PDIM, PIMs, ManLAM, PknG, PtpA) the cellular functions for their survival and persistence (Liu et al., 2017).

In case of *Mtb*, role of APCs in induction of adaptive immune response is well known and *Mtb* has evolved mechanisms to modulate the MHC II expression (Cooper, 2009). It has been seen that purified proteins such as LprA, LprG and LpgH inhibits the MHC II expression (Gehring et al., 2004; Noss et al., 2001; Pecora et al., 2006). Similarly, another protein Hip1 also inhibits MHC II expression. Another important role played by LAM, LM and PIM of *Mtb*, is hampering of MHC II molecule expression

(Saraav et al., 2014). Another way of hampering MHC II expression by *Mtb* is inhibition of Cathepsin S expression through over-expression of IL-10 in host cells (Sendide et al., 2005).

Apoptosis is also known as programmed cell death is a much-regulated process. During this process the apoptosis cell breaks down into membrane-bound bodies known as apoptotic bodies. These apoptotic bodies express phosphatidyl serine on the surface of these apoptotic bodies as a signal for phagocytosis by phagocytic cells (DCs and MΦs) (Lee et al., 2009). *Mtb* obstructs the apoptosis and prompts necrosis to promote the survival and spread of *Mtb* to other nearby cells through PknE (Protein kinaseE), Ndk, nuoG, SecA2 and ptpA (Jayakumar et al., 2008; Sun et al., 2013; Velmurugan et al., 2007). Another secretory protein of *Mtb*, MPT64 is known to inhibit the apoptosis of macrophages (RAW264.7) *in vitro* through up-regulation of bcl-2 (Wang et al., 2014). *Mtb* infected macrophages exhibits Th2 immune response, whereas DCs infected with *Mtb* exhibits Th1 immune response (Khader et al., 2007). It has been reported that infected DCs through the TLR-9 pathway secretes IL-12; whereas, macrophages need TLR-2 for secretion of IL-12 (Pompei et al., 2007). As it is well known that *Mtb* infected DCs secrete IL-12 for Th1 differentiation from naïve T cells, so that an effective immune response can be mounted against it (Vieira et al., 2000). Further, it has been observed that *Mtb* generally utilizes the DCs to modulate the immune response, as *Mtb* infected DCs lost their antigen presentation potential (Jiao et al., 2002). Another protein of *Mtb*, MTSA (10 kDa) impairs the DCs maturation (Sinha et al., 2006). Rv1016c protein of *Mtb* promotes its survival within macrophages and is a component of *Mtb* cell wall (Gonzalez-Zamorano et al., 2009). Acr1 also known as Rv2031c/19 kDa/Hsp16.3/HspX has a molecular weight of ~16 kDa (Sobott et al., 2002). Along with this our group has already shown that latency-associated protein of *Mtb*, Acr1 have diametric role and it impairs the maturation and differentiation of DCs (Amir et al., 2017).

Chapter-4

Materials and Methods

4. MATERIALS AND METHODS

4.1. Antibodies and reagents. All the stated standard chemicals and reagents utilized in the study were procured from Sigma (St. Louis, MO). ELISA Abs and recombinant cytokines were procured from BD Biosciences (San Diego, CA). Abs used for flowcytometry (CD11c-PE-Cy7, F4/80-APC, CD11b-APC-Cy7, CD40-APC, CD80-FITC, MHC II-PerCp-Cy5, CD86-PE, Ly6C-PE-Cy7, Ly6C-PerCP-Cy5.5, Ly6G-PE-Cy5.5, CCR7-APC, Gr-1-APC, PD-L1-PE, TIM-3-APC, FoxP3-APC, CD25-APC-Cy7, CD4-PE and CD8-FITC) were purchased from eBiosciences, Biolegend and BD Biosciences (San Diego, CA). RPMI-1640 and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY). All secondary fluorochrome tagged anti-mouse and anti-rabbit antibodies for confocal microscopy were purchased from Invitrogen Thermo Scientific (Carlsbad, CA). Antibodies for Western blotting were purchased from Cell Signaling Technology (Danvers, MA), BD Biosciences (San Diego, CA), Biolegend (San Diego, CA) and Abcam (Cambridge, UK). All plasticware was procured from BD Biosciences (San Diego, CA), Thermo Fisher Scientific (Carlsbad, CA), Costar™ and Corning™ (Corning, NY), or Falcon (Corning, NY) and Sigma Aldrich (St. Louis, MO).

4.2. Animals. Female C57BL/6 and BALB/c mice (aged 6-8 weeks) were procured from animal house facility of the institute. Institutional Animal Ethics Committee (IAEC) approved all protocols; experiments were performed according to the approved protocols and guidelines issued by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (No. 55/1999/CPCSEA), Ministry of Environment and Forest, Government of India.

4.3. Cloning, expression and purification of MPT64. MPT64 gene was amplified from the genomic DNA of *Mtb* strain H37Ra through polymerase chain reaction by using primers, forward: 5´-AATTGGATCCGTGCGCATCAAGATCTTCATG-3´, reverse: 5´-AATTGAATTCGGCCAGCATCGAGTCGATCG-3´ primers, and cloned into

BamHI and *EcoRI* site of pET28a vector. The plasmid was further transformed into *E. coli* Rosseta (DE3) strain for its expression. The transformed culture was incubated in Studier auto-induction broth containing kanamycin at 37 ^{o}C in a shaker incubator (200 rpm) overnight for protein induction (Studier, 2005). Finally, the cultures were harvested and the MPT64 protein-containing His tag was purified through the Ni-NTA affinity column. Harvested cells were re-suspended in equilibration buffer (NaCl: Tris-HCl: imidazole-500 mM: 10 mM: 5 mM; pH 8.0) and lysed through sonication. The lysed cells were pelleted down at 7,000g at 4 ^{o}C for 0.5 h. The SNs was put onto the Ni-NTA column for protein binding and impurities were removed using wash buffer (NaCl: Tris-HCl: imidazole-500 mM: 10 mM: 10 mM; pH 8.0). Finally, the protein was eluted from the column by elution buffer (NaCl: Tris-HCl: imidazole-500 mM: 10 mM: 300 mM; pH 8.0) and collected into sterile Eppendorf tubes. Excess salt from purified protein was removed through dialysis using a 10 kDa cut-off dialysis membrane. Finally, MPT64 protein was reconstituted in Tris-NaCl buffer and stored at 4 ^{o}C. The purity of MPT64 was confirmed through 12% SDS-PAGE and was >98%. Further, protein concentration was measured by Bradford assay. Endotoxin in MPT64 was <0.05 EU/ml, as estimated by LAL chromogenic endotoxin quantitation kit, as per the manufacturer's protocol (Thermo Scientific, Carlsbad, CA).

4.4. Matrix-assisted laser desorption ionization-time of flight mass analysis and peptide mass fingerprinting. MPT64 (1 mg/ml) was analyzed for its intact molecular mass through MALDI-TOF. MPT64 was assessed through SDS-PAGE. Then a band of purified protein was excised from the gel. The band was washed with type 1 distilled water. Further, the gel was de-stained (25 mM NH_4HCO_3 containing 50% ACN) and dehydration of protein was done with 100% acetonitrile. Gel pieces were dried using a vacuum concentrator and digestion was done at 37 ^{o}C with 0.1% trypsin (diluted in 50 mM NH_4HCO_3) overnight. The MPT64 was extracted with 5% TFA and 50% ACN. The solution of CHCA and dH_2O-ACN (1:1) having 0.1% TFA was used as a matrix. The

peptide extract solution along with the matrix was assessed through MALDI-TOF/MS. The MS spectrum of peptides was submitted to MASCOT server 2.4 with NCBI selected as the database.

4.5. Dendritic cell and macrophage culture. The methodology for culturing of bone marrow-derived DCs and MΦs was followed as per the protocol mentioned by Lutz *et al* and Weischenfeldt *et al* (Lutz et al., 1999; Weischenfeldt and Porse, 2008). BMCs were isolated from the hind limb bones of C57BL/6 mice. RBCs were lysed with ACK lysis buffer. BMCs (2 X 10^6/well) were cultured in RPMI-1640 medium with GM-CSF (2 ng/ml) and IL-4 (4 ng/ml). The cultures were kept for 7 d in CO_2 (5%) incubator at 37 ^oC. After 3 d medium was replenished. Abbreviations used in the text. Complete medium: RPMI-1640+FCS-10% having penicillin-100 U/ml, L-glutamine-100 mM, streptomycin-100 mg/ml and GM-CSF + IL-4. DCs: BMCs cultured with GM-CSF + IL-4; DC^{MPT64}, DC^{CFP-10} and DC^{ESAT-6}: BMCs cultured with GM-CSF + IL-4 along with MPT64, CFP-10 and ESAT-6, respectively. Similarly, for macrophages, BMCs (2 X 10^6/well) were cultured in complete medium with L929 sup-10%. The cultures were kept for 7 d in a CO_2 (5%) incubator at 37 ^oC. After 3 d medium was replenished.

4.6. Cell viability assay. The cell viability of DCs and DC^{MPT64} was checked through PI/Annexin V assay, as per the protocol described previously (Riccardi and Nicoletti, 2006). Briefly, the cells after culturing for 6-7 d, were harvested and washed followed by re-suspended in binding buffer solution [0.01M HEPES (pH 7.4), 2.5 mM $CaCl_2$ and 0.14M NaCl]. Later, cells were incubated with Annexin V-FITC (5 µl/tube) and PI (2 µl/tube) (50 µg/ml) for 15 min in dark at RT. Then, an additional 400 µl binding buffer was added and samples were analysed on a flowcytometer (BD Verse).

4.7. Scanning electron microscopy (SEM). DCs, DC^{MPT64} and DC^{CFP-10} (5 X 10^5/well) were cultured on Poly-L-Lysine glass coverslips and fixed with modified Karnovsky's fixative (MKF) at 4 ^oC for 180 min. Cells were washed and dehydrated with ethanol (30%, 50%, 70%) gradients each

for 0.5 h at 4 ^{o}C. Cells were again dehydrated with ethanol (90% and 100%) for 0.5 h at 25 ^{o}C. Tetra-butyl alcohol was used to dehydrate cells twice for 0.5 h at 25 ^{o}C followed by TBA removal and freeze drying at -120 ^{o}C for 180 min. Finally, pictures were taken at 13.87KX and 2.49KX magnification on a scanning electron microscope (Jeol, Tokyo, Japan).

4.8. Arginase activity assay. Arginase was estimated through arginase activity assay. For 6 d, DCs and DC^{MPT64} (2.5 X 10^5/well) were cultured in a complete medium. The cells were isolated on the 7th day and washed with 1 X PBS; 100 µl of water consisting of 0.1% Triton X-100 and protease inhibitor was added and incubated for 30 min at 37 ^{o}C. Then, Tris-HCL (25 mM), $MnCl_2$ (333 µM) in 100 µl volume (pH 7.5) was added incubated for 10 min at 56 ^{o}C. Then, L-arginine (0.5 mol/L) in 200 µl volume was added and heated for 30 min at 37 ^{o}C. Later, reaction was halted with the solution (H_2SO_4:H_3PO_4:H_2O; 1:3:7) (400 µl). Finally, 50 µl of α-isonitrosopropiophenone was added for colorimetric detection of urea, followed by heating the mixture for 45 min at 56 ^{o}C. Lastly, reaction mixture was incubated at room temperature for 10 min. The urea formed was measured calorimetrically by taking absorbance at 540 nm (Baumann et al., 2020; Corraliza et al., 1994; Cowburn et al., 2016).

4.9. ROS detection. DCs and DC^{MPT64} (5 X 10^5/well) were cultured for 6 d in RPMI-1640+FCS-10%. The cells were harvested on the 7th day and washed with PBS-1X. ROS production was measured using 2,7-dichlorofluorescein diacetate (H_2DCFDA) (2.5 µM) in media. Cells were kept at 37 ^{o}C in 5% CO_2 incubator for 0.5 h after the addition of H_2DCFDA. After washing with PBS-1X the fluorescence intensity (Ex/Em : 485/525 nm) was measured using a microtiter plate reader (BioTek, Winooski, VT)(Ling et al., 2011).

4.10. Nitric oxide estimation. DCs and DC^{MPT64} (5 X 10^5/well) were cultured for 6 d in RPMI-1640+FCS-10% medium. Further, nitric oxide (NO) was measured using Griess reagent in the SNs. Briefly, Griess

reagent (50 µl) was added to culture SNs (1:1) and kept at 25 ^{o}C for 10 min. Then, plates were read at 550 nm. For measuring NO, $NaNO_2$ was used as the standard (Guevara et al., 1998).

4.11. Nile red staining. DCs, DC^{MPT64} and DC^{CFP-10} (5 X 10^5/well) were cultured in complete medium for 6 d. The cell cultures were harvested on 6 d followed by washing with PBS-1X. Nile red dye was added (1:100 of 1 mg/ml) to the cultures and kept at 25 ^{o}C for 5-10 min. Cells were washed 2X with PBS-1X and the presence of Nile red was monitored through flowcytometry and fluorescence microscopy (Nikon Eclipse 80i, Melville, NY)(Greenspan et al., 1985).

4.12. Methylglyoxal staining (CLSM). DCs and DC^{MPT64} (5 X 10^5/well) were cultured and fixed on poly-L-Lysine glass coverslips with 4% PFA (paraformaldehyde). The cells were then incubated at 25 ^{o}C for 10 min. Cells were permeabilised with 0.1 % Triton X-100 for 5 min at RT. Unspecific sites were blocked over the cells by incubating with 2% BSA for 1 h at 25 ^{o}C. Afterwards, culture was incubated with anti-methylglyoxal Ab (1:50) for 1 h at RT, followed by anti-rabbit-Alexa fluor 647 Ab (1:1000 dilution) and Alexa fluor 488 Phalloidin (1:500 dilution) for 1 h at RT. Finally, cells were mounted onto glass slides with slow fade containing DAPI. The cells were then imaged using Nikon A1 confocal microscope (Nikon, Tokyo, Japan) and images were analysed by Nikon-NIS-AR 4.1 image analysis software (Nikon, Melville, NY). DCs cultured overnight with H_2O_2 (50 µM) was a positive control (DC^{pos}). Similarly, culture was set for flowcytometry and cells were acquired on BD-FACS Verse and analyzed analysed on BD-DIVA software.

4.13. Bacterial strains and culture. *Mycobacterium tuberculosis* (H37Rv) was a kind gift from the JALMA Institute, Agra. Mycobacterial strains (H37Rv, GFP-H37Ra) were cultured in Middlebrook 7H9 broth containing glycerol (0.2%), Tween-80 (0.05%) and OADC. Further, the *Mtb* viability was assessed through colony-forming units (CFUs) over Middlebrook 7H11 medium having OADC after 21 days.

The *E. coli* strain DH5α was used for the cloning of MPT64 (Rv1980c). The *E. coli* Rosetta (DE3) strain was used to express MPT64.

4.14. Quantification of cytokines by ELISA. DCs and DC^{MPT64} (5 X 10^5/well) were cultured for 6 d in complete medium. Cytokines were measured in supernatants (SNs) through sandwich ELISA. Initially, plates were coated with anti-Abs (IL-12, IL-6, TNF-α, IL-17, IL-4, IL-10, IFN-γ and TGF-β) at 4 ^oC/ overnight. Blocking was done with BSA (1%) for 2 h. Then, culture SNs were put along with respective recombinant cytokines standard plates were kept at 4 ^oC. The samples were then incubated with respective biotinylated capture Abs 2 h/37 ^oC. Washings were given to remove unbound Abs, and incubated with streptavidin-HRP was done for 45 min/37 ^oC. After each step, regular washing procedures were performed with PBS-Tween-20 (0.05%). Later, H_2O_2 and ultraTMB were added and the reaction was stopped using H_2SO_4 (7%). Reading of plates were taken at 492 nm. Standard plots generated from recombinant cytokines of known concentrations were used to quantify secreted cytokines.

4.15. Expression of surface markers by flowcytometer. DCs, DC^{MPT64} and DC^{CFP-10} (5 X 10^5/well) were cultured in a complete medium for 6 d. The cells were harvested on the 7^{th} day and surface stained with fluorochrome labelled Abs to CD11c, CD11b, F4/80, Ly6c, Ly6G, CCR7, Fox-P3, CD25, CD4, CD8, TCRβ and TCRγ/δ. Cells were acquired through flowcytometry and expression was monitored through BD DIVA software.

4.16. *In vivo* cell migration assay. DCs, DC^{MPT64} and DC^{CFP-10} (2 X 10^6/well) were cultured in 6 well plate complete medium for 6 d. Next, the cells were re-suspended in CFSE (1 ml, 2 µM) and incubated for 8 min at 37 ^oC. Later, FBS (2 ml) was added and cultures were spin at 2000 rpm at RT for 3 min. The cell pellet was washed 2X with PBS-1X+FBS-20%. The CFSE-labelled cells were intravenously injected into

C57BL/6 mice. After 24 h and 48 h, the animals were sacrificed and splenocytes were monitored for the presence of CFSE-labelled DCs by flowcytometer. Results were analysed through BD DIVA software and represented in terms of fold change migration. Later, the expression of CCR7 was monitored on these cells through flowcytometry.

4.17. Soluble antigen uptake assay. DCs and DC^{MPT64} (5 X 10^5/well) were cultured for 6 d in complete medium at 37 ^oC/5% CO_2. Then cells were incubated with FITC-dextran (100 µg/ml) for 30 min/37 ^oC. Washings were given to cells with 1 X PBS containing 1% BSA. Finally, cells were acquired through a flowcytometer and analysed for the expression of dextran-FITC through BD DIVA software.

4.18. Mycobacterial uptake and intracellular survival. DCs and DC^{MPT64} (5 X 10^5/well) were cultured in 24 well plate for 6 d in complete media followed by harvesting at 7^{th} day and infected with *Mtb* strains H37Ra (GFP-*Mtb*) or H37Rv for 4 h at MOI of 1:5 (3 h for *M. smegmatis*) in antibiotics free RPMI-1640 media. Extensive washings with 1 X PBS were given to the cells. Later, to kill the extracellular bacilli, amikacin (2 µg/ml) (for H37Rv) and gentamycin (50 µg/ml) (for H37Ra and *M. smegmatis*) were added to antibiotic-free RPMI-1640 media and added to cell cultures for 60 min at 37 ^oC. To monitor the uptake after 4 h of *Mtb* (GFP-H37Ra, H37Rv, *M. smegmatis*) infection, lysis of cells were done by saponin (0.1%). Plating was done on 7H11 agar plates. After 3 weeks colonies were counted through colony forming units. Expression of uptake and survival of *Mtb* GFP-H37Ra were monitored through flow cytometry. Further, to assess the survival of *Mtb* (H37Rv) within DCs and DC^{MPT64}, infected cells were rested for 72 h at 37 ^oC in 5% CO_2.

4.19. Mycobacterial uptake by cells (CLSM). DCs and DC^{MPT64} (5 X 10^5/well) were cultured over poly-L-Lysine coated coverslips for 6 d in complete medium. Later, *Mtb* GFP-H37Ra bacterial stocks were taken out from -80 ^oC and rapidly thawed, washed and re-suspended in antibiotics free complete medium. Next, cells were infected with *Mtb*

GFP-H37Ra (MOI 1:5) in antibiotics free media for 4 h and kept at 37 ^{o}C in a 5% CO_2. Extensive washings with 1 X PBS were given to remove the extracellular bacteria and treated with gentamycin (50 µg/ml) for 1 h at 37 ^{o}C in 5% CO_2. After washing with 1 X PBS, cells were fixed with 1% PFA. Next, glass coverslips were mounted on slides with slow fade having DAPI. Cells were then imaged and analysed using confocal microscope.

4.20. Methylglyoxal accumulation upon mycobacterial infection (CLSM):

DCs and DC^{MPT64} (5 X 10^5/well) were cultured on sterile coverslips in 12 well plates for 6 d in complete media (RPMI-1640 + 10% FCS). Later, *Mtb* GFP-H37Ra bacterial stocks were taken out from -80 ^{0}C and were rapidly thawed, washed and re-suspended in antibiotics free RPMI-1640 + 10% FCS media. Next, cells were infected with *Mtb* GFP-H37Ra in antibiotics free media for 4 h/37 ^{0}C in 5% CO_2 incubator. Gentamycin (50 µg/ml) was added to the cultures for killing extracellular bacteria. Cells were then 1X PBS washed and fixed with 4% PFA. Fixed cells were permeabilised with Triton X-100 (0.1%) for 90-120 s and immediately washed thrice with 1 X PBS. Non-specific sites in the samples were blocked with 2% BSA for 1 h. Cells were incubated with methylglyoxal monoclonal Ab (1:50 dilution) at RT for 60 min. After that, secondary Alexa fluor 647 anti-rabbit Ab (1:1000 dilution) was added and kept for 60 min at RT. Next, glass coverslips were mounted on slides with slow fade having DAPI. During each steps 1 X PBS washings were given. Pictures were taken using confocal microscope and analysed using Nikon-NIS-AR 4.1 software.

4.21. Glucose uptake assay. DCs and DC^{MPT64} (5 X 10^5/well) were cultured for 6 d in complete medium at 5% CO_2/37 ^{o}C. On the 7^{th,} day culture was re-suspended in a glucose-free complete medium having 2-NBDG (10 µM) and incubated for 30 min/37 ^{o}C in 5% CO_2. After incubation, cells were washed with PBS-1X. Cells were then monitored

for 2-NBDG expression through flowcytometry and data analysed by BD DIVA software. For monitoring 2-NBDG uptake by confocal microscopy, DCs and DC^{MPT64} (5 X 10^5) were cultured on glass coverslips and incubated with 2-NBDG for 30 min/37 ^oC. Finally, after PBS-1X wash, cells were mounted onto glass slides with slow fade having DAPI. Cell images were taken and analyzed using Nikon A1 confocal microscope and Nikon-NIS-AR 4.1 software (Nikon, Tokyo, Japan).

4.22. Isolation of T cells. T cells (CD4 and CD8) were isolated from mouse spleen and lymph nodes by magnetic-activated cell sorting (MACS) through negative selection, as per the manufacturer's instruction (BD Biosciences, San Diego, CA). Spleenocytes were isolated from mice and were processed into a single-cell suspension. Next, T cells (CD4 and CD8) enrichment cocktail was added to these splenocytes. Generally, a mouse CD4 T cell enrichment cocktail (5 µl/1 X 10^6 cells) was added and kept over ice for 15 min followed by centrifugation at 300g for 7 min, and to this pellet streptavidin-magnetic beads (5µl) were added and incubated for 30 min at 6–12 ^oC. BD IMagnet™ was used for CD4 T cells isolation. Similarly, CD8 T cells were isolated using enrichment kit.

4.23. Syngeneic T cell proliferation. T cells (CD4 and CD8) were purified as per protocol mentioned above from the C57BL/6 mice spleenocytes. T cells (CD4 and CD8) were re-suspended in 1 ml PBS (1X) supplemented with 2 µM CFSE and incubated at 37 ^oC for 8 min. Later, extra CFSE was quenched using FCS (2 ml) and cells were washed with PBS-1X twice. T cells were co-cultured with DC^{MPT64} (ratio 10:1) in an anti-CD3 (1 µg/ml) and anti-CD28 (0.5 µg/ml) Abs coated 96 well flat bottom plate for 72 h. T cells proliferation were monitored by CFSE-dye dilution assay through flowcytometry. Data were analysed through BD DIVA software.

4.24. Allogeneic T cell proliferation. T cells (CD4 and CD8) were purified as per protocol mentioned above from the BALB/c mice spleenocytes. T cells (CD4 and CD8) were re-suspended in 1 ml PBS (1X) supplemented with 2 µM CFSE and incubated at 37 ^{o}C for 8 min. Later, extra CFSE was quenched using FCS (2 ml). T cells (BALB/c) were co-cultured with DC^{MPT64} (C57BL/6) (ratio 10:1) in a 96 well flat bottom plate for 72 h. T cells proliferation were monitored by CFSE-dye dilution assay through flowcytometry. Data were examined through BD DIVA software.

4.25. *In vivo* polarization of T cells. DC^{MPT64} and control DCs/DC^{CFP-10} (2 X 10^6/well) were cultured for 6 d in complete media. After harvesting on the 7th day, cells were pulsed for 2 h with ovalbumin (200 µg/ml) and then adoptively transferred (s.c) into mice and repeated a second dose after 7 d. After 3 d, animals were sacrificed and the CD4 T and CD8 T cells expressing $CD25^+$ and $FoxP3^+$ were estimated in the spleen by flowcytometry. Data were examined on BD DIVA software.

4.26. The expression of TCRβ and TCRγ/δ chains and apoptosis of T cells. DC^{MPT64} and control DCs and DC^{CFP-10} were co-cultured with anti-CD3/CD28 stimulated naïve T cells (CD4 and CD8) (DC:T cell, 1:10) for 72 h/37 ^{o}C/5% CO_2. After staining with fluorochrome-labelled anti-TCRβ chain and TCRγ/δ chain Abs, cells were analysed through flowcytometry. Further, the apoptosis in T cells upon culturing with DCs, DC^{MPT64} and DC^{CFP-10} was enumerated through staining with FITC labelled AnnexinV (5 µl/tube) for 15 min in dark. Binding buffer was added after incubation to these cells and monitored by flowcytometry and analysis was done through BD DIVA software.

4.27. Glucose uptake and methylglyoxal transfer in T cells by DCs^{MPT64}. DC^{MPT64} and control DCs and DC^{CFP-10} were co-cultured with anti-CD3/CD28 stimulated naïve T cells (CD4 and CD8) (DC:T cell, 1:10) for 72 h/37 ^{o}C/5% CO_2. These cells were incubated with fluorescent d-glucose analogue 2-NBDG for 0.5 h and methylglyoxal

(MGO) for 72 h/37 ^{o}C/5% CO_2. Cells were fixed with 4% PFA after PBS-1X wash for 10 min/RT. For MGO, cells were kept with anti-MGO Abs (1:50 dilution) on ice for 30 min followed by incubation with secondary Alexa fluor 647-anti-rabbit antibody (1:1000 dilution) at RT for 0.5 h. For 2-NBDG, cells were harvested and cultured in a glucose-free complete media supplemented with 2-NBDG (10 µM) for 30 min/37 ^{o}C/5% CO_2. Finally, the expression of MGO and 2-NBDG was monitored in these lymphocytes through flowcytometry.

4.28. DC^{MPT64} mediated suppression of T cells through NO secretion and in a contact independent way. The suppressive activity of DC^{MPT64} was measured through trans-well plate experiments. CFSE-labelled and anti-CD3 and CD28 Abs stimulated naïve T cells (CD4 and CD8) were poured into the lower portion of transwell plate. DC^{MPT64} and control DC^{CFP-10} and DCs were added into the upper portion of the transwell plate. Further, the same cultures were set in a non -transwell plate. Cells were kept for 72 h/37 ^{o}C/5% CO_2. Furthermore, role of NO-mediated suppression of CD4 T cells and CD8 T cells by DC^{MPT64} was substantiated by treating the cells with iNOS inhibitor (NM: N-monomethyl-L arginine) (20 µM) for 72 h. T cells proliferation were monitored by CFSE-dye dilution assay through flowcytometry and analyzed by BD DIVA software.

4.29. Cell culture and Western blotting. DC^{MPT64} and control DCs (2.5 X 10^6/well) were cultured for 6 d in complete media in a 6 well plate. Cells were harvested, washed on the 7th day, followed by lysis through RIPA buffer containing PMSF, inhibitor cocktail of protease and phosphatase. After protein quantification in lysate, SDS-PAGE gel electrophoresis was performed using these lysates. Next, it was transferred onto PVDF membrane (0.2 microns) and BSA (5%) was used to block unbounded sites. The PVDF membrane was immunoblotted using Abs for IDO, Arg1, STAT-3, STAT-1, iNOS, p65 NF-κB and β-actin as a loading control. During each step, washing was given with PBST

buffer (PBS-1X having Tween 20). Next, the blots were incubated with HRP labelled secondary anti-mouse/rat/rabbit Abs, and developed with chemiluminescence kit (Clarity™ Western ECL substrate; BioRad, Hercules, California). Scanning of the blots was completed with a Bioanalytical imaging system (Azure Biosystems. Inc; c600, Dublin, CA). The images were analysed with Fiji software.

4.30. RT-qPCR for quantification of genes. DC^{MPT64} and control DCs (2.5 X 10^6/well) were cultured for 6 d in complete media in a 6 well plate and total RNA was isolated from these cultures using TRIzol reagent, as per manufacturer's instructions (Invitrogen, Carlsbad, CA). A260/280 value of samples was within the 1.90-2.00 range as quantified by Nano Drop spectrophotometer (BioTek, Winooski, VT). Next, through amplification grade DNase1, RNA samples were made DNA free. Next, cDNA was synthesized from RNA samples using a cDNA synthesis kit. Analysis was performed by the comparative Ct method, whereas Ct values were normalized against the housekeeping control β-Actin gene. Using the comparative Ct method, relative gene expression was calculated as $2(^{-\Delta\Delta}Ct)$, where ΔCt = Ct (gene of interest) -Ct (normalizer = β-actin) and the ΔΔCt = ΔCt (sample) - ΔCt (calibrator). RT-qPCR and data analysis were done by ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Chromos, Singapore). The sequences of primers used are mentioned below.

Gene	**Fwd 5'- -3'**	**Rev 5'- -3'**
Glut1	TCAACACGGCCTTCACTG	CACGATGCTCAGATAGGACATC
Hk2	CAACTCCGGATGGGACAG	CACACGGAAGTTGGTTCCTC
GLO 1	GATTTGGTCACATTGGGATTGC	TTCTTTCATTTTCCCGTCATCAG
SSAO	TGCACATCCCTCATGCAGAA	GGAGGAAGAAGCCCACTGAGT
iNOS	AACGGAGAACGTTGGATTTG	CAGCACAAGGGGTTTTCTT
Tim-3	CGGAGAGAAATGGTTCAGAGACA	TTCATCAGCCCATGTGGAAAT
Pd-l1	TGCGGACTACAAGCGAATCACG	CTCAGCTTCTGGATAACCCTCG
Il-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCATCGTTGTTCATACA
Il-12	GGAAGCACGGCAGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
TGF-β	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

4.31. Statistical analysis. Data analysis was done through one-way analysis of variance (ANOVA) and 't-test' and using Graph Pad Prism 7 software (GraphPad Software, La Jolla, CA). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The 'p-value' of less than 0.05 was considered significant. Data shown are of 2-3 independent experiments.

4.32. Composition of Media and Buffers

RPMI-1640 media	HEPES (10 mM): 2.38 g $NaHCO_3$: 2.2 g L-Glutamine: 290 mg Penicillin: 70 mg Streptomycin: 100 mg Pyruvic acid: 110 mg RPMI (1 sachet): 10.4 g β-mercaptoethanol (2-ME)(0.05 mM): 3.5 µl Dissolve in 800 ml autoclaved millipore water. Adjust pH to 7.4. Make volume up to 900 ml. Filter sterilize and store at 4 ^{0}C
Phosphate Buffered Saline (PBS), 0.15 M, pH 7.2	NaCl: 8.00 g/L KCl: 200 mg/L Na_2HPO_4 (anhydrous): 1.15 g/L KH_2PO_4 (anhydrous): 200 mg/L
10X Phosphate Buffered Saline (PBS)	NaCl: 80 g KCl: 2 g Na_2HPO_4: 11.5 g KH_2PO_4: 2 g Dissolve it in 900 ml water then make up to 1000 ml
ACK Lysis Buffer	NH_4Cl (0.15M): 8.29 g $KHCO_3$ (1 mM): 1.0 g Na_2EDTA (0.1 mM): 37.2 mg The chemicals were dissolved in 1000 ml of distilled water and pH was adjusted to 7.2 with HCl (1N) and stored at 4 ^{0}C
FACS Buffer	2% FBS in 1 X PBS
Fixative Buffer	4% Paraformaldehyde in 100 ml of 1 X PBS (pH 7.4). Briefly, 4 g of paraformaldehyde was dissolved in 80 ml of 1 X PBS and kept on magnetic stirrer with heating around 60 ^{0}C. To this, 10 mM NaOH solution was added dropwise till it forms clear solution. Finally this solution was make up to 100 ml with PBS and aliquoted. Aliquot were made and stored in -20 ^{0}C until use.

Permeabilization buffer	Saponin: 200 mg HEPES: 0.46 mg FBS: 1 ml Dissolve it in 1 X PBS and make up final volume100 ml
ELISA Washing Buffer	PBS: 0.15 M, pH 7.2 Tween-20: 0.05%
ELISA Blocking Buffer	PBS: 0.15 M, pH 7.2 BSA: 1%
ELISA Dilution Buffer	1:1 solution of ELISA blocking buffer and ELISA washing buffer
ELISA Stop Solution	7% H_2SO_4
PI-Annexin Binding Buffer, pH 7.4, 10X	HEPES: 0.1M NaCl: 1.4M $CaCl_2$: 25mM 1 X diluted in double distilled water
7H9 broth preparation (for 900 ml)	7H9 powder: 4.7 g Glycerol: 2 ml Polysorbate 80 (1ml Tween 80): 0.5 g 900 ml purified water ADC: 100 ml Dissolve 7H9 powder in water and autoclaved at 121 ^{0}C for 10-15 mins. Aseptically, added 100 ml of Middlebrook ADC Enrichment to the medium when cooled to 45°C.
7H11 agar preparation (for 900 ml)	7H11 powder: 21 g Glycerol: 5 ml 900 ml purified water OADC: 100 ml Added 7H11 powder in water containing 5 ml glycerol. Autoclaved at 121 ^{0}C for 15 mins. Aseptically, added 100 ml of Middlebrook OADC Enrichment to the medium when cooled to 50-55 ^{0}C.
RIPA buffer	Tris-HCl (1 M, pH 7.4): 1 ml NaCl (5 N): 2.5 ml EDTA (0.5 M): 200 µl TritonX 100 (10%): 500 µl Dissolve in 50 ml of double distilled water

Chapter-5

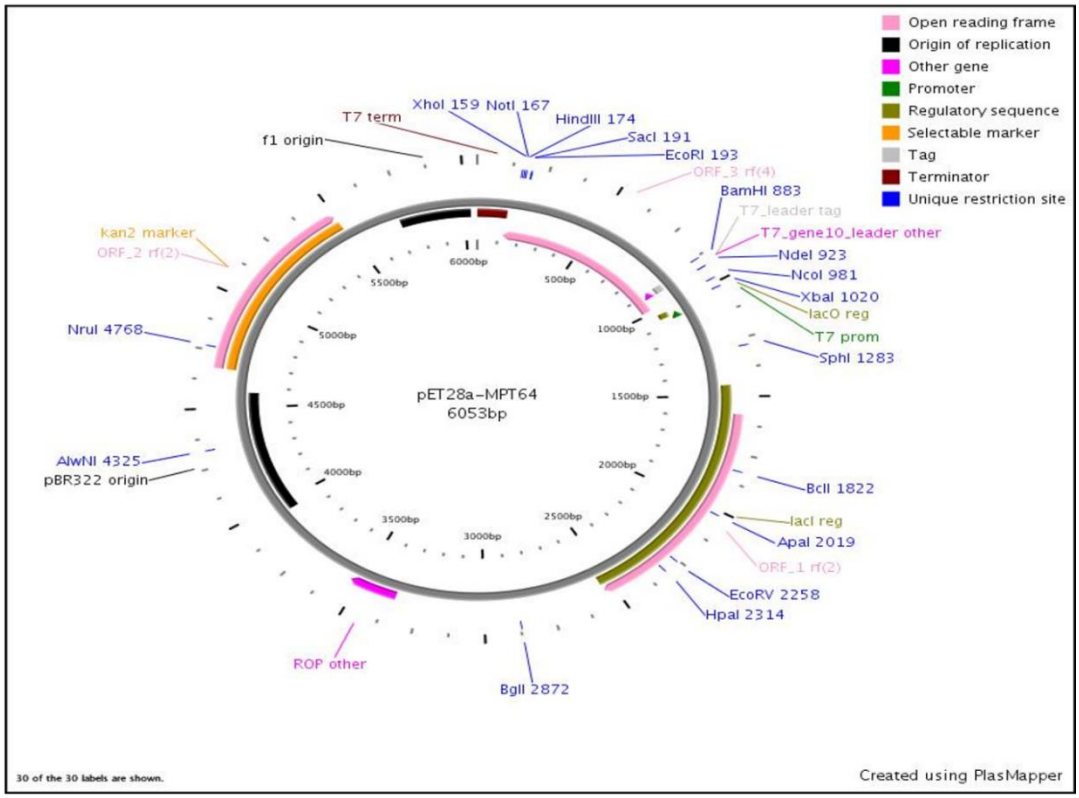
Results and Discussion

5. RESULTS AND DISCUSSION

5.1. Cloning, expression and purification of MPT64.

MPT64 is the secretory protein of *Mtb*, the gene encoding MPT64 (Rv1980c) protein was amplified through PCR by using the genomic DNA of H37Ra as a template. Further, the amplified DNA of MPT64 was cloned and expressed in the pET28a vector between *BamHI* and *EcoRI* sites in *E. coli* and was further transformed into *E. coli* DH5α for purification. Through colony PCR and agarose gel electrophoresis MPT64 gene in plasmid was confirmed (Fig. 1A, B). To express MPT64, the plasmid harboring MPT64 was transformed into *E. coli* Rosseta (DE3). The induction of protein was done by culturing it in Studier auto-induction media followed by purification through Ni-NTA chromatography. The MPT64 protein was purified and eluted in a buffer containing NaCl, Tris-HCl and imidazole through a Ni-NTA column. After collection through the Ni-NTA column, excess salts from purified proteins were removed through dialysis by using a 10 kDa cut-off dialysis membrane. The purity of the MPT64 purified protein was >98%, as assessed by SDS-PAGE (Fig. 1C). Further, endotoxin levels in purified MPT64 protein was measured through Limulus Amoebocyte Lysate assay and was < 0.05 EU/ml. The protein concentration was measured through the Bradford assay. The endotoxin-free purified proteins were used for all subsequent experiments.

A


Open reading frame
Origin of replication
Other gene
Promoter
Regulatory sequence
Selectable marker
Tag
Terminator
Unique restriction site
XhoI 159 NotI 167
T7 term
HindIII 174
f1 origin
SacI 191
EcoRI 193
ORF_3 rf(4)
BamHI 883
T7_leader tag
T7_gene10_leader other
NdeI 923
NcoI 981
XbaI 1020
lacO reg
T7 prom
SphI 1283
kan2 marker
ORF_2 rf(2)
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AlwNI 4325
pBR322 origin
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lacI reg
ApaI 2019
ORF_1 rf(2)
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500bp
1000bp
1500bp
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5000bp
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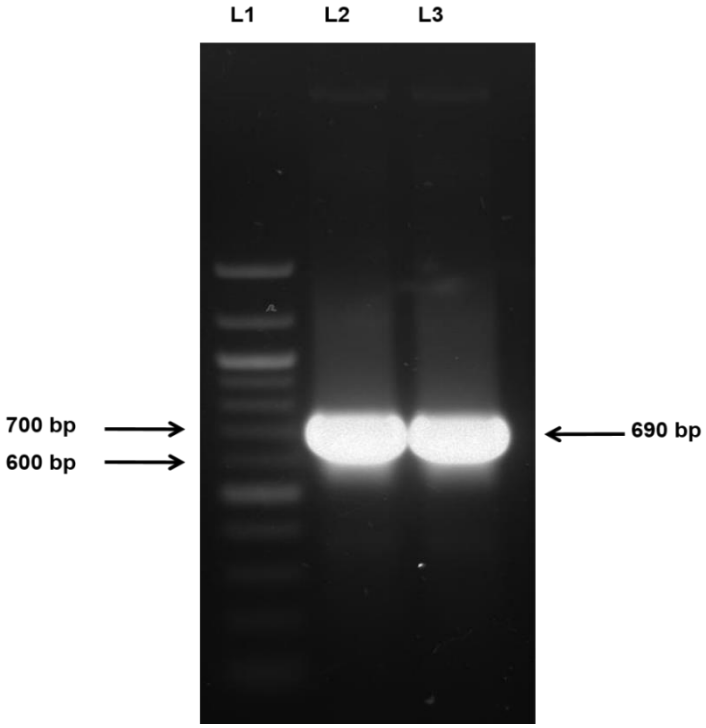
30 of the 30 labels are shown.
Created using PlasMapper

B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L2
L3
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600 bp
690 bp

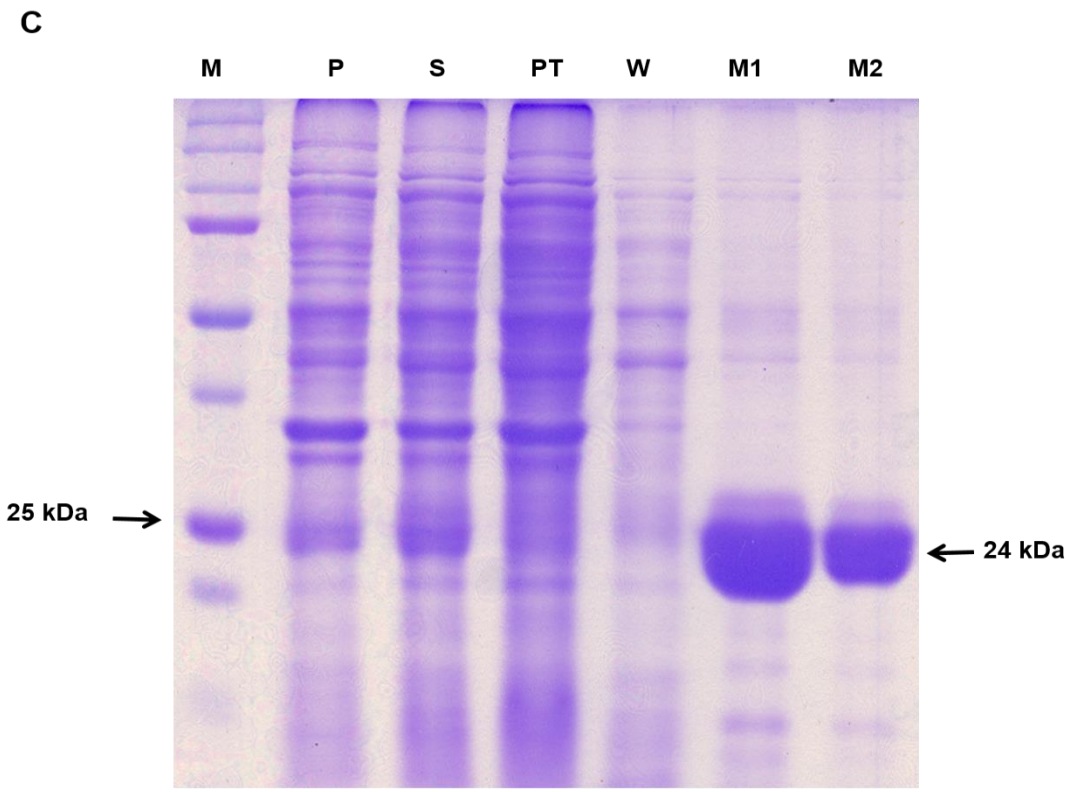


Figure 1. The cloning, expression and purification of MPT64. (A) Diagram showing the vector map of pET28a-MPT64. (B) MPT64 gene was PCR amplified from genomic DNA of *Mtb* (H37Ra), cloned and transformed into pET28a vector and DH5α (*E. coli*); L1: marker, L2 and L3: MPT64 gene. (C) MPT64 was expressed in BL21 (DE3) and was inducted in auto Studier media. Through Ni-NTA chromatography protein was purified and analysed by SDS-PAGE electrophoresis. The protein was visualized by Coomassie Blue G-250 staining; M: Marker, P: Pellet, S: Soup, PT: Pass-Through, W: Wash, M1 and M2: MPT64.

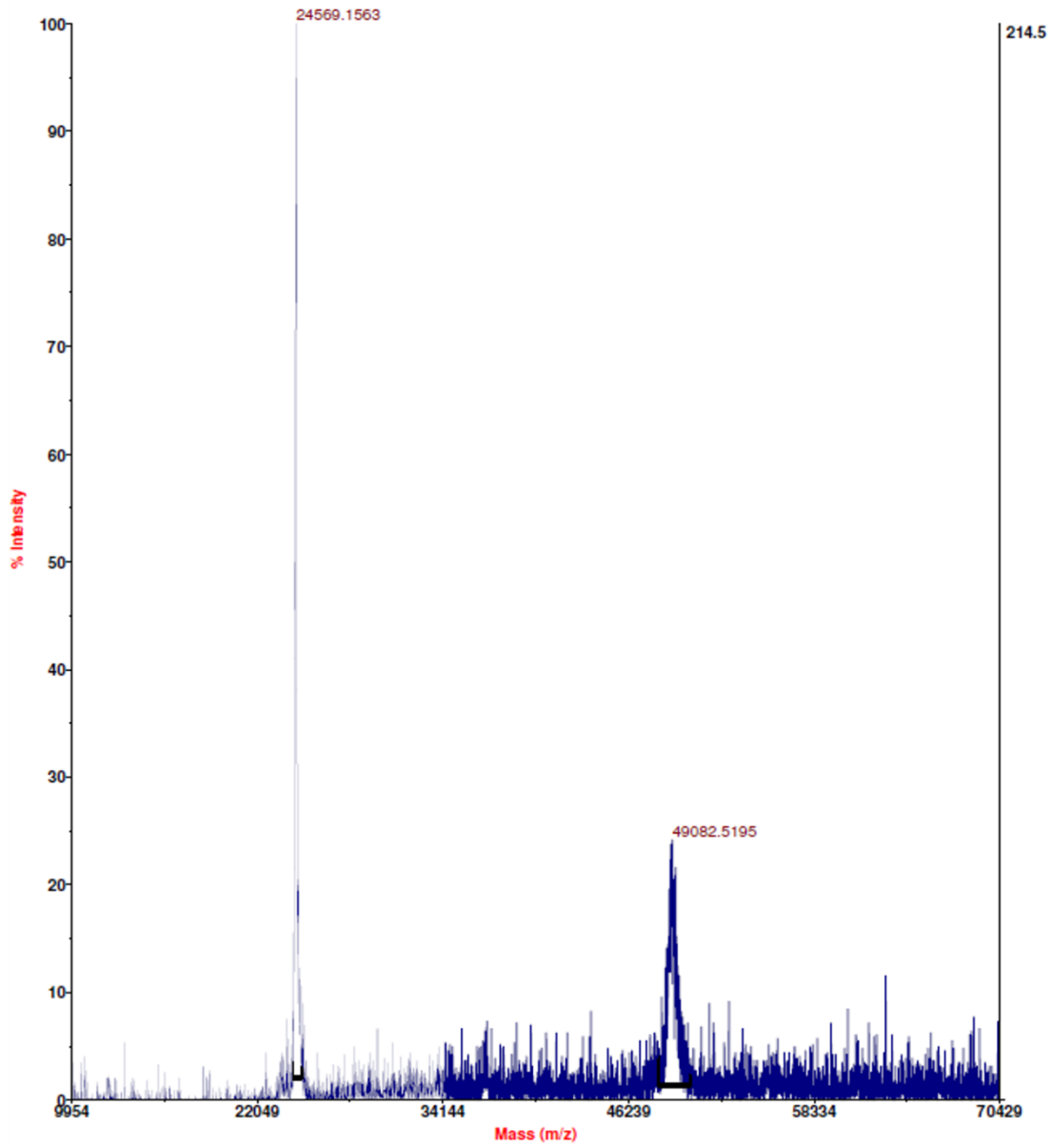
5.2. MALDI and MASCOT of MPT64.

The purity of MPT64 protein purified through Ni-NTA chromatography was >98%, as assessed through SDS-PAGE. Further, the molecular weight of MPT64 protein was elucidated through MALDI-TOF and was observed to be approximately 24,569 Da; which was near to the wild-type MPT64 protein (Wang et al., 2007)(Fig. 2A). The recombinant MPT64 protein was trypsin digested and analyzed through MALDI-TOF/MS. The obtained peptide spectra were further submitted to the

MASCOT database for the identification of peptides/proteins in the NCBI database. The protein will be considered identical or extensively homologous if its sequence coverage with available protein in the database is >31% ($p<0.05$). We observed through MASCOT search that 7 matched polypeptides cover 42% of the MPT64 sequence (gi|15609117; mass: 24839) (Fig. 2B).

Acr1 protein was well identified and characterized in our laboratory previously (Siddiqui et al., 2014). All our experiments were done with the endotoxin-free and purified MPT64, Acr1, ESAT-6 and CFP-10 proteins.

A

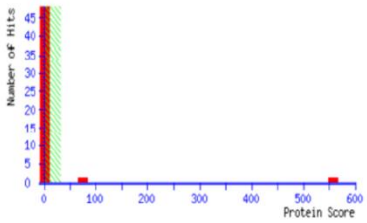


B

Mascot Search Results

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 31 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

1. gi|15609117 **Mass:** 24839 **Score:** 557 **Matches:** 7(6) **Sequences:** 7(6) **emPAI:** 2.42
mpt64 gene product [Mycobacterium tuberculosis H37Rv]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑ 21	1137.6331	1136.6258	1136.5826	0.0432	0	85	2.5e-007	1	U	K.FLSAATSSTPR.E
☑ 28	1171.5590	1170.5517	1170.5094	0.0423	0	74	4.9e-006	1	U	K.AFDWDQAYR.K
☑ 35	1194.6571	1193.6498	1193.6040	0.0458	0	74	3.2e-006	1	U	K.SLENYIAQTR.D
☑ 56	1380.7627	1379.7554	1379.7045	0.0509	1	122	4.3e-011	1	U	R.DKFLSAATSSTPR.E
☑ 101	1638.8253	1637.8180	1636.7845	1.0335	0	24	0.29	1	U	K.VYQNAGGTHPTTTYK.A
☑ 123	2221.1843	2220.1770	2220.1062	0.0708	0	189	6e-018	1	U	R.EAPYELNITSATYQSAIPPR.G
☑ 141	3270.8101	3269.8028	3269.7224	0.0804	0	150	2.4e-014	1	U	R.KPITYDTLWQADTDPLPVVFPIVQGELSK.Q

MASCOT Search Results

Protein View: gi|15609117

mpt64 gene product [Mycobacterium tuberculosis H37Rv]

Database: NCBInr2
Score: 557
Nominal mass (M_r): 24839
Calculated pI: 4.84

Protein sequence coverage: 42%

Matched peptides shown in ***bold red***.

```
  1 MRIKIFMLVT AVVLLCCSGV ATAAPKTYCE ELKGTDTGQA CQIQMSDPAY
 51 NINISLPSYY PDQKSLENYI AQTRDKFLSA ATSSTPREAP YELNITSATY
101 QSAIPPRGTQ AVVLKVYQNA GGTHPTTTYK AFDWDQAYRK PITYDTLWQA
151 DTDPLPVVFP IVQGELSKQT GQQVSIAPNA GLDPVNYQNF AVTNDGVIFF
201 FNPGELLPEA AGPTQVLVPR SAIDSMLA
```

Query	Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
35	65 - 74	1194.6571	1193.6498	1193.6040	0.0458	0	74	3.2e-006	1	U	K.SLENYIAQTR.D
56	75 - 87	1380.7627	1379.7554	1379.7045	0.0509	1	122	4.3e-011	1	U	R.DKFLSAATSSTPR.E
21	77 - 87	1137.6331	1136.6258	1136.5826	0.0432	0	85	2.5e-007	1	U	K.FLSAATSSTPR.E
123	88 - 107	2221.1843	2220.1770	2220.1062	0.0708	0	189	6e-018	1	U	R.EAPYELNITSATYQSAIPPR.G
101	116 - 130	1638.8253	1637.8180	1636.7845	1.0335	0	24	0.29	1	U	K.VYQNAGGTHPTTTYK.A
28	131 - 139	1171.5590	1170.5517	1170.5094	0.0423	0	74	4.9e-006	1	U	K.AFDWDQAYR.K
141	140 - 168	3270.8101	3269.8028	3269.7224	0.0804	0	150	2.4e-014	1	U	R.KPITYDTLWQADTDPLPVVFPIVQGELSK.Q

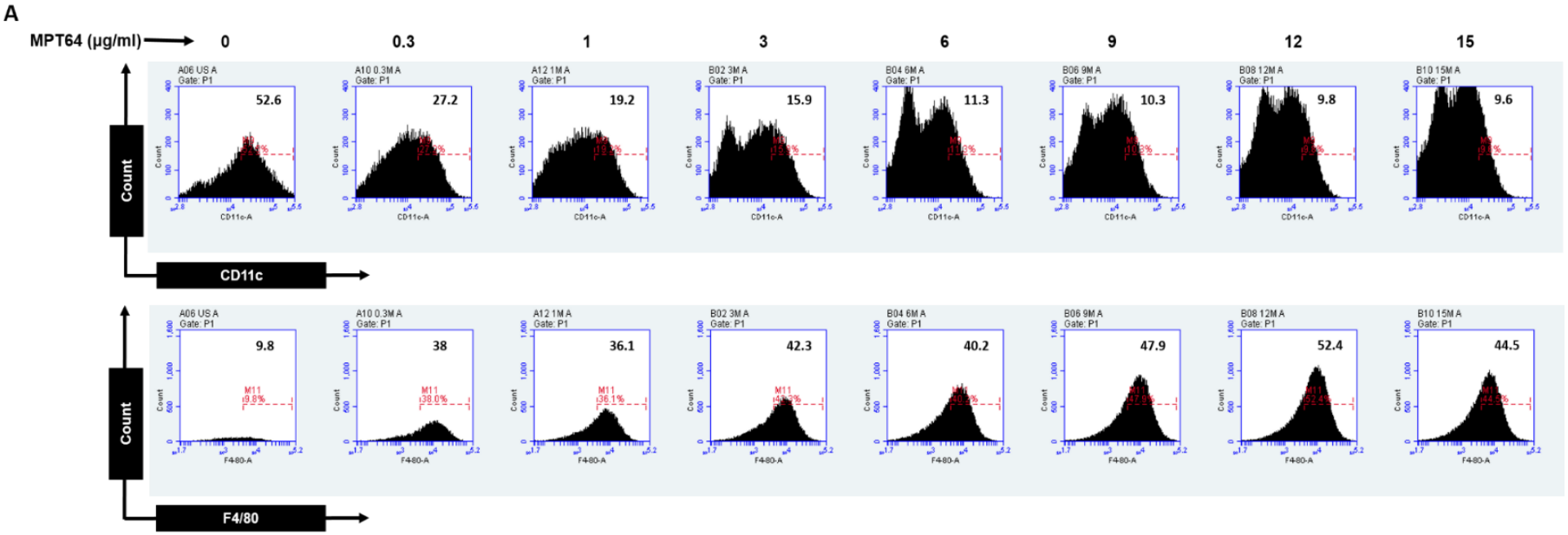
Figure 2. MALDI and MASCOT analysis of MPT64. (A) Intact mass of MPT64 protein. (B) MASCOT score histogram of MPT64 along with peptide summary report of the identified protein. Ion score was -10 X log (P); P represents the chances, where the observed match can be a random event. An ion score of individual > 31 shows an identity or extensive homology ($p<0.05$). Further, MPT64 protein sequence coverage of matched peptides of identified protein has been analyzed through MASCOT.

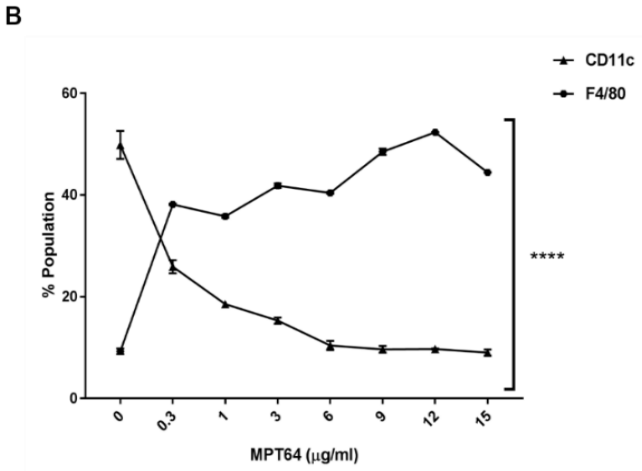
5.3. MPT64 impairs the maturation of DC^{MPT64}. *Mtb* uses an array of approaches to elude its destruction by the host immune system. DCs are the only APCs that can activate naïve T cells. Interestingly, it has been observed that DCs upon *Mtb* infection lose their function (Wolf et al., 2007). Hence, it is crucial to comprehend how *Mtb* uses its proteins to counterbalance the action of DCs. Therefore, we thought to decipher the influence of the exposure of MPT64 on the differentiating DCs. BMCs were cultured in the presence of GM-CSF and IL-4 with (DC^{MPT64}) or without (DCs) MPT64. The control cultures were set using Acr1 (DC^{Acr1}) CFP10 (DC^{CFP10}) and ESAT-6 (DC^{ESAT-6}) proteins of *Mtb*. We noted a decline in the $CD11c^+$ cells, but augmentation in the pool of $F4/80^+$ cells, in a dose-dependent manner (Fig. 3A, B). However, the greatest expansion of $F4/80^+$ cells and lowest percentage of $CD11c^+$ cells was observed with 12 µg/ml of MPT64. Hence, this concentration was used in all the subsequent experiments.

To validate that the decline in the frequency of $CD11c^+$ cells was not because of the overgrowth and death of the cells, we checked their viability. No change in the viability of neither $CD11c^+$ cells nor $F4/80^+$ cells was noted (Fig. 3C). We also checked the specificity of MPT64. To prove this, we used CFP-10 and ESAT-6 proteins of *Mtb.* We noticed that the increase of the $F4/80^+$ cells and decline in the $CD11c^+$ cells were solely associated with a function of MPT64 and not CFP-10 and ESAT-6 proteins (Fig. 3D). Moreover, we observed a decline in $CD11c^+$ cells and an increase in $F4/80^+$ cells upon Acr1 exposure. However,

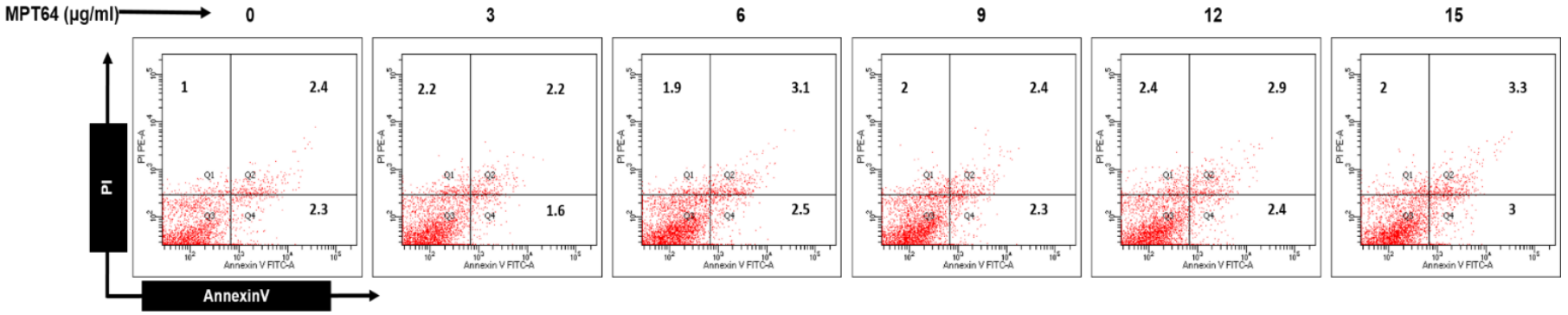
this change was significantly lesser, as compared to MPT64 stimulated cells. Thus, it establishes that MPT64 but no other proteins of *Mtb* are solely responsible for transforming the BMCs under DCs differentiating conditions (IL-4+GM-CSF) into MDSCs.

An optimum surface exhibition of co-stimulatory and MHC molecules is decisive for the presentation of antigen to activate T cells. Hence, we next evaluated these molecules on DC^{MPT64}. DC^{MPT64} exhibited increased CD40, CD80 and CD11b levels but declined MHC II expression (Fig. 3E). We have used purified and endotoxin-free MPT64, CFP-10, and ESAT-6 in all the experiments. We also verified the specificity of the experiments using control CFP-10, ESAT-6 and Acr1 proteins of *Mtb* that were produced under the same conditions.


A
MPT64 (µg/ml)
0
0.3
1
3
6
9
12
15
Count
CD11c
52.6
27.2
19.2
15.9
11.3
10.3
9.8
9.6
F4/80
9.8
38
36.1
42.3
40.2
47.9
52.4
44.5


B
CD11c
F4/80
% Population
MPT64 (µg/ml)
****

C


MPT64 (µg/ml)
0
3
6
9
12
15
PI
AnnexinV
1
2.4
2.3
2.2
2.2
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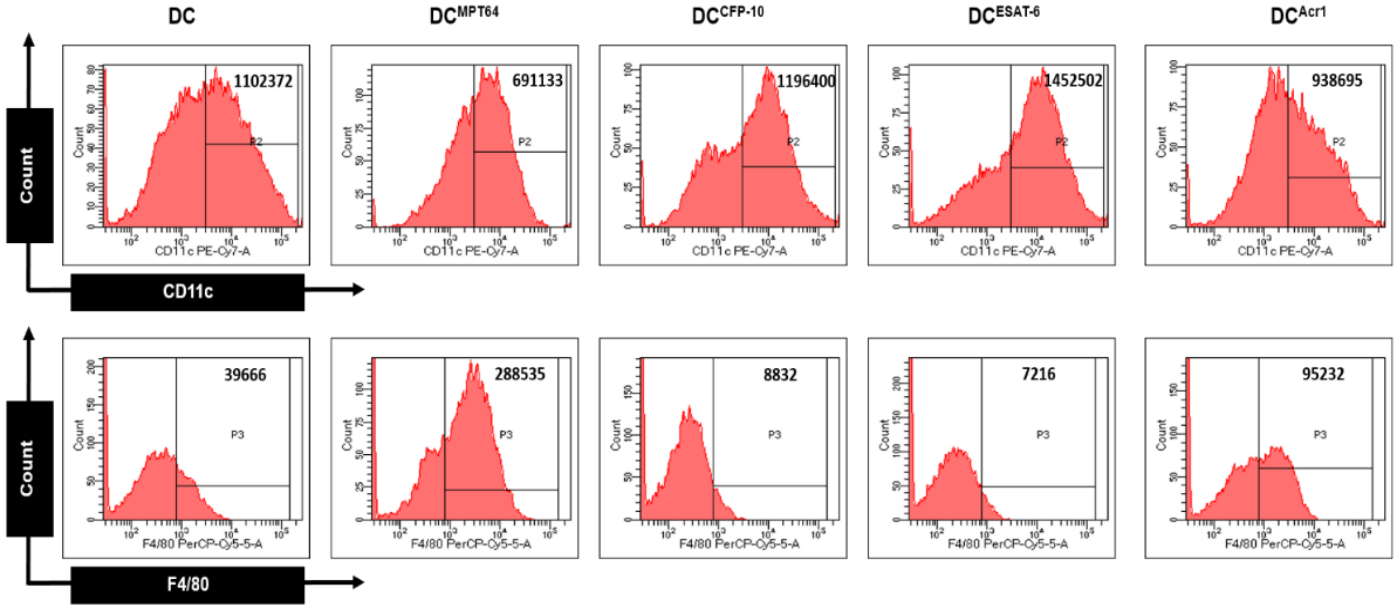
2.4
2.9
2.4
2
3.3
3

D


DC
DC^MPT64
DC^CFP-10
DC^ESAT-6
DC^Acr1
Count
CD11c
F4/80
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P3

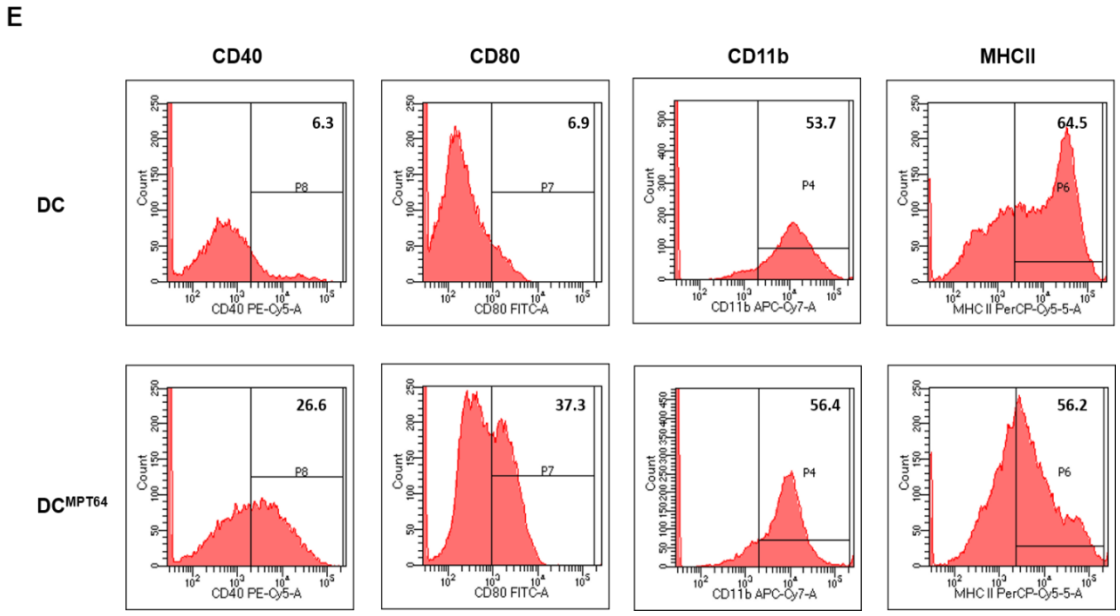


Figure 3. MPT64 impairs the maturation of DC. BMCs cultured with GM-CSF + IL-4 (DCs) along with MPT64 (DC^{MPT64}) were checked for the surface exhibition of (A) CD11c and F4/80 through flowcytometry upon exposure to different concentrations of MPT64 (0-15 µg/ml). Insets of the histogram display percentage positive cells. (B) The line diagram shows dose-dependent expression of CD11c and F4/80 in response to MPT64. (C) DC^{MPT64} viability was assessed through PI/AnnexinV staining. The inset of the histogram shows the percentage of positive cells. (D) The data establishes the specific role of MPT64 in skewing the differentiation of BMCs to myeloid cells and not by other *Mtb* proteins like CFP-10, ESAT-6 and Acr1 by monitoring the expression of CD11c and F4/80 through flow cytometry. Values within the inset of histograms depict integrated mean fluorescence intensity (iMFI). (E) Surface exhibition of CD40, CD80, CD11b and MHC II were assessed through flow cytometry on DC and DC^{MPT64}. Inset of the histogram show percentage of positive cells. Data are the representative of three independent experiments. ****p<0.0001.

5.4. Modulatory effect of MPT64 on macrophages. MΦs are the preferential shelter for the growth and survival of *Mtb* (Fels and Cohn, 1986; Pahari et al., 2017). Most importantly to sustain within the MΦs, this bacillus declines the cell metabolic machinery and shifts it towards a dormant stage from an active stage (Wayne and Hayes, 1996; Wayne and Sohaskey, 2001). Interestingly, it has been shown that MPT64 protein inhibits the apoptosis of macrophages (RAW264.7) and hence renders the survival and persistence of *Mtb* within these cells. Based on these reports, we thought to deduce the modulatory role of MPT64 protein in MΦs generation and differentiation from BMCs. We noticed that early stimulation using MPT64 showed no effect on MΦs generation, as evidenced by no change in the surface expression of F4/80, CD11b and CD11c (Fig. 4A). Thus, it can be inferred from these results that MPT64 does not induce any modulatory effect on the development of MΦs from BMCs. Further, we have shown that early interaction of differentiating MΦs with MPT64 leads to inhibition of apoptosis in a dose-dependent fashion (Fig. 4B). This result validated the recent findings, where it has been shown that MPT64 inhibits the apoptosis of MΦs (RAW264.7) (Wang et al., 2014).

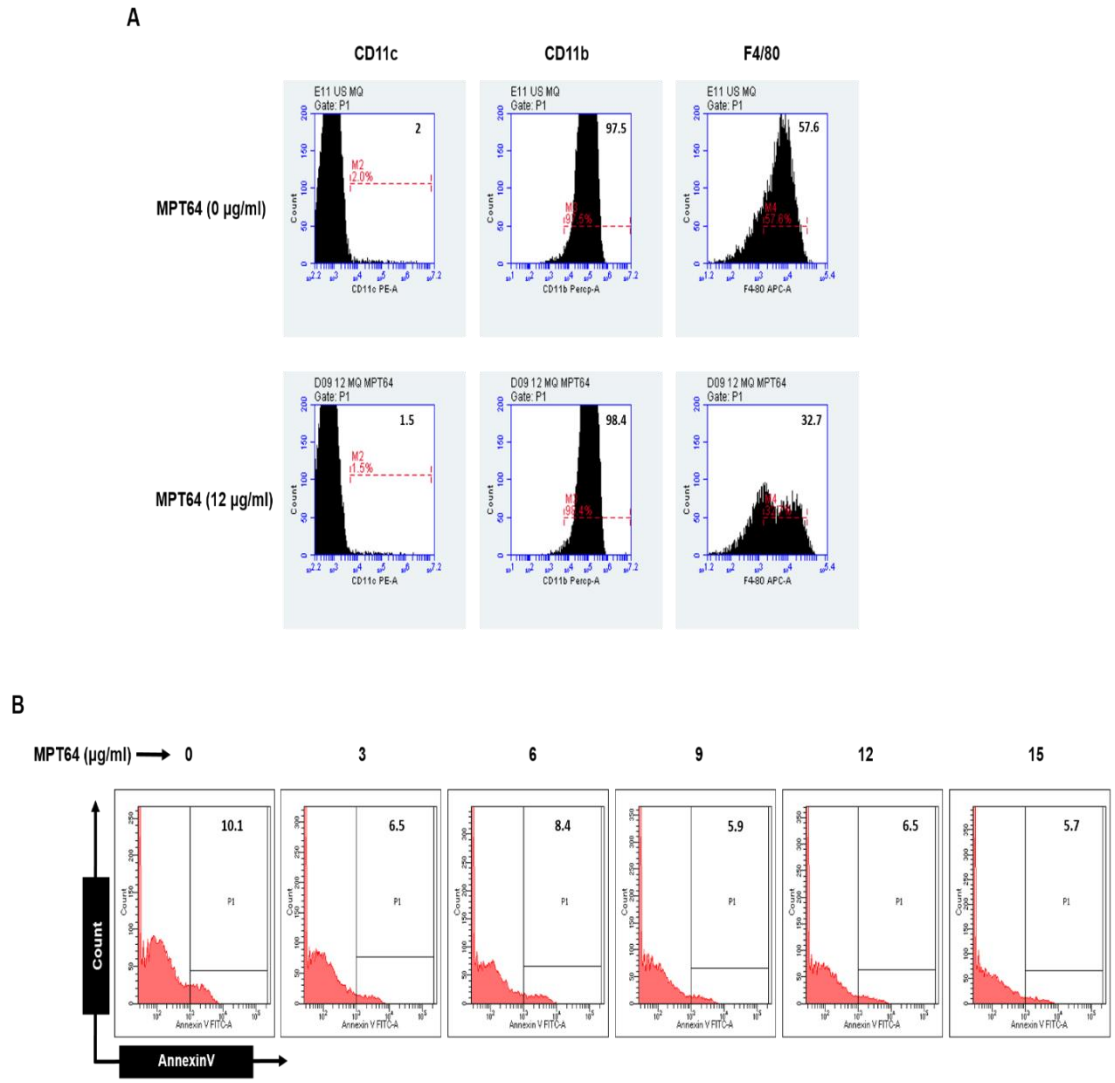


Figure 4. Effect of MPT64 on MΦ generation and differentiation. (A) Bone marrow cells (BMCs) cultured with 10% SNs of L929 cells along with MPT64 (12 µg/ml) were examined for the surface exhibition of CD11c, CD11b and F4/80 through flow cytometry. The insets of the histogram display percentage positive cells. (B) Further, apoptosis in MPT64 untreated and treated MΦs were assessed through Annexin V staining. The inset of the histogram shows percent of positive cells. Data are representative of two independent experiments.

5.5. MPT64 skews the differentiation of DCs to MDSCs. DCs are the sentinel immune cells, which link the innate and adaptive immune systems. During *Mtb* infection, induction of innate immune response is very crucial, so that DCs and MΦs can efficiently uptake, process and present *Mtb* antigens to T cells and generate an effective adaptive immune response. Recently, another cell identified as MDSCs has been localized within the TB granuloma in conjunction with DCs and MΦs. MDSCs have been further sub-categorized into two sub-types viz. M-MDSC and PMN-MDSC are based on a specific set of surface markers. Till now, we observed that DC^{MPT64} have high F4/80, CD11b, CD40, and CD80 and a decline in MHC II, CD11c and CD86 expression. Hence, we were curious to monitor the level of various co-inhibitory and MDSC-specific markers on DC^{MPT64} and control DCs. It has been reported that DCs not only activate an immune response but also suppress it (Popov and Schultze, 2008). PD-L1 and TIM-3 are known for induction of tolerance and are well-established co-inhibitory markers (Sumpter and Thomson, 2011). We noticed that DC^{MPT64} displayed significantly higher levels of PD-L1 ($p<0.05$) and TIM-3 ($p=0.160$), as compared to control DCs (Fig. 5A-D).

MDSCs are heterogeneous cell populations with distinct morphology and functionality in different tissues in inflammatory conditions. It has been observed that MDSCs under inflammatory conditions, share phenotypic expressions of mature myeloid cells, such as DCs (CD11c) and MΦs (F4/80). Hence, more markers are needed to identify these cells from the myeloid cell population. Interestingly, we observed that DC^{MPT64} displayed a higher level of Ly6C, as compared to control DCs and DC^{Acr1} (Fig. 5E). $Ly6C^{hi}$ along with F4/80, CD11b, CD40, CD80, PD-L1 and TIM-3 represents monocytic MDSCs. It can be inferred from these findings that pre-exposure of DCs to MPT64 during their differentiation, endow them with suppressor phenotype.

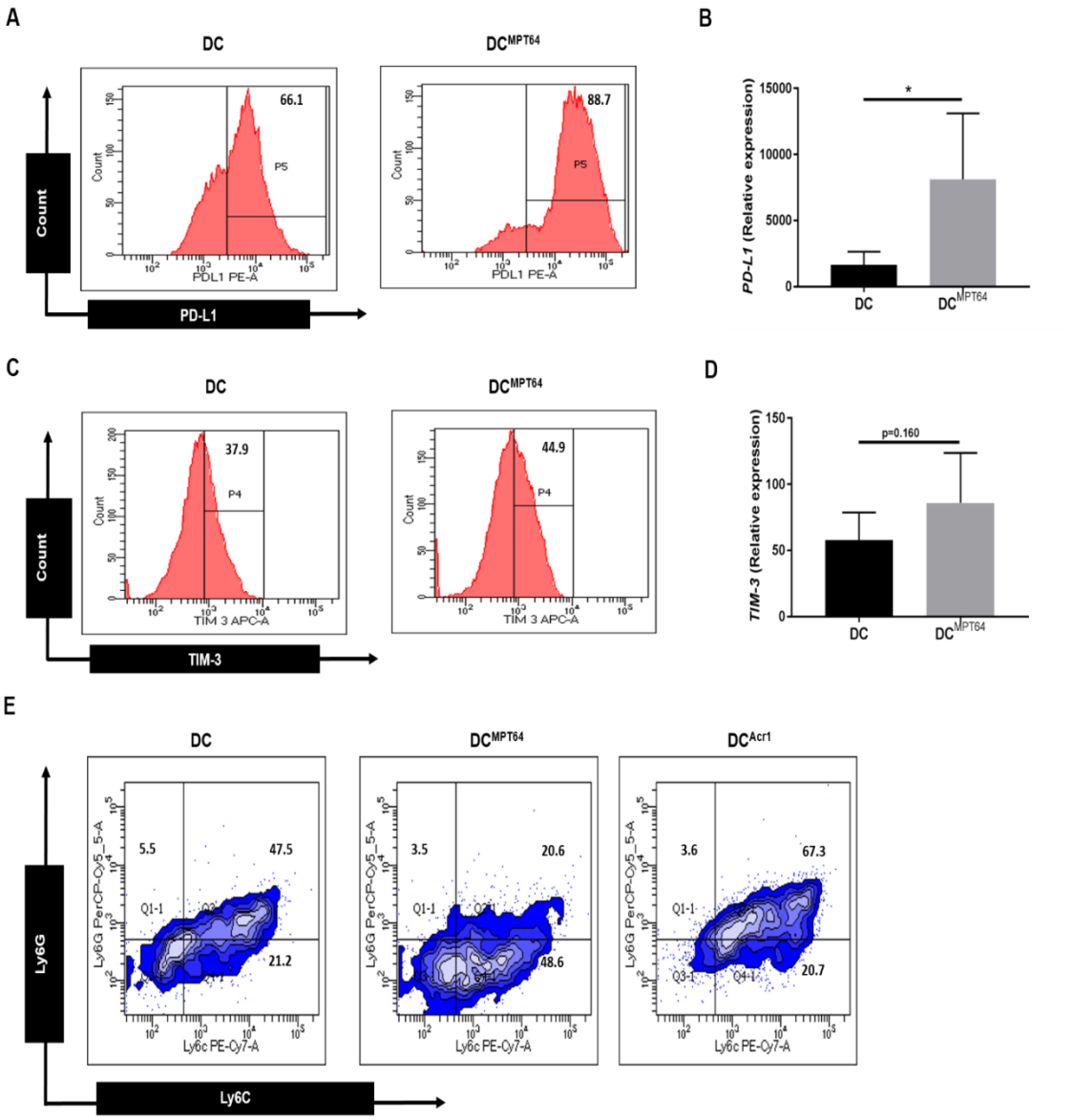


Figure 5. MPT64 promotes the transformation of differentiating DCs into MDSCs. The surface exhibition of PD-L1 and TIM-3 were assessed on the control DCs and DC^{MPT64} through (A, C) flow cytometry; (B, D) quantitative RT-PCR. (E) The surface exhibition demonstration of Ly6C and Ly6G on DC^{MPT64} and control DCs and DC^{Acr1} were monitored by flow cytometry and represented through contour plots. Values within contour plots show per cent positive cells. Data are representative of two to three independent experiments. *p<0.05.

5.6. MPT64 impedes the secretion of pro-inflammatory cytokines by DC^{MPT64}. DCs play a paradoxical part in the initiation and suppression of immune response. DCs maturation is marked on the basis of the expression of co-stimulatory markers along with the production of cytokines. The cytokines secreted by DCs are very critical for turning on and differentiation of T cells towards T helper 1, T helper 2, T helper 17 cells and Treg cells (Zhu et al., 2010). The cytokines like IL-6, IL-12 and TNF-α inhibit the replication of *Mtb*, whereas anti-inflammatory cytokines such as IL-10 and TGF-β encourages it (Domingo-Gonzalez et al., 2016; Redford et al., 2011). The cytokines deliver a third signal essential for optimum instigation of T cells. Therefore, we checked the modulation of the secretion of pro-inflammatory cytokines by DC^{MPT64}. Pre-exposure of DC^{MPT64} to MPT64 exhibited a noteworthy decline in the yield of IL-6 ($p<0.01$), IL-12 ($p<0.0001$) and TNF-α ($p<0.0001$), as compared to control DCs and DC^{CFP-10} (Fig. 6A-C). In contrast, a significantly higher level of IL-10 ($p<0.0001$) was noted in DC^{MPT64} (Fig. 6D). DC^{CFP-10} has been taken as control, as CFP-10 protein is well recognized immunogenic protein. DC^{MPT64} cytokine profile depicts that these cells are anti-inflammatory in nature. A noteworthy upregulation in messenger RNA expression of TGF-β was observed by DC^{MPT64} ($p<0.01$), in comparison to DCs (Fig. 6G). A noteworthy decline in the level of messenger RNA of IL-6 ($p<0.001$) and IL-12 ($p<0.001$) was noticed in DC^{MPT64} (Fig. 6E, F). These results illustrate that MPT64 disables DCs function by obstructing their stimulatory capacity and thereby forming a favorable niche for the survival of *Mtb*.

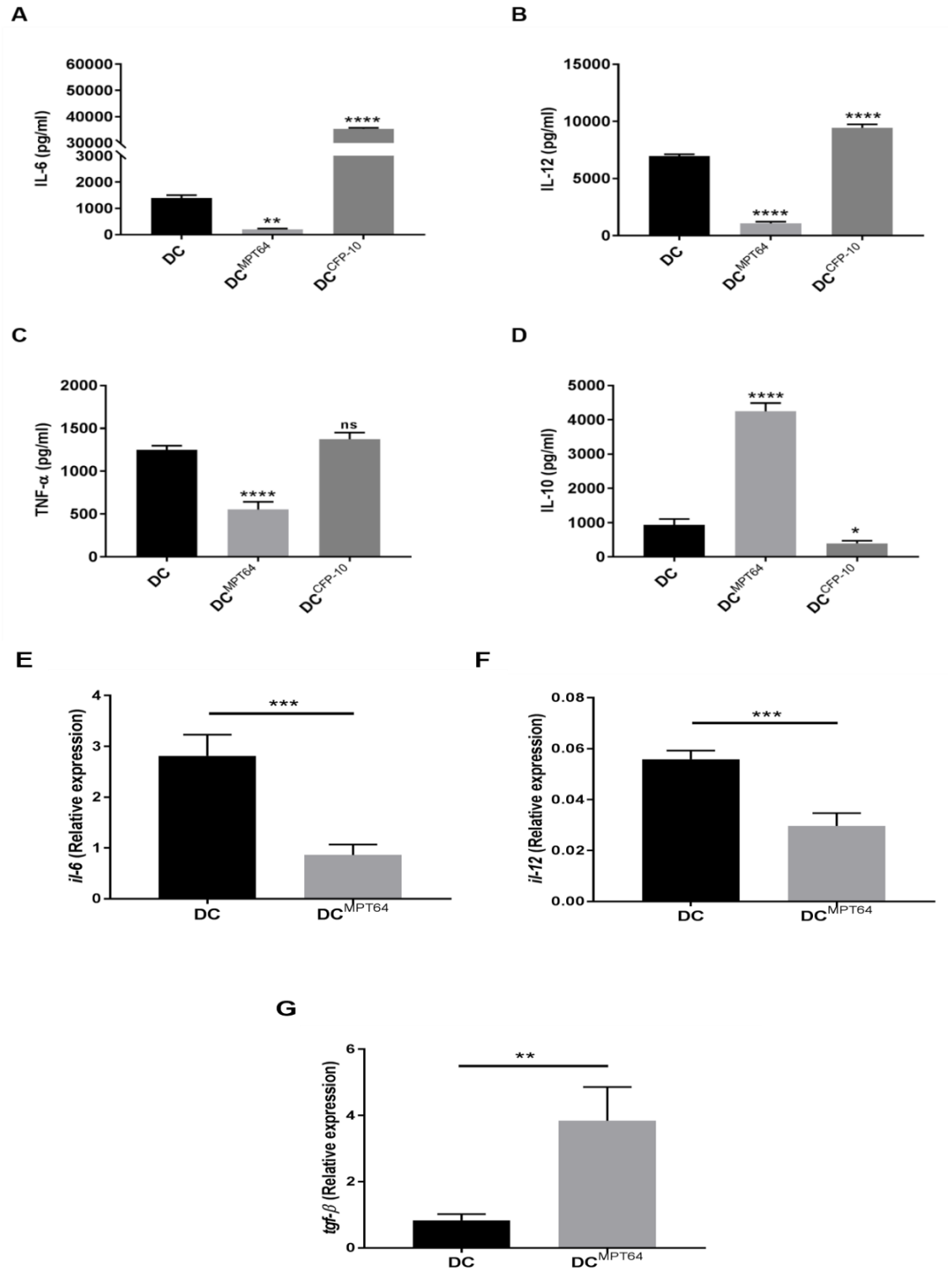
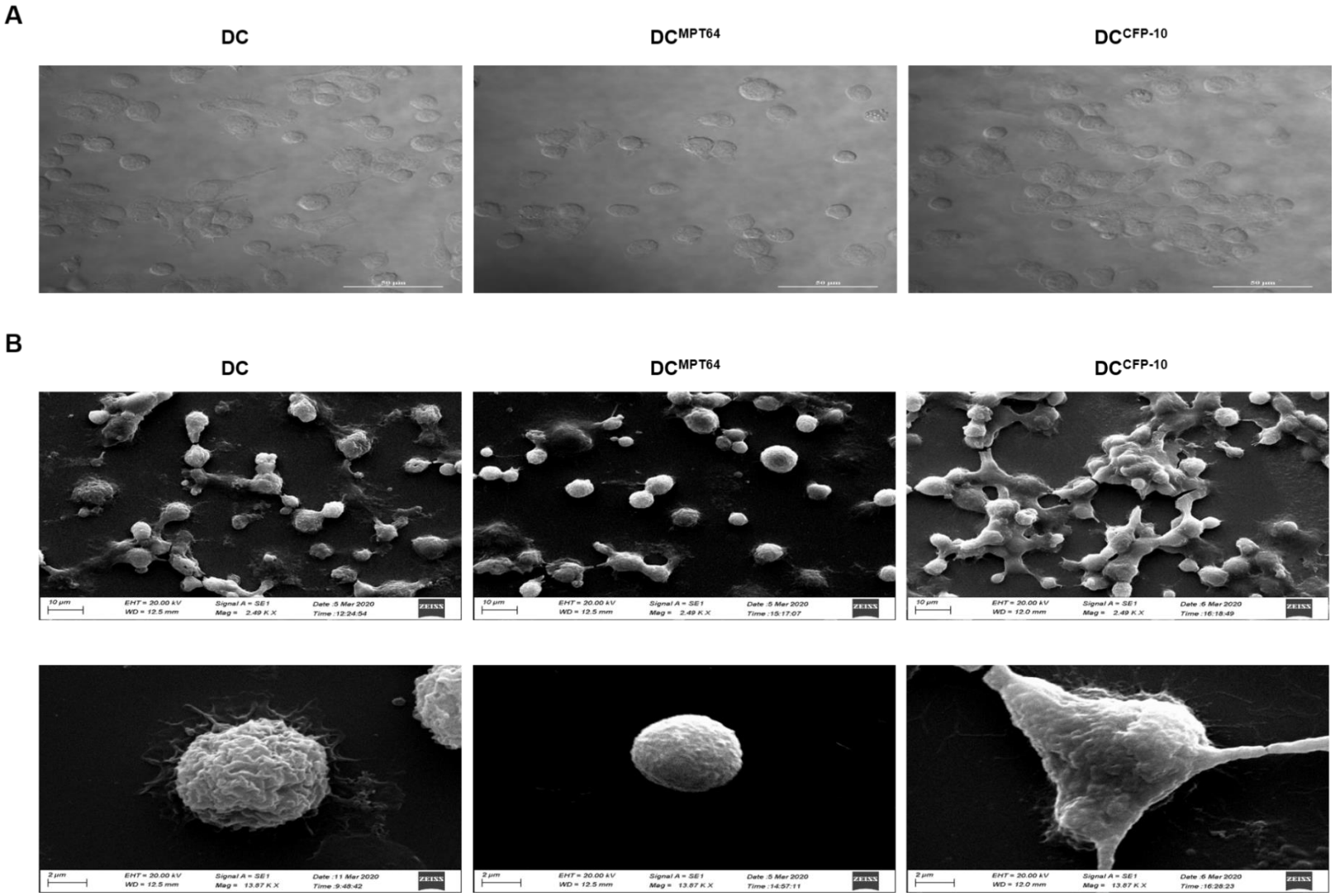


Figure 6. DC^{MPT64} regresses the production of pro-inflammatory IL-6, IL-12 and TNF-α but augments anti-inflammatory IL-10 cytokines. DC^{MPT64} and control DC^{CFP-10} and DCs were cultured for 6 days in the complete medium. Then, SNs were assessed for the estimation of (A) IL-6; (B) IL-12; (C) TNF-α; (D) IL-10 through ELISA.

Data represented as mean ± SD are of three wells and from 2 independent experiments. Further, qRT-PCR was performed to evaluate the expression of (E) IL-6; (F) IL-12; (G) TGF-β in DC^{MPT64}. DCs and DC^{CFP-10} were taken as controls. Data are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.7. MPT64 induces morphological changes in DC^{MPT64}. DC bridges innate immunity with adaptive immunity (Prendergast and Kirman, 2013). DC imparts a decisive role in provoking immune system against *Mtb* infection (Banchereau and Steinman, 1998). Activated DCs looks morphologically vivid with elongated dendrites. They robustly capture antigens for processing and presentation to T cells (Warne et al., 1991). DCs have many long arborizing projections on their surface known as dendrites, which helps in the capturing and processing of antigen in the periphery and lymphoid organs to prime T cells (Gil Del Rio, 1975). Hence, we next monitored the morphology of DC^{MPT64}. We observed that DC^{MPT64} appeared withered with absence of dendrites, oval in shape and smaller size, as compared to control DCs. Exposure of CFP-10 activates DCs, as demonstrated by massive enhancement in the size and extended dendrites (Fig. 7B). These changes were further validated by confocal imaging (Fig. 7A). This corroborates with our previous observations that MPT64 prompts the generation of myeloid suppressive cells. Myeloid suppressor cells have shrunken morphology with lesser efficacy of activating adaptive immune response.


A
DC
DC^MPT64
DC^CFP-10
B
DC
DC^MPT64
DC^CFP-10

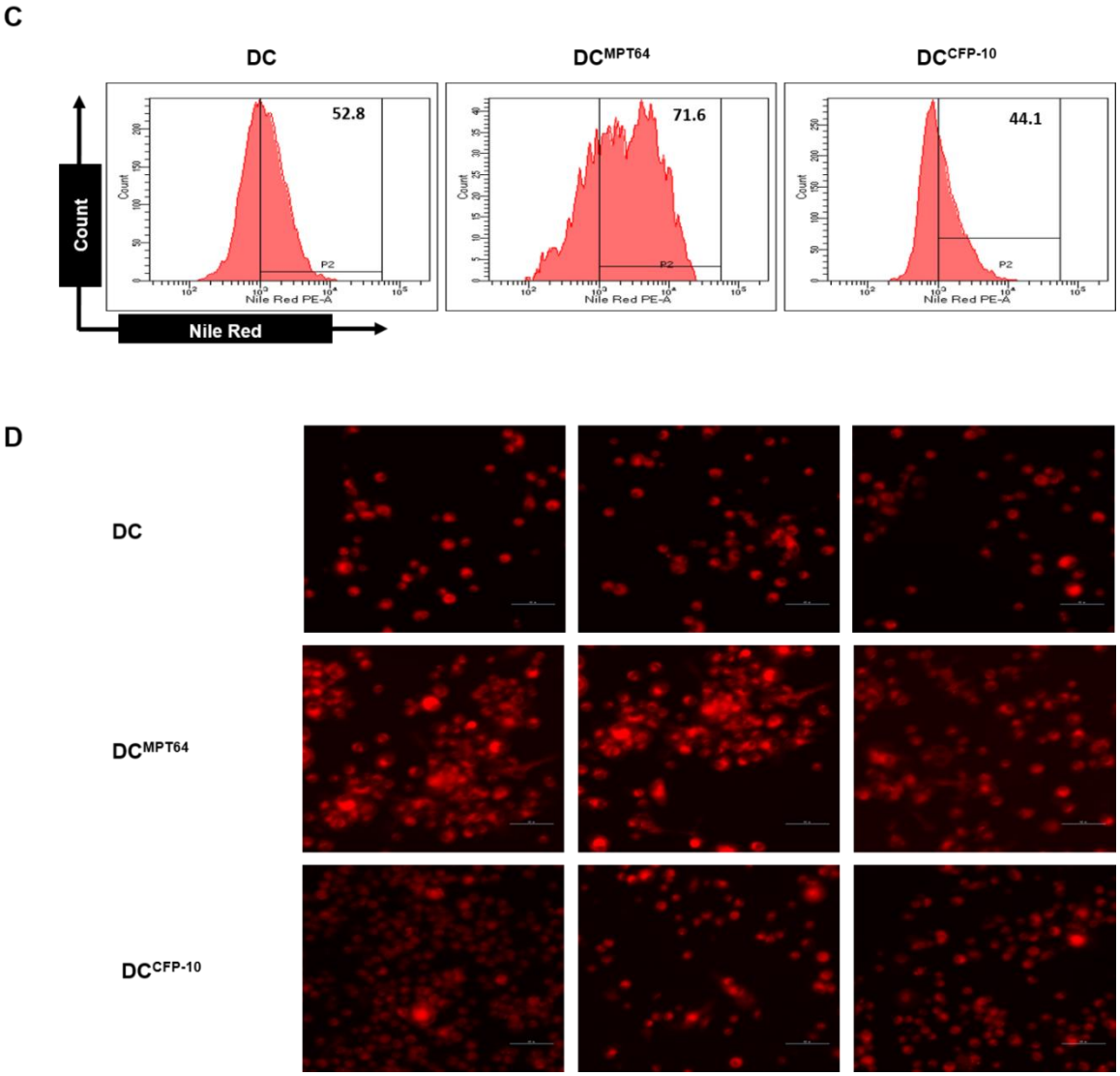


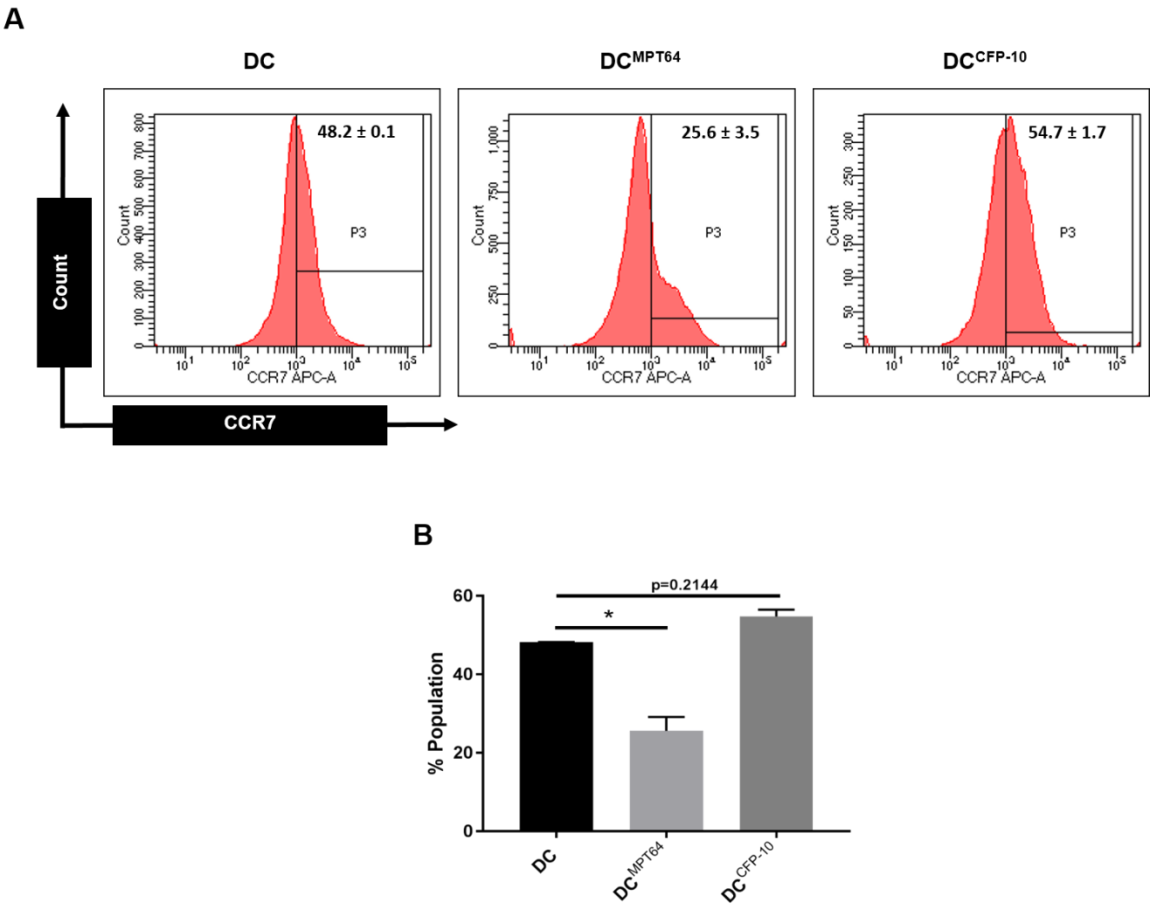
Figure 7. Induction of morphological changes and lipid accumulation in DC^{MPT64}. (A) DC^{MPT64} and control DC^{CFP-10} and DCs were cultured on poly-L-Lysine pre-coated glass coverslips in 12 well plates for 6 d. Later, cells were imaged through confocal microscopy (60X) to study the changes in their morphology (scale bar: 50 µm). (B) DCs, DC^{MPT64} and DC^{CFP-10} were cultured over poly-L-Lysine treated sterile coverslips for 6 d. Later, the cells were fixed with modified Karnovsky's fixative and imaged though SEM. Pictures were clicked at 13.97K (scale bar-2 µm). Data are from two independent experiments. (C) DC^{MPT64}, DC^{CFP-10} and DCs were Nile red dye stained for monitoring the lipid deposition within cells through flowcytometry. (D) DCs, DC^{MPT64} and DC^{CFP-10} were Nile red dye stained and the red staining specifies the presence of lipid in the cells. Images were clicked through

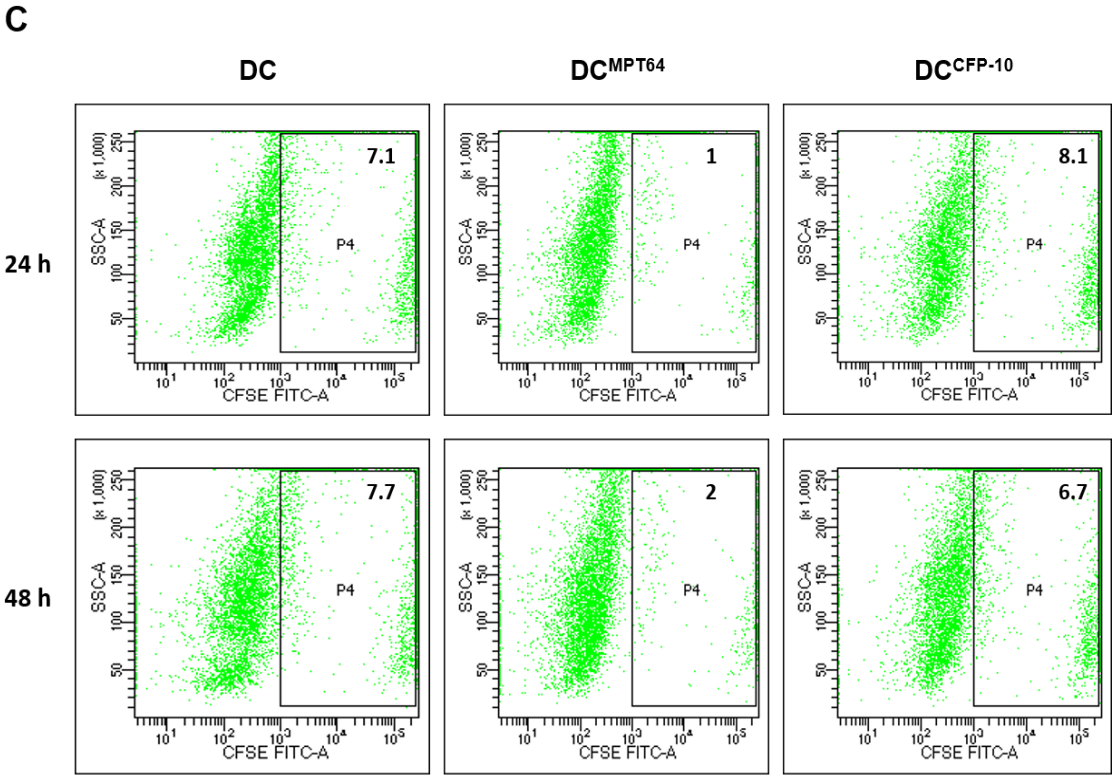
fluorescence microscope. DCs and DC^{CFP-10} were taken as control. Data are representative of two to three experiments.

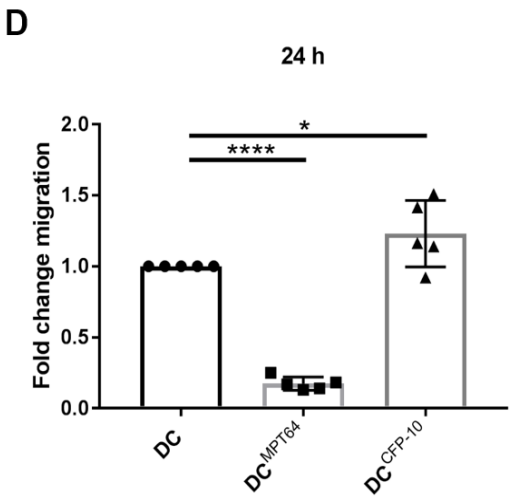
5.8. MPT64 instigates lipid accumulation in DC^{MPT64}. *Mtb* conventionally infects and prefers to be phagocytosed by the macrophages. *Mtb* within foamy macrophages trigger metabolic changes for its survival (Watanabe and Gilmour, 1996). Lipid depots within the host cells act as an energy reservoir for the survival of *Mtb* (Daniel et al., 2011). Lipids within MΦs augment the expression of PGE_2 and COX-2, which ultimately dampens the Th1 immunity (D'Avila et al., 2006; Knight et al., 2018). MDSCs have high lipid content and act as a nutrient-rich niche for *Mtb* (Hossain et al., 2015). Further, the lipid within DCs, hampers their functionality (Herber et al., 2010). Hence, we planned our experiments to check whether lipid accumulation within DC^{MPT64} curbs their functionality. We observed augmented lipid deposition in DC^{MPT64}, as compared to control DCs and DC^{CFP-10} by both flowcytometry and fluorescence microscopy (Fig. 7C, D). It can be inferred from these findings that MPT64 encourages lipid accumulation in DC^{MPT64}, which will help in the survival of *Mtb* and suppression of DC^{MPT64}.

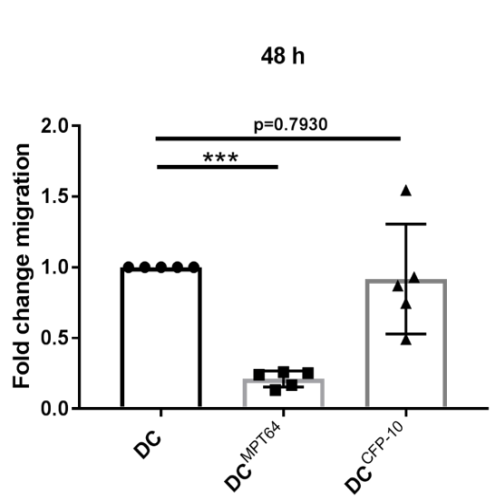
5.9. MPT64 inhibits the migratory function of DC^{MPT64}. After capturing antigens from the place of infection, DCs travel to the secondary lymphoid organs to activate pathogen-reactive T cells. Such DCs express an optimum level of CCR7, a receptor that is responsible for their migration (van Vliet et al., 2007). Therefore, migration of antigen-loaded DCs to lymphoid organs is very crucial for the induction of adaptive immune response. Till now, we have observed that DC^{MPT64} exhibits a suppressive phenotype with high co-inhibitory markers and lesser CD86 and MHC II molecules. Hence, we were eager to know, whether DC^{MPT64} have retained their migratory potential or not. DC^{MPT64} exhibited a noteworthy ($p<0.05$) decline in the expression of CCR7, but DC^{CFP-10} showed a significant increase in the surface appearance of

CCR7 (p=0.2144), compared to DCs (Fig. 8A, B). Further, to examine the migratory potential of DC^{MPT64} *in vivo*, we adoptively transferred CFSE-labelled DC^{MPT64} through an intravenous route into mice. A significant drop was apparent in the migration of CFSE-DC^{MPT64} to the spleen after 24 h ($p<0.0001$) and 48 h ($p<0.001$). Whereas, CFSE-DC^{CFP-10} showed higher migration to the spleen after 24 h ($p<0.05$) and 48 h ($p=0.7930$), as compared to control DCs (Fig. 8C-E). Like the spleen, the same pattern of CFSE-DC^{MPT64} migration was observed in lymph nodes after 24 h and 48 h (Fig. 8F-H). This DC^{MPT64} displayed a significantly lower level of CCR7 in the spleen ($p<0.01$) and lymph nodes ($p<0.001$) (Fig. 8I-L). These observations corroborate our initial finding of a decrease in the presence of CCR7 on DC^{MPT64}, and therefore limit their migration to secondary lymphoid organs.

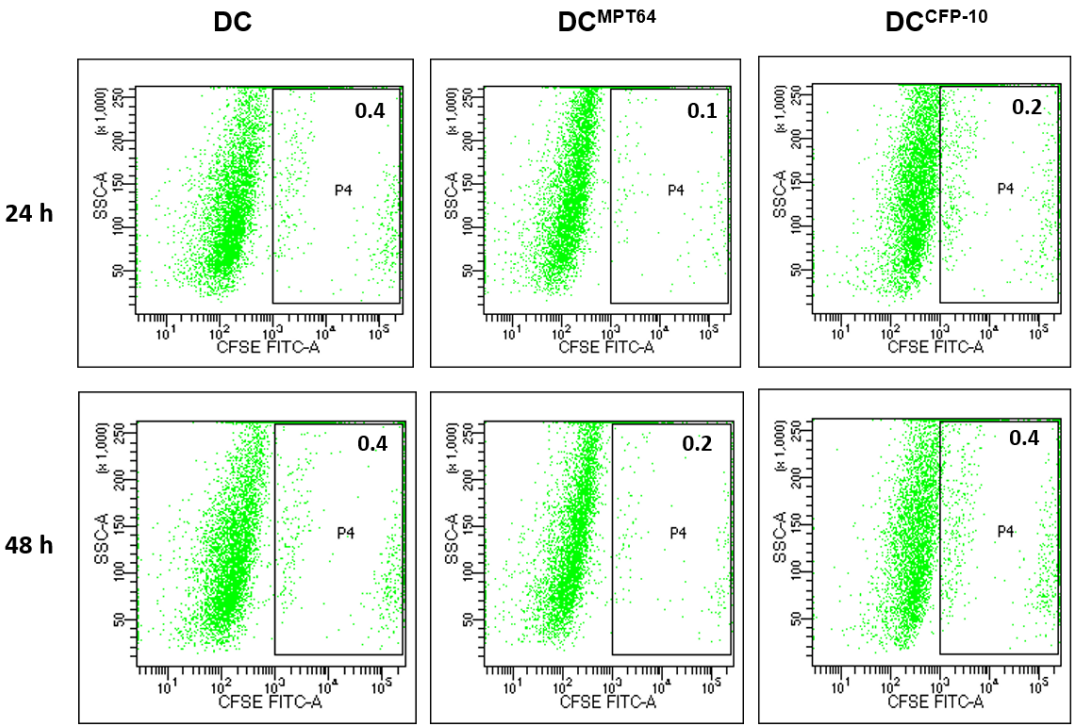



C
DC
DC^MPT64
DC^CFP-10
24 h
48 h
7.1
1
8.1
7.7
2
6.7
P4
SSC-A
CFSE FITC-A

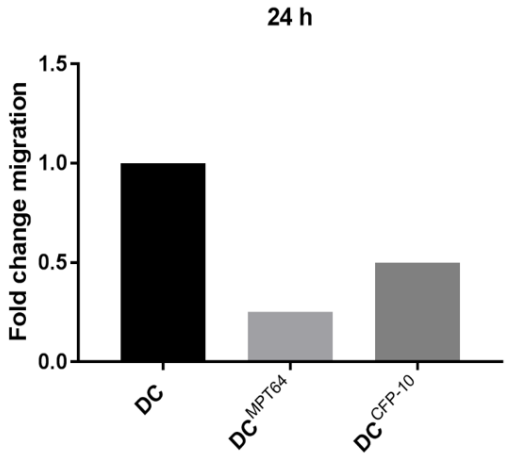

D
24 h
Fold change migration
*
****
DC
DC^MPT64
DC^CFP-10


E
48 h
Fold change migration
p=0.7930
***
DC
DC^MPT64
DC^CFP-10

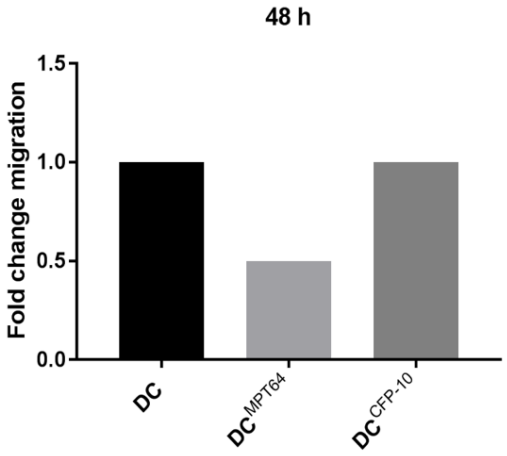
F


DC
DC^MPT64
DC^CFP-10
24 h
48 h
0.4
0.1
0.2
0.4
0.2
0.4
P4
SSC-A
CFSE FITC-A

G


24 h
Fold change migration
1.5
1.0
0.5
0.0
DC
DC^MPT64
DC^CFP-10

H


48 h
Fold change migration
1.5
1.0
0.5
0.0
DC
DC^MPT64
DC^CFP-10

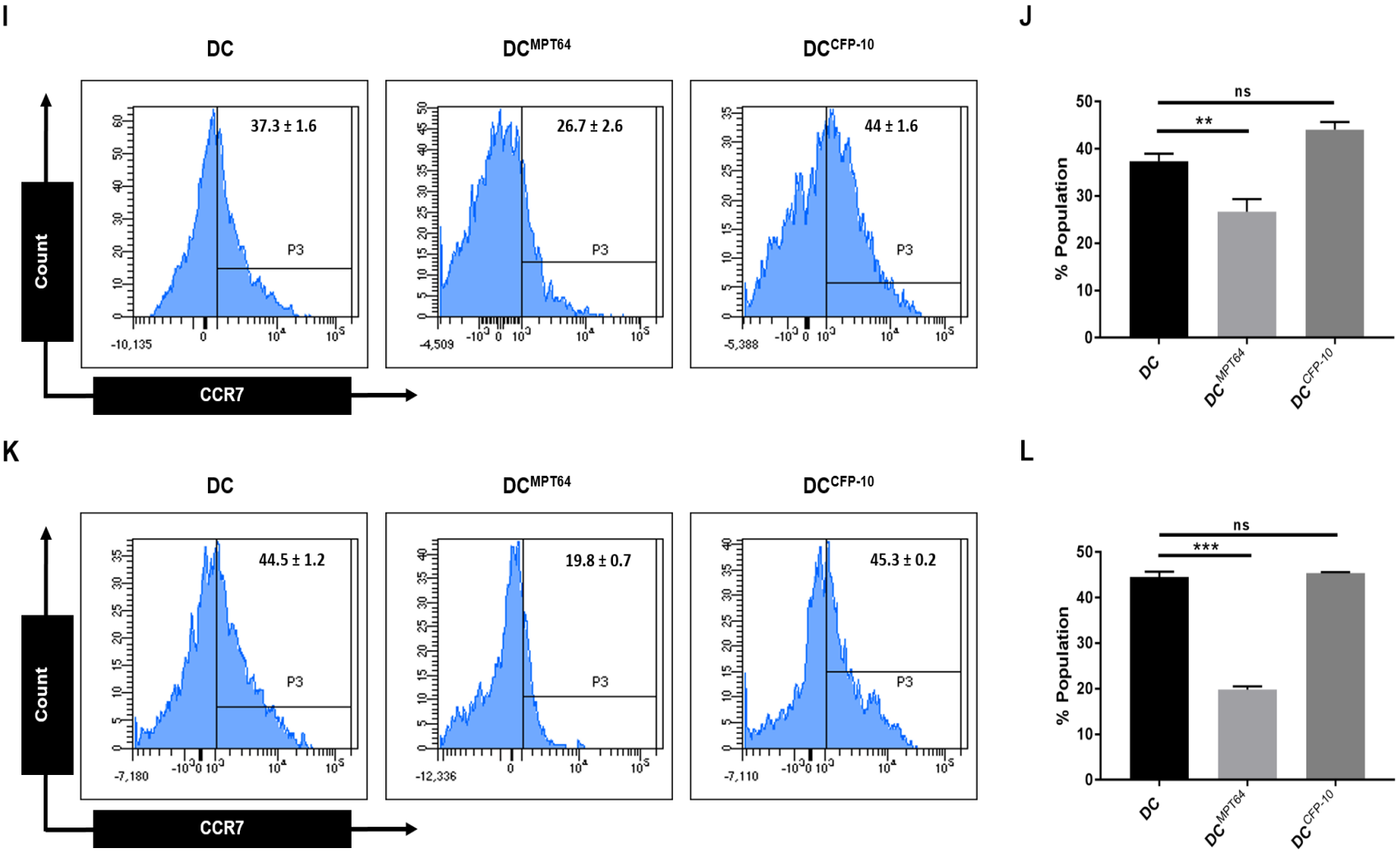
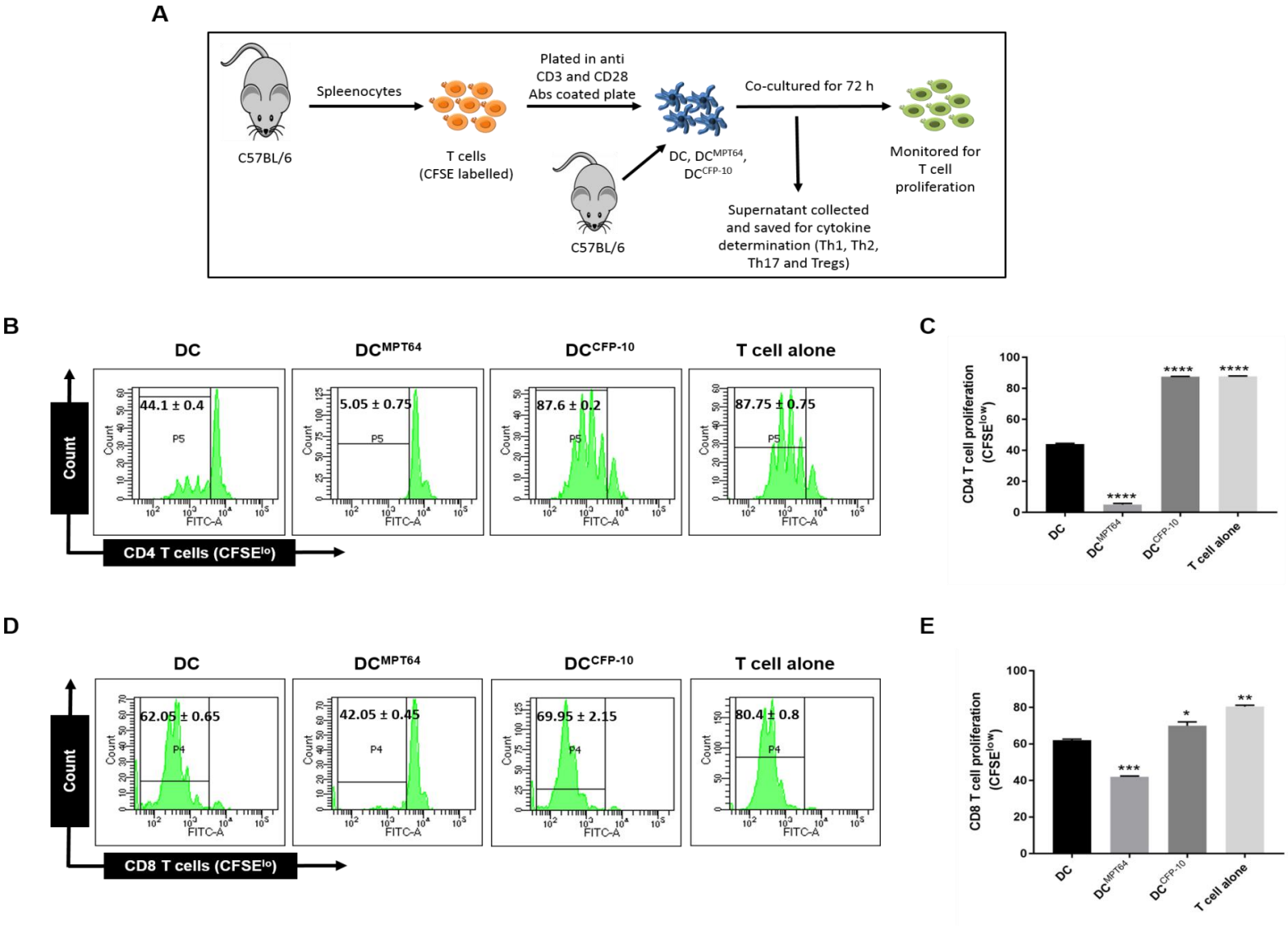

I
DC
DC^MPT64
DC^CFP-10
Count
CCR7
37.3 ± 1.6
26.7 ± 2.6
44 ± 1.6
P3
J
% Population
ns
**
DC
DC^MPT64
DC^CFP-10
K
44.5 ± 1.2
19.8 ± 0.7
45.3 ± 0.2
L
***

Figure 8. MPT64 restricts the migratory potential of DC^{MPT64}. (A, B) DC^{MPT64}, DC^{CFP-10} and DCs were monitored for the surface expression of CCR7. Data are represented as percentage positive cells (mean ± SEM) through flowcytometer histograms and bar diagrams. (E-F) DC^{MPT64}, DC^{CFP-10} and DCs were CFSE-labelled and injected i.v. in the mice. After 24 h and 48 h, presence of $CFSE^{+}$ cells was monitored in the (C-E) spleen; (F-H) lymph nodes. The data are represented as histograms (C, F), scatter plots (D, E) and bar diagrams (G, H). Inset of histograms show percentage positive cells and scatter plots depict fold change migration of DC, DC^{MPT64} and DC^{CFP-10}. Each dot denotes an individual animal. Bar diagrams represent pooled data of all animals at 24 h and 48 h and describe fold change migration of DCs, DC^{MPT64} and DC^{CFP-10}. The surface expression of CCR7 was observed ex-vivo on DC^{MPT64} migrated to the spleen (I, J) and lymph nodes (K, L). The percentage positive (mean ± SEM) DC^{MPT64} is illustrated through a flowcytometry and bar diagram. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: non-significant.

5.10. MPT64 abates the efficacy of DC^{MPT64} to activate T cells. Complete elimination of a pathogen requires both innate and adaptive immunity. Upon recognition of pathogens by DCs, MΦs and B cells, it leads to an induction of primary immune response through secretion of antibodies, cytokines and other soluble mediators. Pathogen-captured DCs migrate to the secondary lymphoid organs to turn on the pathogen-reactive T cells (Lenschow et al., 1996). Prohibition of the turning on of T cells has been referred to as a 'gold standard of MDSCs (Hongo et al., 2014). Since DC^{MPT64} exhibited MDSCs-like phenotype, we were interested to notice whether DC^{MPT64} also suppresses the proliferation of T cells. We co-cultured DC^{MPT64} with syngeneic T cells (CD4 and CD8) and observed a noteworthy decline in the proliferation of both CD4 T cells ($p<0.0001$) and CD8 T cells ($p<0.001$). On the contrary, DC^{CFP-10} co-cultured with syngeneic T cells showed a

significant augmentation in the proliferation of both CD4 T cells ($p<0.0001$) and CD8 T cells ($p<0.01$) (Fig. 9A-E).

These results were further supported by observing a substantial fall in the proliferation of allogeneic T cells. We co-cultured DC^{MPT64} with allogeneic T cells and observed a noteworthy decline in the proliferation of both CD4 T cells ($p<0.05$) and CD8 T cells ($p<0.001$). On contrary, DC^{CFP-10} co-cultured with allogeneic T cells revealed an increase in the proliferation of both CD4 and CD8 T cells ($p<0.01$) (Fig. 9F-J). These findings suggest the suppressive nature of DC^{MPT64}, as has been reported in the case of MDSCs, and therefore DC^{MPT64} tend to inhibit immune response.


A
C57BL/6
Spleenocytes
T cells
(CFSE labelled)
Plated in anti
CD3 and CD28
Abs coated plate
DC, DC^MPT64,
DC^CFP-10
C57BL/6
Co-cultured for 72 h
Monitored for
T cell
proliferation
Supernatant collected
and saved for cytokine
determination (Th1, Th2,
Th17 and Tregs)
B
DC
DC^MPT64
DC^CFP-10
T cell alone
Count
44.1 ± 0.4
5.05 ± 0.75
87.6 ± 0.2
87.75 ± 0.75
P5
FITC-A
CD4 T cells (CFSE^lo)
C
CD4 T cell proliferation
(CFSE^low)
****
D
62.05 ± 0.65
42.05 ± 0.45
69.95 ± 2.15
80.4 ± 0.8
P4
CD8 T cells (CFSE^lo)
E
CD8 T cell proliferation
(CFSE^low)
***
*
**

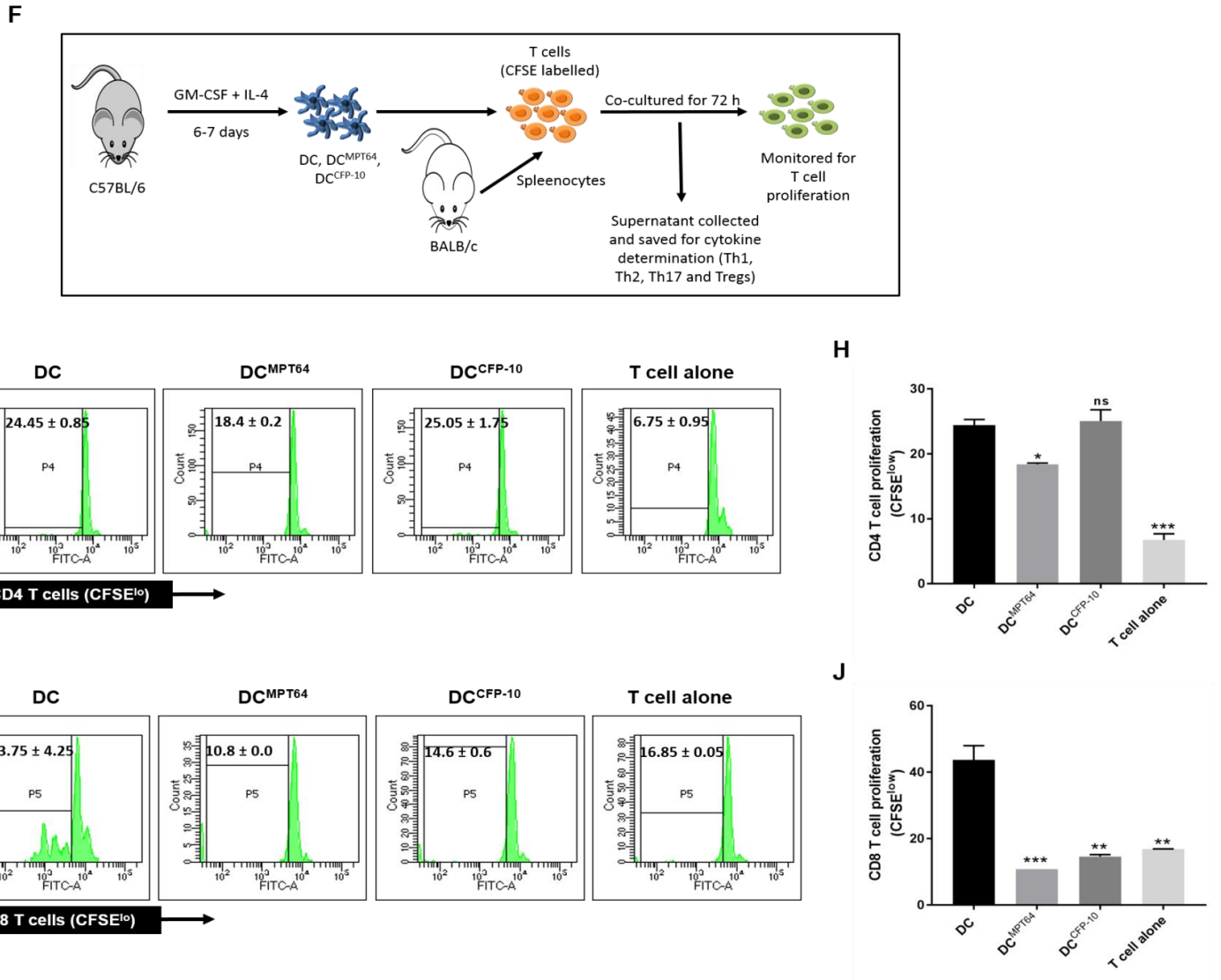
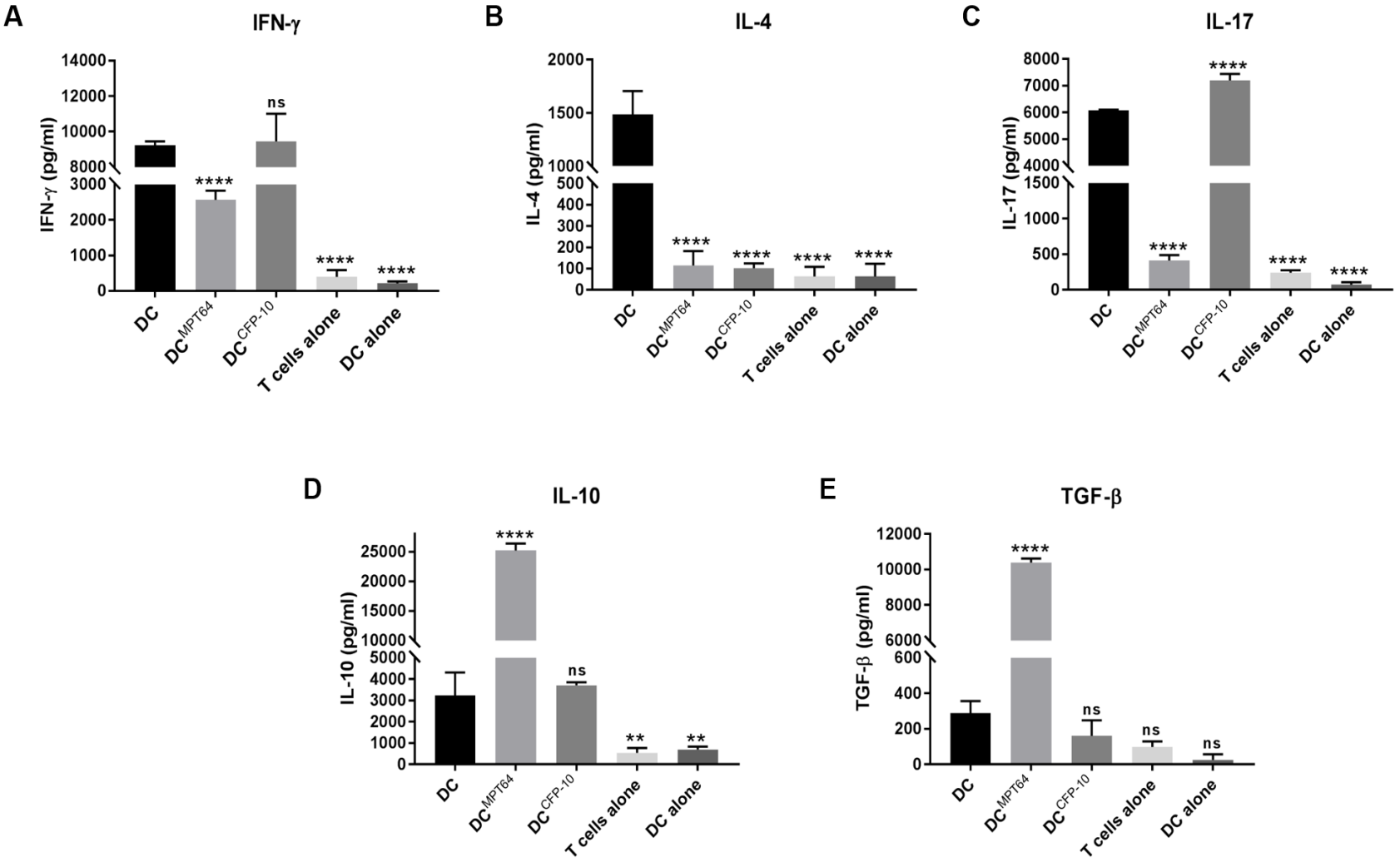

F
C57BL/6
GM-CSF + IL-4
6-7 days
DC, DC^MPT64, DC^CFP-10
T cells (CFSE labelled)
Co-cultured for 72 h
Monitored for T cell proliferation
BALB/c
Spleenocytes
Supernatant collected and saved for cytokine determination (Th1, Th2, Th17 and Tregs)
G
DC
DC^MPT64
DC^CFP-10
T cell alone
24.45 ± 0.85
18.4 ± 0.2
25.05 ± 1.75
6.75 ± 0.95
Count
FITC-A
CD4 T cells (CFSE^lo)
H
CD4 T cell proliferation (CFSE^low)
ns
I
43.75 ± 4.25
10.8 ± 0.0
14.6 ± 0.6
16.85 ± 0.05
CD8 T cells (CFSE^lo)
J
CD8 T cell proliferation (CFSE^low)

Figure 9. MPT64 impairs the function of DC^{MPT64} to activate lymphocytes. (A, F) Schematic representation of experimental methodology. The proliferation of syngeneic CD4 T cells and CD8 T cells by DC^{MPT64}. CFSE-labelled syngeneic naïve (B, C) CD4 T cells; (D, E) CD8 T cells were stimulated with anti-CD3 and anti-CD28 Abs and co-cultured with syngeneic DC^{MPT64} and control DC^{CFP10} and DCs (T cell: DCs; 10:1 ratio). Allogeneic naïve (G, H) CD4 T cells; (I, J) CD8 T cells isolated from BALB/c mice were co-cultured with allogeneic (C57BL/6) DCs (T cell: DCs; 10:1 ratio). After 72 h, T cell proliferation was enumerated by CFSE-dye dilution assay. The bar diagram represents the percentage of positive cells (mean ± SEM), and is from two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: non-significant.

5.11. MPT64 diminishes the efficacy of DC^{MPT64} to differentiate T cells. The adaptive immune system has a very compelling role in limiting the spread of *Mtb*. Consequent to *Mtb* infection, innate immune cells, on one hand, curtail the infection and on the other side provide a safe shelter for the survival and persistence of the bacterium. Lymphocytes have a noteworthy part in the pathogenesis and development of the immunity (Cooper, 2009). Upon stimulation by DCs, T cells release an array of cytokines like IL-17, IFN-γ, TNF-α, IL-10, TGF-β and IL-4. These cytokines have a robust role in the induction and differentiation of Th1 cells, Th2 cells, Th17 cells and Tregs cells. T helper1 cells and T helper17 cells play a central role in protecting against *Mtb* (Chackerian et al., 2001). On contrary, T helper 2 cells and Tregs promote the progression of the disease (Cardona and Cardona, 2019; McLaughlin et al., 2020). Thus, we were curious to know, which T cells are being chiefly induced by DC^{MPT64}. Consequently, we harvested the supernatants from the DC^{MPT64} co-cultured with syngeneic CD4 T cells and estimated the number of cytokines released. We noticed significant decline in IFN-γ (p<0.0001), IL-17 (p<0.0001)

and IL-4 ($p<0.0001$), but increase in IL-10 ($p<0.0001$) and TGF-β ($p<0.0001$). Contrary to this, we noted significant upregulation in the secretion of IL-17 ($p<0.0001$), and IL-4 ($p<0.0001$) with a slight increase in IFN-γ and no significant secretion of IL-10 and TGF-β by DC^{CFP-10} (Fig. 10A-E). As noticed in the case of syngeneic CD4 T cells, similar reduction in IFN-γ ($p<0.0001$), IL-17 ($p<0.0001$) and IL-4 ($p<0.0001$), but an increase in the IL-10 ($p<0.0001$) and TGF-β ($p<0.0001$) yield by allogeneic CD4 T cells was detected. On the contrary, we observed a significant increase in the secretion of IL-17 ($p<0.0001$), IFN-γ ($p<0.0001$) and no significant secretion of IL-4, IL-10 and TGF-β by DC^{CFP-10} (Fig. 10F-J). Therefore, the results signify that DC^{MPT64} not only suppresses the proliferation and differentiation of Th1 cells (IFN-γ), Th2 cells (IL-4) and Th17 (IL-17) cells but supports TGF-β and IL-10 secreting Tregs.


A
IFN-γ
IFN-γ (pg/ml)
14000
12000
10000
8000
3000
2000
1000
0
****
ns
****
****
DC
DC MPT64
DC CFP-10
T cells alone
DC alone
B
IL-4
IL-4 (pg/ml)
2000
1500
1000
500
400
300
200
100
0
****
****
****
****
DC
DC MPT64
DC CFP-10
T cells alone
DC alone
C
IL-17
IL-17 (pg/ml)
8000
7000
6000
5000
4000
1500
1000
500
0
****
****
****
****
DC
DC MPT64
DC CFP-10
T cells alone
DC alone
D
IL-10
IL-10 (pg/ml)
25000
20000
15000
10000
5000
4000
3000
2000
1000
0
****
ns
**
**
DC
DC MPT64
DC CFP-10
T cells alone
DC alone
E
TGF-β
TGF-β (pg/ml)
12000
10000
8000
6000
600
400
200
0
****
ns
ns
ns
DC
DC MPT64
DC CFP-10
T cells alone
DC alone

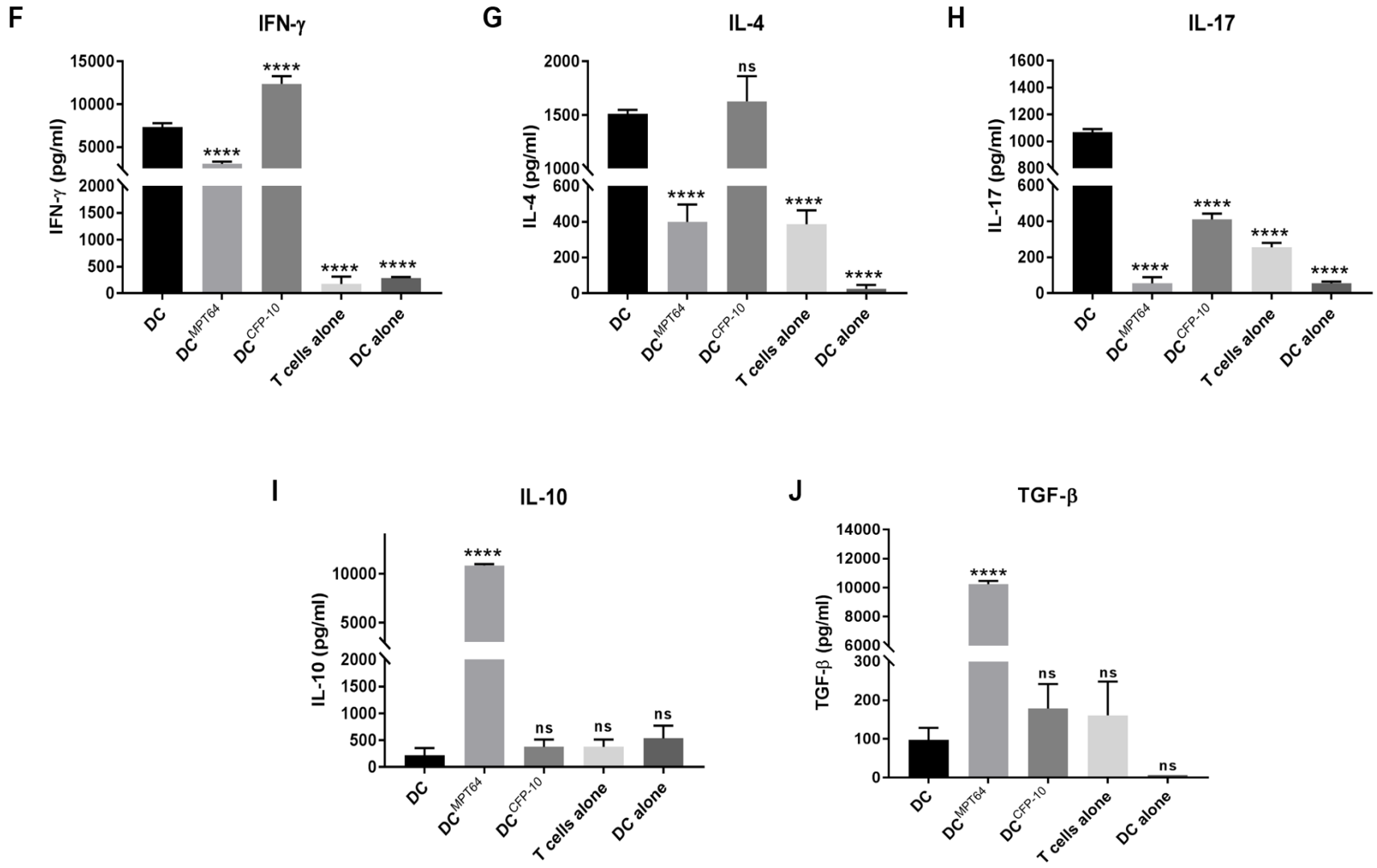

F
IFN-γ
IFN-γ (pg/ml)
15000
10000
5000
2000
1500
1000
500
0
****
****
****
****
****
DC
DC^MPT64
DC^CFP-10
T cells alone
DC alone
G
IL-4
IL-4 (pg/ml)
2000
1500
1000
600
400
200
0
****
ns
****
****
DC
DC^MPT64
DC^CFP-10
T cells alone
DC alone
H
IL-17
IL-17 (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
****
****
****
****
DC
DC^MPT64
DC^CFP-10
T cells alone
DC alone
I
IL-10
IL-10 (pg/ml)
10000
5000
2000
1500
1000
500
0
****
ns
ns
ns
DC
DC^MPT64
DC^CFP-10
T cells alone
DC alone
J
TGF-β
TGF-β (pg/ml)
14000
12000
10000
8000
6000
300
200
100
0
****
ns
ns
ns
DC
DC^MPT64
DC^CFP-10
T cells alone
DC alone

Figure 10. MPT64 stimulated DC^{MPT64} abates the differentiation of CD4 T cells into Th1 cells, Th2 cells and Th17 cells and augments the pool of Tregs. Naïve T cells (CD4 and CD8) isolated from syngeneic (C57BL/6) or allogeneic (BALB/c) mice were co-cultured with DC^{MPT64} (C57BL/6) (T cell: DCs; 10:1 ratio), as mentioned in the legend of Figure 9. Subsequent to 72 h of co-culture, the SNs were harvested from the (A-E) syngeneic cultures; (F-J) allogeneic cultures for the secretion of IFN-γ, IL-4, IL-17, IL-10 and TGF-β by ELISA. Data are from 3 wells and represented as mean ± SD. **p<0.01, ****p<0.0001, ns: non-significant.

5.12. MPT64 induces *in vivo* generation of Tregs by DC^{MPT64}. Tolerance of immune response has been associated with the development of chronic infections like TB and cancer. Conventionally, the immune system of the host always balances between the generation of immune response and tolerance. DCs not only stimulate innate and adaptive immunity but impart tolerance too (Popov and Schultze, 2008; Shklovskaya and Fazekas de St Groth, 2007). Till now, we have noticed that DC^{MPT64} exhibits high PD-L1, TIM-3, IL-10 and TGF-β expression. Both PD-L1 and TIM-3 are well known for the induction of tolerance in DCs (Alderton, 2012; Sumpter and Thomson, 2011). Moreover, we have shown that DC^{MPT64} have the efficacy to decrease the activation and differentiation of T cells (CD4 and CD8). Based on these findings, we were eager to know whether DC^{MPT64} can evoke *in vivo* generation of FoxP3$^+$ Tregs. Hence, OVA pulsed-DC^{MPT64} were adoptively transferred twice at the gap of 7 d into the mice via the subcutaneous route (Fig. 11A). Intriguingly, we observed a significant increase in the percentage of CD4$^+$CD25$^+$FoxP3$^+$Treg cells ($p<0.05$)(Fig. 11B, C) and CD8$^+$CD25$^+$FoxP3$^+$Treg cells ($p<0.05$)(Fig. 11D, E). These results further substantiated earlier findings demonstrating that MDSCs promote Tregs generation in TB infection (Yang et al., 2014). These results suggest that *Mtb* may be using its MPT64 protein to evade the immune response by engendering Tregs.

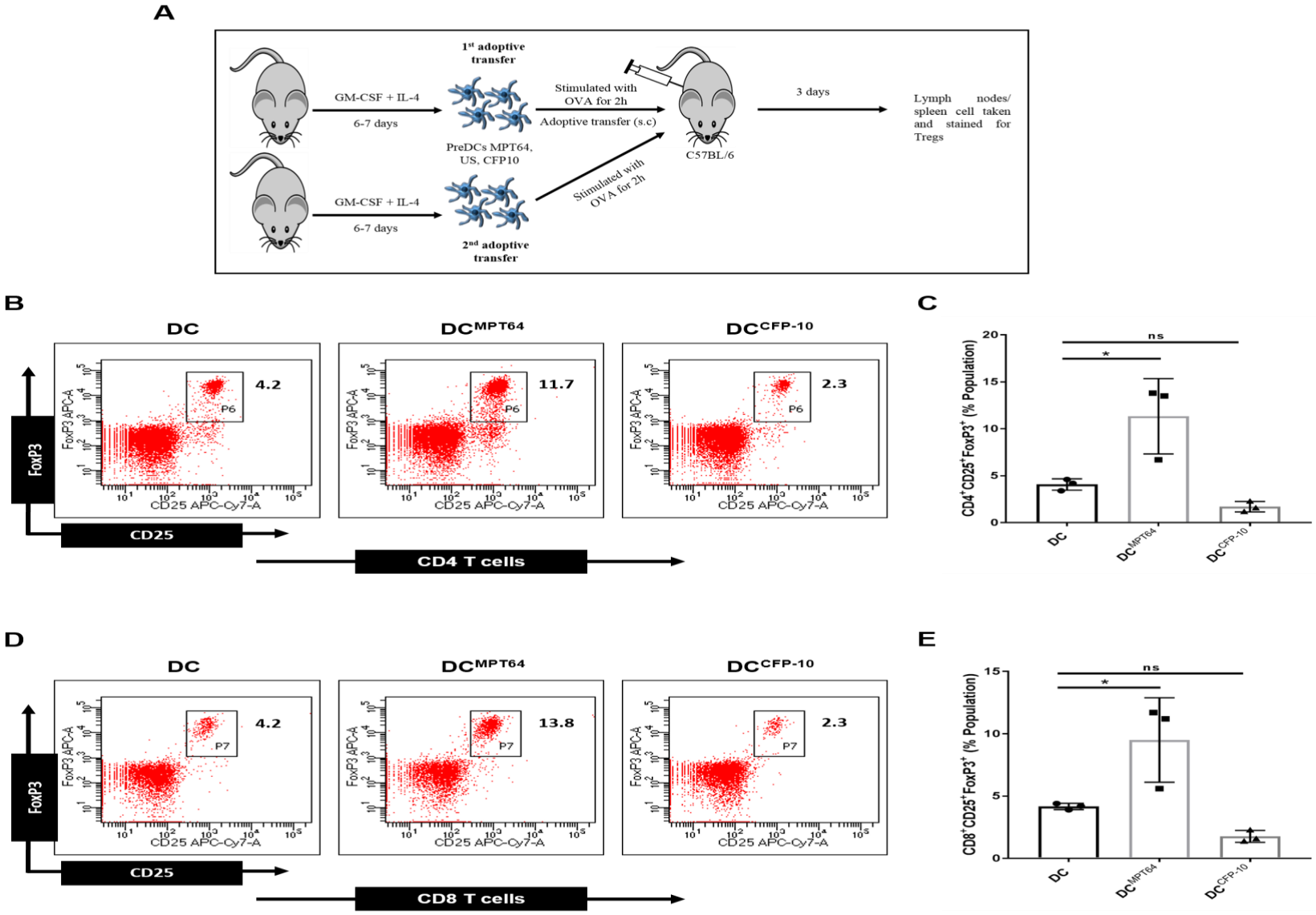
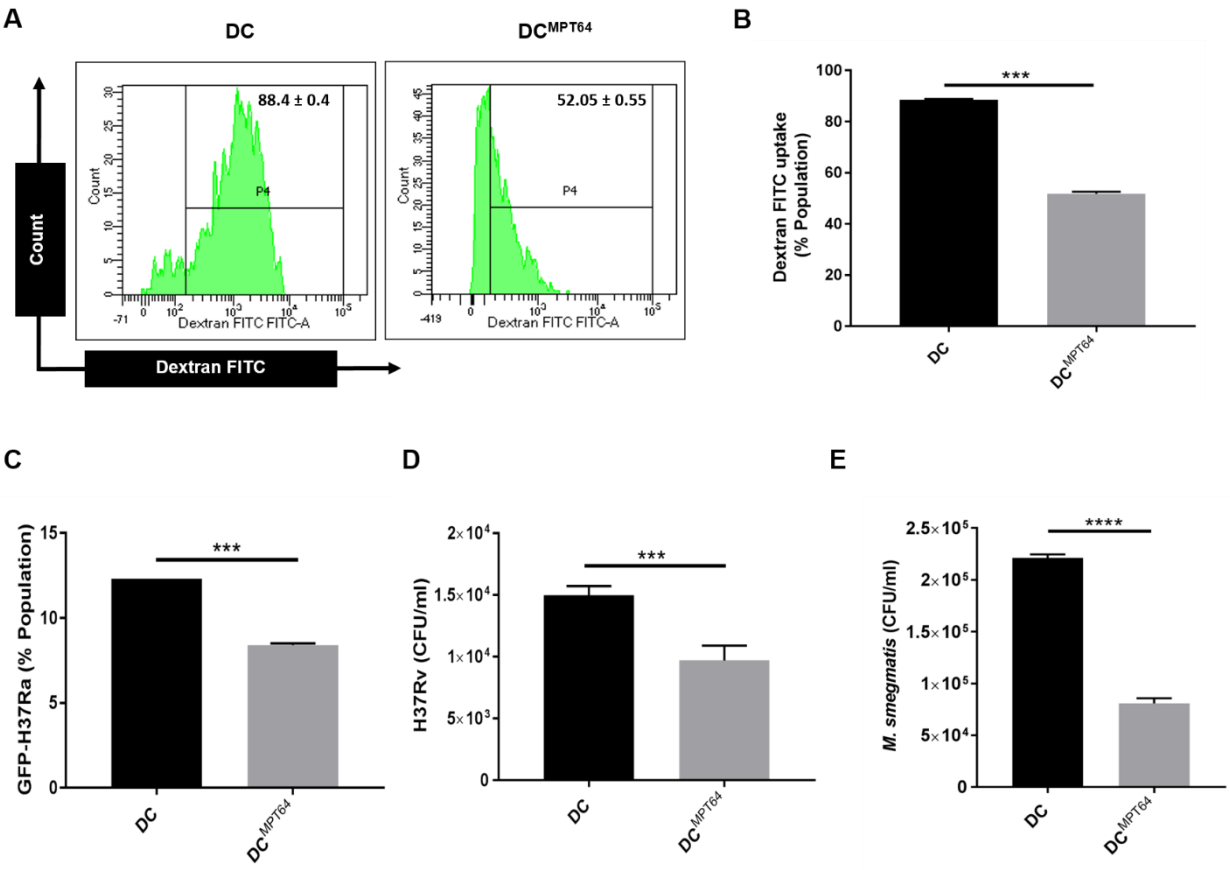

A
1st adoptive transfer
GM-CSF + IL-4
6-7 days
PreDCs MPT64, US, CFP10
Stimulated with OVA for 2h
Adoptive transfer (s.c)
C57BL/6
3 days
Lymph nodes/ spleen cell taken and stained for Tregs
GM-CSF + IL-4
6-7 days
2nd adoptive transfer
Stimulated with OVA for 2h
B
DC
DCMPT64
DCCFP-10
4.2
11.7
2.3
P6
FoxP3
CD25
CD4 T cells
C
ns
*
CD4+CD25+FoxP3+ (% Population)
D
4.2
13.8
2.3
P7
CD8 T cells
E
CD8+CD25+FoxP3+ (% Population)

Figure 11. MPT64 stimulated DC^{MPT64} supports the *in vivo* generation of Tregs. (A) Diagrammatic representation of the experimental design. Antigen-pulsed DC^{MPT64}, DC^{CFP-10} and DCs were injected (s.c) twice at an interval of 7 d in the mice. Then, 3 d later, mice were sacrificed and the pool of (B) CD4 T cells; (D) CD8 T cells expressing CD25^{+} and FoxP3^{+} were enumerated by flowcytometry and depicted as (B, D) dot plots; (C, E) bar diagrams. Each dot in the scatter plot signifies data from one mouse. The values (mean ± SEM), in the inset, are percentage positive cells and representative of 3 independent experiments. *p<0.05, ns: non-significant.

5.13. MPT64 impairs the ability of DC^{MPT64} to phagocytose mycobacteria but promotes intracellular survival of the bacteria. DCs and MΦs are the most potent innate immune cells with strong phagocytic functions. Upon uptake of the pathogen, DCs translocate pathogenic epitopes in association with the MHC I or II complex on its surface with enhanced expression of co-stimulatory molecules and cytokines (van Vliet et al., 2007). We observed that DC^{MPT64} showed less expression of MHC II. Moreover, the literature suggests that tolerogenic DCs and MDSCs are suppressive cells with impaired phagocytic function. Like alveolar macrophages, *Mtb* can also persist in MDSCs (Agrawal et al., 2018; Knaul et al., 2014). So, we were curious to check the DC^{MPT64} ability to phagocytose soluble and particulate antigens. We observed that DC^{MPT64} showed remarkable inhibition (p<0.001) in the uptake of dextran-FITC, a soluble form of an antigen (Fig. 12A-B). *Mtb* infected macrophages and DCs showed deterioration in engulfing antigens. To check this, we infected DC^{MPT64} with *Mtb* GFP-H37Ra and *Mtb* H37Rv and *M. smegmatis* (MOI, 1:5) for 4 h (3 h for *M. smegmatis*) and examined their uptake and survival by flowcytometry (GFP-H37Ra) and CFUs (H37Rv and *M. smegmatis*). DC^{MPT64} indicated a significant decline in the phagocytosis of both the virulent (H37Rv) (p<0.001) and avirulent (H37Ra) (p<0.001) strains of mycobacteria (Fig. 12C, D). These results were further confirmed, when we noted a substantial

(p<0.0001) drop to engulf *M. smegmatis*, another strain of mycobacteria (Fig. 12E). Impairment in the uptake of GFP-H37Ra by DC^{MPT64} was further confirmed through confocal imaging (Fig. 12F). Moreover, we have shown that GFP-H37Ra was localized inside the cell and not on the surface, as confirmed through Z-stacking of confocal imaging (Fig. 12G). Next, we were curious to know whether DC^{MPT64}will become a safe niche for the survival of *Mtb*. Consequently, we monitored the presence of virulent (H37Rv) and avirulent (GFP-H37Ra) strains of mycobacteria in DC^{MPT64} after 72 h of post-infection. The results were quite fascinatingly to note that the H37Rv (p<0.0001) and H37Ra (p<0.05) exhibited remarkably higher growth in the DC^{MPT64}, as compared control DCs even after 72 h of post-infection (Fig. 13A, B). The results indicate that *Mtb* exploits its MPT64 protein to establish its survival in the DCs by skewing their differentiation to MDSCs.



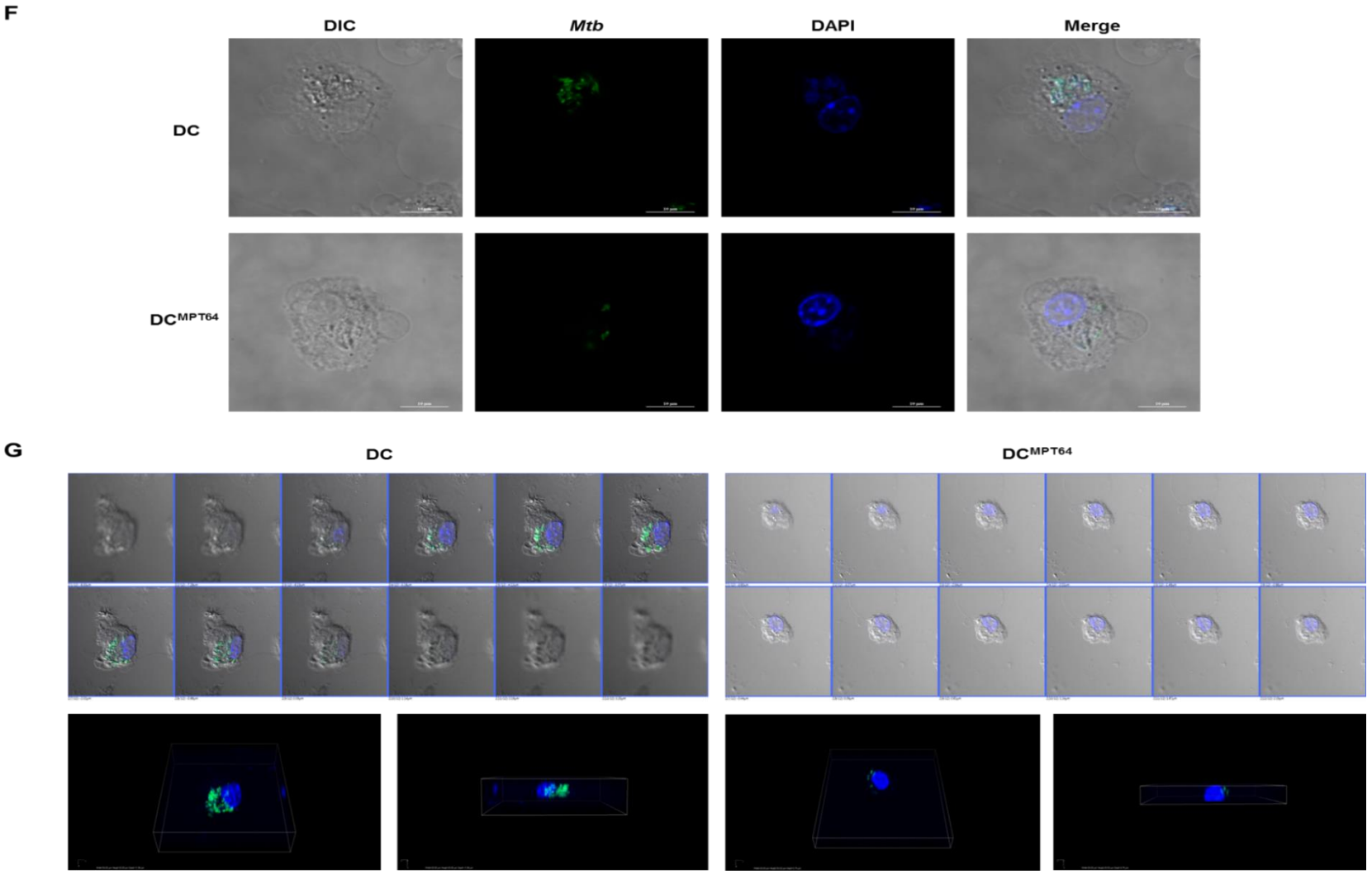

F
DIC
Mtb
DAPI
Merge
DC
DC^{MPT64}
G
DC
DC^{MPT64}

Figure 12. MPT64 restricted *Mtb* uptake by DC^{MPT64}. (A, B) DC^{MPT64} and DCs were monitored for antigen (Dextran-FITC) uptake by flowcytometry and depicted through histograms and bar diagrams. The DC^{MPT64} and control DCs were infected with (C) GFP-H37Ra; (D) H37Rv; (E) *M. smegmatis*; and further monitored the phagocytosis by (C) flow cytometer and (D-E) CFUs. The control DCs and DC^{MPT64} were infected with GFP-H37Ra, H37Rv for 4 h (MOI - 1:5) and *M. smegmatis* for 3h (MOI - 1:5). The extracellular bacilli were killed by incubating with gentamycin/amikacin for 1 h. (F) GFP-H37Ra uptake by DC^{MPT64}and control DCs was monitored through confocal microscopy in 2D and (G) 3D (Z stacking) (scale bar: 10 µM; magnification: 60X). The blue color signified the DAPI staining nucleus and the green is GFP-H37Ra. The data (mean ± SD) depicted as bar diagrams are indicative of percentage population and CFU/ml of *Mtb* and representative of triplicate samples and 2 independent experiments. ***p<0.001, ****p<0.0001.

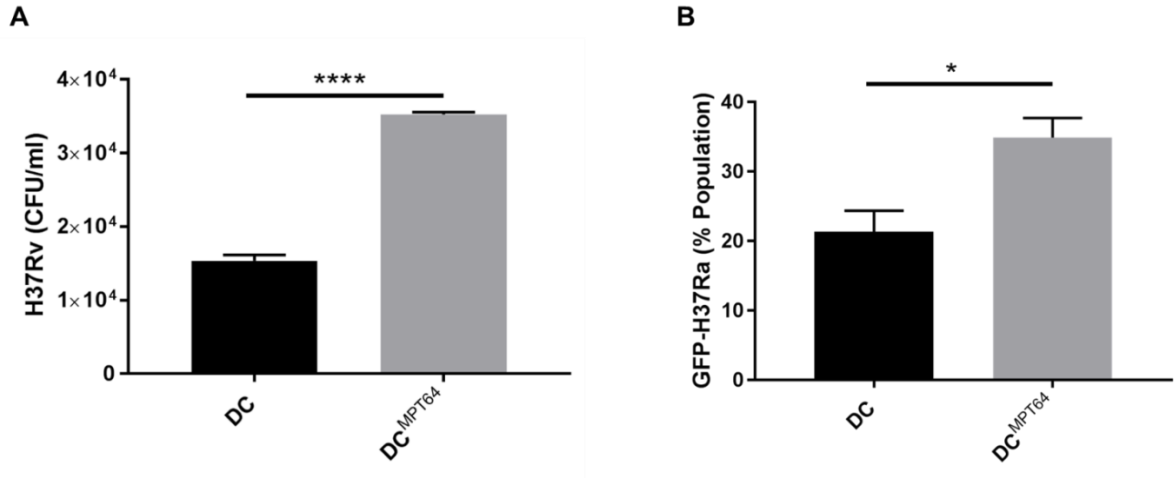
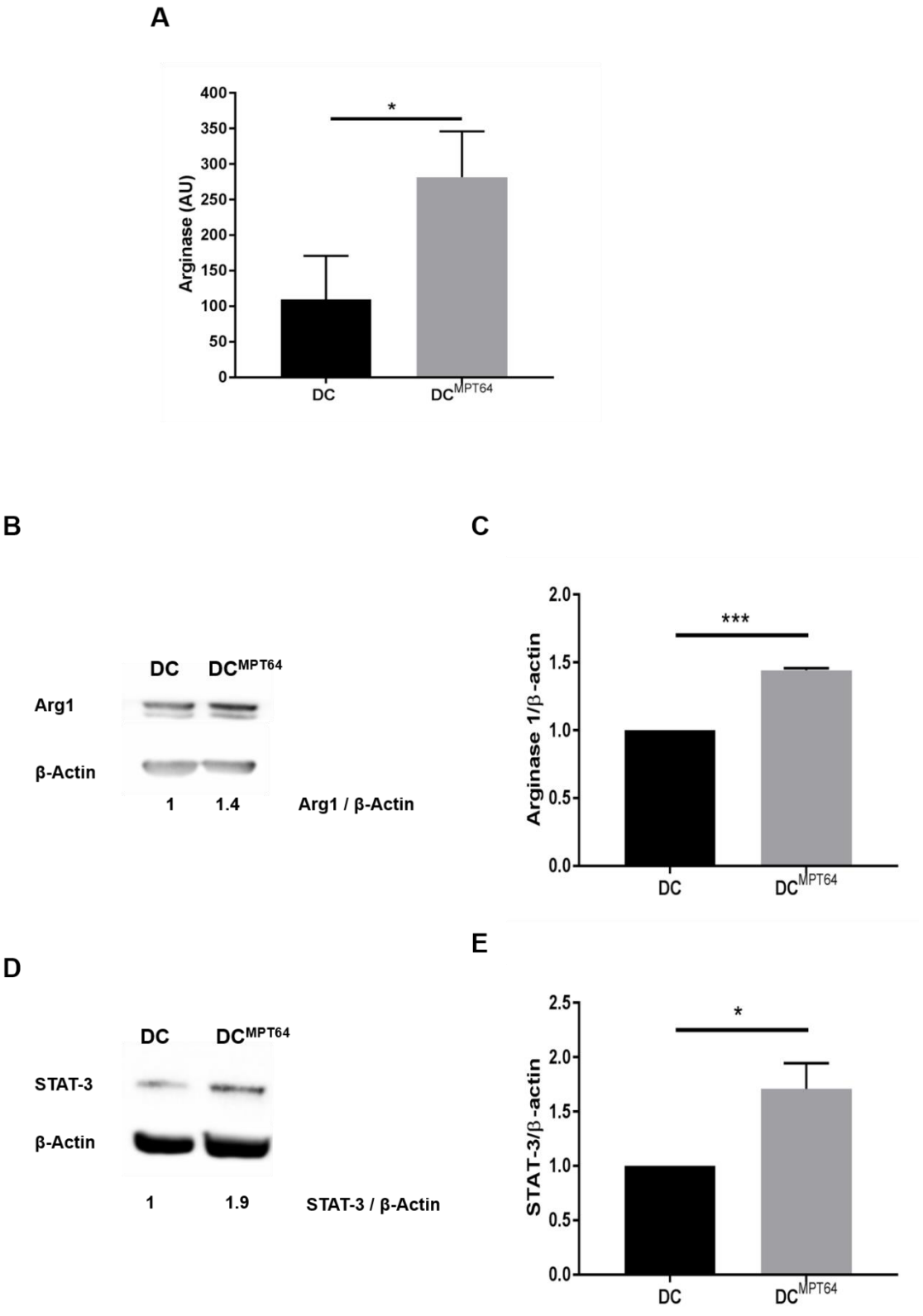


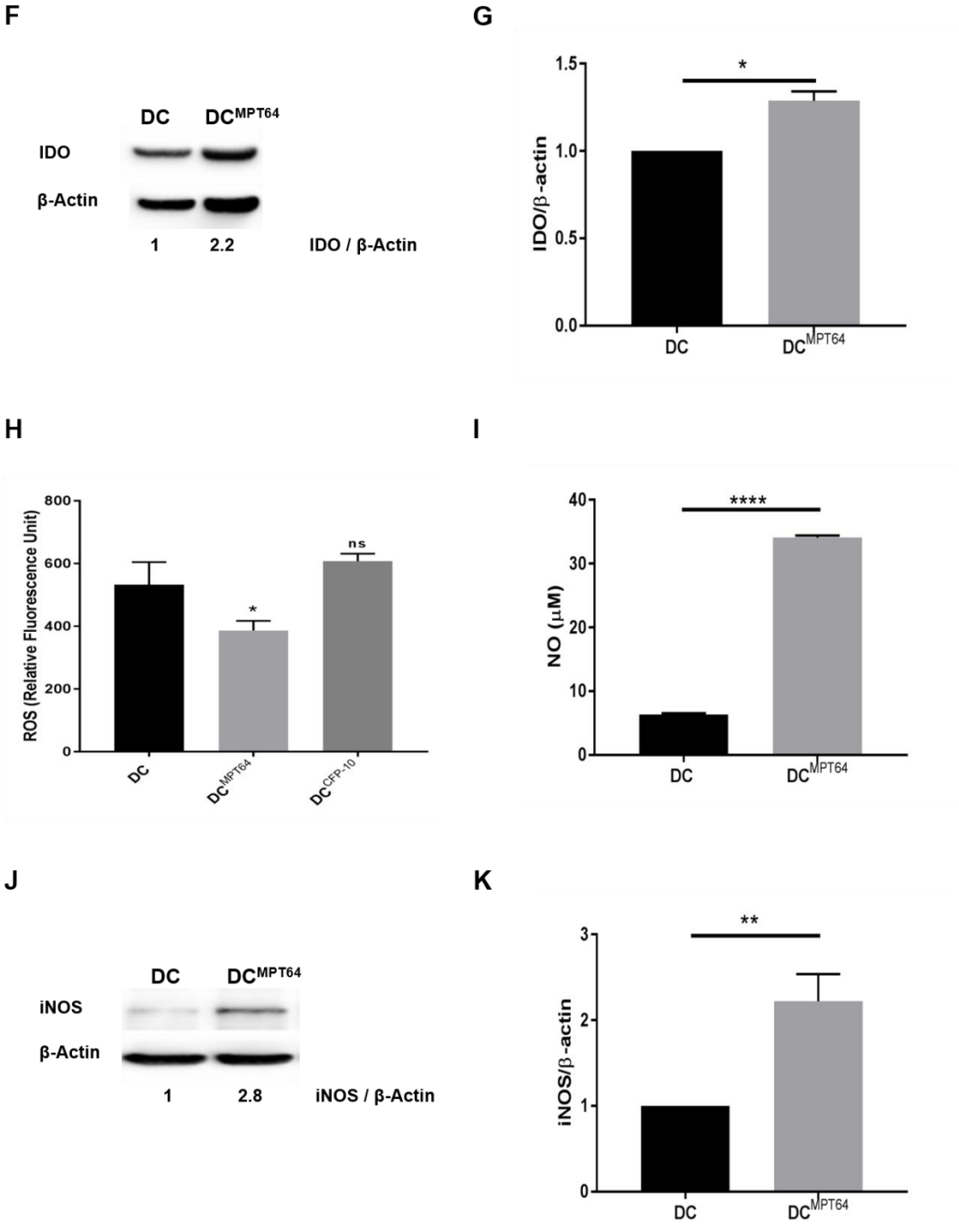
Figure 13. MPT64 augments intracellular *Mtb* survival in DC^{MPT64}. The DC^{MPT64} was infected with H37Rv and H37Ra (MOI - 1:5) for 4 h. The extracellular bacilli were killed by incubating the cultures with amikacin for 1 h. DC^{MPT64} were further cultured for 72 h. (A) Later, the infected DC^{MPT64} were lysed and the survival of *Mtb* (H37Rv) was estimated by CFUs. (B) Survival of GFP-H37Ra was monitored in DC^{MPT64} and control DCs by flow cytometer and shown as bar diagram (mean ± SD) are indicative of percentage population. The data (mean ±

SD) presented as bar diagrams are indicative of CFU/ml of *Mtb* and representative of triplicate samples and 2 independent experiments. *p<0.05, ****p<0.0001.

5.14. *Mtb* induces immunosuppression of DC^{MPT64} by utilizing its MPT64 protein. Innate immune cells control *Mtb* infection through various mechanisms such as phagocytosis, opsonization, inflammasome activation and producing various inflammatory mediators (Lerner et al., 2015; Yang et al., 2014). Hence, we did an extensive analysis of the moieties that are responsible for MDSCs mediated immunosuppression and noticed that the DC^{MPT64} expressed $CD11c^{lo}$, $CD11b^{hi}$, $F4/80^{hi}$, $CD40^{hi}$, $CD80^{hi}$, $MHCII^{lo}$, $Ly6C^{hi}$, $Ly6G^{lo}$, $PD\text{-}L1^{hi}$, $TIM\text{-}3^{hi}$, $IL\text{-}10^{hi}$, $TGF\text{-}\beta^{hi}$, $IL\text{-}6^{lo}$, $IL\text{-}12^{lo}$, $TNF\text{-}\alpha^{lo}$, $CCR7^{lo}$, the phenotype, which is well-established in imparting immuno-suppression. Further, based on their surface markers, these cells have been characterized as M-MDSCs. To further confirm the immunosuppressive nature of the DC^{MPT64}, we analysed more molecules responsible for the inhibition of the immune system. We observed an elevated yield ($p<0.05$) of the arginase in DC^{MPT64}, as tested by biochemical assay (Fig. 14A). These results were further substantiated by Western blotting since arginase levels were significantly enhanced ($p<0.001$) (Fig. 14B, C). The higher quantity of arginase was well correlated with STAT-3 expression ($p<0.05$) in DC^{MPT64} (Fig. 14D, E). STAT-3 controls arginase-1 in MDSCs (Vasquez-Dunddel et al., 2013). MDSCs produce a high amount of arginase, which contributes in suppressing immunity (Grzywa et al., 2020). We next examined indoleamine 2, 3-dioxygenase (IDO) and ROS in DC^{MPT64}. IDO and ROS are important immunosuppressive molecules generated by MDSCs (Fleming et al., 2018; Kumar et al., 2016; Platten et al., 2012). DC^{MPT64} showed augmentation in IDO ($p<0.05$), compared to DCs (Fig. 14F-G). DC^{MPT64} showed substantial regression in the ROS ($p<0.05$), compared to DCs (Fig. 14H). We noted a significant increase in the NO ($p<0.0001$)

released by DC^{MPT64} (Fig. 14I). This finding was further validated through considerable enhancement ($p<0.01$) in the protein levels of iNOS (Fig. 14J, K). A noticeable increase in STAT-1 expression was also manifested ($p<0.05$) (Fig. 14L, M). STAT-1 is required for iNOS activation. These experiments established the specificity of our results. NF-κB has a crucial part in managing the immune response during infection. It translocates to the nucleus and promotes the release of pro-inflammatory cytokines, chemokines and fosters the migration of the cells to the site of infection. Consequently, we examined NF-κB in DC^{MPT64} and observed a remarkable reduction of NF-κB, as compared to control DCs ($p<0.001$) (Fig. 14N, O). In essence, the modulation in the level of the immunosuppressive molecules produced by the DC^{MPT64}, illustrates that *Mtb* skillfully exploits its MPT64 protein to skew the differentiation of DCs to MDSCs.


A
Arginase (AU)
400
350
300
250
200
150
100
50
0
*
DC
DC^MPT64
B
DC
DC^MPT64
Arg1
β-Actin
1
1.4
Arg1 / β-Actin
C
Arginase 1/β-actin
2.0
1.5
1.0
0.5
0.0
***
DC
DC^MPT64
D
DC
DC^MPT64
STAT-3
β-Actin
1
1.9
STAT-3 / β-Actin
E
STAT-3/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
*
DC
DC^MPT64


F
DC
DC^MPT64
IDO
β-Actin
1
2.2
IDO / β-Actin
G
IDO/β-actin
1.5
1.0
0.5
0.0
*
DC
DC^MPT64
H
ROS (Relative Fluorescence Unit)
800
600
400
200
0
*
ns
DC
DC^MPT64
DC^CFP-10
I
NO (μM)
40
30
20
10
0
****
DC
DC^MPT64
J
DC
DC^MPT64
iNOS
β-Actin
1
2.8
iNOS / β-Actin
K
iNOS/β-actin
3
2
1
0
**
DC
DC^MPT64

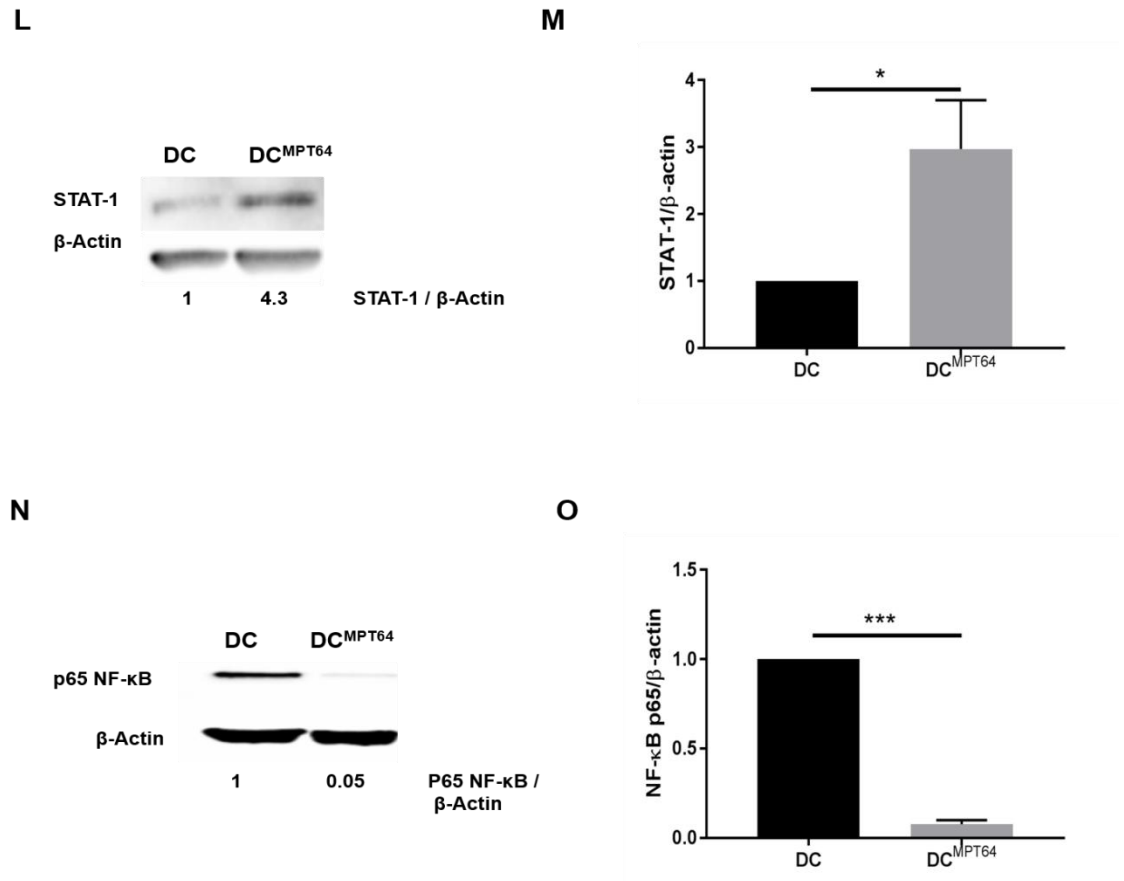
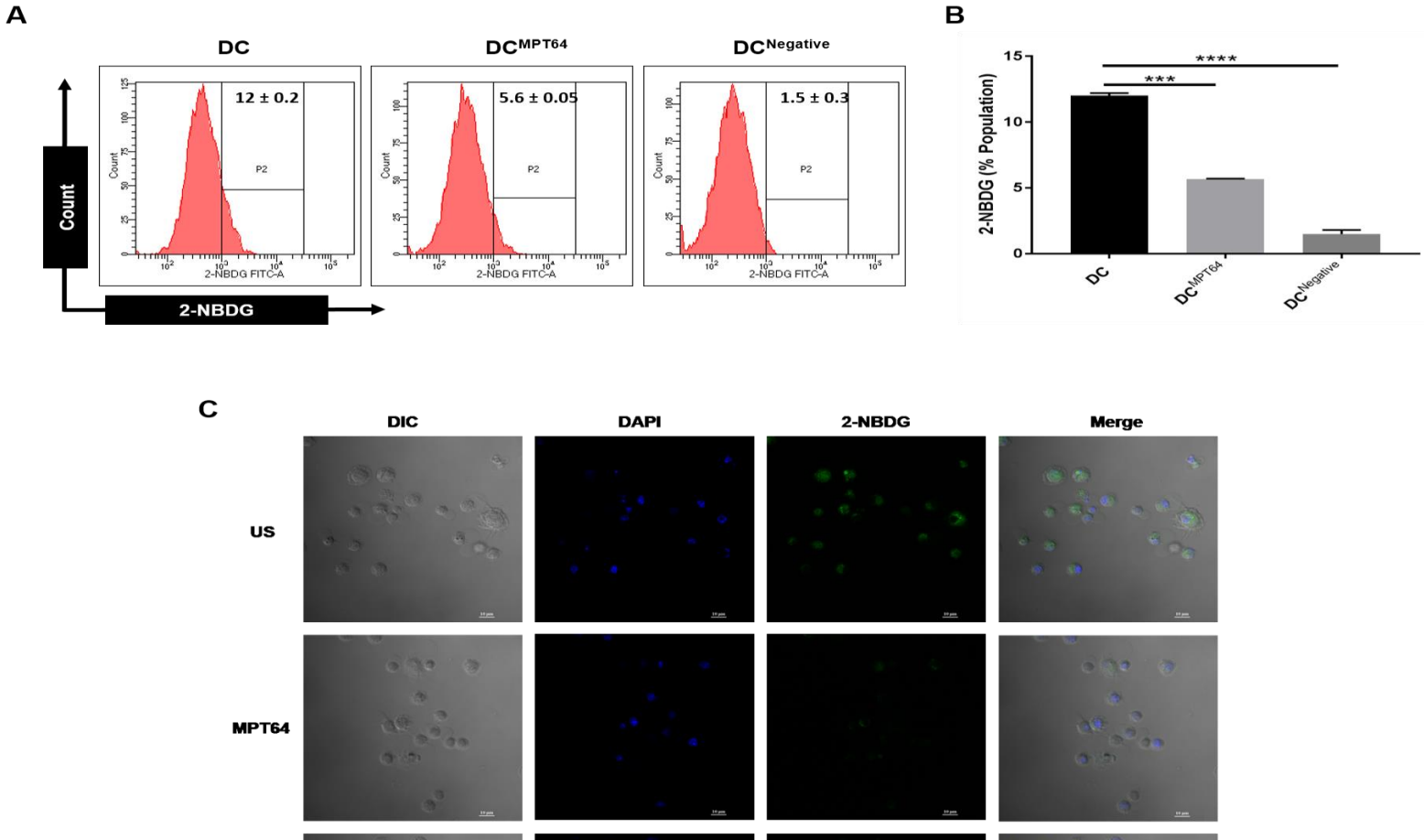


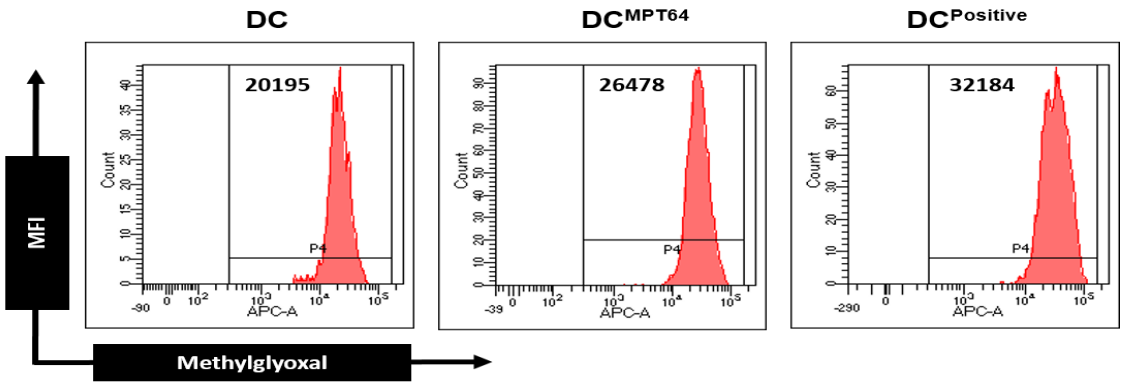
Figure 14. MPT64 upregulated Arg1, IDO, and iNOS but inhibited ROS and NF-κB expression in DC^{MPT64}. DC^{MPT64} and control DC^{CFP-10} and DCs were cultured for 6 d in a complete medium. Later, (A) arginase was estimated by arginase activity assay. The cell lysate was used for Western blotting for the expression of (B) arginase 1; (D) STAT-3; (F) IDO; (J) iNOS; (L) STAT-1; (N) NF-κB p65. The densitometric analysis of (C) arginase 1; (E) STAT-3; (G) IDO; (K) iNOS; (M) STAT-1; (O) NF-κB p65 normalized with β-actin was denoted as bar diagrams. (H) ROS was analysed by labelling with oxidation-sensitive dye H2DCFDA. (I) The SNs of DCs and DC^{MPT64} cultures were used to estimate NO. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: non-significant.

5.15. Acquisition of dormant metabolic phenotype by DC^{MPT64}. Immune surveillance against infections depends upon the proper activation of immune response in the tissues (Spitzer et al., 2017). Various mechanisms have been delineated for immune surveillance against infections and elicitation of the optimum immune response (Williams and Bevan, 2007). Surprisingly, along with activation of effector immune cells, it is also very crucial to focus on various other possible regulatory or inhibitory mechanisms; so that a proper immune response can be maintained against any pathogen by the host. The generation of MDSCs has been well reported during chronic infections and the cancer (Bennett et al., 1978; Gabrilovich and Nagaraj, 2009; Wang et al., 2010). MDSCs have a diametric role in TB infection, on one side it provides a safer resort for *Mtb* survival and on the other side slashes the adaptive immune response. *Mtb* utilizes lipids as a potent nutritional source (Daniel et al., 2011; Guirado et al., 2015) for its survival and progression within the host cell. During TB infection, MDSCs shift their glycolysis machinery to fatty acid oxidation (Al-Khami et al., 2016). Therefore, we analyzed the expression of metabolic components of DC^{MPT64}. Initially, we monitored the glucose uptake potential of DC^{MPT64} by using glucose analogue 2-NBDG and noted a significant decline ($p<0.001$) in the glucose (2-NBDG) uptake by DC^{MPT64}, when compared to control DCs. A negative control was also kept, where the DCs were cultured in media supplemented with glucose (DC^{neg}), which exhibited a significant impairment in glucose uptake ($p<0.0001$)(Fig. 15A, B). Further, this result was validated by confocal imaging and a similar reduction in glucose (2-NBDG) uptake by $DC^{MPT64}$was noticed (Fig. 15C). Recently, an accumulation of methylglyoxal (MGO) in MDSCs, responsible for their suppressive nature has been reported (Baumann et al., 2020). Likewise, we confirmed significantly high ($p<0.001$) MGO in DC^{MPT64} by flowcytometry. DCs stressed with H_2O_2 served as a positive control and

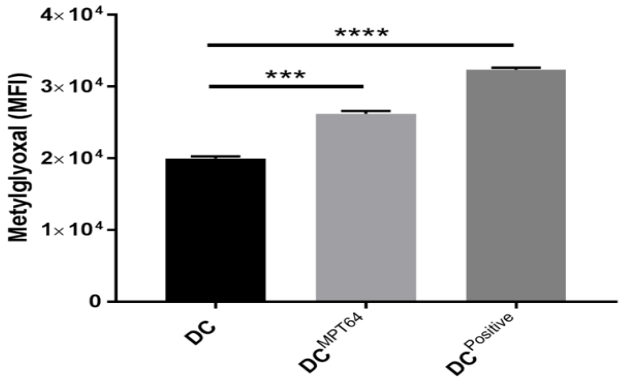
showed higher MGO expression ($p<0.0001$) (Fig.15D, E). Presence of high MGO in DC^{MPT64} was further corroborated by confocal imaging (Fig.15F). Intriguingly, we observed a substantial reduction in Glut-1 ($p<0.05$) in DC^{MPT64} (Fig. 15G). Glut-1 is the main glucose transport channel in immune and non-immune cells for glucose transport. Additionally, a striking reduction in the expression of hexokinase 2 ($p<0.05$) enzyme was detected in DC^{MPT64} (Fig. 15H). Noteworthy augmentation in the messenger RNA of semicarbazide-sensitive amine oxidase (SSAO) ($p<0.01$) was identified in DC^{MPT64} (Fig. 15I). Further, we monitored the expression of glyoxalase 1 in DC^{MPT64}. It has been reported that intracellular MGO was regulated by glyoxalase and glutathione (Rabbani and Thornalley, 2008). Marked reduction in the glyoxalase 1 ($p<0.01$) in DC^{MPT64} was noticed (Fig. 15J). These findings agree with our previous results of MGO. MGO generation occurs through the by-products (glyceraldehyde3-phosphate and dihydroxyacetone phosphate) of glycolysis from acetone and from aminoacetone by SSAO (Lyles and Chalmers, 1992; Phillips and Thornalley, 1993; Ray and Ray, 1983). Together, these results imply that DCs encounter with MPT64 during their differentiation renders them metabolically dormant and suppressive in function.

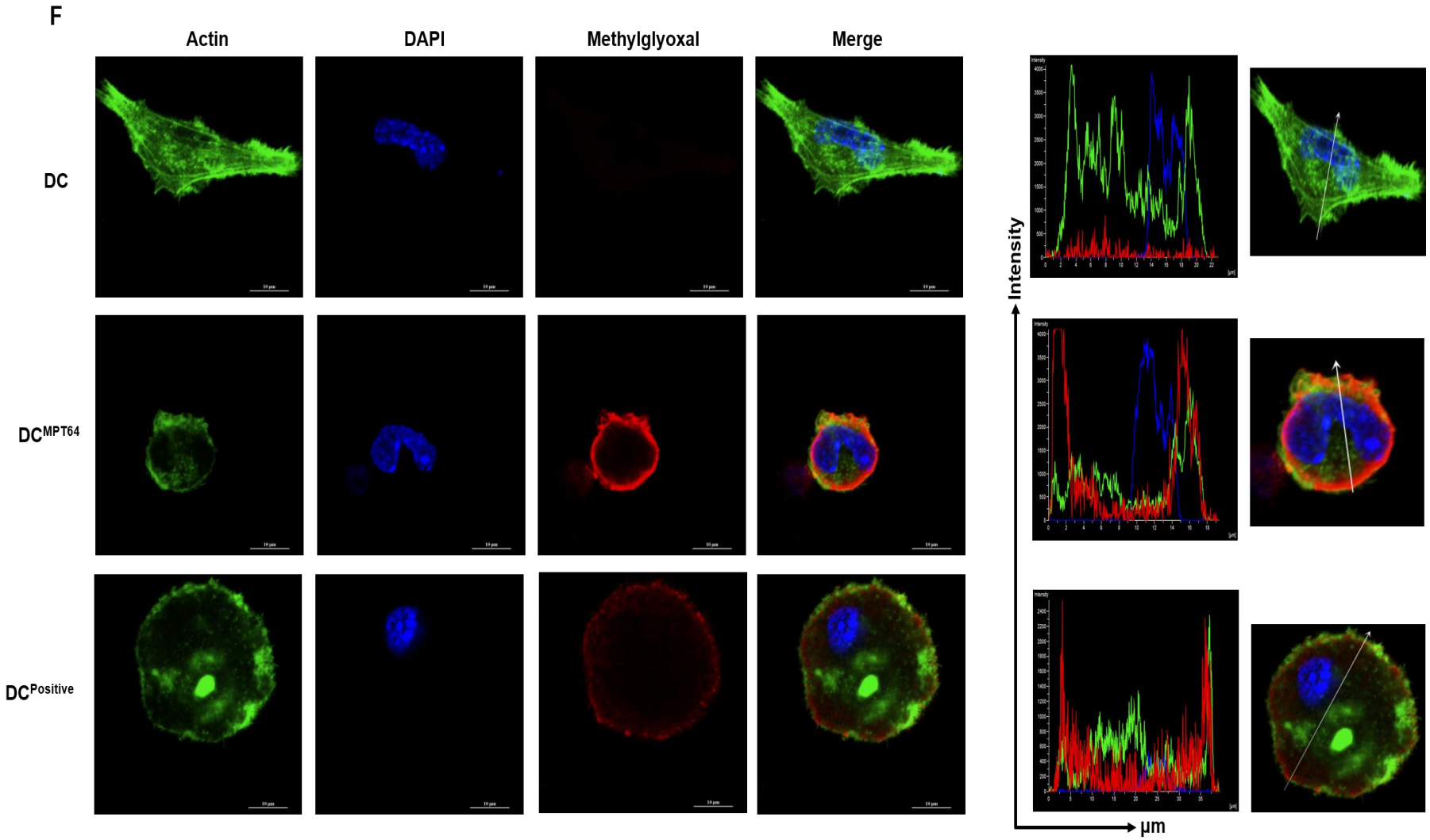

A
DC
DC^MPT64
DC^Negative
Count
2-NBDG
12 ± 0.2
5.6 ± 0.05
1.5 ± 0.3
P2
2-NBDG FITC-A
B
2-NBDG (% Population)
****
***
DC
DC^MPT64
DC^Negative
C
DIC
DAPI
2-NBDG
Merge
US
MPT64
Negative

D


DC
DC^MPT64
DC^Positive
20195
26478
32184
P4
Count
APC-A
MFI
Methylglyoxal

E


Metylglyoxal (MFI)
4×10^4
3×10^4
2×10^4
1×10^4
0
****
***
DC
DC^MPT64
DC^Positive


F
Actin
DAPI
Methylglyoxal
Merge
DC
DC^MPT64
DC^Positive
Intensity
µm

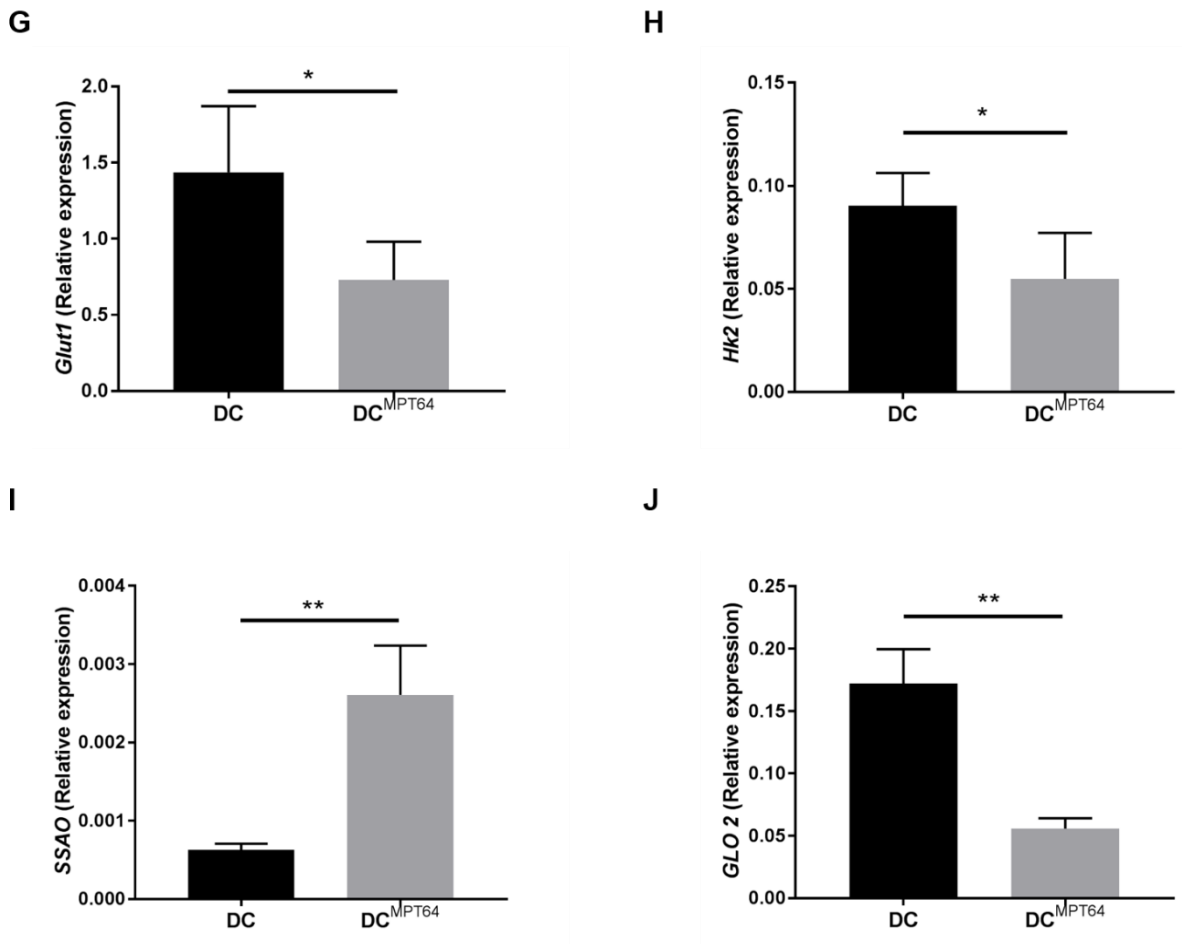


Figure 15. Acquisition of dormant metabolic phenotype by DC^{MPT64} upon MPT64 exposure. DC^{MPT64} were metabolically characterized and studied for the (A-B) impairment of the uptake of glucose analogue 2-NBDG by flowcytometry. The histogram and bar diagrams show the percentage of positive cells (mean ± SEM). DCs cultured with glucose was taken as a negative control (DC^{neg}). (C) The decreased level of glucose uptake was monitored by confocal microscopy (scale bar: 10 µM, magnification: 60X). (D) The expression of methylglyoxal was examined by flowcytometry. H_2O_2 treated DCs (DC^{pos}) were kept as a positive control. Data represented as histograms showing MFI and (E) bar diagrams. (F) DC^{MPT64} were stained with anti-methylglyoxal Abs (red), DAPI (blue) to visualize the nucleus and phalloidin (green) for actin. The images were obtained through a confocal microscope (scale bar: 10 µm, magnification: 60X). The histogram (right side) depicts the

intensity of methylglyoxal, actin and DAPI, as indicated by white arrows. (G-J) The expression of Glut1, Hk2, SSAO and GLO1 was monitored by RT-qPCR. The data are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.16. *Mtb* promotes the accumulation of MGO in DC^{MPT64}. *Mtb* utilizes carbohydrates, lipids and amino acids of the host cells for its survival (Lovewell et al., 2016). The presence of suppressor cells is noticed during chronic *Mtb* infection. MPT64 protein of *Mtb* generates metabolically dormant MDSCs during an early encounter with BMCs. Hence, we thought to check the presence of a suppressor moiety methylglyoxal in the DC^{MPT64}. DC^{MPT64} and control DCs were infected with *Mtb* GFP-H37Ra for 4 h. Interestingly, we observed that *Mtb* infected DCs (DC + *Mtb*) shows enhanced generation and accumulation of MGO, as compared to uninfected DCs. Surprisingly, we noticed that upon infecting DC^{MPT64} with *Mtb* (DC^{MPT64} + *Mtb*) there was augmentation in the expression of MGO (Fig. 16A). Hence, it can be inferred from this result that MPT64 might be one of the components of *Mtb,* which bolsters the generation and accumulation of MGO in DCs.

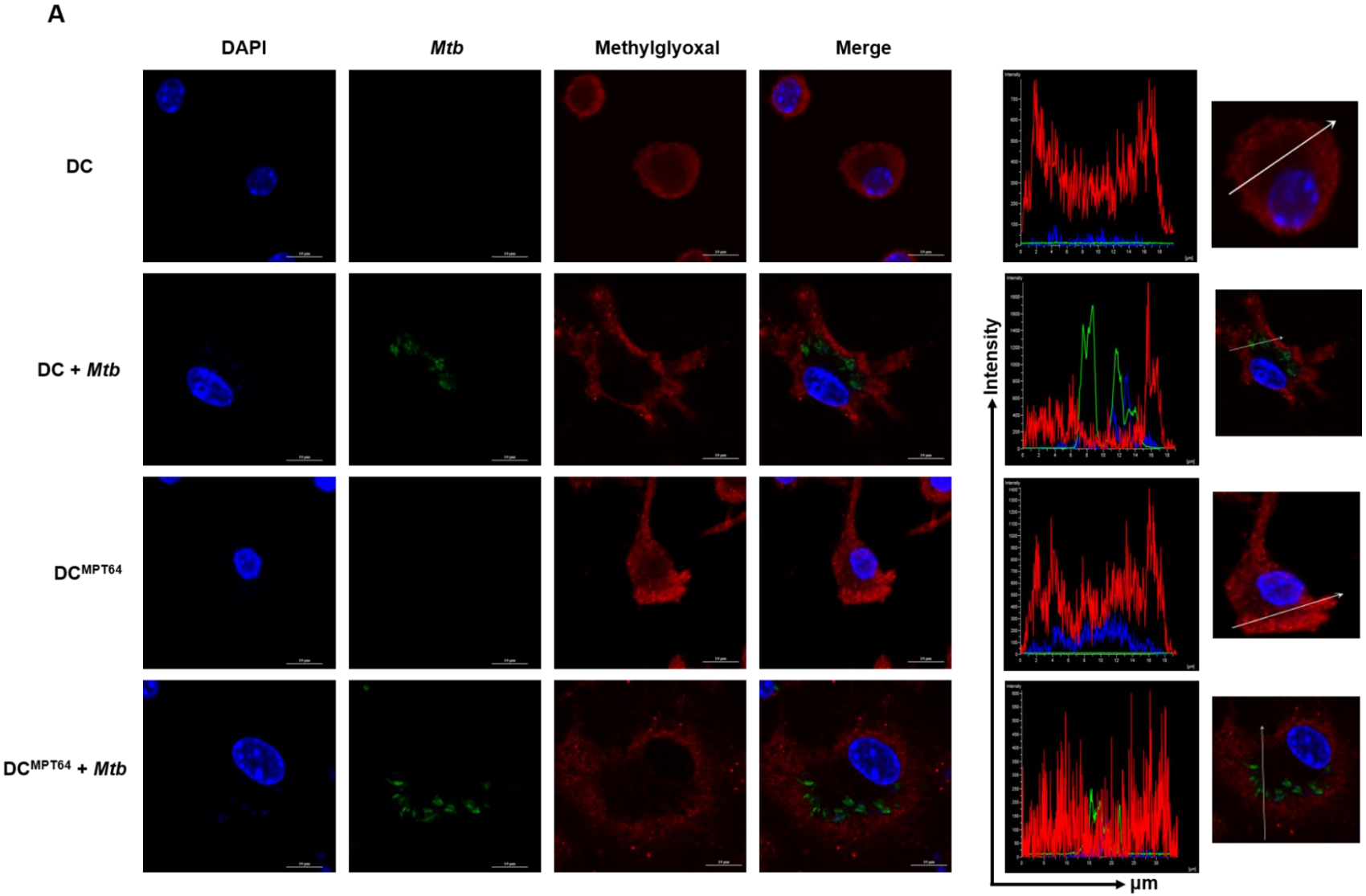
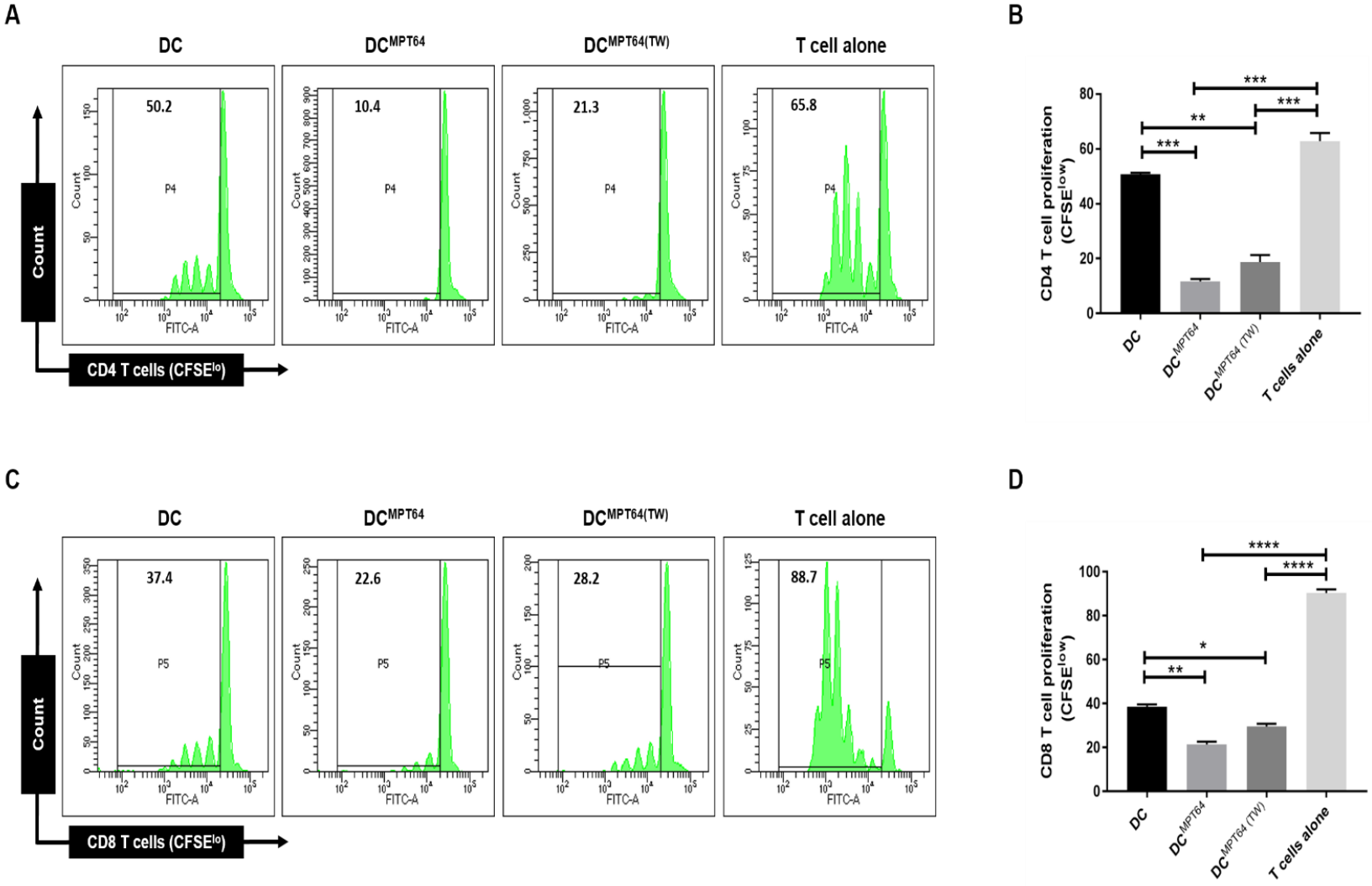

A
DAPI
Mtb
Methylglyoxal
Merge
DC
DC + *Mtb*
DC^{MPT64}
DC^{MPT64} + *Mtb*
Intensity
µm

Figure 16. *Mtb* augments the generation and accumulation of methylglyoxal in DCs. (A) The DC^{MPT64} and control DCs were infected with GFP-H37Ra for 4 h (MOI; 1:5). The extracellular bacilli were killed by incubating with gentamycin for 1 h. The expression of methylglyoxal was examined by confocal imaging. DC^{MPT64} were immunostained with anti-methylglyoxal Ab (red) along with DAPI (blue) to visualize the nucleus. The images were obtained through a confocal microscope (scale bar: 10 μm, magnification: 60X). The histogram depicts the intensity of methylglyoxal, GFP-H37Ra and DAPI, as indicated by white arrows. DC + *Mtb*: DCs infected with *Mtb*; DC^{MPT64} + *Mtb*: DC^{MPT64} infected with *Mtb*. Data is from two experiments.

5.17. DC^{MPT64} inhibits the proliferation of T cells through the secretion of NO in a contact-dependent and independent manner. Till now, the results suggest that DC^{MPT64} secretes soluble mediators, as well as display certain molecules on its surface, which are responsible for imparting suppressive activity. Hence, we were curious to know whether the DC^{MPT64} suppressed the T cells proliferation in a contact-dependent or independent manner. DC^{MPT64} were co-cultured with CFSE labelled T cells (CD4 and CD8), either in a normal tissue plate (contact-dependent) or trans-well culture plate (contact independent). We noticed a statistically noteworthy decline in CD4 T cells ($p<0.001$) and CD8 T cells ($p<0.01$) proliferation, when cultured with DC^{MPT64} in a contact-dependent fashion. We noticed a significant decline in the CD4 T cells ($p<0.01$) and CD8 T cells ($p<0.05$) proliferation, when cultured with DC^{MPT64} in contact independent mode (Fig. 17A-D). It can be inferred from these experiments that DC^{MPT64} impedes the proliferation of T cells through the molecules expressed on its surface, as well as through soluble mediators.

Nitric oxide inhibits T cell proliferation (Hardy et al., 2008). NO was produced in a higher amount by DC^{MPT64} (Fig. 14I-K). We observed that DC^{MPT64} significantly inhibited the proliferation of CD4 T cells ($p<0.01$) and CD8 T cells ($p=0.396$). Consequently, we wanted to examine if NO may be a soluble factor that was responsible for deterring the T cells proliferation. Therefore, the iNOS inhibitor N-monomethyl-L-arginine (NM) was put to the cultures of DC^{MPT64} and T cells. A significant suppression in the proliferation of CD4 T cells ($p=0.0899$) and CD8 T cells ($p=0.077$) was noted, when cultured with DC^{MPT64} (Fig. 17E-H). Thus, it may be concluded that the NO produced by DC^{MPT64} may be one of the factors that are responsible for restricting the expansion of T cells.


A
DC
DC^MPT64
DC^MPT64(TW)
T cell alone
50.2
10.4
21.3
65.8
P4
Count
FITC-A
CD4 T cells (CFSE^lo)
B
CD4 T cell proliferation (CFSE^low)
DC
DC^MPT64
DC^MPT64 (TW)
T cells alone
C
37.4
22.6
28.2
88.7
P5
CD8 T cells (CFSE^lo)
D
CD8 T cell proliferation (CFSE^low)

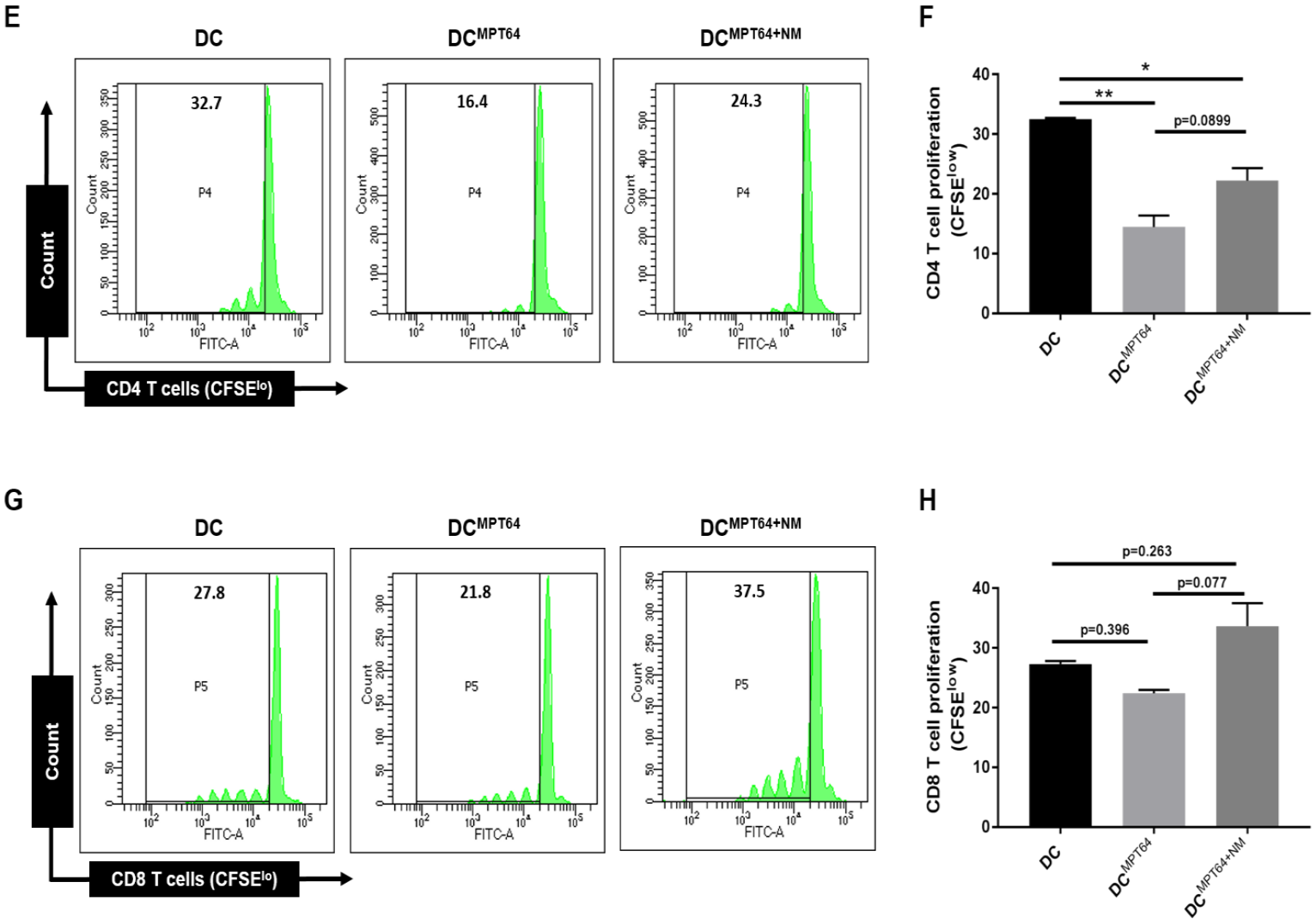
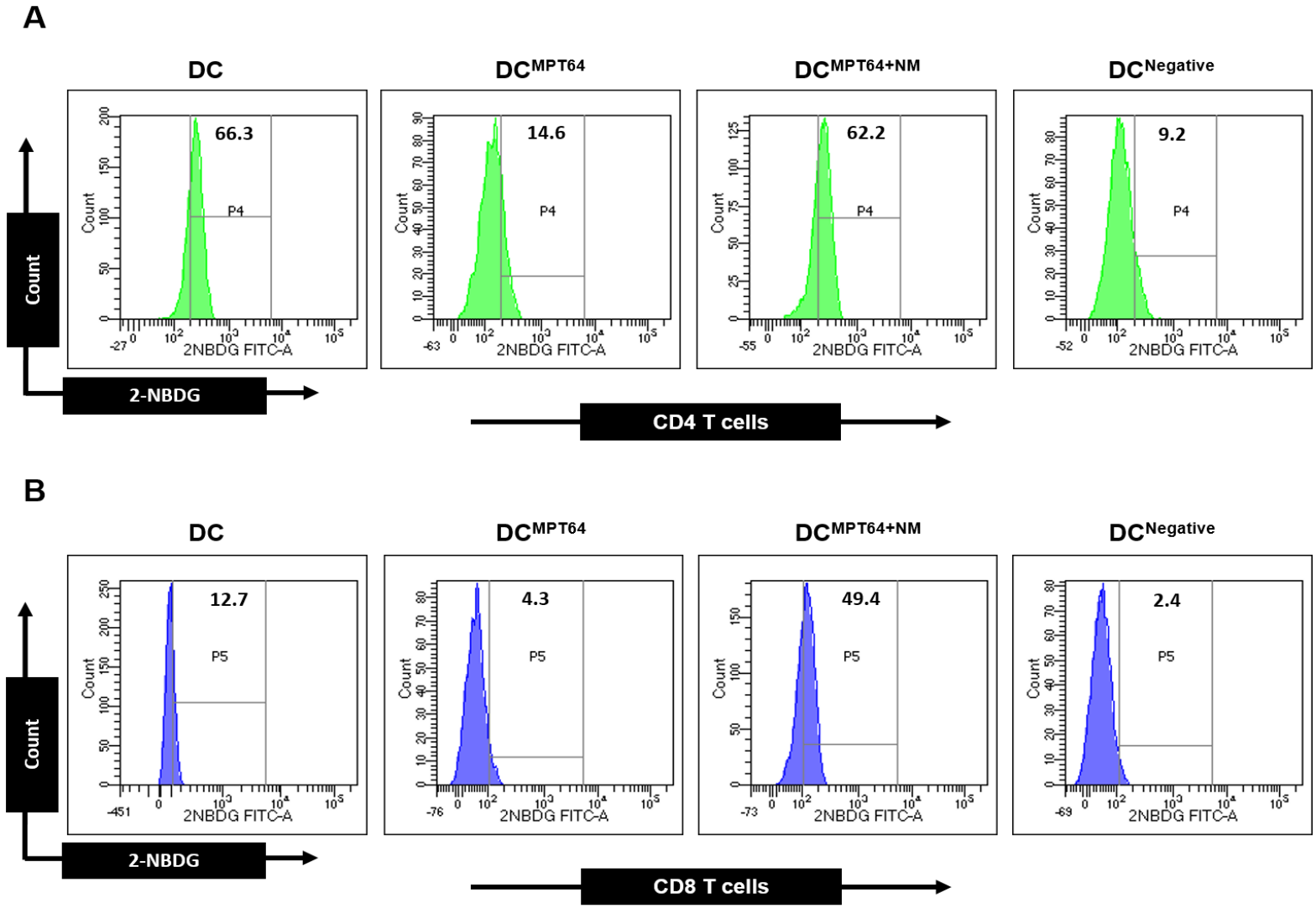

E
DC
DC^MPT64
DC^MPT64+NM
32.7
16.4
24.3
P4
Count
FITC-A
Count
CD4 T cells (CFSE^lo)
F
CD4 T cell proliferation (CFSE^low)
*
**
p=0.0899
DC
DC^MPT64
DC^MPT64+NM
G
DC
DC^MPT64
DC^MPT64+NM
27.8
21.8
37.5
P5
Count
FITC-A
CD8 T cells (CFSE^lo)
H
CD8 T cell proliferation (CFSE^low)
p=0.263
p=0.077
p=0.396
DC
DC^MPT64
DC^MPT64+NM

Figure 17. DC^{MPT64} released NO and inhibited the proliferation of CD4 T cells and CD8 T cells in a contact-independent manner. DC^{MPT64} and control DCs were co-cultured in 'regular' and 'trans-well' (TW) plates with CFSE-labelled and anti-CD3 and CD28 Abs stimulated naïve (A, B) CD4 T cells; (C, D) CD8 T cells. Similarly, DC^{MPT64} and control DCs were co-cultured with CFSE-labelled and anti-CD3 and CD28 Abs stimulated (E, F) CD4 T cells; (G, H) CD8 T cells with or without iNOS inhibitor (NM). The cultures were set for 72 h and the proliferation was determined by flowcytometry. (F, H) The results (mean ± SEM) are also illustrated as the percentage of $CFSE^{lo}$ CD4 T cells and CD8 T cells through a bar diagram. The data presented here are of 2-3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.18. MPT64 stimulated DC^{MPT64} downregulates the expression of T cell receptors and augments T cell apoptosis. Till now, we have noticed that MPT64 exposed DCs (DC^{MPT64}) skew their differentiation towards MDSCs. DC^{MPT64} exhibited significant expression of co-inhibitory markers and acquired metabolically quiescent morphology with impaired T cell activation potential. Moreover, DC^{MPT64} augments the survival of mycobacteria. MDSCs exhibit high expression of iNOS and arginase, which leads to the depletion of L-arginine with the generation of superoxide. Subsequently, superoxide interacts with NO and forms $ONOO^-$ and reactive nitrogen species (RNS), which ultimately leads to nitrosylation of TCR followed by apoptosis of T cells (Bronte et al., 2003; du Plessis et al., 2013). The adaptive immune system has a central role in limiting the progression of TB disease. Therefore, we were interested in knowing whether metabolically rendered DC^{MPT64} also induces a metabolically quiescent stage in T cells. To demonstrate this, DC^{MPT64} were cultured together with T cells (CD4 T cells or CD8 T cells) and we observed a statistically noteworthy reduction in the glucose uptake by CD4 T cells and CD8 T cells. Moreover, these CD4 T cells and CD8 T cells showed significant expression of methylglyoxal upon culturing with DC^{MPT64} (Fig.18A-D).


A
DC
DC^MPT64
DC^MPT64+NM
DC^Negative
66.3
14.6
62.2
9.2
P4
Count
2NBDG FITC-A
Count
2-NBDG
CD4 T cells
B
DC
DC^MPT64
DC^MPT64+NM
DC^Negative
12.7
4.3
49.4
2.4
P5
Count
2NBDG FITC-A
Count
2-NBDG
CD8 T cells

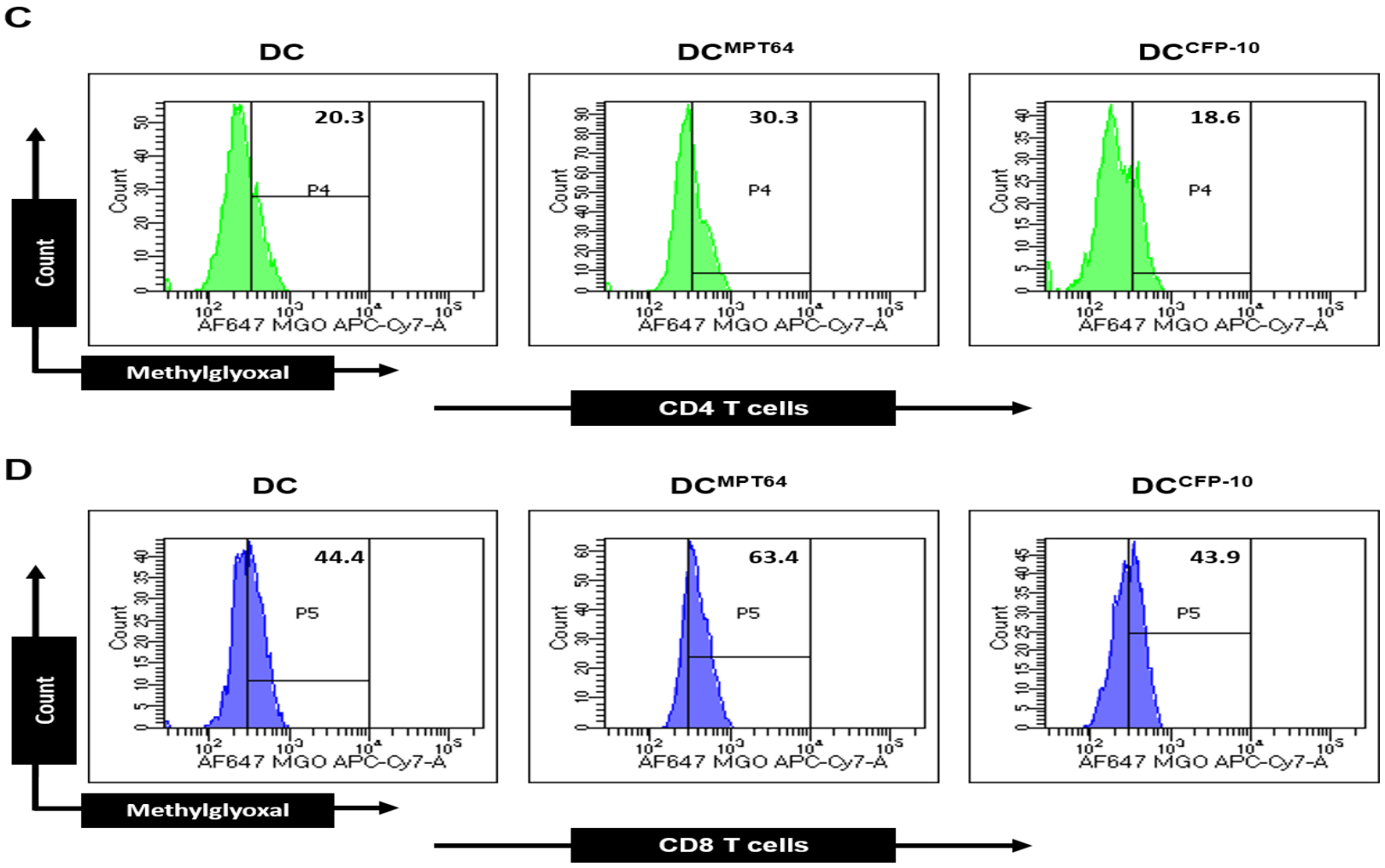
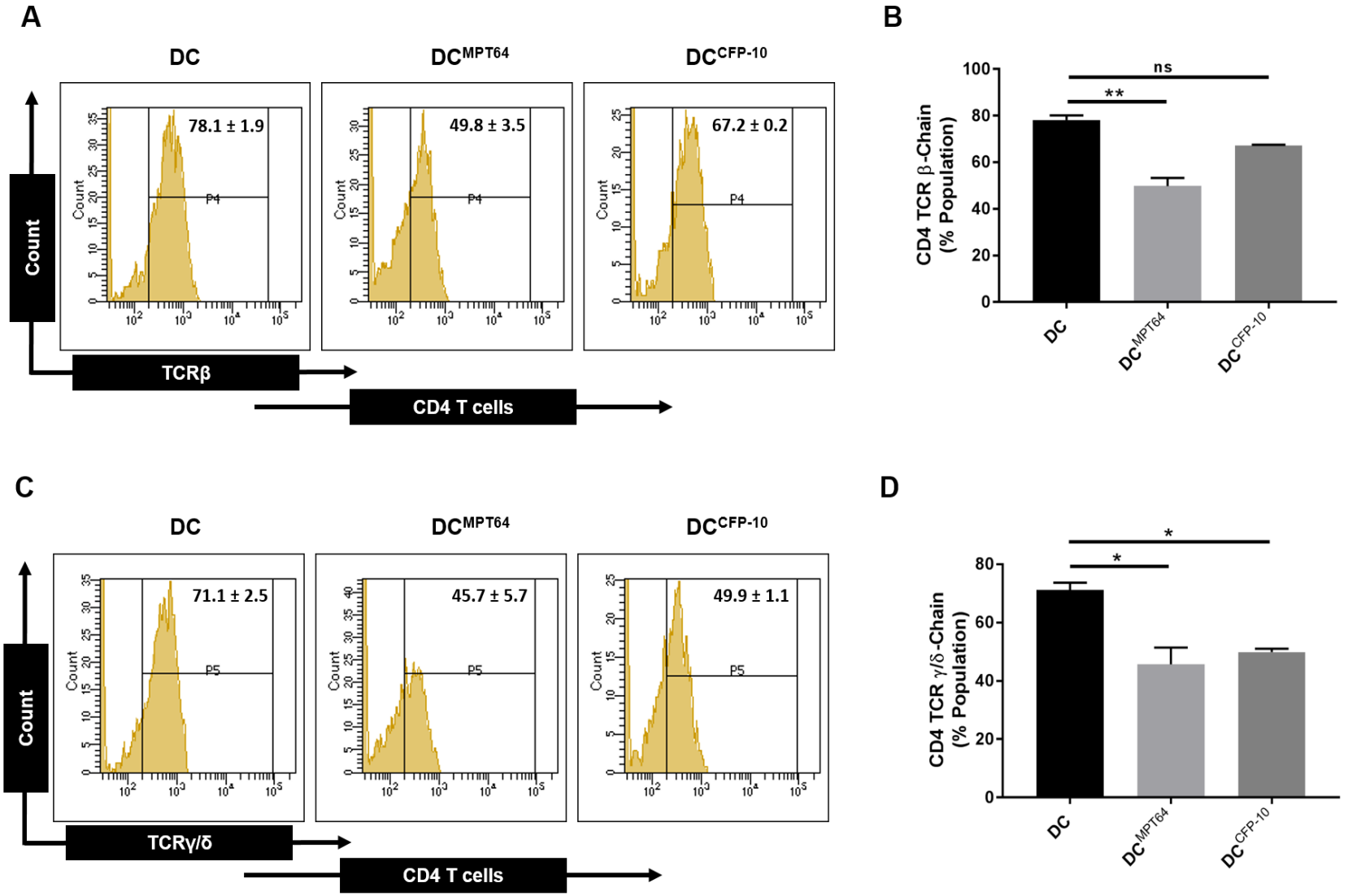
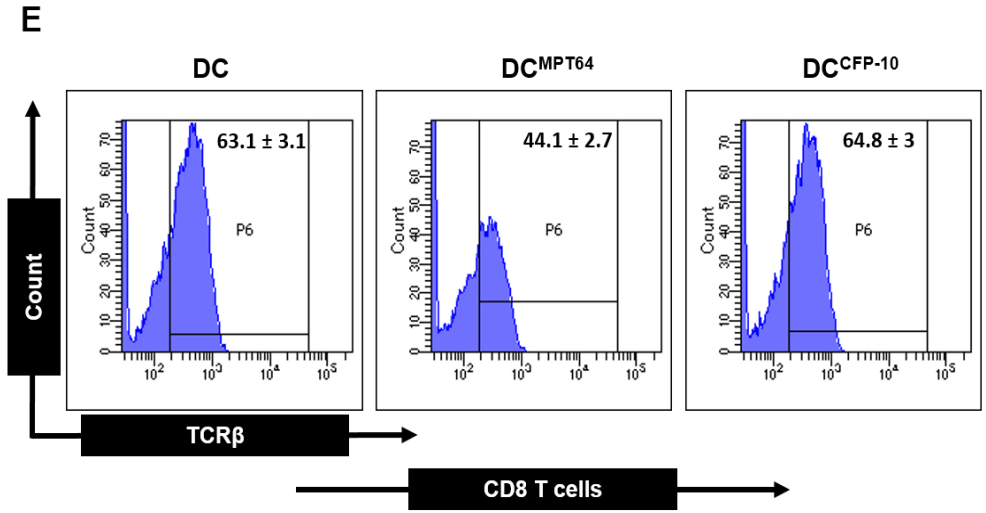
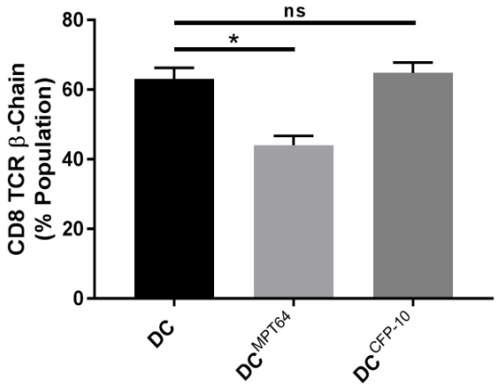

C
DC
DC^MPT64
DC^CFP-10
20.3
30.3
18.6
P4
Count
AF647 MGO APC-Cy7-A
Methylglyoxal
CD4 T cells
D
DC
DC^MPT64
DC^CFP-10
44.4
63.4
43.9
P5
Count
AF647 MGO APC-Cy7-A
Methylglyoxal
CD8 T cells

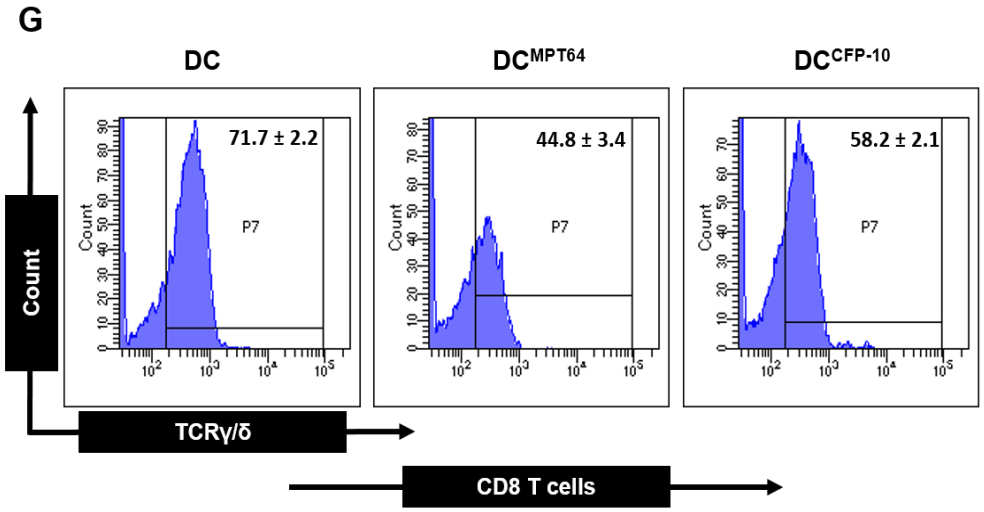
Figure 18. T cells acquire a metabolically dormant phenotype on interaction with DC^{MPT64}. DC^{MPT64} and control DCs were co-cultured with the anti-CD3 and CD28 Abs stimulated naïve (A, C) CD4 T cells; (B, D) CD8 T cells and assessed for the uptake of (A-B) glucose analogue (2-NBDG); (C, D) methylglyoxal through flowcytometry. Inset values of histograms refer to the percentage of positive cells for 2-NBDG and methylglyoxal uptake. DCs cultured in glucose-conditioned media were used as a negative control (DC^{neg}) for glucose uptake assay. Data shown are representative of 2-3 independent experiments.

After deciphering the mechanism of suppression through NO by DC^{MPT64}, we were curious to know whether DC^{MPT64} instigated a suppressive effect on T cells through cognate interaction, as well. T cell through its T cell receptor (TCR) recognizes peptide-MHC complex over the surface of DCs. TCR is made up of α and β or γ and δ chains made of glycoproteins. TCR chains get downregulated in the presence of high NO and ROS (Hardy et al., 2008). We noticed significant downregulation in the surface exhibition of TCR-β chain ($p<0.01$) and TCR γ/δ ($p<0.05$) of CD4 T cells upon co-culturing with DC^{MPT64} (Fig. 19A-D). Similarly, we observed a noteworthy reduction in the TCR β chain ($p<0.05$) and TCR γ/δ units ($p<0.01$) of CD8 T cells (Fig. 19E-H). The possible reason for the impairment in TCR expression might be due to the generation of $ONOO^-$ or RNS by DC^{MPT64}. Literature suggests that myeloid suppressor cells induce apoptosis of T cells. Likewise, we noticed apoptosis in CD4 T cells ($p<0.01$) and CD8 T cells ($p<0.001$), when co-cultured with DC^{MPT64} (Fig. 20A-D). No change was seen with the control DCs. These results confirm that MPT64 impairs the function DCs through cognate interaction, as well.


A
DC
DC^MPT64
DC^CFP-10
78.1 ± 1.9
49.8 ± 3.5
67.2 ± 0.2
P4
Count
TCRβ
CD4 T cells
B
CD4 TCR β-Chain (% Population)
ns
**
DC
DC^MPT64
DC^CFP-10
C
71.1 ± 2.5
45.7 ± 5.7
49.9 ± 1.1
P5
TCRγ/δ
D
CD4 TCR γ/δ-Chain (% Population)
*


E
DC
DC^MPT64
DC^CFP-10
63.1 ± 3.1
44.1 ± 2.7
64.8 ± 3
P6
Count
TCRβ
CD8 T cells


F
CD8 TCR β-Chain (% Population)
ns
*
DC
DC^MPT64
DC^CFP-10


G
DC
DC^MPT64
DC^CFP-10
71.7 ± 2.2
44.8 ± 3.4
58.2 ± 2.1
P7
Count
TCRγ/δ
CD8 T cells

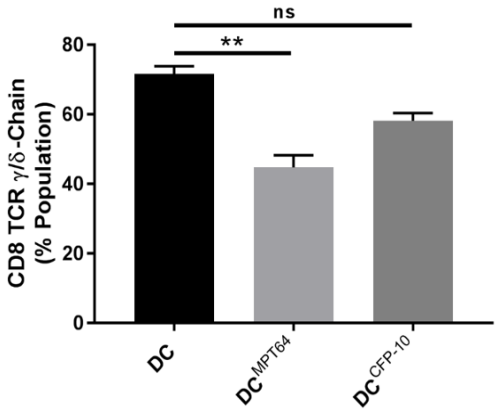
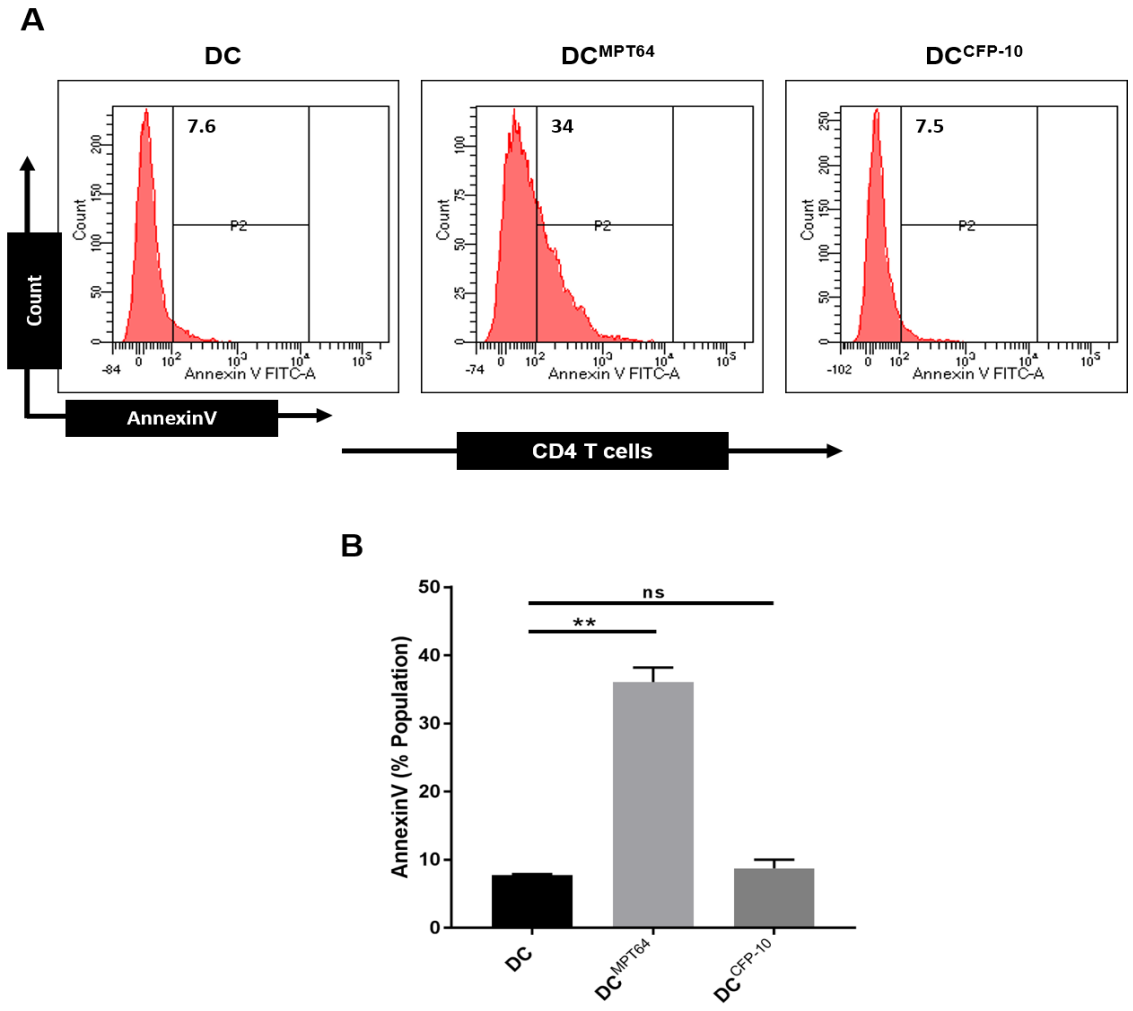

H
CD8 TCR γ/δ-Chain (% Population)
ns
**
DC
DC^MPT64
DC^CFP-10

Figure 19. DC^{MPT64} on interaction with T cells downregulates the expression of T cells receptor. DC^{MPT64} and control DCs were cultured with the anti-CD3 and CD28 Abs stimulated naïve (A-D) CD4 T cells; (E-H) CD8 T cells for 72 h. Later, the cells were analysed for the expression of (A, B) TCRβ and (E, F) TCRγ/δ chains on CD4 T cells and; (C, D) TCRβ and (G, H) TCRγ/δ chains on CD8 T cells by flowcytometry. The data (mean ± SEM) was expressed as histograms and bar diagrams are the percent positive cells and representative of two to three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: non-significant.



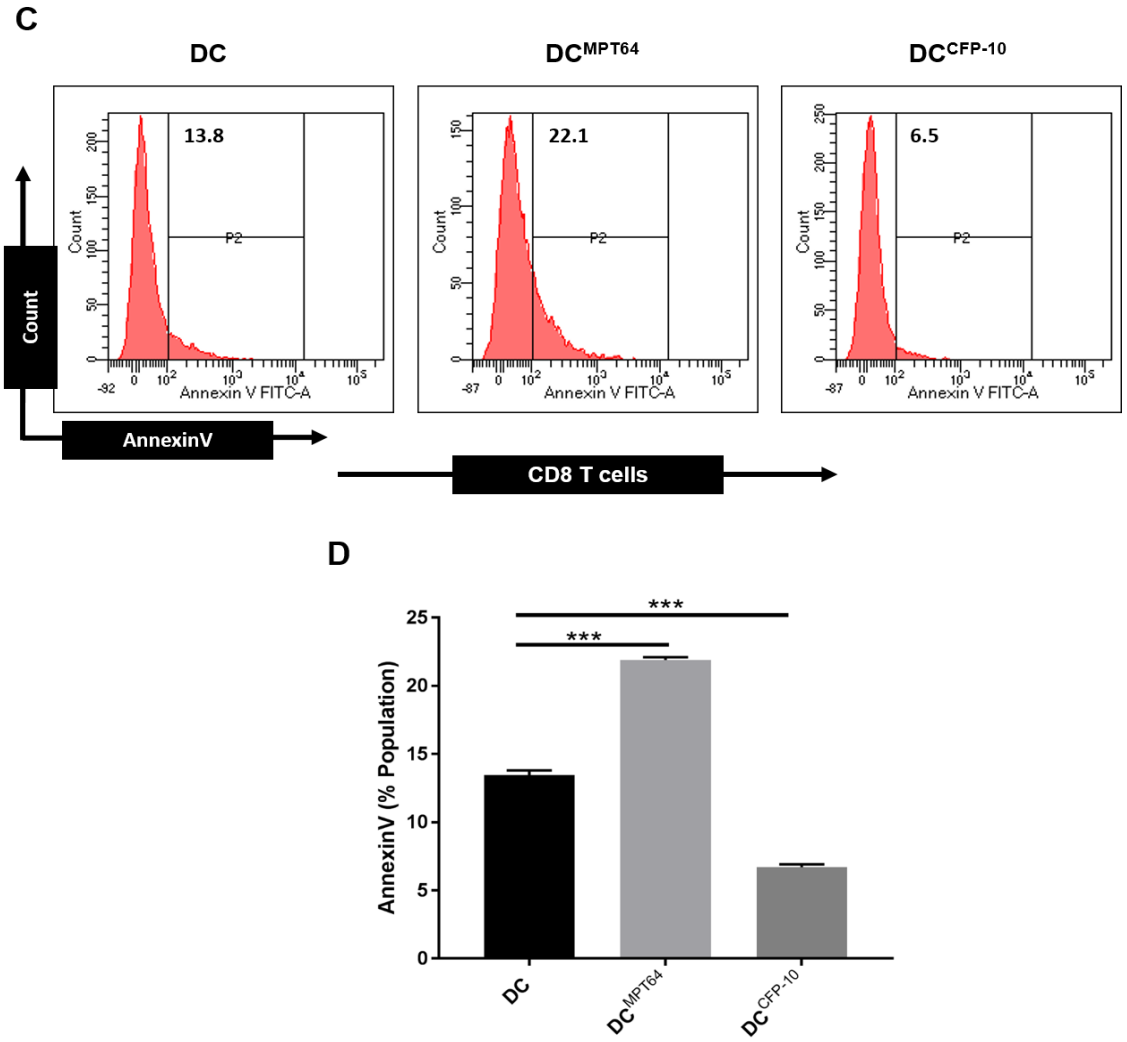


Figure 20. DC^{MPT64} induces apoptosis in T cells. DC^{MPT64} and control DCs were cultured with the naïve (A, B) CD4 T cells; (C, D) CD8 T cells for 72 h. Later, CD4 T cells and CD8 T cells were monitored for apoptosis by staining with Annexin V through flow cytometry. Data (mean ± SEM) denoted as histograms and bar diagrams are the percent positive cells and representative of two independent experiments. **p<0.01, ***p<0.001, ns: non-significant.

5.19. DISCUSSION

Mycobacterium tuberculosis (Mtb) has evolved various strategies to survive and persist within the inimical milieu of host cells. Nearly 2 billion people worldwide have been infected by this pathogen and it still remains a major cause of mortality and morbidity. *Mtb* generally infects through aerosols and attains a dormant stage in the host just after infection and resides over there for years (Zaman, 2010). These latently infected individuals serve as a reservoir of *Mtb* and are a matter of great concern for society. Being an intracellular pathogen, *Mtb* deceives the immune system and sustains within the host through the secretion of numerous factors that attunes the expression of various genes (Collins, 1997; Mukamolova et al., 2010). Therefore, it is imperative to understand how *Mtb* through its components inflects the host immune response. Plentiful secretory proteins of *Mtb* have been demonstrated to modulate the cells of the immune system (Beatty and Russell, 2000). *Mtb* produces nearly 257 proteins that are secretory in nature (Malen et al., 2007). Intriguingly, it has been hypothesized that *Mtb* secretory proteins are the first to interact with the host immune system (Jiang et al., 2013). The major part of the *Mtb* secretome is constituted of the Ag85 family, ESAT-6 family, MPT63, MPT64, Acr1 and lipoproteins.

Acr1 is a latency-associated protein of *Mtb* and elicits both humoral and cell-mediated immunity. Moreover, Acr1 protein has enormous potential to act as a vaccine candidate against TB. Recently, our group has shown that Acr1 protein fine tunes the generation and maturation of DCs and MΦs (Amir et al., 2017; Mubin et al., 2018; Siddiqui et al., 2014). Further, MPT64 (Rv1980c) is secreted by actively dividing *Mtb* (Gaillard et al., 2011; Wang et al., 2007). MPT64 belongs to the new family of beta grasp proteins with a molecular weight of approximately 24 kDa (Sehnal et al., 2021). During the active phase of TB, a high concentration of MPT64 has been observed in the sputum and serum of the patients (Mehaffy et al., 2017). Furthermore, MPT64 is abundantly present in the granulomas, as compared to non-granulomas areas

(Baba et al., 2008). This signifies that MPT64 distinguishes the active and latent stages of TB. Because of its immunodominant nature, MPT64 protein is recognized by Th1 cells in humans and hence, can act as a potential vaccine candidate (Bayne et al., 1990; Mustafa, 2010; Sibley et al., 2014). Simultaneously, the role of MPT64 in diagnostics has been widely explored (Sypabekova et al., 2019). Additionally, literature has shown a new facet of MPT64, where it inhibits apoptosis in macrophages and multinucleated giant cells (Mustafa et al., 2008; Wang et al., 2014). Although, MPT64 has been studied to some extent in terms of its modulatory effect on MΦs, MGCs and ECs; its role in DCs and its precursors remains explored (Latchumanan et al., 2002).

Recently, significant progress has been made in understanding the role of innate immune cells in various chronic infections. Among innate immune cells, DCs play a cardinal role in the clearance of pathogens and connecting innate immunity with adaptive immunity. *Mtb* first infects MΦs and DCs (Cooper, 2009). Intriguingly, *Mtb* impedes DCs maturation (Hanekom et al., 2003). However, alveolar MΦs phagocytose *Mtb* and initiates a cascade of immune responses that can limit the spread of this pathogen, whereas DCs upon phagocytose of *Mtb*, migrates to the draining lymph nodes, where it primes naïve T cells (Krishnaswamy et al., 2013). Hence, we became curious to know the impact of MPT64 on the pathogenesis of TB.

MPT64 protein was isolated, expressed, cloned and purified from *Mtb*. The purified proteins were characterized through SDS-PAGE, MALDI and MASCOT; and were endotoxin-free. Interestingly, we have observed that pre-exposure of differentiating bone marrow cells (BMCs) to MPT64 (DC^{MPT64}) transformed them into a phenotype of myeloid-derived suppressor cells (MDSCs). We observed a decline in CD11c and up-regulation in F4/80 expression, in a dose-dependent manner. CD11c is a prominent marker of DCs and F4/80 of macrophages (MΦs) (Dos Anjos Cassado, 2017; Singh-Jasuja et al., 2013). The highest expression of F4/80 and lowest of CD11c on BMCs was observed at 12

µg/ml of MPT64. Further, to validate that decline in the population of $CD11c^+$ cells was not due to cell death, we stained DCs pre-exposed to MPT64 (DC^{MPT64}) with PI/AnnexinV and observed no change in the viability. The expression of co-stimulatory molecules by DCs imparts a very compelling role in the optimum activation of naïve T cells. Both, CD40 and CD80 important costimulatory molecules present on DCs. It is well noticed that CD80 has a stronger binding affinity for CTLA-4, as compared to CD28 (Pentcheva-Hoang et al., 2004). The binding of CD80 with CTLA-4 generates a negative impact on T cell activation and ultimately hampers immunity against tumours (Leach et al., 1996; McCoy and Le Gros, 1999; Walunas et al., 2011). Moreover, a surface exhibition of CD80 and CD86 by APCs is involved in the priming and activation of T cells. Interaction between CD80 with CTLA-4 leads to anergy in T cells, as observed by a state of unresponsiveness (Marengere et al., 1996). Similarly, the expression of CD40 on APCs is very important for interaction with CD40L on T cells, to mount an effective T cell response (Elgueta et al., 2009; van Kooten and Banchereau, 1997). This interaction of CD40-CD40L leads to the generation of inflammatory responses in autoimmune diseases (Elgueta et al., 2009). This shows that elevation in the expression of CD40 and CD80 might have a negative regulatory role in the activation and differentiation of T cells, as well as DCs. We next analyzed the expression of CD11b, CD40, CD80, CD86 and MHC II on DC^{MPT64}. We observed enhanced expression of CD11b, CD40 and CD80 on DC^{MPT64} as compared to control DCs. On the contrary, we noticed a decline in the appearance of MHC II and CD86 on DC^{MPT64} as compared to control DCs. Similar to CD40 and CD80; CD86 is also one of the crucial co-stimulatory molecules, present on the APCs. Interaction of CD86 with CD28 present on T cells is needed for the activation of T cells during antigen presentation by APCs (Nakajima et al., 1997). Moreover, DCs or other APCs utilize the Ag-MHC complex for antigen presentation. Upon activation in response to any pathogen, DCs display the MHC-Ag

complex on their surface (van Vliet et al., 2007). After engulfing the pathogen, DCs migrate to secondary lymphoid organs and in conjunction with MHC II present the processed antigen to T cells (Lenschow et al., 1996). Interestingly, *Mtb* has evolved multiple mechanisms to modulate the MHC II expression (Cooper, 2009). Proteins like LprA, LprG, LprH, and Hip1 are well known to inhibit the expression of MHC II and hence have a role in the *Mtb* pathogenesis (Gehring et al., 2004; Madan-Lala et al., 2014; Pecora et al., 2006).

Interestingly, we observed that the pre-exposure MPT64 inhibits the differentiation of BMCs into DCs (DC^{MPT64}). In contrast, it was observed that MPT64 fails to suppress the function of differentiated DCs, since no change in the expression of CD11c, CD11b, F4/80 and CD86 was noticed. We noticed that augmentation in $F4/80^{+}$ cells and downregulation in $DC11c^{+}$ cells was solely because of MPT64 and not CFP-10 and ESAT-6 proteins. Therefore, establishing the specificity of MPT64 function.

Recently, it was shown that TB granulomas consist of a new innate cell population known as MDCSs along with other cells like DCs, MΦs and T cells. Chronic *Mtb* infection elicits the generation of MDSCs (Bennett et al., 1978; Kato and Yamamoto, 1982). These MDSCs were initially noticed in cancer and their role in tumour progression is well documented. Contrary to this, MDSCs role in the field of bacterial infections remains unexplored. MDSCs are sub-categorized into monocytic MDSC (M-MDSC) and granulocytic/polymorphonuclear MDSC (PMN-MDSC), based on their morphology and surface markers (Bronte et al., 2016; Dolcetti et al., 2010; Movahedi et al., 2008). We observed that DC^{MPT64} expressed $CD11c^{lo}$, $CD11b^{hi}$, $F4/80^{hi}$, $CD40^{hi}$, $CD80^{hi}$, $MHCII^{lo}$. Further, we analyzed the expression of Ly6C and Ly6G on DC^{MPT64}. We observed DC^{MPT64} expressed $Ly6C^{hi}$ with $Ly6G^{lo}$, as compared to control DCs. In the case of pre-exposure of DCs to Acr1, no change in Ly6C was noticed, suggesting the failure of Acr1 to

generate M-MDSC. Intriguingly, MPT64-induced DC^{MPT64} transforms into M-MDSCs, as evidenced through $CD11b^{hi}$, $F4/80^{hi}$, $CD40^{hi}$, $CD80^{hi}$, $Ly6C^{hi}$, $CD11c^{lo}$, $MHCII^{lo}$. Literature suggests that MDSCs are present within the TB granulomas and promote the *Mtb* pathogenesis (Dorhoi and Du Plessis, 2017). Since, M-MDSC expresses $Gr\text{-}1^{dim/+}CD11b^{+}Ly6C^{+}Ly6G^{-/lo}CD40^{+}CD80^{+}MHCII^{lo}CD86^{lo}$ (Bronte et al., 2016; Gabrilovich, 2017); similar pattern was apparent in the case of DC^{MPT64}. Therefore, we were curious to examine the role of DC^{MPT64} in augmenting the suppression of immune response and pathogenesis of *Mtb*. Further, we observed that DC^{MPT64} expressed co-inhibitory $PD\text{-}L1^{hi}$ and $TIM\text{-}3^{hi}$ markers. Both PD-L1 and TIM-3 are well-defined co-inhibitory markers and are responsible for the induction of the tolerance (Alderton, 2012; Sumpter and Thomson, 2011). Noteworthy, $PD\text{-}L1^{hi}$ on DCs have a negative regulatory effect on the functionality of T cells (Kushwah and Hu, 2011). PD-L1 on APCs interacts with PD1 on naïve T cells and promotes their differentiation towards Tregs (Hassan et al., 2015). $PD\text{-}L1^{hi}$ has been reported in TB patients responsible for the suppression of T cell activation (Agrawal et al., 2018). Our findings signify that MPT64 pre-stimulation of DCs (DC^{MPT64}) imparts a suppressive phenotype.

The cytokines secreted in response to infection also play a critical part in the disease progression or suppression. DCs and MΦs upon *Mtb* infection promote the secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-12 to control the infection (Sasindran and Torrelles, 2011; Seder et al., 1993). Contrary to this, anti-inflammatory cytokines (IL-10 and TGF-β) have a decisive role in the pathogenesis of *Mtb*. MDSCs secrete anti-inflammatory cytokines and restrain the adaptive immune response (Haist et al., 2021). DC^{MPT64} showed a significant decline in the secretion of IL-6, IL-12 and TNF-α cytokines and augmented the secretion of IL-10 and TGF-β. This finding indicates that MPT64 may impede DCs function by suppressing the production of pro-inflammatory cytokines and fostering the pathogenesis of *Mtb*.

The presence of long arborizing projections on the surface of DCs helps in capturing antigen and priming naïve T cells in the lymphoid organs. Activated DCs are morphologically large with elongated dendrites. DC^{MPT64} were shrunken in shape with an absence of dendrites. The presence of lipids depots within immune cells act as an energy reservoir for feeding *Mtb* (Daniel et al., 2011). Furthermore, it has been reported that lipid deposition within DCs, suppresses their functional activity (Herber et al., 2010). MΦs with high lipid content inhibit Th1 and Th17 immunity through augmentation of PGE_2 and COX-2 (D'Avila et al., 2006; Knight et al., 2018). Both Th1 cells and Th17 cells are responsible for the protection against *Mtb*. MDSCs have high lipid reservoir that nourishes *Mtb* (Hossain et al., 2015). We observed high lipid deposition in DC^{MPT64}, as compared to control DCs. Pathogen-captured DCs migrate to the secondary lymphoid organs to present the processed antigen to activate naïve T cells (van Vliet et al., 2007). The presence of chemokine receptor CCR7 on DCs is a master receptor involved in their migration (van Vliet et al., 2007). Migration of DCs to the site of infection after capturing antigen relies heavily on the CCR7 expression (Liu et al., 2021; Saban, 2014). We detected a decline in the expression of CCR7 on DC^{MPT64}. Further, we corroborated this finding in *vivo* and noticed a reduction in the migration of DC^{MPT64} to the spleen and lymph nodes of the mice. Therefore, it can be inferred from these findings that a decrease in the expression of CCR7, CD86, and MHC II with upregulation in co-inhibitory markers and high lipid content in DC^{MPT64}, impedes their migratory function and subsequently fail to activate naïve T cells. Furthermore, DC^{MPT64} suppressive nature confirmed the inhibition of the activation of Th1 cells, Th17 cells and Th2 cells. Inhibition of T cells by MDSCs has been referred to as the 'gold standard' to consider them as suppressive cells (Hongo et al., 2014). DC^{MPT64} promoted *in vitro* and *in vivo* activation of Tregs. MDSCs too preferably activate Tregs and Bregs (Lei et al., 2015; Park et al., 2016).

Like alveolar macrophages, MDSCs have been identified as another hiding niche for *Mtb* (Agrawal et al., 2018; Knaul et al., 2014). Numerous proteins of *Mtb* have been reported to hamper the antigen processing and presentation by APCs (Dolasia et al., 2021). We observed that DC^{MPT64} with MHC^{lo} exhibited a decrease in phagocytosing mycobacteria. However, the growth of *Mtb* was highly enhanced, suggesting that DC^{MPT64} is a safer shelter for the survival and persistence of *Mtb*.

We also tried to decipher the mechanism to understand the immunosuppressive role of MDSCs. It has been observed that within MDSC, L-arginine was utilized through enhanced activity of iNOS and arginase 1 (Bronte and Zanovello, 2005; Rodriguez and Ochoa, 2008; Zoso et al., 2014). We noticed that IDO^{hi} imparts tolerogenic function into DC^{MPT64} through the generation of Tregs. Further, an elevated yield of NO by iNOS imparted inhibition of JAK/STAT5 signaling. This ultimately inhibits T cells function (Dilek et al., 2012). High expression of iNOS, arginase 1 and IL-10 by M-MDSC induces Tregs generation (Huang et al., 2006). Till now, we observed that DC^{MPT64} expressed $CD11c^{lo}$, $CD11b^{hi}$, $F4/80^{hi}$, $CD40^{hi}$, $CD80^{hi}$, MHC II^{lo}, $Ly6C^{hi}$, $Ly6G^{lo}$, PD-$L1^{hi}$, TIM-3^{hi}, IL-10^{hi}, TGF-β^{hi}, IL-6^{lo}, IL-12^{lo}, TNF-α^{lo}, $CCR7^{lo}$ and are phenotypically as well as morphologically suppressive in nature as that of M-MDSCs. Based on these findings, we analysed more molecules responsible for revealing the immunosuppressive nature of DC^{MPT64}. Surprisingly, we observed that DC^{MPT64} expressed high arginase activity, as compared to control DCs. Furthermore, to corroborate this finding, we observed that DC^{MPT64} expressed a high level of arginase 1, iNOS, STAT-1, STAT-3, and IDO with reduced expression of ROS. High NO secretion was verified through the augmented level of iNOS. These results suggest that the suppressive mechanism operating in DC^{MPT64} is through the high presence of iNOS/NO and arginase 1.

Next, we delineated the metabolic state of DC^{MPT64} during *Mtb* infection. *Mtb* within the foamy macrophages utilizes lipids as a source of energy for its survival (Peyron et al., 2008). Moreover, upon the shortage of carbon source, *Mtb* induces glyoxylate shunt for its survival (Russell et al., 2009). MDSCs during cancer or any infection shift their glycolysis machinery to fatty acid oxidation (Al-Khami et al., 2016). We noted that DC^{MPT64} showed reduced glucose (2-NBDG) uptake and downregulation of Glut-1 expression. As Glut-1 is the main glucose transport channel in immune and non-immune cells. This signifies that low glucose uptake by DC^{MPT64} is because of a reduction in the Glut-1 channel. Additionally, we observed a fall in the hexokinase 2 enzyme, suggesting that the glycolysis cycle is compromised in DC^{MPT64}. Hence, DC^{MPT64} acquired a metabolically dormant phenotype. Recently, accumulation of methylglyoxal (MGO) in MDSCs has been reported, which imparts a suppressive characteristic (Baumann et al., 2020). Surprisingly, we observed high MGO and semicarbazide-sensitive amine oxidase (SSAO) in DC^{MPT64}. Basically, MGO generation occurs through glyceraldehyde3-phosphate and dihydroxyacetone phosphate of glycolysis; from acetone and aminoacetone by SSAO (Lyles and Chalmers, 1992; Phillips and Thornalley, 1993; Ray and Ray, 1983).

The intracellular MGO level is regulated by glyoxalase and glutathione (Rabbani and Thornalley, 2008). We observed a decline in glyoxalase 1 in DC^{MPT64}. It can be deduced from these results that DC^{MPT64} have an impaired glycolysis cycle that leads to the generation of advanced glycation end products (MGO) from the byproducts of glycolysis and aminoacetone. Intriguingly, we have shown that *Mtb* infection augments the accumulation of MGO in DC^{MPT64}. Hence, it can be inferred from these results that MPT64 protein might be one of the *Mtb* components responsible for stimulating the accumulation of MGO. Together, these results show that pre-exposure of differentiating DCs to MPT64 renders them metabolically inert and induce unresponsiveness in the T cells. Interestingly, we observed significantly reduced glucose

uptake and increased MGO by T cells on interaction with DC^{MPT64}. It can be inferred that DC^{MPT64} transfers the quiescent stage in T cells through the transfer of MGO. The inhibition in the function of T cells was through cognate and non-cognate interactions.

Since we observed that DC^{MPT64}secretes a high amount of NO as compared to control DCs. And it is well known that a high amount of NO has an inhibitory effect on lymphocyte proliferation (Gabrilovich et al., 2012). Consequently, we examined the role of NO in imparting suppression on T cells in a contact-independent manner. Intriguingly, we observed that upon co-culturing DC^{MPT64} with T cells (CD4 and CD8) along with iNOS inhibitor, N-monomethyl-L-arginine (NM), the proliferative potential of CD4 T cells and CD8 T cells were resumed. Hence, NO produced by DC^{MPT64} might be one of the components that limit T cell functionality. Next, we observed that contact-dependent interaction between DC^{MPT64} and T cells (CD4 and CD8) inhibits T cell proliferation. Moreover, we noted that NO secreted by DC^{MPT64} have an inhibitory role. Surprisingly, we observed that there was a significant decline in the expression of TCR (β-chain and γ/δ chain) of CD4 T cells and CD8 T cells during cognate interaction with DC^{MPT64}. T cells recognize peptide-MHC complex and are very crucial for the induction of adaptive immune response. This TCR is generally composed of α and β or γ and δ chains. Moreover, it has been reported that NO and ROS, downregulate the TCR expression (Hardy et al., 2008). It is well known that iNOS leads to depletion of L-arginine followed by a low amount of superoxide formation. This superoxide further reacts with NO and forms $ONOO^-$ and RNS that nitrosylates TCR. Subsequently, it is well known that MDSCs through high MGO, NO and arginase 1 inhibit T cell proliferation and augment apoptosis (Baumann et al., 2020; Bronte et al., 2003; du Plessis et al., 2013; Rodriguez et al., 2005). Similarly, we observed that DC^{MPT64} induces apoptosis induction in CD4 T cells and CD8 T cells.

Chapter-6

Summary

6. SUMMARY

Mycobacterium tuberculosis (*Mtb*) is a smart and clever pathogen since it can persist in the intimidating environment of the host by taming and tuning the immune system. Nearly, 1/4th of the world's population is infected by this pathogen. *Mtb* adopts various immune evasion strategies to survive in the hostile milieu of the host cell. *Mtb* inhibits the fusion of phagosome-lysosome, prompts necrosis and inhibits autophagy and apoptosis, obstructs APCs functions, and impairs the generation of ROS/RNI (Cambier et al., 2014; Lerner et al., 2015; Mehra et al., 2013; Queval et al., 2017; Velmurugan et al., 2007). It suggests that *Mtb* has co-evolved with the host and is able to successfully weaken the immune system of the host (Ernst, 2018; Zhai et al., 2019).

Further, *Mtb* can elicit the generation of myeloid-derived suppressor cells (MDSCs) that fail to activate T cells (Ribechini et al., 2019). In conjunction with T regulatory cells (Tregs), MDSCs have been reported in TB granulomas to suppress the function of T cells and therefore promote the advancement of the disease. MDSCs utilize nitric oxide, arginase 1, and methylglyoxal (MGO) with other constituents that cause nitrosylation of T cell receptors, apoptosis of T cells, and induction of Tregs (Baumann et al., 2020; El Daker et al., 2015; Knaul et al., 2014). The vast detrimental effect of MDSCs is mediated by the strong suppression of Th1 immunity along with a decline in the secretion of innate cytokines. Consequently, it becomes crucial to decode the function of unexplored proteins exploited by *Mtb* to stimulate the generation of MDSCs to avoid its destruction by the immune system.

DCs and MΦs are the main innate cells responsible for curtailing *Mtb* infection and induction of immunity. Macrophages upon infection with *Mtb* initiate a cascade of defense mechanisms to kill the bacterium. Contrary to this, it also becomes a protective niche for the

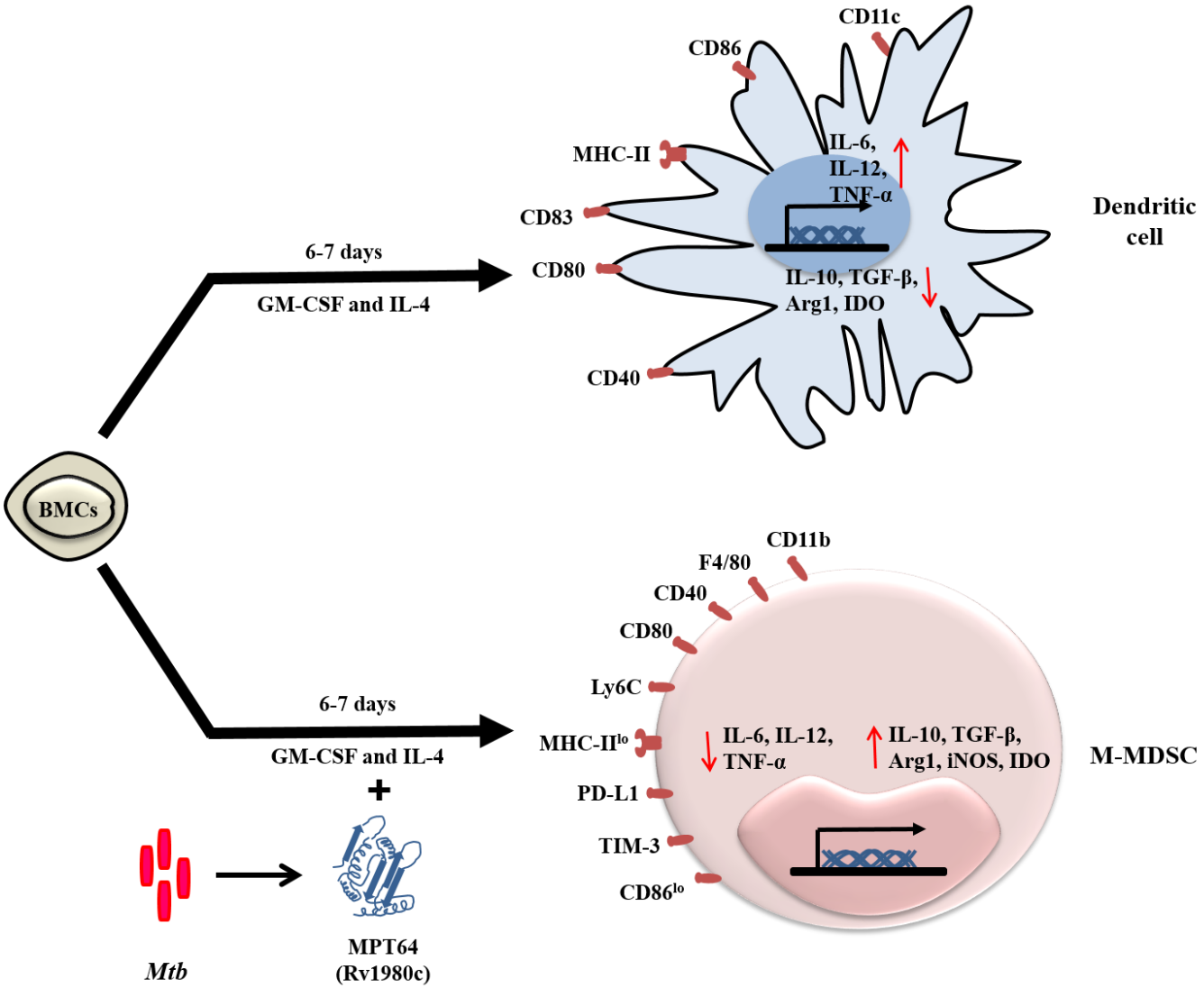
persistence and survival of *Mtb* (McClean and Tobin, 2016). DCs have a sentinel role in linking innate and adaptive immunity by activating naïve T cells (Prendergast and Kirman, 2013).

Various molecules of *Mtb* are endowed with an immunomodulatory potential (Amir et al., 2017; Chatterjee et al., 2011; Siddiqui et al., 2014; Simeone et al., 2012). Through apoptosis, macrophages limit the spread of *Mtb* but some components of *Mtb* antagonize this process and survive in the inimical milieu (Jayakumar et al., 2008; Mustafa et al., 2008; Sun et al., 2013).

MPT64 protein is secreted by actively dividing *Mtb* and its role is not much studied in the modulation of the immune system. MPT64 (Rv1980c) is a secretory protein having a molecular weight of ~24 kDa. In active TB patients, a high level of MPT64 has been observed in the sputum and serum (Mehaffy et al., 2017). Recently, it has been reported that MPT64 inhibits the apoptosis of MΦs (Mustafa et al., 2007; Wang et al., 2014); and the MPT64 mutant *Mtb* strain generates less bacterial burden in mice compared to the wild-type *Mtb* (Stamm et al., 2019). It is also unclear how *Mtb* exploits MPT64 in tuning and taming of differentiating DCs (DC^{MPT64}) to suppress their activation and differentiation. Hence, it will be quite interesting to decipher the role of MPT64 on DCs and MΦs along with its role in the pathogenesis of *Mtb*. Surprisingly, we have not observed any significant effect of MPT64 on the activation and differentiation of MΦs. Rather, we noticed that it augments the apoptosis in MΦs, which corroborates with recently published reports. Moreover, MPT64 has no effect on fully matured DCs. Intriguingly, we noted that pre-exposure of MPT64 on the differentiating DCs (DC^{MPT64}) skews them towards myeloid-derived suppressor cells (MDSCs). Subsequently, we thought to monitor the influence of MPT64 on differentiating DCs (DC^{MPT64}) and the major findings observed were the (i) decline in the ratio of cells expressing CD11c,CD86 and MHC II; (ii) enhancement in percentage of cells expressing $F4/80^{hi}$,$CD11b^{hi}$,$CD40^{hi}$,$CD80^{hi}$,$Ly6G^{lo}$,$Ly6C^{hi}$, $PD\text{-}L1^{hi}$,

TIM-3^{hi}; (iii) decrease in the yield of IL-6, IL-12, TNF-α with augmentation in IL-10 and TGF-β cytokines; (iv) reduced cell size with high lipid content; (v) diminished activation and proliferation of CD4 T cells and CD8 T cells; (vi) inhibition in the differentiation of Th1 cells, Th2 cells and Th17 cells; (vii) augmented *in vivo* generation of T regulatory cells; (viii) reduced phagocytosis of mycobacteria; (ix) persistence and promotion of the intracellular growth of *Mtb*; (vii) impaired migratory potential with degeneration in expression of CCR7; (viii) augmented secretion of IL-10 and TFG-β with reduction in secretion of IFN-γ, IL-4 and IL-17 by the CD4 T cells; (ix) high level of nitric oxide (NO), arginase 1, IDO-1, STAT-1, STAT-3; (x) reduced expression of p65 (NF-κB) and ROS; (xi) low glucose uptake and expression of Glut-1 and hexokinase 2; (xii) high MGO and SSAO with reduced expression of glyoxalase 1; (xiii) quiescent morphology, as verified through reduced glucose uptake and accumulation of MGO; (xiv) suppression in T cells in a contact dependent and independent manner through transfer of MGO and NO; (xv) hampers TCR expression in CD4 T cells and CD8 T cells followed by apoptosis.

In the present study, we have explored the role of MPT64 on the differentiation of DCs (DC^{MPT64}), and observed that it led to the development of M-MDSCs. DC^{MPT64} were functionally inert and failed to optimally activate and differentiate CD4 T cells and CD8 T cells. Further, high lipid and methylglyoxal content, and reduced glucose consumption by DC^{MPT64}, rendered them metabolically quiescent, and therefore reduced their ability to phagocytose *Mtb*, and provided a safer shelter for the intracellular survival of the *Mtb*. The mechanism identified in compromising the function of DC^{MPT64} was through the increased production and accumulation of methylglyoxal and NO. This study suggests that *Mtb* utilizes its MPT64 protein to evade the host immune system (Figure E).


CD11c
CD86
MHC-II
CD83
CD80
CD40
IL-6,
IL-12,
TNF-α
IL-10, TGF-β,
Arg1, IDO
Dendritic
cell
6-7 days
GM-CSF and IL-4
BMCs
CD11b
F4/80
CD40
CD80
Ly6C
MHC-IIlo
PD-L1
TIM-3
CD86lo
IL-6, IL-12,
TNF-α
IL-10, TGF-β,
Arg1, iNOS, IDO
M-MDSC
6-7 days
GM-CSF and IL-4
+
Mtb
MPT64
(Rv1980c)

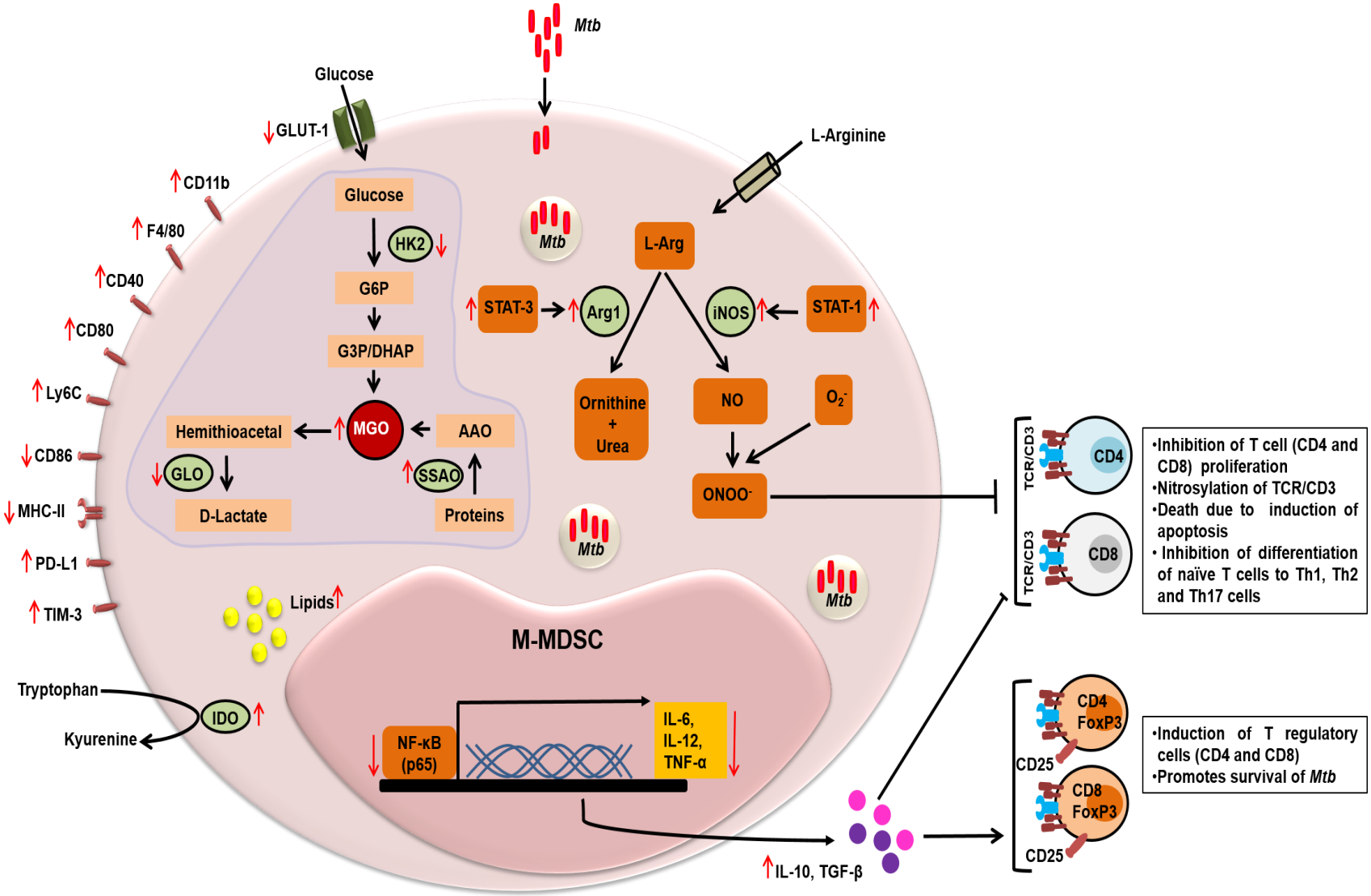

Mtb
Glucose
GLUT-1
L-Arginine
CD11b
F4/80
CD40
CD80
Ly6C
CD86
MHC-II
PD-L1
TIM-3
Glucose
HK2
G6P
G3P/DHAP
Hemithioacetal
MGO
AAO
GLO
SSAO
D-Lactate
Proteins
Mtb
L-Arg
STAT-3
Arg1
iNOS
STAT-1
Ornithine + Urea
NO
O_2^-
ONOO-
Mtb
Mtb
Lipids
M-MDSC
Tryptophan
IDO
Kyurenine
NF-κB (p65)
IL-6, IL-12, TNF-α
IL-10, TGF-β
TCR/CD3
CD4
TCR/CD3
CD8
•Inhibition of T cell (CD4 and CD8) proliferation
•Nitrosylation of TCR/CD3
•Death due to induction of apoptosis
• Inhibition of differentiation of naïve T cells to Th1, Th2 and Th17 cells
CD4 FoxP3
CD25
CD8 FoxP3
CD25
•Induction of T regulatory cells (CD4 and CD8)
•Promotes survival of Mtb

Figure E. Proposed mechanism of skewing BMCs towards M-MDSCs by MPT64 protein of *Mtb* under DCs differentiation conditions. Pre-exposure of BMCs to MPT64 under DC differentiating conditions (GM-CSF + IL-4) promotes the generation of M-MDSCs, as evidenced by the enhanced expression of CD11b, Ly6C, F4/80, CD40, CD80, PD-L1, TIM-3 and reduction in the expression of CD86 and MHC II. Enhanced expression of STAT-1 elicits the up-regulation of iNOS followed by NO secretion; increased STAT-3 leads to arginase 1 that utilizes L-arginine and generates urea and ornithine. Induction of iNOS and arginase 1 leads to the generation of $ONOO^-$ and RNS that induces malfunctioning of TCR and inhibits T cell proliferation followed by apoptosis. Augmentation of IDO leads to consumption of tryptophan from the cell and surroundings, and therefore deprivation of amino acids in the cell and its microenvironment. Reduction in glucose uptake along with a decline in the expression of Glut1 and Hk2 leads to the formation of MGO, as supported by high levels of SSAO and low expression of GLO1. Augmentation in the secretion of IL-10 and TGF-β leads to Tregs differentiation and inhibition of Th1 cells, Th2 cells and Th17 cells. Downregulation in pro-inflammatory response is marked by reduced expression of p65 NF-κB. Higher lipid accumulation works as a source of nutrition for *Mtb* survival in the M-MDSC, as demonstrated by the higher survival rate of *Mtb*.

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Publications

Publications

1. Kaur G, **Singh S**, Nanda S, Zafar MA, Malik JA, Arshi MU, Lamba T, Agrewala JN (2022). Fiction and facts about BCG imparting trained immunity against COVID-19. *Vaccines* 2022, 10, 1006. https://doi.org/10.3390/vaccines10071006. (I.F: 4.4)

2. Aqdas M, Maurya SK, Pahari S, **Singh S**, Khan N, Sethi K, Kaur G, Agrewala JN (2021). Immunotherapeutic Role of NOD-2 and TLR-4 Signaling as an Adjunct to Antituberculosis Chemotherapy. *ACS Infect. Dis.*, 7, 11, 2999-3008. (IF: 5.0)

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vaccines

MDPI

Review

Fiction and Facts about BCG Imparting Trained Immunity against COVID-19

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Abstract: The Bacille Calmette-Guérin or BCG vaccine, the only vaccine available against *Mycobacterium tuberculosis* can induce a marked Th1 polarization of T-cells, characterized by the antigen-specific secretion of IFN-γ and enhanced antiviral response. A number of studies have supported the concept of protection by non-specific boosting of immunity by BCG and other microbes. BCG is a well-known example of a trained immunity inducer since it imparts 'non-specific heterologous' immunity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for the recent pandemic. SARS-CoV-2 continues to inflict an unabated surge in morbidity and mortality around the world. There is an urgent need to devise and develop alternate strategies to bolster host immunity against the coronavirus disease of 2019 (COVID-19) and its continuously emerging variants. Several vaccines have been developed recently against COVID-19, but the data on their protective efficacy remains doubtful. Therefore, urgent strategies are required to enhance system immunity to adequately defend against newly emerging infections. The concept of trained immunity may play a cardinal role in protection against COVID-19. The ability of trained immunity-based vaccines is to promote heterologous immune responses beyond their specific antigens, which may notably help in defending against an emergency situation such as COVID-19 when the protective ability of vaccines is suspicious. A growing body of evidence points towards the beneficial non-specific boosting of immune responses by BCG or other microbes, which may protect against COVID-19. Clinical trials are underway to consider the efficacy of BCG vaccination against SARS-CoV-2 on healthcare workers and the elderly population. In this review, we will discuss the role of BCG in eliciting trained immunity and the possible limitations and challenges in controlling COVID-19 and future pandemics.

Keywords: innate immunity; BCG; SARS-CoV-2; COVID-19; vaccines

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1. Introduction

Vaccines provide a long-lived pathogen-specific protective immunity. However, some vaccines, viz., influenza, oral polio, MMR (measles, mumps, rubella), smallpox, measles, BCG, etc., can also provide non-specific cross-protection against other pathogens. The non-specific cross-protection against unrelated diseases has been described for other vaccines such as influenza, oral poliovirus, smallpox, and measles vaccines. These heterologous effects emerge from vaccine-induced immunomodulation. Various studies have shown non-specific protective effects after immunization with an unrelated vaccine or microbial antigens (Table 1). This *de facto* immunological memory occurs in innate immune cells and has been termed 'trained immunity'. A deeper understanding of the mechanism of trained immunity-based vaccines may result in the next generation of broad-spectrum

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Article

Immunotherapeutic Role of NOD-2 and TLR-4 Signaling as an Adjunct to Antituberculosis Chemotherapy

Mohammad Aqdas, Sudeep Kumar Maurya, Susanta Pahari, Sanpreet Singh, Nargis Khan, Kanupriya Sethi, Gurpreet Kaur, and Javed Naim Agrewala*

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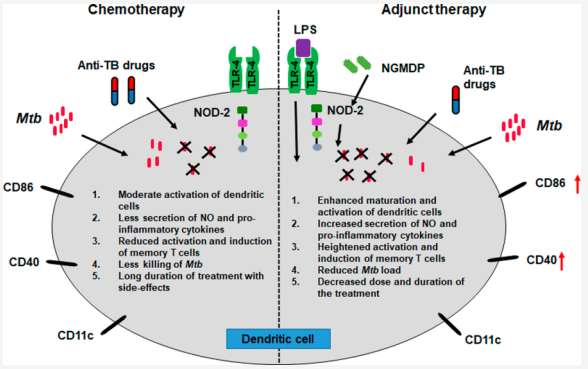
ABSTRACT: Tuberculosis (TB) treatment is lengthy and inflicted with severe side-effects. Here, we attempted a novel strategy to reinforce host immunity through NOD-like receptor (NOD-2) and Toll-like receptor (TLR-4) signaling in the murine model of TB. Intriguingly, we noticed that it not only bolstered the immunity but also reduced the dose and duration of rifampicin and isoniazid therapy. Further, we observed expansion in the pool of effector ($CD44^{hi}$, $CD62L^{lo}$, $CD127^{hi}$) and central ($CD44^{hi}$, $CD62L^{hi}$, $CD127^{hi}$) memory CD4 T cells and CD8 T cells and increased the intracellular killing of *Mycobacterium tuberculosis* (*Mtb*) by activated dendritic cells [$CD86^{hi}$, $CD40^{hi}$, IL-6^{hi}, IL-12^{hi}, TNF-α^{hi}, nitric oxide $(NO)^{hi}$] with significant reduction in *Mtb* load in the lungs and spleen of infected animals. We infer that the signaling through NOD-2 and TLR-4 may be an important approach to reduce the dose and duration of the drugs to treat TB.



KEYWORDS: *Mycobacterium tuberculosis, antibiotics, host-directed therapy*

Tuberculosis (TB) continues to kill 1.6 million people annually. The emergence of multidrug (MDR) resistant strains of *Mycobacterium tuberculosis* (*Mtb*) has made the situation even worse.[1] The "End TB" strategy by the World Health Organization aims to eliminate the TB epidemic globally by 2035 with 90% reduction in disease incidences and 95% dropping the mortality rate.[2] More effective therapeutics or radical changes in the current regimens are the challenges for the scientific community in order to reduce the transmission of TB.[3,4] Vaccination and drug treatment remain the primary remedial measures against TB. BCG is the most widely used vaccine against TB, yet its protective efficacy remains controversial due to its variable potency from 0% to 85%.[5,6] Although the current regimen for TB provides a 95% cure, its side effects and prolonged duration lead to patient non-compliance with the treatment.[7]

Various interventions have been tried in supplementing chemotherapeutic regimens, such as vaccines,[8,9] detoxified pathogen extract in liposomes (RUTI),[10] DNA plasmids,[11,12] cytokines,[13] immunoglobulins,[14] and bacterial antigens to make the treatment more effective.[15] More recently, host-directed therapy [HDT] is gaining considerable attention as it improves the efficacy of drugs.[16−18] Consequently, HDT can be an effective approach to bolster immunity against *Mtb*, thereby complementing the potency of the drugs. Moreover, HDT can reduce the dose and duration of the treatment and thus the cost and complications associated with the drugs will decrease. Further, the conjunction of drugs with HDT will substantially improve the efficacy of the drugs.[19] Chemotherapy can kill *Mtb*, whereas stimulation of the host immunity via receptors of innate immunity can effectively eliminate the residual bacteria. Consequently, multiple approaches against *Mtb* consisting of anti-TB drugs and HDT would effectively control TB.

Innate immune cells of the body are the first line of defense. They identify pathogen associated molecular patterns (PAMPs) of invading germs through their pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), nod-like receptors (NLRs), and C-type lectin receptors (CLRs). Although TLRs widely contribute in protection against *Mtb*, yet other molecules like the NLR family are reported to be effective against TB.[20−24] Moreover, the synergism between TLRs and NLRs significantly boosts the immune response.[25,26] Dendritic cells (DCs) can activate both innate and adaptive immunity and play crucial roles in bridging these two fundamental arms of immunity. Therefore, it is quite imperative for DCs to be activated to elicit an effective innate and adaptive immune response against any pathogen.

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ORIGINAL RESEARCH
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Cumulative Signaling Through NOD-2 and TLR-4 Eliminates the *Mycobacterium Tuberculosis* Concealed Inside the Mesenchymal Stem Cells

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For a long time, tuberculosis (TB) has been inflicting mankind with the highest morbidity and mortality. Although the current treatment is extremely potent, a few bacilli can still hide inside the host mesenchymal stem cells (MSC). The functional capabilities of MSCs are known to be modulated by TLRs, NOD-2, and RIG-1 signaling. Therefore, we hypothesize that modulating the MSC activity through TLR-4 and NOD-2 can be an attractive immunotherapeutic strategy to eliminate the *Mtb* hiding inside these cells. In our current study, we observed that MSC stimulated through TLR-4 and NOD-2 (N2.T4) i) activated MSC and augmented the secretion of pro-inflammatory cytokines; ii) co-localized *Mtb* in the lysosomes; iii) induced autophagy; iv) enhanced NF-κB activity *via* p38 MAPK signaling pathway; and v) significantly reduced the intracellular survival of *Mtb* in the MSC. Overall, the results suggest that the triggering through N2.T4 can be a future method of immunotherapy to eliminate the *Mtb* concealed inside the MSC.

Keywords: tuberculosis, mesenchymal stem cell, NOD-2, TLR-4, autophagy

INTRODUCTION

Tuberculosis (TB) is the cause of 2 million deaths each year and an estimated 1.8 billion people with latent disease worldwide (Mwaba et al., 2020). It is one of the top 10 diseases in terms of high morbidity and mortality worldwide (Herbert et al., 2014). Currently, drug-resistant TB is a major threat to mankind and quite common in TB endemic countries viz., India and China. Even though the available drugs remain the mainstay for the treatment of TB, certain limitations, such as their narrow therapeutic index and the associated toxicities, dilute their effectiveness (Forget and Menzies, 2006; Trauner et al., 2014). Due to its long duration, many patients fail to abide by the current regimen and quit before the completion of the course. This leads to the development of the very lethal drug-resistant TB (Munro et al., 2007). Innate and adaptive immune responses are responsible for protecting against invading pathogens. Early events include the engulfment of *Mtb* by the alveolar macrophages and dendritic cells, followed by their bactericidal mechanisms, such as the generation of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI)

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RESEARCH ARTICLE Open Access

A multiple T cell epitope comprising DNA vaccine boosts the protective efficacy of Bacillus Calmette–Guérin (BCG) against *Mycobacterium tuberculosis*

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Abstract

Background: Approximately 80% - 90% of individuals infected with latent *Mycobacterium tuberculosis (Mtb)* remain protected throughout their life-span. The release of unique, latent-phase antigens are known to have a protective role in the immune response against *Mtb*. Although the BCG vaccine has been administered for nine decades to provide immunity against *Mtb*, the number of TB cases continues to rise, thereby raising doubts on BCG vaccine efficacy. The shortcomings of BCG have been associated with inadequate processing and presentation of its antigens, an inability to optimally activate T cells against *Mtb*, and generation of regulatory T cells. Furthermore, BCG vaccination lacks the ability to eliminate latent *Mtb* infection. With these facts in mind, we selected six immunodominant CD4 and CD8 T cell epitopes of *Mtb* expressed during latent, acute, and chronic stages of infection and engineered a multi-epitope-based DNA vaccine (C6).

Result: BALB/c mice vaccinated with the C6 construct along with a BCG vaccine exhibited an expansion of both CD4 and CD8 T cell memory populations and augmented IFN-γ and TNF-α cytokine release. Furthermore, enhancement of dendritic cell and macrophage activation was noted. Consequently, illustrating the elicitation of immunity that helps in the protection against *Mtb* infection; which was evident by a significant reduction in the *Mtb* burden in the lungs and spleen of C6 + BCG administered animals.

Conclusion: Overall, the results suggest that a C6 + BCG vaccination approach may serve as an effective vaccination strategy in future attempts to control TB.

Keywords: T cells, Epitopes, DNA vaccine, BCG, Tuberculosis

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ORIGINAL ARTICLE

Predominance of M2 macrophages in gliomas leads to the suppression of local and systemic immunity

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Abstract

Glioblastoma is a highly prevalent and aggressive form of primary brain tumor. It represents approximately 56% of all the newly diagnosed gliomas. Macrophages are one of the major constituents of tumor-infiltrating immune cells in the human gliomas. The role of immunosuppressive macrophages is very well documented in correlation with the poor prognosis of patients suffering from breast, prostate, bladder and cervical cancers. The current study highlights the correlation between the tumor-associated macrophage phenotypes and glioma progression. We observed an increase in the pool of M2 macrophages in high-grade gliomas, as confirmed by their CD68 and CD163 double-positive phenotype. In contrast, less M1 macrophages were noticed in high-grade gliomas, as evidenced by the down-regulation in the expression of *CCL3* marker. In addition, we observed that higher gene expression ratio of *CD163/CCL3* is associated with glioma progression. The Kaplan–Meier survival plots indicate that glioma patients with lower expression of M2c marker (CD163), and higher expression of M1 marker (CCL3) had better survival. Furthermore, we examined the systemic immune response in the peripheral blood and noted a predominance of M2 macrophages, myeloid-derived suppressor cells and PD-1^+ CD4 T cells in glioma patients. Thus, the study indicates a high gene expression ratio of *CD163/CCL3* in high-grade gliomas as compared to low-grade gliomas and significantly elevated frequency of M2 macrophages and PD-1^+ CD4 T cells in the blood of tumor patients. These parameters could be used as an indicator of the early diagnosis and prognosis of the disease.

Keywords Glioma · Tumor-associated macrophages · CD163 · CCL3 · PD-1

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Abbreviations

Abs	Antibodies
DAB	(3,3′-diaminobenzidine)
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGG	High-grade glioma
HLA-DR	Human Leukocyte Antigen—DR isotype
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-10R	Interleukin-10 receptor
LGG	Low-grade glioma
LPS	Lipopolysaccharides
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
TAM	Tumor-associated macrophages
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-beta

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Reinforcing the Functionality of Mononuclear Phagocyte System to Control Tuberculosis

*Susanta Pahari, Gurpreet Kaur, Shikha Negi, Mohammad Aqdas, Deepjyoti K. Das, Hilal Bashir, Sanpreet Singh, Mukta Nagare, Junaid Khan and Javed N. Agrewala**

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The mononuclear phagocyte system (MPS) constitutes dendritic cells, monocytes, and macrophages. This system contributes to various functions that are essential for maintaining homeostasis, activation of innate immunity, and bridging it with the adaptive immunity. Consequently, MPS is highly important in bolstering immunity against the pathogens. However, MPS is the frontline cells in destroying *Mycobacterium tuberculosis* (*Mtb*), yet the bacterium prefers to reside in the hostile environment of macrophages. Therefore, it may be very interesting to study the struggle between *Mtb* and MPS to understand the outcome of the disease. In an event when MPS predominates *Mtb*, the host remains protected. By contrast, the situation becomes devastating when the pathogen tames and tunes the host MPS, which ultimately culminates into tuberculosis (TB). Hence, it becomes extremely crucial to reinvigorate MPS functionality to overwhelm *Mtb* and eliminate it. In this article, we discuss the strategies to bolster the function of MPS by exploiting the molecules associated with the innate immunity and highlight the mechanisms involved to overcome the *Mtb*-induced suppression of host immunity. In future, such approaches may provide an insight to develop immunotherapeutics to treat TB.

Keywords: mononuclear phagocyte system, tuberculosis, monocyte, macrophage, dendritic cell, pattern recognition receptors, infection, immunotherapy

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INTRODUCTION

Despite of the fact that efficient anti-tuberculosis (TB) drugs are available, TB remains to ruin public health globally. Reports suggest that one-third of the populace is infected with *Mycobacterium tuberculosis* (*Mtb*), almost 10.4 million active cases and around 1.8 million deaths in 2016 (1). The occurrence of threat is further complicated due to acquired immunodeficiency syndrome pandemic, the appearance of multidrug-resistant (MDR), extensively drug-resistant, as well as totally drug-resistant *Mtb* strains (2). Vaccines are the most effective strategy to control and eliminate any disease (3, 4). Ironically, bacillus Calmette–Guérin (BCG) is the most controversial vaccine because of its variable efficacy worldwide (5). Moreover, it protects only children but not adults (6). Therefore, an urgent necessity and the challenge for the scientific society are to improve the current drug regimen or develop alternative stratagems against TB.

Our immune system is quite complex and complicated, comprising of innate as well as adaptive branch of immunity. Innate immunity is the primary and foremost line of defense against intruding pathogens (7). Innate immunity was initially believed to be non-specific and considered to be of lesser importance for the immune function. On the other hand, adaptive immunity is allied with

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Bolstering Immunity through Pattern Recognition Receptors: A Unique Approach to Control Tuberculosis

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The global control of tuberculosis (TB) presents a continuous health challenge to mankind. Despite having effective drugs, TB still has a devastating impact on human health. Contributing reasons include the emergence of drug-resistant strains of *Mycobacterium tuberculosis (Mtb),* the AIDS-pandemic, and the absence of effective vaccines against the disease. Indeed, alternative and effective methods of TB treatment and control are urgently needed. One such approach may be to more effectively engage the immune system; particularly the frontline pattern recognition receptor (PRR) systems of the host, which sense pathogen-associated molecular patterns (PAMPs) of *Mtb*. It is well known that 95% of individuals infected with *Mtb* in latent form remain healthy throughout their life. Therefore, we propose that clues can be found to control the remainder by successfully manipulating the innate immune mechanisms, particularly of nasal and mucosal cavities. This article highlights the importance of signaling through PRRs in restricting *Mtb* entry and subsequently preventing its infection. Furthermore, we discuss whether this unique therapy employing PRRs in combination with drugs can help in reducing the dose and duration of current TB regimen.

Keywords: tuberculosis, immunomodulation, innate cells, innate molecules, pattern recognition receptors

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INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB). It remains a major health problem worldwide. It is responsible for over 10.4 million cases and 1.8 million deaths annually (1). About one-third of the global population is infected with *Mtb* but only 5–10% succumb to disease (2, 3). The failure of BCG vaccine to protect against TB, AIDS-pandemic, and the emergence of drug resistance of *Mtb* has further exacerbated the problem (4). The current lengthy regimen for TB treatment is full of complexity and inflicts patients with severe side-effects (5, 6). Hence, it is imperative to design novel and unique strategies that can overcome the problems associated with current treatment.

Host immunity successfully imparts optimum protection against the majority of pathogens (7–11). However, the success of *Mtb* to establish pathogenicity is due to its unique ability to skillfully tame and tune host immune responses and reside in the hostile environment, waiting for the right moment to take over the host immunity (11). Although our immune system sufficiently protects 90–95% of latently infected individuals from developing the disease, the remaining 5–10% of individuals are unable to restrict the growth of *Mtb*. T cells of adaptive immunity are known to play an important role in controlling the survival of the bacterium during latency, as well as in disease progression (12). Importantly, the role of innate immunity is considered more crucial than adaptive

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A Facile Approach for Synthesis and Intracellular Delivery of Size Tunable Cationic Peptide Functionalized Gold Nanohybrids in Cancer Cells

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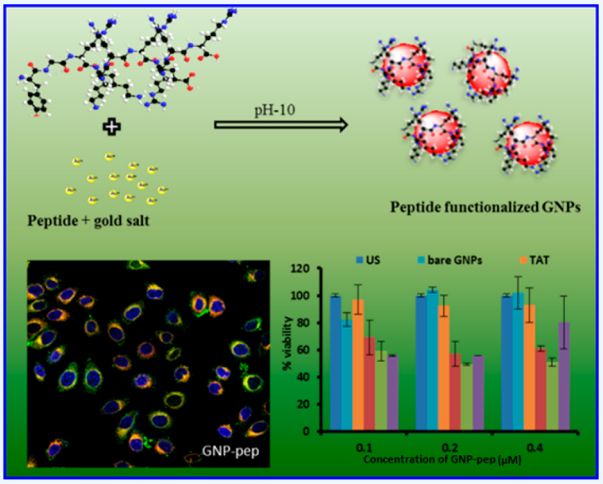
Supporting Information

ABSTRACT: Peptide-based drug delivery systems have become a mainstay in the contemporary medicinal field, resulting in the design and development of better pharmaceutical formulations. However, most of the available reports employ tedious multiple reaction steps for the conjugation of bioactive cationic peptides with drug delivery vehicles. To overcome these limitations, the present work describes a one-step approach for facile and time efficient synthesis of highly cationic cell penetrating peptide functionalized gold nanoparticles and their intracellular delivery. The nanoconstruct was synthesized by the reduction of gold metal ions utilizing cell penetrating peptide (CPP), which facilitated the simultaneous synthesis of metal nanoparticles and the capping of the peptide over the nanoparticle surface. The developed nanoconstruct was thoroughly characterized and tested for intracellular delivery into HeLa cells. Intriguingly, a high payload of cationic peptide over gold particles was achieved, in comparison to conventional conjugation methods. Moreover, this method also provides the ability to control the size and peptide payload of nanoparticles. The nanoconstructs produced showed enhanced cancer cell penetration (μM) and significant cytotoxic effect compared to unlabeled gold nanoparticles. Therefore, this novel approach may also have significant future potential to kill intracellular hidden dreaded pathogens like the human immunodeficiency virus, *Mycobacterium tuberculosis*, and so forth.



INTRODUCTION

Over the past few years, medicinal chemistry has seen a tremendous rise in the use of functional peptides. Peptides are enriched with unique properties such as small size, high specificity, limited toxicity, ease of synthesis, and facile surface modification. Owing to their high biocompatibility and diverse nature, peptides have gained considerable impetus and become an integral part of targeted drug delivery systems. Among peptides, CPPs, in particular, have gained a lot of attention in recent years. CPPs are peptides used to increase the cellular internalization of high molecular weight molecules, for instance, DNA, proteins, and fat-insoluble drugs.[1] It has been reported that the transcriptional activator of transcription (TAT) protein of Human Immunodeficiency Virus-1 (HIV-1) could be efficiently internalized into cells when present in the surrounding tissue culture media.[2−4] The domain which is responsible for this translocation is a short basic region comprising 47−57 residues (YGRKKRRQRRR) bearing six arginine and two lysine molecules. These positively charged residues facilitate the cellular uptake by initial interactions of the peptide with negatively charged residues of heparin sulfate proteoglycans.[5−7] TAT is the most frequently used CPP for functionalization of nanoparticles to increase their overall efficiency and specificity as delivery systems.[8,9] Among various nanoparticles, gold nanoparticles (GNPs) are of significant interest as a delivery vehicle due to their considerable proven biocompatibility and unique optical properties[10] induced by surface plasmon resonance (SPR) of the GNP surface.[11] Moreover, the ability to functionalize the GNPs by multiple ligands (chemical and biomolecules) with high loading capacity

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Tuberculosis Vaccine: Past Experiences and Future Prospects

21

Gurpreet Kaur, Deepjyoti K. Das, Sanpreet Singh, Junaid Khan, Mohammad Sajid, Hilal Bashir, Mohammad Aqdas, Shikha Negi, Uthaman Gowthaman, and Javed N. Agrewala

Abstract

Vaccines are the best prophylactic measure that have eradicated several diseases like polio, measles, and small pox. Currently, efficient vaccines are unavailable for many dreaded diseases, including tuberculosis (TB). TB is caused by an intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*) and is responsible for about two million deaths and nine million new cases annually. The WHO has announced TB as a global emergency in the wake of emerging multidrug-resistant, extremely drug-resistant, and totally drug-resistant strains of *Mtb*. An alarming increase in the number of TB cases around the world and its co-occurrence with HIV has further complicated the problem. Additionally, BCG has failed in reducing the global TB burden, despite its widespread usage. Interestingly, BCG protects children from TB, indicating that it has sufficient antigenic repertoire to protect against *Mtb*. In contrast, failure to protect adults, suggests BCG inability to generate long-lasting immunological memory. Further, protection rendered by BCG against pulmonary TB in adults is highly inconsistent, varying from 0% to 85% (Andersen and Doherty, Nat Rev Microbiol 3:656–62, 2005). Furthermore, its efficacy is least in TB-endemic countries. Studies conducted in Malawi, India, and other endemic countries have concluded that BCG induces inadequate protection. The probable reasons suggested are the

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