THE ROLE AND MECHANISM OF PARAHYDROXYBENZOIC ACID DERIVATIVES IN BOVINE TB PATHOGENESIS

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By

Olivia Ihedioha

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ABSTRACT

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* imposes a significant financial burden on the global cattle industry. Although considerable effort is being spent developing vaccines for bTB disease prevention and control, a licensed vaccine for use in cattle has yet to emerge. Mycobacterial cell envelope antigens are known to interact with the host immune system and contribute to TB pathogenesis. Thus, a better understanding of the effects of *M. bovis* cell envelope antigens on the bovine immune system will aid the rational development of effective bTB vaccines. In this thesis, I first report a comparative analysis of the immune-stimulatory effects of various fractions of the *M. bovis* cell envelope on bovine dendritic cells (bDCs), which are key immune cells required for TB control by the host. I found that the cell surface sugar extract (CSSE) fraction is the least immune-stimulatory suggesting that this fraction might contain an immunosuppressive molecule. Given that CSSE fractions of the *M. tuberculosis* complex are enriched in phenolic glycans, including *para*-hydroxy benzoic acid derivatives (*p*-HBADs), which are known to have immunosuppressive properties, I then examined the immunomodulatory effects of *p*-HBAD-1, the major *p*-HBAD made by *M. bovis* on bDCs. I found that *p*-HBAD-1 has opposing effects in non-primed and IFN-γ primed bDCs *in vitro*. In non-primed bDCs, *p*-HBAD-I induces a tolerogenic response, while a pro-inflammatory response is observed with IFN-γ primed bDCs. These findings suggest that *M. bovis p*-HBAD-I is an immune-regulatory molecule that might have a dual function in bTB pathogenesis. Finally, I successfully prepared plasmid constructs required to disrupt the gene encoding a key enzyme involved in *p*-HBAD synthesis in *M. bovis*. These will be used in future efforts to generate an *M. bovis* mutant deficient in *p*-HBAD 1 for detailed studies in animal models of TB.

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ABBREVIATIONS

Acronym	Definition
ANOVA	Analysis of variance
ADCC	Antibody-dependent cell-mediated cytotoxicity
AG	Arabinogalactan
APC	Antigen presenting cells
AP-1	Activation protein 1
BCG	Bacillus Calmette Guerin
bDCs	Bovine dendritic cells
BPPD	Bovine purified protein derivatives
bTB	Bovine TB
CFP-10	Culture filtrate protein-10
CW	Cell wall
CCR	Chemokine receptors
CPL	Chorismate pyruvate ligase
CD	Cluster of differentiation
CLRs	C-type lectin receptors
CXCL9	Chemokine C-X-C motif ligand 9
CMI	Cell-mediated immunity
CWL	Cell wall lipid
CXCL10	Chemokine C-X-C motif ligand 10
CSSE	Cell surface sugar extract
CyAn ADP	CyAn Advanced Digital Processing (ADP)
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin
DCOs	Double cross over events
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside triphosphates
DPG	Diphosphatidyl glycerol
Dos-R	Dormancy survival regulator
ESAT-6	Early secretory antigenic target-6
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal Regulated Kinase
E.coli	*Escherichia coli*
FACS	Fluorescence Activated Cell Sorting
FACOLA	FCM buffer consisting of 0.03% sodium azide and 0.2% gelatin in PBS pH 7.3
FBS	Fetal Bovine Serum
GAS	Interferon gamma activated Site
gDNA	Genomic deoxyribonucleic acid
GFP	Green flouresent protein
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GMM	Glucose monomycolate
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-γ	Interferon gamma
IFN-γR	Interferon Gamma Receptor

IGRA	Interferon-gamma release assays
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRF3	Interferon regulatory factor-3
IP-10	IFN-γ-induced protein-10
IRAK-4	IL-1 receptor-associated kinase-4
IRF3	Interferon regulatory factor-3
IKK	Inhibitor of nuclear factor kappa B kinase
IKB	Inhibitor of nuclear factor kappa B
JNK	c-Jun Terminal Kinase
kDa	Kilodaltons
LM	lipomannan
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LBP	LPS binding protein
LP	Lipoprotein
M. bovis	*Mycobacterium bovis*
MIG	Monokine induced by IFN-γ
M. tb	*Mycobacterium tuberculosis*
MAPK	Mitogen-Activated Protein Kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MyD88	Myeloid Differentiation Primary Response Protein 88
MDPs	Macrophage/DC progenitors
MMG	Mono-mycolyl glycerol
ManLam	mannose-capped lipoarabinomannan
NFκB	Nuclear Factor κB
NK	Natural killer
NKT	Natural killer T-cells
NLR	NOD-like Receptor
NO	Nitric Oxide
OXPHOS	Oxidative phosphorylation
OL	Outermost capsular layer
ODN	Oligodeoxynucleotides
p-HBAD-I	Parahydroxybenzoic acid derivatives -I
p-HBAD-II	Parahydroxybenzoic acid derivatives -II
PM	Plasma membrane
PGE_2	Prostaglandin E_2
PAMPs	Pathogen Associated Molecular Patterns
PG	Peptidoglycan
PBS	Phosphate Buffered Saline
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST-G	PBS-T + 0.5% gelatin
PBST	PBS + 0.10% Tween 20
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pattern Recognition Receptor
PCR	Polymerase chain reaction
PD1	Programmed cell death protein
Poly (I:C)	Polyinosinic-polycytidylic acid

PNPP	*p*-Nitrophenyl Phosphate
PE	Phenylethanolamine
PE	Phycoerythrin
PIMs	Mono/di-acylated phosphatidyl inositol
PDIMs	Phthiocerol dimycocersates
PAT	Pentacyl trehalose
PGL	Phenolic glycolipid
RLR	RIG-like Receptor
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
RPMI	Rosewell Park Memorial Institute Medium
RT	Room temperature
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
rIFN-γ-	Recombinant interferon-gamma
SDP	Suicide delivery plasmid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src Homology 2
SOCS-3	Suppressor of Cytokine Signaling-3
SOCS-1	Suppressor of Cytokine Signaling-1
STAT	Signal Transducer and Activator of Transcription Protein
ssRNA40	Single stranded-RNA
SCOs	Single cross over events
TB	Tuberculosis
TGFβ	Transforming growth factor-beta
TCR	T-cell receptor
TBST	Tris-Buffered Saline
TST	Tuberculin skin tests
TGF-β	Transforming Growth Factor β
Th	T-Helper
TIR	Toll Interleukin Receptor
TIRAP	TIR-domain Containing Adaptor Protein
TLR	Toll-like Receptor
TRM	Tissue resident memory T- cells
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRIF	TIR Domain Containing Adaptor Inducing IFN-β
TDM	Trehalose dimycolate
TMM	Trehalose monomycolate
TAE	Tris base+ acetic acid +EDTA
TRAF6	TNF receptor superfamily
TAG	Triacyl glycerol
TCR	T- cell receptor
TAK1	Transforming growth factor-β (TGF-β)-activated kinase 1
UV	Ultraviolet
µg	Microgram
WHO	World Health Organization
WT	Wild Type
γδ T	Gamma delta T- cells
X-gal-	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranosid

CHAPTER 1. Introduction and literature review

1.1 *Mycobacteria tuberculosis* complex

The *Mycobacterium tuberculosis* complex (MTBC) consists of *M. tuberculosis* (causative agent of human tuberculosis), *Mycobacterium bovis* (*M. bovis*), *M. bovis* Bacillus Calmette-Guérin (live attenuated TB vaccine strain), *Mycobacterium microti*, *Mycobacterium africanum*, *Mycobacterium canetti and Mycobacterium caprae* [1]. Although some species have 99.9% similarity in nucleotide sequences, they have different abilities to induce macrophage cell death [2]. *M. tuberculosis* is 99.95 % genetically identical to *M. bovis* [2, 3]. Genetic analysis showed that *M. bovis* lacks certain trehalose-containing glycolipids on its cell wall, which could affect the virulence and adaptability within the host cells. The loss of some of these trehalose-containing glycolipids was related to distortion of the surface-exposed acyltrehaloses signaling system [4, 5]. The reduction of this signaling system in *M. bovis* has been linked to less virulence in humans [5].

1.2 *Mycobacterium bovis*

Mycobacterium bovis is a slow-growing, facultative intracellular, aerobic, and Gram-positive bacterium. It is the causative agent of bovine tuberculosis (bTB). Despite cattle being the preferred host for *M. bovis*, it can also infect and cause disease in humans and other animals such as swine, bison, and cervids (deer and elk) [6]. Indeed as a zoonotic disease, bTB is recognized as a major hindrance to global efforts to eradicate TB by the year 2030 [7]. Thus, the human TB burden cannot be decreased without controlling bTB in animal reservoirs. High-risk individuals are those in direct contact with infected animals,

such as farmers and veterinarians [8]. The most frequent route of transmitting the disease is through inhalation of aerosols containing bacilli, and risk exposure is highest in the enclosed areas [9]. Other methods of contracting the disease are drinking unpasteurized milk products from an infected cow, direct contact with the excreta of infected animals, and sharing common water or feed sources [9]. Clinically the disease is characterized by debilitating conditions, cough, decreased milk production, and labored breathing.

1.2.1 Prevalence and financial implication in the cattle industry

Based on the report from the Worldwide Animal Health Information Database of OIE [10], an estimate of 91 out of 182 countries publicized the existence of bTB infection in cattle between 2015–2017. Widespread bTB has been reported in Central and South America, Middle East countries and some parts of Asia [10]. An estimated 30 countries in Africa have also reported the presence of bTB.

In most developed countries, bTB has been successfully controlled by applying test-and-slaughter schemes, milk pasteurization and meat inspection in abattoirs [10]. However, in some developed countries (Ireland, UK, New Zealand), eradication of bTB is challenging despite the implementation of extensive control [11, 12]. In these countries, elimination of bTB is challenging in wildlife (source of infection) when compared to domestic animals resulting in inadequate eradication of the disease [13]. Despite the low prevalence in developed countries, the costs of bTB are primarily associated with trade barriers for live animals. Also, the financial costs of executing compulsory bTB control programs are high [14]. Other costs can negatively impact consumer trust, adverse market reactions, the farming industry, and the country's reputation.

In most developing countries, eliminating bTB remains a problem where the implementation of preventive measures is non-existent [15]. In countries with endemic disease, bTB threatens their national economies by reducing productivity and affecting animal product international trade [16]. Also, the costs of bTB are primarily associated with losses in livestock production, including lower meat/milk production and a high mortality rate [17].

1.2.2 Diagnosis

The diagnosis of bTB is based on clinical ante-mortem and post-mortem tests [18]. The principal ante-mortem tests for bTB diagnosis are immune-based assays to detect cell-mediated immune (CMI) response. This includes tuberculin skin tests (TST) and interferon-gamma release assays (IGRAs) [19]. Commercially available IGRAs for cattle use include the Bovigam assay and the BIO-RAD bovine interferon-gamma ELISA Kit [20, 21]. In addition to IFN-γ, other biomarkers of CMI responses have emerged as potential TB tests for humans and cattle. These include interleukin-1β (IL-1β), interleukin-2 (IL-2), chemokine C-X-C motif ligand 9 (CXCL9), chemokine C-X-C motif ligand 10 (CXCL10), also known as IFN-γ-induced protein-10 (IP-10), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), interleukin-17 (IL-17), and interleukin-22 (IL-22)[22]. Recently, a whole blood culture system was used for evaluation of antigen-specific cytokine/chemokine gene and protein expression, and data obtained further confirm that CXCL9, CXCL10, IL-21, IL-13, and several acute-phase cytokines may serve as diagnostic host biomarkers of *M. bovis* infection in cattle [22].

The synchronous presence of infection in the herd of *Mycobacterium avium subsp. paratuberculosis* or non-pathogenic environmental mycobacteria [23], or co-infection with *Fasciola hepatica* [24], may compromise specificity and sensitivity values, thus affecting the reliability of diagnostic tools. Additionally, *M. bovis* infection is not detectable in animals with a depressed cell-mediated immune response [25]. Therefore, gross analysis of the carcass at slaughterhouses during post-motem inspection allows the confirmation of bTB in herd test reactors and provides additional data concerning infected animals that have not reacted in field tests.

During post-mortem diagnosis, all cattle intended for human consumption are exposed to routine meat inspection. Carcasses are assessed for typical gross and histopathological lesions resulting from bTB in naturally infected animals [26-28]. Gross pathology of bTB manifests as a chronic inflammatory disease with the formation of a granulomatous lesion affecting lungs, lymph node, intestines, liver and spleen [27]. These granulomatous lesions are characterized by different sizes, superficially or deeply located, caseous or calcified nodules bulging from the mucous or serous surface. The tissue analysis procedure via histopathology is as follows: paraffin-embedded, formalized tissues are finely sectioned and stained with Haematoxylin/Eosin and Ziehl Neelsen stains.

1.2.3 Control

Considering the zoonotic potential of bTB and its economic impact on animal production, many industrialized countries established specific eradication programs, which aimed to control the infection in susceptible animal hosts, thus reducing the risk for human infection [29, 30]. Several countries adopt bTB control programs, which constitute ‘tuberculin test

and slaughter' to control the bovine disease [29]. However, the incidence of bTB continues to increase (due to the presence of *M. bovis* wildlife reservoir) despite the use of test and slaughter control policy, highlighting the urgent need for a better control strategy [31]. An effective strategy for bTB control could involve cattle vaccination combined with a highly specific and sensitive diagnostic test that discriminates *M. bovis*-infected from vaccinated cattle. The ideal bTB vaccine would induce protective immunity without tuberculin skin test reactivity [32, 33].

So far, the only vaccine available for humans and bTB is the live attenuated Bacille Calmette Guerin (BCG) [34]. BCG derived from the serial passage of *M. bovis* confer protection against mycobacteria via the induction of Th1 responses [35-37]. Though BCG remains the standard against which the efficacy of any novel vaccine is judged, it is unlikely to fulfill the criteria defined for an ideal cattle vaccine. A reason for this is because BCG vaccination to control bTB can also sensitize cattle to the tuberculin skin test. That way, vaccinated cattle respond positively to the skin test [38]. The tuberculin test primary antigenic target is bPPD antigen, and it is present in both *M. bovis* and BCG vaccines [38]. This challenge can be overcome by applying a diagnostic antigen whose genes are lacking from the BCG genome. Thus, antigens whose genes are expressed by *M. bovis* but are lacking in environmental mycobacteria or BCG constitute candidates for diagnostic antigens that are more specific and better defined than PPD. This candidate antigenic target can help differentiate the BCG vaccine from *M. bovis* infection [38]. Primary antigenic targets (ESAT-6 and CFP-10) encoded by genes located on the RD1 region of the *M. bovis* genome but deleted from the BCG genome have been shown to discriminate between vaccinated and infected cattle successfully [39].

Other challenges incurred using BCG as a vaccine in cattle are due to a high degree of variability in the ability of BCG to protect against infection with *M. bovis* as demonstrated in field trials and challenge cattle experiments [40]. The variability in protection is associated with different BCG strains (with variable genetic content) exhibiting different protective efficacies [41]. Therefore, developing a new vaccine or boosting the immunogenicity of BCG as well as identifying candidate diagnostic antigens that can differentiate *M. bovis* infected and BCG vaccinated cattle is vital for bTB control. Many vaccine development approaches, such as MTBVAC (live attenuated vaccine), subunit, and modified BCG vaccines, are currently being explored for safer and more efficacious TB vaccines than BCG. These approaches have been successful in developing a large number of vaccine candidates included in the TB vaccine pipeline and are at different stages of clinical trials in humans[42]

1.3 *Mycobacterium bovis* cell wall antigens

The unique cell envelope of all members of *M. bovis* consists of three major layers: the plasma membrane (PM), the cell wall (CW), and the outermost capsular layer (OL) [43]. This thick multilayer cell envelope is highly hydrophobic with a very low cellular permeability. Therefore, it acts as a barrier against many classes of hydrophilic antibacterial drugs and chemotherapeutic agents. This has been implicated in the mycobacteria's ability to develop resistance to several anti-tubercular drugs [44]. The plasma membrane appears similar in structure to that of other bacteria, and it is composed of an asymmetrical bilayer of phospholipids decorated with glycoproteins and transmembrane proteins. Surrounding the plasma membrane is the cell wall consisting of

two segments: a lower segment of peptidoglycan (PG), which is covalently linked to the arabinogalactan (AG) layer [43]. The AG layer consists of galactofuranose, oligosaccharides and arabinofuranose, esterified to mycolic acids [43]. Lastly, the upper segment of the cell wall is made up of intercalating glycolipids and waxes [43]. Collectively, the cell wall surface is studded with various proteins, glycoproteins and glycolipids [45]. These cell envelope antigens exert a robust immunomodulatory effect because they are the first to interact with host immune cells.

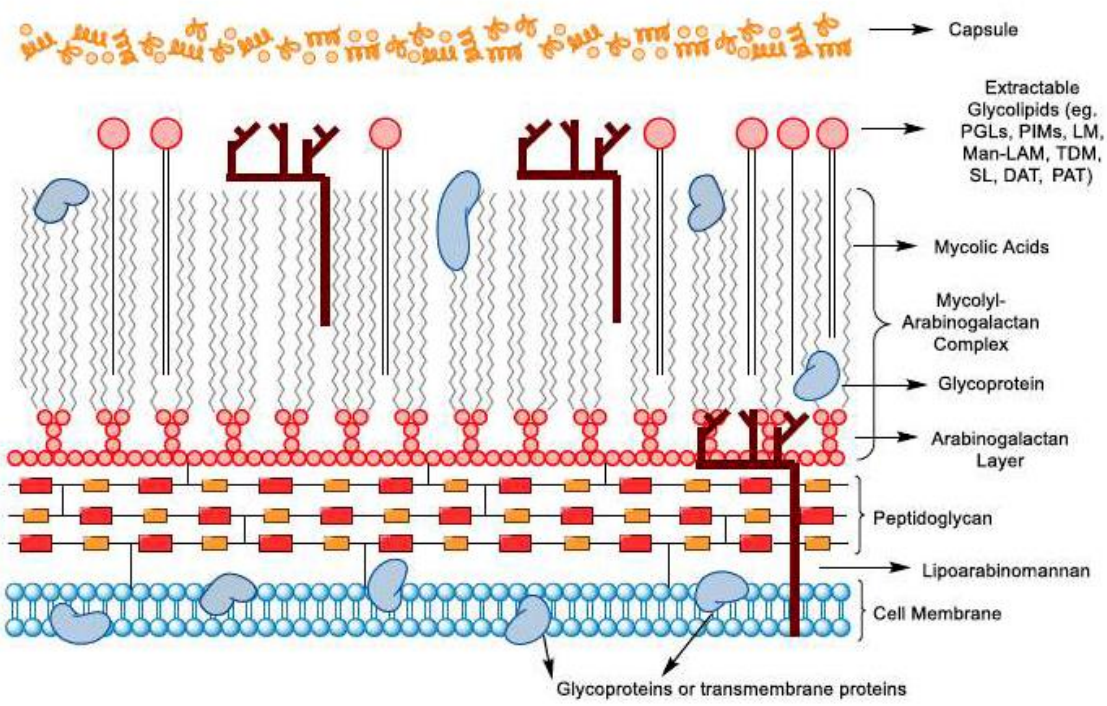

Figure 1.1: Diagrammatic representation of the cell envelope *of all members of M. tb complex.* The plasma membrane, cell wall, and outer capsule are depicted along with their comprising biomolecules [46]

1.3.1 Bovine purified protein derivatives (bPPD)

The first purified protein derivatives were developed by Florence B. Seibert in the US in 1934 [47]. Bovine purified protein derivatives contain a complex mixture of proteins, including the antigens ESAT-6 and CFP10, which are the antigens used in the *M. tb* specific IFN-γ release assays [48]. The primary eradication program for bTB involves the ability to diagnose and identify affected animals correctly. Thus, the eradication of *M. bovis* is dependent on the "test and slaughter" program, where cattle are consistently tested using the tuberculin skin test, which utilizes purified protein derivatives derived from *M. bovis*, and reacting animals are slaughtered [49]. Bovine purified protein derivatives in the skin test lack specificity as some of its antigenic components are present in non-pathogenic environmental mycobacteria and BCG.

The injection of bPPD induces inflammation resulting in a delayed-type of hypersensitivity response in TB-infected animals. The "delayed" response occurs within 3 days following exposure to antigen. Previous studies have reported that CD4+, CD8+, and γδ T cells are the primary T-cell subsets responding to bPPD stimulation in cattle experimentally infected with *M. bovis* [50]. Consistent with data obtained from cattle experimentally infected with *M. bovis*, another study reported a Th1 cytokine profile in response to bPPD stimulation in cattle naturally infected with *M. bovis* [51].

1.3.2 Cell wall lipids (CWL)

Many CWL has been implicated in the virulence of mycobacteria. These CWL consist of a complex mixture of polar, apolar lipids and glycolipids. The identified polar lipids

include diphosphatidyl glycerol (DPG), glucose monomycolate (GMM), phenylethanolamine (PE), and mono/di-acylated phosphatidyl inositol mannosides (PIMs) [52]. In contrast, the apolar Lipids consist of phthiocerol dimycocersates (PDIMs), trehalose dimycolate (TDM) and trehalose monomycolate (TMM), pentacyl trehalose (PAT), triacyl glycerol (TAG), phenolic glycolipid (PGL), and mono-mycolyl glycerol (MMG) [52]. These mycobacterial CWL are well known to regulate the function of a variety of innate immune cells [52-54]. Recent studies demonstrated that polar lipid fractions can alter the cytokine profile of bovine macrophages and DCs [52]. Furthermore, exposure of bovine APCs to polar lipids hinders antigen presentation by down-regulating MHC-II and other co-stimulatory molecules [52]. Thus, these data demonstrate that pathogenic mycobacterial polar lipids can block the ability of antigen-presenting cells to induce an appropriate immune response to an invading pathogen.

Similar to polar lipids, apolar lipids such as TDM have been reported to promote a tolerogenic phenotype in bone marrow-derived murine DCs activated with mycobacterial antigens and Toll-like receptor agonists, resulting in low expression of DCs activation makers and altered cytokine production [55]. Moreover, PGL expression is associated with increased mycobacterial virulence by downregulating host phagocyte inflammatory responses [56, 57]. In addition, PGL-1 and PGL-tuberculosis produced by the clinical isolates of *M. tb* block TLR2 agonist-driven activation NF-κB and cytokine production [58], as well as decreased TRIF dependent TLR4 signaling in macrophages resulting in limited pro-inflammatory and bactericidal responses [59]. Collectively, these studies

suggest that CWL contributes to the pathogenicity of mycobacteria by blocking the generation of robust innate immune responses.

1.3.3 Cell surface sugar extract (CSSE)

Mycobacterial-derived CSSE fractions, enriched in glycolipids and glycans, play prominent roles in the evasion of host immune responses [46, 60]. For example, Phosphatidylinositol mannosides derived from *M. tuberculosis* have been shown to inhibit LPS activation of human DCs by reducing the up-regulation of MHC II molecules and pro-inflammatory cytokine production [61]. Moreover, *M. tuberculosis*-derived LM reportedly blocks the biosynthesis of TNF-α in human macrophages by destabilizing TNF mRNA transcripts, thereby allowing *M. tuberculosis* to evade host immune response and potentially increase its virulence [62]. Furthermore, ManLAM has been shown to block IL-12 production by human DCs previously stimulated with LPS [63] and inhibit LPS-induced DCs maturation [64]. Recently, it was reported that Di-*O*-Acyl-trehalose promotes a tolerogenic phenotype in bone marrow-derived murine DCs activated with mycobacterial antigens, resulting in low expression of DCs activation markers and altered cytokine production [55]. These studies suggest that CSSE fraction contributes to host evasion by mycobacteria.

1.3.4 Phenolic glycoconjugates

Phenolic glycoconjugates belong to CSSE antigenic class, and it consists of phenolic glycolipid (PGL) and structurally related parahydroxybenzoic acid derivatives (*p*-HBADs). Both share the same glycosylated aromatic nucleus. The aromatic core is derived

from the methyl ester of *p*-hydroxybenzoic acid (*p*-HBA) and synthesis of both *p*-HBADs and PGL is thought to proceed from *p*-hydroxybenzoic acid [65]. The glycosyl moieties in *p*-HBADs are identical to mycoside B in PGL-tb, and display potent immunomodulatory activities [65]. While few mycobacterial clinical isolates synthesize PGL, *p*-HBADs are secreted by all mycobacteria strains [65, 66]. It has been shown that *M. bovis* and BCG produce only *p*-HBAD-I (monosaccharide), while *M.tb* generates both *p*-HBAD-I and II (trisaccharide) (Stadthagen et al., 2005). Moreover, Scanlan *et al.* reported the chemical synthesis of *p*-HBADs, accompanied by biological studies on the immunological effects of the molecules on immune cells [65]. They showed that the synthesis of *p*-HBADs involves chorismate pyruvate lyase enzyme during host infection and is released in culture filtrates [67]. The immune-modulatory role of *p*-HBADs has been studied in mycobacteria [68]. Previous studies have shown that *M.tb* mutant defective in the synthesis of *p*-HBAD-I and II promote a more robust inflammatory response than wild-type strains [68]. Furthermore, *in vitro* studies with mouse splenocytes and bone marrow-derived macrophages showed that *p*-HBAD-I and II derived from *M. tuberculosis* strain can suppress host immune response and enhance TB pathogenesis [69]. A recent report demonstrates that *M. tuberculosis* derived *p*-HBAD-I and related structures affect acute macrophage activation by inhibition of pro-inflammatory response and reduction of bactericidal nitric oxide production following BCG vaccination [70]. Thus, this indicates that the presence of *p*-HBAD-I and related molecules could be undermining the innate protective response.

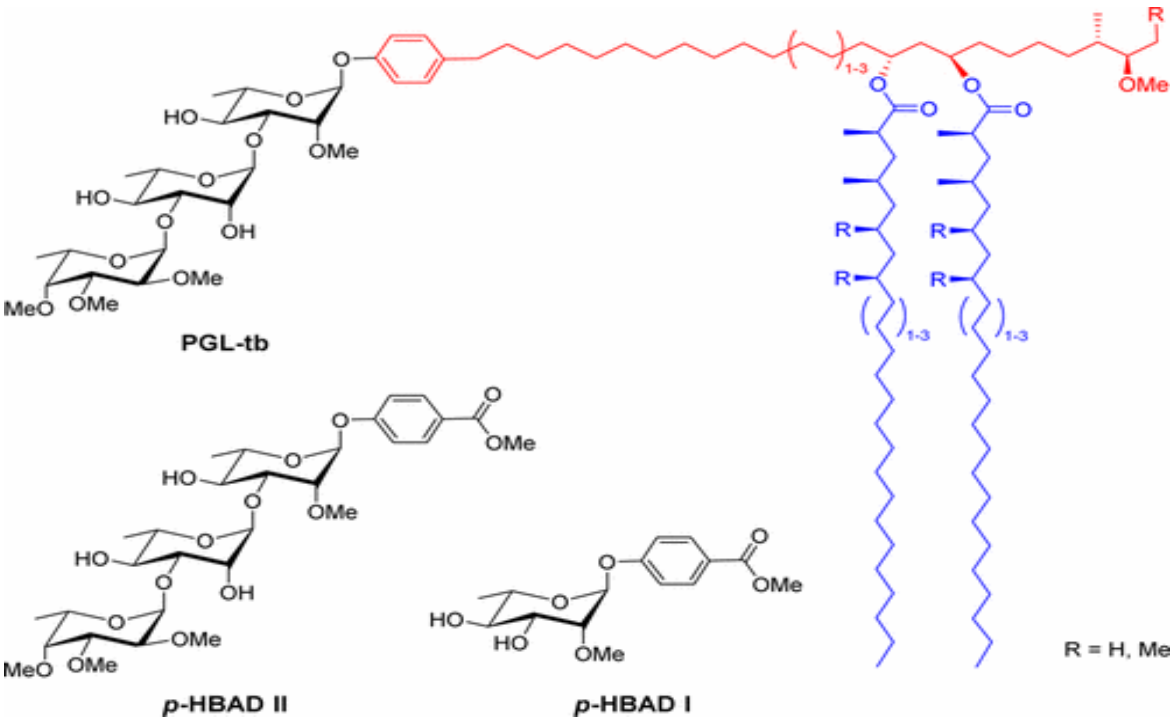

Figure 1.2: Structures of phenolic glycolipid of *M. tb* (PGL-tb) and structurally related glycans, *para*-hydroxybenzoic acid derivatives I and II (*p*-HBAD I and *p*-HBAD II) [46].

1.4 Pathogenesis of *Mycobacterium bovis*

Infection is initiated following inhalation of a droplet containing bacilli of size ranging between 1-5μm in diameter [71]. Inhaled bacilli avoid the bronchi's defense mechanism and penetrate the terminal alveoli, where they are recognized and phagocytized by resident alveolar macrophages [71]. Bacterial components are detected by host receptors expressed on alveolar macrophages and other immune cells via Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and C-type lectins [72, 73].

During the first ten days of infection with *M. bovis*, alveolar macrophages switch towards an M2 phenotype with an anti-inflammatory response, which enables bacilli to manipulate host immune responses to favor their survival [74]. Towards the end of ten days post-infection, infected alveolar macrophages transmigrate from the alveolar space to the lung interstitium via the help of cytokine released by alveolar epithelial cells. In the interstitium, infected alveolar macrophages release chemokines and cytokines, which attract monocytes and other inflammatory cells. During the acute phase of infection, signals from *M. bovis* infection polarize interstitial macrophages to the M1 phenotype. Switch over of M2 macrophages to M1 phenotype is accompanied by aerobic glycolysis resulting in enhanced pro-inflammatory response, subsequent elimination of non-virulent mycobacteria, and formation of primary TB lesion [74].

However, virulent *M. bovis* evade interstitial macrophage microbicidal mechanisms via its virulence factor known as ESAT- 6, resulting in the induction of adaptive immune response. DCs mediate induction of adaptive immune response against mycobacterial infection. Infected DCs migrate to the regional lymph node and prime T-cells by up-regulation of chemokine receptor 7 (CCR7) (Worbs, Hammerschmidt [75]. Depending on the host immune response, primed T-cells and other immune cells accumulate at the infection site resulting in total clearance of infection or granuloma formation. Though immunocompetent hosts clear off the bacteria. However, in the case of immunocompromised hosts, virulent bacilli capable of surviving escape killing and persist in the chronic phase [76]. Thus, the host protects itself by switching from host resistance to host tolerance by the formation of granuloma. The granuloma wall off tubercle bacilli from the rest of the lung tissue, thereby limiting the spread of bacteria and provides a

microenvironment for interactions among macrophages and other immune cells as well as cytokines produced by these cells.

Two distinct types of granulomatous lesions exist (chronic and necrotic granulomas) depending on the host immune response [77]. Histopathological studies have demonstrated that the classic chronic granuloma comprises epithelial macrophages, neutrophils, and other immune cells surrounded by fibroblasts [78]. The chronic granuloma with neutral pH consists of dead macrophages and other immune cells with non-replicating *M. bovis* residing inside macrophages in the hypoxic center [78]. The necrotic granuloma with acidic pH possesses actively replicating bacilli surrounded by immune cells [79]. Infected cattle with chronic or necrotic granuloma manifest signs of latent or active infection, respectively. While latently infected cattle show no sign and rarely transmit diseases, actively infected animals show signs and transmit the disease to both immunocompetent and immunocompromised cattle. Thus, the establishment of both latent or active TB infection depends on host immune system.

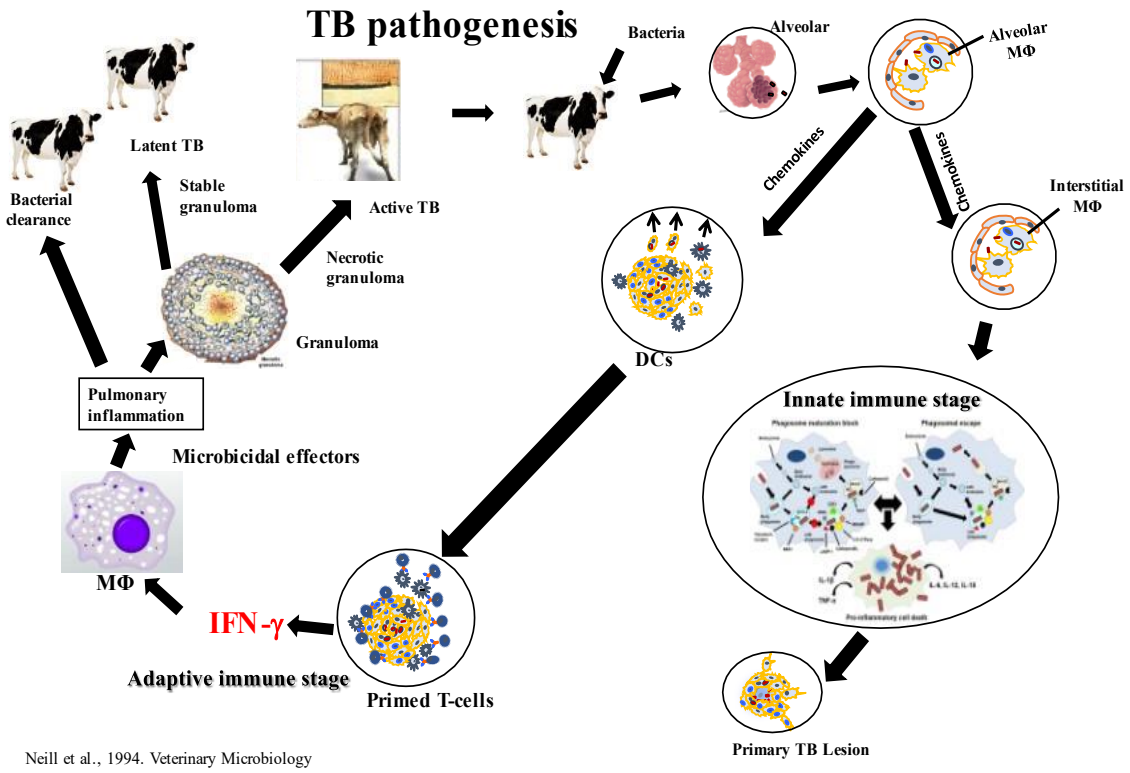

Figure 1.3: Pathogenesis of bovine TB- modified from Neil et al. 1994 [80]

1.5 The Immune System

The immune system protects the host via a highly regulated network of cells and molecules, which work synergistically. Upon encountering a threat, the immune system responds with diverse mechanisms to control the threat. The immune system is split up into two separate arms, the innate and the adaptive immune system [81]. While innate immune cells respond quickly to invading pathogens, adaptive immune cells exhibit a delayed response and form immunological memory [82]. It is established that both innate and acquired immune defenses are involved in defending the host against *M. bovis* infections and vary depending on the host species, the virulence of an *M. bovis* strain, and host genetics [83].

1.5.1 Innate immune response to *M. bovis*

Alveolar macrophage (AM) is a subset of tissue-resident macrophages that reside within the lung airspace and play crucial roles in lung homeostasis, surfactant metabolism, and tissue repair [84]. Alveolar macrophages are the first cell type to encounter *M. bovis*. In mice, a productive *M. tuberculosis* infection starts with an infection of AM that resides in the lung alveoli [85]. Depleting AM prior to infection reduces the bacterial burden in the lungs and increases survival, suggesting that AM forms a replicative niche early after infection [86]. The reverse is seen in the growth-restrictive environment within M1 interstitial macrophages. In addition to eliciting inflammatory cytokine in interstitial macrophages, pathogen recognition by innate immune cells triggers a cascade of cellular events such as phagocytosis, autophagy, apoptosis, and induction of adaptive immunity. All these cellular events contribute to the elimination of invading pathogens [87].

During phagocytosis, various host cell receptors are involved in the uptake of *M. bovis* by macrophages [88]. Ultimately, the fusion of mycobacteria-containing phagosomes with lysosomes results in bacterial killing. The importance of IFN-γ during *M. tuberculosis* infection has been attributed to its ability to activate microbicidal mechanisms of macrophages, most notably via the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and expression of the enzyme inducible nitric oxide synthase (iNOS) [89]. iNOS catalyzes the production of the bactericidal/static radical NO, which is critical for controlling *M. tuberculosis* infection, as Nos2–/– mice are highly susceptible to infection [90].

1.5.2 Cell-mediated immune response against *M. bovis* infection

Cell-mediated immunity activates phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Although innate immune responses eliminate invading pathogens by the induction of adaptive immunity, dendritic cells recruited to the site of mycobacterial infection mediate this effector function. These dendritic cells bridge innate and adaptive immunity, traveling from sites of infection and inflammation to secondary lymphoid tissues for activation of T-cells. Antibody-based depletion of CD11c+ cells, which transiently eliminates both classical and monocyte-derived DCs, results in defective CD4 T-cell priming and increased susceptibility to *M. tb* infection, demonstrating the importance of CD4 T-cells and DCs for host defense [91].

The accumulation of IFN-γ producing Th1 cells at the inflammatory site mediate protective immunity by activating mycobacteria-infected macrophages. Although it is assumed that IFN-γ producing CD4 T-cells are required for induction of protective immunity, however, it has been reported that IFN-γ is not a reliable correlate of TB protection because IFN-γ levels poorly correlate with TB protection [92]. Recently, the induction of polyfunctional lung tissue-resident memory CD4 T-cells (CD4+TRM) responses has been established to protect against mycobacterial infection [93, 94]. The protective response of CD4+TRM cells against mycobacterial infection is partly based on reports demonstrating their localization at the site of pathogen infection [93].

1.6 Innate immune cells

The innate immune cells develop from the bone marrow-derived multipotent hematopoietic stem cells [95]. The establishment of mycobacterial infection depends on its early interaction with host innate immune cells, such as natural killer (NK) cells, neutrophils, and phagocytes (monocytes, macrophages, and dendritic cells) [96]. These innate immune cells express various pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs). All these receptors play a role in pathogen recognition, uptake of mycobacteria, and initiation of intracellular signaling events that result in antimicrobial function [97]. Thus, the innate immune cells, signaling pathways, and cellular functions that are involved in the early phases of *M. bovis* infection are crucial in limiting disease and serve as potent regulators of antigen-specific adaptive immunity

1.6.1 Macrophages

1.6.1.1 Lung macrophages origin and development

The lung consists of two main types of macrophages that reside in different anatomical compartments: interstitial and alveolar macrophages [98]. Macrophages are classified based on their origin as either derived from fetal/embryonic precursors that primarily self-renew or from adult blood monocytes that evolve from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow [99]. Earlier studies on mice have demonstrated that alveolar macrophages are predominantly of embryonic origin in a steady-state, as they are nurtured and sustained independently of circulating monocyte [100]. Specifically, mouse

alveolar macrophages evolve from fetal monocytes that seed into the lung and differentiate after birth into mature alveolar macrophages under the influence of GM-CSF, TGF-β, and PPAR [101, 102].

In contrast, interstitial macrophages have a mixed origin, originating from blood and lung monocytes in mice with a minor early contribution from yolk sac macrophages [103]. The mixed origin may relate to distinct populations of interstitial macrophages that occupy specific niches in the lung. The monocytic origin of interstitial lung macrophages may explain why their energy metabolism relies on glycolysis, whereas alveolar macrophages mainly use fatty acid oxidation [74]

1.6.1.2 Macrophage phenotypes and role in mycobacterial infection

Through the process of polarization, macrophages can change their phenotypic and functional profiles [104]. Macrophage polarization is the process by which macrophages respond to stimuli from the local microenvironment and acquire a specific functional phenotype. Based on the secretion of certain specific cytokines and switch in cell metabolism, naïve macrophages (M0) can polarize to either classical/pro-inflammatory (M1) or alternative/anti-inflammatory (M2) phenotype [105, 106].

The M1 phenotype is obtained following exposure to a pathogenic molecule or cytokines (tumor necrosis factor-α/Interferon-gamma) [107]. Thus, M1 phenotypes are associated with an increase in aerobic glycolysis, which is essential for the optimal production of pro-inflammatory cytokines and suppresses the anti-inflammatory cytokine, resulting in reduced bacterial load. The mediators of host metabolism such as hypoxia-inducible

factor-1 (HIF-1), glucose transporters (Glut-1), and mammalian target of rapamycin (mTOR)) enhance induction of the glycolytic enzymes and metabolic shift to aerobic glycolysis [108]. The M1 macrophages are critical in host defense, and the mediators they produce can lead to host-tissue damage. Hence, their activation must be tightly controlled.

However, M2 macrophages are induced upon exposure to IL-10/IL-14/IL-13 or TGF-b. *In vitro* treatment of macrophages with IL-4 and IL13 (alternative activation) results in the failure of antigen presentation to T-cells, and therefore minimal cytokines are produced from these macrophages. Alternatively, activated macrophages are also less efficient in producing toxic oxygen/ nitrogen radicals and killing of intracellular pathogens than classically activated macrophages [109]. The M2 macrophages depend on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) for energy generation. This phenotype is associated with enhanced fatty acid metabolism [110]. The accumulated lipid serves as a nutrient for bacterial growth and survival by down-modulation of immune responses via anti-inflammatory cytokine release [110]. Other signaling proteins associated with M2 polarization include a signal transducer and activator of transcription 6 (STAT 6), GATA binding protein (GATA 3), suppressor of cytokine signaling (SOCs 1& 3) and arginase 1[111]. All these molecules regulate the anti-inflammatory profile of M2 macrophages, allowing the resolution of excess inflammation and tissue repair**.**

1.6.2 Dendritic cells

1.6.2.1 Dendritic cells development and activation

Dendritic cells, so named because of their distinctive dendritic processes, are professional antigen-presenting cells localize in tissues that are in communication with the skin and mucosal surfaces [112]. Dendritic cells are infrequent and are not abundantly distributed within the tissues as macrophages [113]. Like other phagocytes, DCs develop from macrophage/DCs progenitors (MDPs) in the bone marrow [114]. Four primary stages of DC development have been identified, including bone marrow progenitors, circulating precursor DCs, and tissue-resident immature or mature DCs. Precursor DCs patrol and circulate in the blood before populating peripheral tissues (e.g., the lungs) and lymphoid tissues. Granulocyte monocyte colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) can induce DCs differentiation from precursors cells [115].

Dendritic cells are described as either immature or mature based on their phenotypic characteristics and function. In steady-state, DCs are immature and are characterized by the high capacity to capture antigens, low expression of activation markers, and decreased secretion of cytokines [116]. Following exposure to an antigen, immature DCs undergo transformational changes and become mature DCs, which is associated with changes in their phenotypic and functional characteristics [117]. Inflammatory stimuli, such as lipopolysaccharide (LPS) and TNF-α can also facilitate DCs maturation [118]. Mature DCs lack the capability of antigen uptake. However, they are known for antigen presentation and have a higher capacity for activating naïve T-cells to initiate adaptive immune responses [119]. Thus maturation of DCs results in upregulation of major

histocompatibility complex (MHC) molecules [120], co-stimulatory molecules (CD40, CD80, and CD86), lymph node-homing chemokines (CCR7), and increased production of pro-inflammatory cytokines required for T-cell activation [119].

The pro-inflammatory cytokines produced by the mature DCs polarize T-cell response into different types of cell-mediated immunity [121]. Thus, according to the allergen sources, with pro-inflammatory cytokines and participation of costimulatory signals, DCs induce the polarization and differentiation of distinct types of T-cell responses that align well with different classes of pathogens (e.g., Th1, Th2, Th17, or regulatory T cells). Interleukin 12 (IL-12) production by DCs is crucial for the differentiation of naïve T-cells into Th1 cells that produce IFN-γ, an important cytokine needed to control mycobacterial infection [122]. In contrast, lack of IL-12 production by DCs results in Th2 response and susceptibility to mycobacterial infection [123].

1.6.2.2 Role of DCs in mycobacteria infection

Pathogen-associated molecular patterns (PAMPs) induce an inflammatory response initiated via pattern recognition receptors (PRRs) expressed on dendritic cells [121]. The early wave of proinflammatory cytokines and chemokines released by DCs inhibits pathogen spread and prime T-cells to eradicate the invading pathogen [121]. For the early pro-inflammatory response, activation of DCs is triggered by PRR signals. These signals transform resting DCs into strong antigen-presenting cells capable of boosting the expansion and differentiation of naive pathogen-specific T-cells to effector T-cells [121, 124]. Following infection, DCs are required for induction of the cell-mediated immune response by promoting the dissemination of mycobacteria and its antigen from the infection

site to the local draining lymph node [125]. Chemokine homing receptors (CCR7 and CCR8) expressed on monocyte-derived DCs promote migration from lung tissue into the local draining lymph nodes [126, 127]. Following transportation of mycobacteria to draining lymph node by myeloid DCs, only tissue-resident DCs presents antigen and prime CD4 T-cells in the lymph node [128, 129]. Within the granuloma, the role of DCs varies depending on the stage of infection. DCs within acute granulomas upregulate the expression of costimulatory molecules, promote priming of naïve T-cells, and reactivation of newly arrived IFN-γ producing Th1 cells [129]. However, DCs in chronic lesions promote latent TB infection by inducing a high level of PD-L1 expression and do not support the reactivation of newly recruited T-cells, thereby decreasing protective T-cell responses, thus acting as a shield that facilitates mycobacterium survival [128]. Although mycobacteria infect both macrophages and DCs, infected DCs lack the ability to control mycobacteria efficiently as macrophages [130, 131]. This suggests that infected DCs, may serve as a long-term reservoir for mycobacteria, thereby promoting their survival within the granuloma during chronic infection. The DCs shield may explain why mycobacteria adapt for long-term survival in granulomatous lesions.

1.6.2.3 DC activation markers

The full activation of T-cells requires two signals. The primary signal is mediated by antigen-presenting molecules, while the secondary signal is provided through costimulatory molecules. The up-regulation of antigen-presenting and costimulatory molecules occurs nearly simultaneously.

1.6.2.3.1 Antigen-presenting molecules (MHCI and II) and mycobacterial infection

Mature DCs express high levels of MHC I and II molecules. The class II transactivator (CIITA) is a master regulator of MHC gene expression. It induces *de novo* transcription of MHC class II genes and enhances constitutive MHC class I gene expression [132]. The role of MHC molecules is to bind pathogen-derived peptide fragments and display them on the cell surface for detection by the appropriate T-cells via T-cell receptors [133]. Dendritic cells are highly efficient at antigen presentation and adaptive immune cell activation due to their ability to determine if the antigen is endogenous or exogenous [134]. To communicate with CD8 T-cells, MHC-I presents endogenous peptides to CD8 T-cells by the classical pathway via the endoplasmic reticulum (ER) and the transporter associated with antigen processing (TAP) complex [135]. The classical TAP-dependent MHC-I pathway is critical in host defense against *M. tuberculosis* [136]. In addition, mice lacking β2-microglobulin (β2m), a component of the MHC-I chain, cannot stably present antigens on the surfaces of their cells. Recent studies using β2m knockout (KO) mice have shown that MHC-I plays a vital role in host defense against several intracellular bacterial pathogens, including *M. tuberculosis* and *Listeria monocytogenes* [135, 136]. Furthermore, the evidence that *M. tuberculosis* infection induces severe disease and a significant reduction of CD8 T-cells in MHC-I-deficient β2 $m^{-/-}$ mice indicates that MHC-I-restricted antigen-specific CD8 T-cells are crucial for host defense against *M. tb* infection.

Also, DCs are the only antigen-presenting cells with the unique ability to undergo cross-presentation and display exogenous antigens on MHC-I molecules to activate CD8 T-cells [137]. The intracellular pathway for DCs cross-presentation is still under debate. However,

two pathways proposed for the cross-presentation of exogenous antigens are the cytosolic and vacuolar pathways [138]. In the cytosolic pathway, an exogenous antigen undergoes phagocytosis and is transported to the phagosome, followed by exportation to the cytosol, where it is degraded to a peptide by the proteasome [139]. From there, the antigenic peptide can be re-imported into the phagosome (phagosomal loading) or transported to the ER (ER loading) for MHC-I loading and cell-surface antigen presentation [140]. In the vacuolar pathway, exogenous antigens undergo phagocytosis followed by transportation to the phagosome for degradation. The antigenic peptides are loaded on the MHC class I molecule in the phagosome then transported to the cell surface for antigen presentation [141].

In contrast to MHC-I molecules, MHC-II molecules are present only on the surfaces of antigen-presenting cells, such as dendritic cells, B-cells, and macrophages. MHC-II molecules are transmembrane αβ heterodimers, and they present exogenous antigens from phagocytosed compounds to CD4 T-cells, which is critical for the expansion and function of CD4 T-cells during host immune responses [137, 142]. *In vitro* exposure to *M. tuberculosis* or its lipoproteins drives DCs maturation and the increased expression of co-stimulatory and MHC class II molecules [143, 144]. Despite the proposal that TLR stimulation increases DCs antigen presentation, a mycobacterial infection might interfere with MHC-II antigen processing and the presentation of *M. tuberculosis* antigens by DCs. For instance, ManLAM derived from *M. tuberculosis* is a DC-SIGN ligand, which inhibits DCs maturation [145]. In addition, Some *in vitro* studies indicate that infection of DCs with *M. bovis* BCG ultimately leads to loss of MHC-II molecules, making DCs another possible niche for immune evasion by mycobacteria [124]. Furthermore, *in vivo* infection

of lung DCs after aerosol administration of GFP-expressing *M. bovis* BCG led to poor expression of MHC-II molecules relative to expression in uninfected DCs from the same lung [146].

1.6.2.3.2 Costimulatory molecules (CD40, CD80 and CD86) and mycobacterial infection

The ligands CD80 and CD86 are predominantly expressed on APCs, such as B cells, dendritic cells, macrophages, and monocytes. The expression of these molecules is upregulated in response to inflammatory stimuli. However, the timing and pattern of expression of these molecules differ. While CD86 is expressed at moderate levels at steady-state and upregulated rapidly in response to inflammatory stimuli, CD80 is found at low levels in the absence of inflammation and is upregulated more slowly than CD86 after activation [147]. Also, CD86 is expressed as a monomer, while CD80 is a dimer with stronger binding affinities than CD86 to CD28. CD28 was one of the first costimulatory molecules to be identified and is a surface protein expressed on T-cells. Engagement of CD28 with CD80 and CD86 provides a second signal required for optimal T-cell activation and differentiation [148]. Antigen presentation to T-cells in the absence of costimulation can lead to clonal T-cell anergy[149].

Several studies demonstrated the role of mycobacteria in downregulating the expression of co-stimulatory molecules on APCs [128, 150]. A recent study, albeit for BCG, shows that MHC-II, CD80, CD86, and CD40 are down-regulated during the chronic phase of infection [128]. The down-modulation of CD80/CD86 in the chronic phase of infection suggests that mycobacteria may exploit this pathway to anergize the T-cells. Another study reported

that the expression of costimulatory and MHC II molecules are downregulated in macrophages infected with fluorescent reporter bacteria [128, 146]. In addition, it has been shown that cell wall lipid trehalose 6, 6′-dimycolate (TDM) of *M. tuberculosis* inhibits the expression of costimulatory molecules on the surface of the macrophages [151]. The importance of CD80/CD86 in controlling mycobacterial infection was further demonstrated in CD80/CD86 double knockout mice [152]. In contrast, others suggested the augmentation of costimulatory molecules upon infection [153]. This discrepancy may be primarily dependent on the strain, system, or time-point of the study.

1.6.2.4 Regulatory DCs and effect on the immune response to mycobacterial infection

The immune system exists in equilibrium between tolerance and effective inflammatory responses. Recent findings suggest that innate immune cells (in particular DCs) are essential players in the induction of effective immunity and tolerance [154]. Immunogenic DCs mediate effective innate and adaptive immunity primarily due to their remarkable capacity to process and present antigens through major histocompatibility complexes to T-cells [155]. Unlike immunogenic DCs, regulatory DCs can induce tolerance resulting in T-cell anergy and deletion [156]. Several factors including DCs phenotype, antigen detection receptors, DCs maturation state, and exposure to microbial and soluble inflammatory factors, play a crucial role in the induction of regulatory DCs [157-160]. Soluble inflammatory factors such as TGFβ, IL-10, or PGE_2 are the key players in the induction of regulatory DCs by inhibiting the upregulation of DCs activation markers [161, 162].

Regulatory DCs can be characterized by the combination of surface marker expression (PD-L1, PD-L2, B7-H3, B7-H4, CD103, ILT3/4) and cytokine (IL-10, IL-1β, TGF-β)

production [163]. Other immunoregulatory factors produced by regulatory DCs include indoleamine 2,3 dioxygenase (IDO), arginase I and inducible nitric oxide synthase (iNOS) [163]. In addition, regulatory DCs express a normal level of MHC-II, while expression of the costimulatory molecules is low or deficient [164-166]. By the lack of costimulatory molecules, regulatory DCs are not fitted to provide T-cells with the necessary signal two required for full T-cell activation.

Considering the prolonged coevolution of the tubercle bacilli with humans, the bacillus has evolved the capacity to persist in the host tissue in a dormant state. This ability primarily depends on cell wall glycolipids, which target antigen-presenting cells, thereby dampening effective T-cell immunity [61, 167]. A recent study demonstrated that mycobacterial glycolipid Di-*O*-acyl trehalose induces a tolerogenic phenotype in DCs by altering DCs maturation, leading to the expansion of regulatory T-cells [55]. In a murine model, regulatory DCs in chronic mycobacterial granulomas block protective T-cell response via the PD-1:PD-L signaling pathway, thereby promoting prolonged survival of bacteria [128]. This suggests that the regulatory DCs contributes to the induction of immune tolerance and survival of mycobacteria.

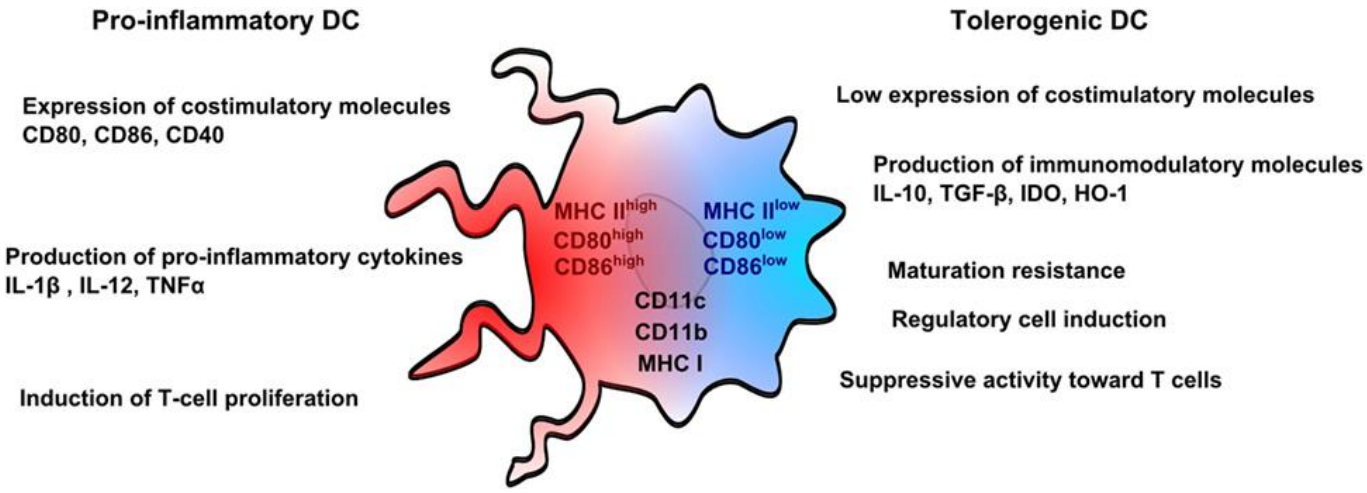

Figure 1.4: Immunogenic and tolerogenic DC profile. Modified from Marin et al. 2018 [168]

1.6.2.4.1 Inhibitory markers expressed by regulatory DCs

1.6.2.4.1.1 Programmed death-ligand 1 (PD-L1) and role in mycobacterial infection

Two major PD-1 ligands exist namely PD-L1 and PD-L2, and they differ in their affinities for PD-1. PD-L2 exhibits a stronger affinity for PD-1 as compared to PD-L1 [169]. While expression PD-L2 is inducible in fewer cell types (mainly antigen-presenting cells), PD-L1 is broadly or constitutively expressed on hematopoietic and non-hematopoietic cells [170]. The inflammatory cytokines control the expression of PD-L1 and PD-L2. In particular, PD-L1 expression on APCs is up-regulated following stimulation with IFN-γ and toll-like receptor ligands [171, 172]. Studies have shown that DCs can suppress T-cell responses by providing inhibitory signal via binding of PDL-1 on DCs with the PD-1 molecules expressed on T- cells [173]. Thus, the PD1/PDL-1 pathway is a key signaling pathway that terminates immune responses resulting in immune tolerance [174]. The PD-1/PD-L pathway produces inhibitory signals that control both central and peripheral T-cell tolerance. Also, it has been demonstrated that the PD-1/PD-L pathway controls peripheral

T-cell tolerance by limiting the activation, expansion, and function of self-reactive T-cells [175]. The elimination of PD-L1/PD-I signaling pathway results in the breakdown of tolerance and promotes autoimmunity.

In mycobacterial infection, $CD4^+$ T cells exert strong cytokine production at the initial stage of infection, but these robust cytokine responses are diminished during the later stage of infection [176, 177]. This suggests that an antigen-specific T-cell response is suppressed by some inhibitory mechanism, thereby allowing mycobacteria persistence in the host. The PD-1/PD-L1 signaling pathway has been reported to impair the protective Th1 immune response in the later stage of infection with *M.bovis* bacillus Calmette–Guérin resulting in bacteria persistence in host tissue [178]. A recent study further confirms that *M. tuberculosis* antigens suppress Th1 immune response and facilitate lung cancer metastasis via the PD-1/PDL-1 signaling pathway [179]. This indicates that the PD-1/PD-L1 signaling pathway blockade may benefit patients with *M .tb* or other chronic infections.

1.6.2.4.1.2 Indoleamine 2,3-dioxygenase and role in mycobacterial infection

Indolamine-2,3-dioxygenase (IDO2) is a cytosolic immune-regulatory enzyme that plays a role in immunological tolerance [180]. The IDO2 oxidizes/converts the essential amino acid (tryptophan) to kynurenine (Kyn) by cleaving the 2,3-double bond of the indole ring [181]. The absence of tryptophan due to catabolism by IDO2 prevents T-cell proliferation due to starvation. Thus, T-cells are highly sensitive to a low concentration of tryptophan resulting in cell cycle arrest [182]. The IDO2 enzyme expressed in various tissues and antigen-presenting cells (DCs and macrophages) become up-regulated in response to

inflammation (specifically bacterial lipopolysaccharides and cytokines such as IFN-γ) [183]. It has been demonstrated that human dendritic cells expressing IDO2 are capable of blocking T-cell proliferation, suppress effector T-cells, and promote differentiation of naïve T-cells to regulatory T-cells [184-186]. The expression and activation of IDO2 create a local immunosuppressive micro-environment that promotes pathogen survival [187]. The role of IDO2 in immune regulation is observed in various disease states such as autoimmune disorders, tolerance in transplantation, and response to infection.

Expression and activation of IDO2 have been demonstrated in both mice and non-human primates with mycobacterial infection [188]. In mice, enhanced activation of IDO2 has been linked to poor TB outcomes [189]. In non-human primates, IDO2 induction in the granuloma is associated with active TB disease [190]. In humans exposed to *M. tuberculosis*, significant IDO expression levels have been reported [191, 192]. This evidence from both human and animal studies indicates that high expression of IDO2 in the granuloma compromises T-cell function, thereby supporting mycobacterial proliferation and survival [188, 190]. Blocking the expression and activity of IDO2 via gene silencing plays a role in reducing pathogen burden, minimize TB pathology and enhance host survival [188]. In addition, the inhibition of IDO2 is associated with the restoration of T-cell proliferation and functions [188]. These findings indicate that IDO2 activation plays a vital role in *M. tuberculosis* pathogenesis

1.7 Innate immune receptors

Sensing and recognition of pathogens by the innate immune system is mediated by a number of germline-encoded receptors, which detect pathogen-associated molecular

patterns (PAMPs) released by the invading pathogens. The receptors involved in detecting these structures are called pattern recognition receptors (PRRs), expressed on the cell surfaces of innate immune cells [193]. PRRs are broadly divided into two: Transmembrane proteins (Toll-like receptors and C-type receptors) and cytoplasmic proteins (Retinoic acid-inducible gene I-like receptors and NOD-like receptors) [193, 194]. The detection of PAMPs by PRRs induces an inflammatory response, resulting in a rapid innate immune response. In addition, the signal from the activated receptors plays a crucial role in the expression of costimulatory and MHC molecules on professional antigen-presenting cells, resulting in the generation of an effective immune response against pathogens.

1.7.1 Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) receptors

DC-SIGN is mainly expressed on immature DCs, macrophages and karyocytes, and is one of a few lectin-like receptors involved in initiating signaling cascades after binding ligands [195]. The expression of DC-SIGN is mainly induced by IL-4 [196]. As with other lectin-like receptors, DC-SIGN can successfully direct foreign antigens to late endosomal/lysosomal compartments for efficient processing and presentation to T-cells, specifically CD4 T-cells [197], and in some cases naïve and memory CD8 T-cells [198].

Active participation of DCs to peripheral tolerance depends on how DCs sense or detect foreign antigens. For instance, DCs activation via Toll-like receptors (mainly TLR-4 and TLR-2) induces an immunogenic response. In contrast, DC-SIGN (lectin-like receptors) favor tolerogenic DCs that produce anti-inflammatory factors and activate regulatory T-cells [159]. Many pathogens exploit DC-SIGN binding to evade host immune response.

The DC-SIGN has been identified and characterized as a key receptor for *M. tuberculosis* in human DCs and alveolar macrophages [160, 199]. This lectin-like receptor is capable of sensing mycobacterial-derived mannose-containing molecules such as mannose-capped LAM (Man-LAM), lipomannan (LM), arabinomannan, glycoproteins, PIMs, and α-glucan. Thus, *M. tuberculosis* utilizes its ability to interact with DC-SIGN to evade immune detection [200]. For instance, the activation of dendritic cells by ManLAM via DC-SIGN impairs maturation and induces the anti-inflammatory cytokine IL-10 [160]. In this manner, the bacillus prevents the proper activation of DCs, given that IL-10 inhibits the expression of co-stimulatory molecules and the production of IL-12, which are essential for the activation of Th1 cells. A recent study showed that macrophages found in tuberculous pulmonary lesions of non-human primates express DC-SIGN, which enable *M. tb* to parasitize macrophages, and also turn off the pro-inflammatory response in these cells to prevent potential immunopathology [200]. Therefore, mycobacteria target DC-SIGN to infect DCs and shift the Th1- versus Th2 cell balance towards Th2 in favor of the pathogen's persistence.

1.7.2 Toll-like receptors (TLRs)

TLRs are members of the type-1 transmembrane receptor family that are evolutionarily conserved proteins among vertebrates and invertebrates [201]. It belongs to one of the most essential and functionally characterized pattern recognition receptors playing a crucial role in innate immunity [202]. TLRs, named after the *Drosophila melanogaster* toll protein, are membrane-bound sensors present on cell plasma membranes and endosomes [203]. Prototypical TLR proteins are structurally characterized by two major domains, namely-

an extracellular domain that contains hydrophobic tandem leucine-rich repeat (mediates the recognition of pathogen molecular patterns) and the cytoplasmic tail that contains a highly conserved region called the Toll/IL-1 receptor (TIR) signaling domain, required for signal transduction [204].

The TLR family is highly expressed in immune-responsive tissue such as macrophages, dendritic cells, spleen cells, and cells of the tissues exposed to the external environment such as lungs and the gastrointestinal tract [205]. They recognize PAMPs, which are highly expressed by microbial pathogens or danger-associated molecular patterns (DAMP) [206]. Upon binding PAMPs, TLRs activate the NF-κB signaling pathway and induce the transcription of pro-inflammatory genes [204]. During mycobacterial infection, there is evidence that mycobacterial components are detected by TLR2, TLR4 and TLR9 [207]

1.7.2.1 Toll-like receptor 2 (TLR2)

TLR2 signaling is induced by forming a heterodimer with its coreceptors (TLR1 or TLR6) which increases the diverseness of molecules detected by the receptor [208]. Following ligand stimulation, TLR2 heterodimers initiate a MyD88-dependent intracellular signaling cascade [209]. This signaling pathway induces nuclear translocation of nuclear factor-kappa B (NF-κB) to activate gene transcription and subsequent cytokine production [210]. The signaling cascade also induces mitogen-activated protein kinases (MAPKs) that can promote transcription of inflammatory genes via activation protein 1 (AP-1) [210, 211].

A growing number of studies have shown that TLR2 is involved in macrophages/DCs recognition and responses to a variety of mycobacterial cell wall glycolipids such as

lipoarabinomannan (LAM), phosphatidylinositol mannoside (PIM), Lipomannan (LM), Trehalose 6,6'-dimycolate (TDM) [88, 207, 212]. Aside lipoglycan binding, TLR2 also recognizes glycoprotein present or secreted from the mycobacterial cell wall. One of these glycoproteins is the 19 kDa mycobacterial lipoprotein [213, 214]. In addition to mycobacterial cell wall glycolipids and lipoproteins, secreted proteins such as ESAT-6 and TB10.4 induce pro-inflammatory cytokine production in a cell line via TLR2 signaling [215, 216]. Furthermore, recent findings indicate that *M. bovis*-derived protein activates the NF-κB pathway via TLR2 in macrophage cell lines [217]. Thus, the broad ligand specificity of TLR2 indicates a crucial role for TLR2 activation in eradicating mycobacterial infection.

1.7.2.2 Toll-like receptor 4 (TLR4)

As a complex glycoprotein, TLR4 structurally has both extracellular domains containing leucine-rich repeats (LRRs) and the intracellular TIR domain. The LRRs play a role in ligand detection resulting in activation of TRIF- or MYD88-dependent intracellular signaling cascades [218]. TLR4 is known for recognizing gram-negative bacteria-derived lipopolysaccharide (LPS) [219]. In the serum, LPS first binds to LPS binding protein (LBP) [220, 221]. LBP catalysis the transfer of LPS to CD14 receptor. CD14 receptor is a co-receptor for TLR4, and it is a membrane-bound pattern recognition receptor that primarily recognizes LPS [222]. However, CD14 lacks an intracellular domain and thus is incapable of transducing cytoplasmic signals [223]. To compensate for the limitation of intracellular signal transduction, CD14 interacts with TLR4 to recognize LPS. The complex (LPS-CD14-MD-2 proteins) formed associate with the extracellular domain of TLR4 to initiate

intracellular signaling events that lead to inflammatory cytokine production [224]. MD-2, also known as Lymphocyte antigen 96, is a molecule that confers LPS responsiveness on TLR4. The binding of LPS/MD-2/CD14 complex to TLR4 leads to TLR4 dimerization and conformational changes in the TIR domain interface. This enhances the recruitment of adaptor proteins that binds to the intracellular TIR-domain [225]. TLR4 activation triggers two intracellular signaling pathways: myeloid differentiation factor 88 (MyD88)-dependent and independent pathways.

In *M. tuberculosis*, TLR4 recognizes cell wall lipids, glycoproteins, and secreted proteins as TLR2. Similar to TLR2, Lipomanan induces the production of pro-inflammatory cytokines in macrophages in a TRL4-dependent manner [226]. Thus, whereas some *M. tuberculosis* strains activate mainly TLR2, others also activate TLR4, resulting in different cytokine profiles [227]

1.7.2.3 Toll-like receptor 9 (TLR9)

Synthetic CpG-DNA derived from bacterial DNA activates innate immune cells such as macrophages and DCs via its specific receptor TLR9 localized on the phagosomal membrane [228]. Mice lacking TLR9 are highly susceptible to a low-dose aerosol *M. tb* infection than wild-type controls [229]. Furthermore, IL-12p40 production is abolished in TLR2/TLR9$^{-/-}$ cells infected with *M. tuberculosis*, indicating a synergistic effect of TLR2 and TLR9 activation in cytokine production [229]. Aside from TLR2 and 4 recognizing BCG, TLR9 also contributes to the recognition of *M. bovis* BCG by Flt3-ligand generated DCs [230].

1.8 TLR signaling

The binding of TLRs to its ligands activates specific downstream intracellular signaling cascades that initiate host defense response [231]. The interaction of PAMPs with TLR results in the production of pro-inflammatory cytokines and type 1 interferon [232]. Signaling via TLR depends on the type of stimulus, the TLR stimulated, and the downstream adaptor molecule recruited to the TIR domain [233]. Thus, TLR signaling involves two distinct pathways: the MyD88-dependent pathway (employed by all TLRs apart from TLR3 resulting in the generation of inflammatory cytokine) and TRIF-dependent pathway (utilized by TLR3 and 4 leading to interferon regulatory factor-3 (IRF3) transcription factor activation and subsequent increase of genes encoding type 1 interferons (IFNs) and costimulatory molecules) [234]. Following activation of MyD88, IL-1 receptor-associated kinase-4 (IRAK-4) are recruited through the death domains to TLR4. The recruited IRAK4 binds to its receptor complex leading to activation of IRAK1, thereby inducing the kinase activity of IRAK1[235]. The phosphorylated IRAK1 dissociates and binds to tumor necrosis factor receptor-associated factor-6 (TRAF6) through 3 major conserved binding domains [236, 237]. The downstream signaling of the TNF receptor superfamily is mediated by TRAF6 [237]. The complex formed by IRAK1 and TRAF6 dissociates from the TLR4 receptor cytoplasmic domain and later form a complex with three adapter molecules, which are TAK1-binding protein 1, 2 and 3 (TAB1, TAB2 and TAB3) and transforming growth factor-β activated kinase 1 (TAK1). Also, TRAF6 phosphorylation leads to activation of MKK and IKK complex resulting in the subsequent phosphorylation of NF-κB and MAP kinases.

1.9 Categories of Toll-like receptor agonists

PAMPs are agonists of PRRs expressed by innate immune cells. Immune system stimulation via TLRs activates innate immune cells and promotes inflammatory responses, which are critical for host protective mechanisms [238]. As such TLRs recognize lipopolysaccharide (LPS), Single stranded-RNA (ssRNA40), flagellin and CpG oligodeoxynucleotides to initiate intracellular signaling events that result in the activation of a variety of proinflammatory immune responses [239]. Due to the ability of TLR agonists to activate the immune system, promising approaches to control infectious disease involve regulation of the host's innate immune cells using agonists that bind to its receptor and execute an agonist function. Because of TLR agonist immunostimulatory activity, TLR agonists are being utilized as cancer immunotherapeutics and vaccine adjuvants.

1.9.1 Lipopolysaccharide (LPS) - (a TLR4 agonist)

Multiplication of the gram-negative bacteria within the host results in the release of LPS into the circulation. Lipopolysaccharide, a proinflammatory endotoxin is a component of the outer membrane of Gram-negative bacteria that robustly activates different circulating cell types [240]. Dendritic cells and macrophages, which are LPS-responsive cells, get activated following the interaction of LPS with circulating LPS-binding protein and CD14 [241]. It has been demonstrated that LPS triggers the induction of NF-κB-dependent proinflammatory mediators such as interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), interleukin (IL)-1[242, 243].

1.9.2 Flagellin - (a TLR5 agonist)

Flagellin, a major component of bacterial flagella, is a virulence factor detected by the innate immune system [244]. It has been reported that mammalian TLR5 senses bacterial flagellin from both gram-negative and gram-positive bacteria resulting in the activation of the receptor and subsequent phosphorylation of the NF-κB signaling events [245]. There is growing evidence suggesting that Salmonella flagellin is a potent inducer of pro-inflammatory cytokines in monocytes following interaction between flagellin and the surface receptor localize on innate immune cells [244, 246]. Also, flagellin is known as a potent adjuvant. The adjuvant activity depends on the ability of flagellin to induce the production of proinflammatory responses in both innate and non-immune cells, which is essential for the activation of adaptive immune responses [247, 248]. Dendritic cells are among the innate immune cells activated by flagellin and depending on the type of DCs involved, the flagellin-DCs interaction is either direct or indirect. Thus, myeloid-derived DCs directly respond to flagellin via TLR5, unlike the indirect response of splenic DCs to flagellin [249].

1.9.3 Polyinosinic-polycytidylic acid-Poly (I:C) - (a TLR3 agonist)

Viral proteins and nucleic acids activate TLRs to induce the production of chemokines, cytokines, and IFNs. Poly (I:C) is a synthetic analog of double-stranded RNA, which serves as a molecular pattern associated with cells infected with RNA virus [250]. Both viral double-stranded RNA and its analog poly (I:C) are potent inducers of type I IFNs (IFN-α and -β), which are the key cytokines in antiviral host defense. Induction of type I IFNs by poly (I:C) occurs via activation of TLR-3, resulting in the protection of mice and rhesus

monkeys from viral infection [251, 252]. Furthermore, TLR3 recognizes dsRNA and resultant signal transduction activates the nuclear factor-kappaB (NF-κB), resulting in pro-inflammatory cytokine production [253]. Unlike other TLRs, TLR3 mRNA expression is restricted to DCs subsets and intestinal epithelial cells fibroblasts [254]. Only $CD11c^+$ DC subset was shown to respond to poly (I:C), while plasmacytoid DC are stimulated by immunostimulatory bacterial DNA (CpG DNA) through TLR9 [255].

1.9.4 Imiquimod and Single stranded-RNA (ssRNA40) - (a TLR7 agonist)

Imiquimod (an immune response modifier) activates innate and adaptive immune responses by binding to TLR7, with subsequent activation NF-κB resulting in cytokine release [256]. Imiquimod exposure causes activation of immune cells to produce antiviral cytokines, particularly IFN-α, TNF-α, and interleukin (IL)-12, IL-10, IL-1, IL-6, and IL-8 [257]. Imiquimod also triggers IFN-γ production via T-cell stimulation, thereby promoting cell-mediated immunity [258]. IFN-γ released stimulates cytotoxic T lymphocytes, which is essential in the clearance of virally infected cells [259]. Thus, besides its anti-viral activity, imiquimod causes apoptosis of skin cancer cells, implicating its anti-tumoral activity [260]. The ability of Imiquimod to induce Interferon-alpha and other cytokines explains its acute antiviral and antitumor effects. Single-stranded RNA (ssRNA) is also known as the natural ligand of endosomal localized TLR7 [261, 262]. Influenza virus-derived ssRNA has been demonstrated to induce the production of proinflammatory cytokines in plasmacytoid DC following detection by TLR7 [263].

1.9.5 Pam_3CSK_4 – (a TLR2 agonist)

Pam_3CSK_4 ($Pam_3CysSerLys_4$), is a synthetic triacylated lipopeptide (LP) that mimics bacterial lipopeptides [264]. Detection of Pam_3CSK_4 is mediated by TLR2, which synergizes with TLR1 via the cytoplasmic domain, triggers the signaling events resulting in activation of NF-κB [265]. Pam_3CSK_4 promotes Th1 responses, thereby serving as a beneficial antiallergic effect on allergic rhinitis [266]. Thus, the binding of TLR2 by its agonist Pam_3CSK_4 decreased allergic inflammation and skewed Th2 response toward Th1 response. This suggests that activation of TLR2 by Pam_3CSK_4 activates the antigen-presenting cells to produce cytokines such as IL-12, which induces IFN-γ producing Th1 response, resulting in impaired Th2 cell differentiation and weakened eosinophilic airways inflammation. Other studies demonstrated that PAM_3CSK_4 instruct or bias the commitment of haematopoietic stem cells to favour myeloid cell, which correlate to the selective upregulation of transcription factors known to facilitate myeloid commitment [267]. This suggest that TLR2 agonists can shift the developing lineage committed progenitors to enhance the generation of effector cells associated with innate immune system.

1.9.6 CpG oligodeoxynucleotides (ODN) - (a TLR9 agonist)

Synthetic CpG ODN is the ligand for TLR9, and activation occurs via endosomal uptake of CpG ODN by TLR9 [268]. Following TLR9 activation, two different TLR9 signaling cascades occur namely- IFNα and NF-κB signaling pathway. Interaction of TLR9 and CpG ODNs stimulate the innate immune system through direct and indirect induction of IFNs, chemokines, and pro-inflammatory cytokines [269]. Innate immune cells such as plasmacytoid DCs and natural killer cells are efficiently activated by CpG ODNs.

Furthermore, CpG ODNs stimulate the B-cell arm of the adaptive immune system, resulting in antibody production and the activation of antibody-dependent cell-mediated cytotoxicity (ADCC) [269]. This suggests that the overall immune responses induced by CpG ODNs should be beneficial for controlling numerous diseases where robust innate and adaptive immune responses serve as a requirement.

CpG ODNs activation of the immune system has been shown to have a broad range of benefits. For instance, CpG ODNs serve as a vaccine adjuvant and also treat viral, bacterial, and parasitic diseases [269]. Furthermore, the antitumor activity of CpG ODNs has also been demonstrated in a mouse model [269]. Therefore, CpG ODNs represent targeted immune-modulatory drugs with a broad range of potential applications.

1.10 Immune signaling pathways for cytokine production

The primary signaling pathways known to induce cytokine production in immune cells include the mitogen-activated protein kinase pathway (MAPKs), JAK-STAT and NF-κB pathway. MAPKs and STATs are cytoplasmic proteins that play a critical role in immune regulation, cytokine production and inflammatory response [270, 271]. Hence, the activation of MAPKs, STAT and NF-κB pathways initiate a cascade of intracellular signaling events resulting in the expression of various pro-inflammatory genes.

1.10.1 Extracellular signal-regulated kinases (ERK) pathway

Mitogen-activated protein kinases are highly conserved group of serine/threonine protein kinases that mediate intracellular signaling events required to accomplish various fundamental cellular processes like differentiation, proliferation, apoptosis, motility, stress

response and survival [272, 273]. Conventional MAPKs consist of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPKs, c-Jun amino-terminal kinase (JNK), and Big MAPKs (BMK) [273, 274].

ERK is the first mammalian MAPKs to be characterized and exhibit different isoforms from ERK1-ERK8, but ERK 1and ERK2 are the most important among them. The two phosphorylation sites (tyrosine and threonine) are phosphorylated in order to activate the ERK1/2 signaling cascade, which occurs exclusively through MEK1 and MEK2 [275]. ERK1/2 shares 83% amino acid sequence identity, and they are highly conserved throughout eukaryotic cells. The ERK1/2 is primarily activated by the cell surface receptor enzyme (receptor tyrosine kinases), and they respond to various extracellular stimuli, including mitogens, cytokines, growth factors, bacterial products and environmental stressors [276]. Following stimulation, ERK1/2 phosphorylates different substrates in many cellular locations, resulting in the induction of ERK1/2 dependent cellular processes [277].

1.10.2 Signal transducer and activator of transcription (STAT) signaling pathway

The biological effects of IFN-γ are manifested via its interaction with the IFN-γ receptor complex localizes on the cells. The heterodimeric receptor complex consists of a ligand-binding alpha subunit (IFNGR1) and a signal-transducing beta subunit (IFNGR2). There is higher affinity binding of IFN-γ to IFNGR1 when compared with IFNGR2. The interaction between the receptor and ligand initiates a signaling event that subsequently induces host protective immune responses [278]. IFN-γ binding crosslinks IFNGR1 and

IFNGR2 and phosphorylates Janus Kinases (JAK1 and JAK2). The inactive cytosolic transcription factor STAT1 becomes phosphorylated by the activated JAKs [279].

All STATs have seven well-defined domains, including an N-terminal conserved domain and a C-terminal transactivation domain. The amino-terminal region prevents the dimerization of STATs in their inactive state [280]. SH2 domain is critical for recruiting STATs to activate receptor complexes and interaction with Janus (JAK) and Src kinases. It is the most conserved domain among STATs, and it plays a vital role in STAT signaling by facilitating homodimerization and heterodimerization, which are crucial for nuclear localization and DNA binding activities [281]. In an inactive or unstimulated cell, STATs are inactive and exists in an unphosphorylated state in the cytoplasm. Receptor tyrosine phosphorylation occurs upon cytokine stimulation and serves as a docking site for STATs through the SH2 domains leading to the reorientation of STAT proteins resulting in homodimerization or heterodimerization. Once phosphorylated, the dimerized STATs translocate to the nucleus and bind to specific regulatory sequences to activate or repress transcription of target genes. Although the classical JAK-STAT pathway is usually initiated by tyrosine phosphorylation, most vertebrate STATs also contain a second phosphorylation site, which is a serine, and serine phosphorylation also regulates STAT transcriptional activities [282]. STAT1 phosphorylation occurs at Tyr701, resulting in STAT1 dimerization, nuclear translocation and DNA binding. STAT1 can also be phosphorylated at serine (ser727) and serine phosphorylation is required for the maximal induction of STAT1 mediated gene activation. Phosphorylated STAT1 translocate to the nucleus and binds to GAS (gamma activated sequences) elements in the promoter region and mediates protective immunity by transcription of IFN-γ associated genes. Activated

STAT1 phosphorylation subsequently activates T-bet transcription factor, representing the onset of T-cell polarization to Th1 subset [283]. Recruitment of additional transcription factor Runx3, which utilizes IFN-γ promoter as its binding site, induces IFN-γ expression and simultaneous silencing of the *IL-4* gene [284]. Hence, IFN-γ plays a crucial role in maintaining T-bet expression and suppresses the capability of Th1 cells to produce IL-4 [273].

1.10.3 Nuclear factor-kappa B (NF-κB) signaling pathway

The NF-κB protein represents Rel family, and it is associated with five structurally related members, which include RelA (p65), C-Rel, RelB, p105 (NF-κB1) and p100 (NF-κB2) [285]. Members of NF-κB are characterized by the presence of Rel homology domain (RHD), which is essential for binding to cognate DNA elements. The phosphorylation of NF-κB consists of two major signaling pathways, namely- the canonical and noncanonical pathways [286]. In contrast to the canonical NF-κB signaling pathway, which responds to various ligands of pattern-recognition receptors (PRRs), cytokine receptors, T-cell receptor as well as TNF receptor superfamily members [287], the non-canonical NF-κB pathway only responds to a specific group of stimuli such as ligands of a subset of TNF receptor superfamily members [286].

Under homeostatic conditions, the inactive NF-κB is localized in the cytoplasm due to its interaction with IKB proteins. The binding of IKB proteins to inactive NF-κB inhibits its nuclear translocation, resulting in cytoplasmic confinement. However, following TLR stimulation with a pathogenic molecule or cytokine, the ensuing signal transduction results

in NF-κB activation [288]. This is achieved via the inducible degradation of IKB by activated IKK, resulting in nuclear translocation of canonical NF-κB [289]. The primary role of NF-κB is the mediation of pro-inflammatory gene induction in innate immune cells and the regulation of T-cells effector function [290]. In addition, it has been reported that NF-κB regulates the activation of inflammasomes [291].

1.11 Role of signaling pathways in mycobacterial infection

M. tuberculosis-complex PAMPs bind various PRRs expressed on innate immune cells resulting in the activation of different signaling pathways [292, 293]. Although mycobacteria infection leads to profound production of proinflammatory cytokines, the intracellular signaling pathways leading to the production of these cytokines are well studied. An increasing number of studies have revealed the role of MAPKs, STAT and NF-kB family proteins in mycobacteria-induced proinflammatory cytokine production [294-297]. However, there are inconsistent reports on the role of these proteins in mycobacteria-induced cytokine production. One study shows that TLR2-ERK signaling in *M. tuberculosis*-infected macrophages drives anti-inflammatory responses and inhibits Th1 polarization of responding T-cells [298]. In addition, *M. tuberculosis* uses the TLR2-ERK signaling pathway in macrophages to facilitate its intracellular survival and prevent efficient elimination by antigen-specific IFN-γ-producing T-cells [299].

In contrast, protective immune responses in seen in studies where activation of p38 and ERK mediates IFN-γ production from T-cell in response to *M. tuberculosis* infection, a defective pathway in patients with TB [300]. Also, Jung et al. revealed that ESAT6 directly induces IL-6 production in macrophages by activating STAT3 [301]. Furthermore, *M.*

tuberculosis induces the expression of proinflammatory and regulatory cytokines in human monocytes infected with *M. tuberculosis* via NF-*κ*B and MAPK dependent mechanisms [302]. A recent study reported that *M. tuberculosis* activation of MAPKs and NF-κB via the TLR4 dependent pathway is critical in producing pro-inflammatory cytokines in DCs [303]. To date, no study has addressed the role of ERK and NF-κB proteins in pro-inflammatory cytokine production following stimulation of bDCs with *M. bovis* cell surface antigen. In studies II and III, I addressed this gap in knowledge and provided strong evidence to show that ERK and NF-κB proteins play a critical role in *M. bovis*-induced proinflammatory cytokine production in DCs

1.12 Cytokines

Cytokines are water-soluble proteins and glycoproteins produced by hematopoietic and non-hematopoietic cells to influence the activity of other cells [304]. They are critical in regulating immune cells by mediating cell to cell communication through autocrine and paracrine pathways. In a homeostatic condition, cytokine production is limited. However, with infectious conditions, cytokine induction is in response to the causative agent [305]. Interaction of *M. tuberculosis* ligands with the receptors on innate immune cells causes cell activation and cytokine production, modulating innate and adaptive immune response to *M. tuberculosis* [306]. It has been reported that cytokines produced in response to a pathogen could be beneficial or harmful to the host [307]. Thus, the production of cytokines in response to *M. tuberculosis* infection is not always helpful to the host protection, instead some of these immune modulators promote pathogen survival [308]. Therefore following *M. tuberculosis* infection, two major groups of cytokines are released, namely

proinflammatory cytokines (beneficial to the host) and anti-inflammatory cytokines (harmful to the host).

1.12.1 Proinflammatory Cytokines

Proinflammatory cytokines are usually released by innate immune and non-immune cells following exposure to a pathogenic molecule. These cytokines promote both local and systemic inflammatory responses, and severe inflammatory response due to excessive generation of these cytokines results in tissue damage [307]. After encountering *M. tuberculosis* antigen by immune cells, several pro-inflammatory cytokines (IL-12, IL-1, TNF, IL-6, IL-23, and IL-18) are produced, which control mycobacterial infection by induction of Th1 immunity [309].

1.12.1.1 Interleukin 12 (IL-12) and control of mycobacterial infection

Interleukin 12 is a pro-inflammatory cytokine composed of two subunits (p35 and p40 subunits). While p35 is constitutively expressed at a low level, however the expression of p40 is induced with microbial stimulation [310]. The co-expression of these two subunits within a cell is required to produce a bioactive IL12p70 cytokine. Production of IL-12 following infection is critical for the induction of IFN-γ producing Th1 responses against intracellular pathogens. Hence, the binding of IL-12 to its receptor expressed on naïve CD4 T-cells induces intracellular signaling cascades leading to activation and differentiation of naïve CD4 T-cells to IFN-γ producing Th1 subset [310]. Furthermore, IL-12 inhibits the polarization of Th2 and associated cytokines (IL-4 and IL-10).

It has been reported that microbial stimuli are not potent enough to induce the production

of bioactive IL-12 cytokine. Thus, secondary signals in the form of CD40-CD40 ligand interaction are required to promote the production of bioactive IL-12 [310-312]. During IL-12 signaling, communication between cells occurs via CD40-CD40L interaction and this interaction is bi-directional, which allows signaling to both APCs and T-cells [313]. Thus, aside from CD40-CD40L interaction resulting in increased production of bioactive IL-12 from APCs, the interaction of CD40-CD40L also induce T-cell proliferation and IFN-γ production [313].

Following *M. tuberculosis* infection, IL-12 produced by DCs synergizes with IL-18 to induce IFN-γ production from Th1 subset, which is essential for host protection against *M. tuberculosis* infection [314]. It has been revealed that IL-12-deficient mice are highly susceptible to *M. tuberculosis* infection [315]. Mutations in IL-12 and its receptor have also been demonstrated in individuals suffering from mycobacterial disease due to the attenuated production of IFN-γ [316]. This suggests that IL-12 protects the host against mycobacterial infection via induction of IFN-γ, thereby serving as a link between adaptive and innate host immune responses [317].

1.12.1.2 Tumor necrosis factor-alpha (TNF-α) and mycobacterial infection

Tumor necrosis factor-alpha glycoprotein is another proinflammatory cytokine produced by phagocytic cells. There are two isoforms of TNFs, namely TNF-β and TNF-α. Both TNF-α and TNF-β induce an immune response by binding to surface receptors TNFR1 and TNFR2, respectively. In homeostatic conditions, TNF-α detection is rare. However, increased TNF levels are detected following infection or during malignant conditions

[318].

In mycobacterial infections, TNF-α plays a significant role in protecting the host [319]. TNF-α is the primary cytokine that maintains granuloma containing the infectious foci, thereby preventing dissemination [320]. In a mouse model, TNF-α is essential in protecting the host from developing active TB by containment of latent infection within granuloma [320]. Mice lacking either TNF-α production or its receptor are highly susceptible to *M. tuberculosis* infection [321]. In addition, mice lacking TNF-α receptors or treated with an anti-TNF antibody are at high risk of infection with mycobacteria [322]. It has been shown that the neutralization of TNF-α activity during latent TB infection triggers TB reactivation in C57BL/6 mice resulting in host tissue damage and death [320]. Other studies also reported that TNF-α signaling blockage increases mortality in zebrafish infected with *M. marinum* due to increased intracellular bacterial growth [323]. These findings indicate that TNF-α is required for maintaining the integrity of granuloma structure, thereby inhibiting bacterial dissemination.

1.12.1.3 Role of interleukin 6 (IL-6) in mycobacterial infection

IL-6 is a multifunctional cytokine. IL-6, together with TNF-αand IL-1β, initiates early pro-inflammatory responses [324]. Mice studies demonstrated that IL-6 might play multiple roles and contribute positively and negatively to host defense against *M. tuberculosis* infection. It has been shown that IL-6 plays a vital role in protection against murine *M. tuberculosis* infection [325] due to the influence of the CD4+ T cells response [326]. *M. tuberculosis*-infected IL-6-deficient animals show an impaired Th1 response and increased

bacterial loads, indicating a requirement for IL-6 in host resistance to *M. tuberculosis* infection [325, 327].

In contrast, IL-6 is also associated with the pathogenesis of many chronic inflammatory diseases, including tuberculosis [328, 329]. For instance, IL-6 secreted by *M. tuberculosis*-infected macrophages suppresses the responses of uninfected macrophages to IFN-γ, resulting in increased proliferation of bacteria [330]. To understand which cellular process induced by IFN-γ is actually counteracted by IL-6, Dutta et al. studied the role of IL-6 on IFN-γ induced autophagy formation in virulent *M. tuberculosis* infection. They observed that IL-6 inhibits both IFN-γ and starvation-induced autophagy in *M. tuberculosis*-infected macrophages [331]. Further studies revealed that increased levels of IL-6 in the lungs, along with increased levels of IL-1β, is significantly correlated with tuberculosis progression in genetically susceptible mice [328]. The downregulation of IL-6R expression on CD4 T-cells in patients with active pulmonary TB is associated with decreased Th17 phenotype response, suggesting a role for IL-6 in the progression of TB in humans [332].

1.12.1.4 Role of interleukin 1 beta (IL-1β) in mycobacterial infection

The pro-inflammatory cytokine IL-1β is a key mediator of inflammation and plays a vital role in the host's resistance to *M. tuberculosis* infections [333]. IL-1β directly kills *M. tuberculosis* in murine and human macrophages and promotes the recruitment of anti-microbial effector molecules. The role played by IL-1β in host resistance has been demonstrated by the significantly reduced survival of IL-1β-/- or IL1R-/- mice following mycobacterial infection [334-336]. The increased susceptibility of mice lacking critical mediators of IL-1 signaling suggests that the initial production of IL-1 upon *M.*

tuberculosis infection is essential for establishing protective immune responses necessary for disease control [337].

In contrast, IL-1β has been implicated in TB disease severity. Production of IL-1 is regulated after the onset of adaptive immunity via multiple mechanisms, including IFNγ production, which acts via the induction of nitric oxide synthase 2 (NOS2) [338, 339]. In susceptible mice, persistent IL-1 signaling can contribute to the accumulation of disease-promoting neutrophils, and genetic variants that result in higher IL-1β production are associated with increased disease severity and neutrophil accumulation in humans [339, 340]. This suggests that IL-1 blocking may ameliorate inflammation-induced tissue damage and improve the treatment outcome in TB infected hosts.

1.12.1.5 Interferon-gamma (IFN-γ) and biological significance in mycobacterial infection

Interferon-gamma is the primary subtype of type 2 IFNs, and it is established as a macrophage activating factor due to its role in activating macrophage physiologic activities. IFN-γ production is mainly by Th1 subset of CD4 T-cells, cytotoxic CD8 T-cells, NK cells and NKT cells [341, 342]. Also, it can be produced by DCs, macrophages, and B-cells [343]. IFN-γ has been demonstrated to have numerous immunomodulatory functions such as inflammatory activity and regulation of macrophage intracellular microbicidal effect [343]. Some of the immunomodulatory effects of IFN-γ on macrophages include induction of cytokine and nitric oxide production, which facilitates the killing of intracellular bacteria [344]. In addition, IFN-γ reverts the blockage of

phagosome-lysosome fusion caused by *M. tuberculosis* as part of its survival mechanism, as well as induction of autophagy to promote clearance of *M. tuberculosis* [345]. Furthermore, IFN-γ boosts human macrophage apoptosis in a NO-dependent manner [346].

Several studies showed that IFN-γ orchestrates the induction of cellular immunity, which is the hallmark of TB infection. Thus, IFN-γ induces the expression of antigen-presenting molecules on DCs, thereby increasing antigen presentation and induction of protective host immune response. It has been demonstrated that a non-functional IFN-γ or IFN-γR signaling pathway enhances host susceptibility to less virulent strains. Also, dysfunctional IFN-γR promotes disseminated infection in children vaccinated with BCG [347]. Knock-out mice lacking IFN-γ exhibit increased mycobacterial growth and developed necrotic granulomas [348]. The increased mycobacterial burden and host susceptibility are related to dampened cell-mediated immunity and poor expression of nitric oxide synthase 2 (NOS2) [349].

1.12.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines are the immune modulators that control pro-inflammatory cytokine induction, thereby inhibiting the inflammatory process [350]. These cytokines exhibit their immunomodulatory role by blocking the proinflammatory cytokine production or by interfering with their biological effects [350]. Thus, over-production of anti-inflammatory cytokines may compromise host protection induced by the proinflammatory cytokines. The three major anti-inflammatory cytokines secreted by immune cells include interleukin 4 (IL-4), interleukin 10 (IL-10), and transforming growth beta (TGF-β) [350].

1.12.2.1 Interleukin 10 and regulation of mycobacterial infection

Interleukin 10 is a well-established anti-inflammatory cytokine produced by different immune cells [351]. It blocks immunopathology emanating from excessive activity of DCs and Th1 cells by down-regulating the production of IL-12 and TNF-α [351]. Excessive IL-10 production blocks the pro-inflammatory response to mycobacterial infection [352], which aids in pathogen escape. Production of IL-10 also supports mycobacterial survival by inhibiting CD4 T-cell responses and antigen presentation by mycobacteria-infected DCs [353]. Thus, DCs transportation of mycobacterial antigens to the draining lymph nodes was shown to be blocked by IL-10 [354]. Moreover, IL-10 inhibits DCs mediated T-cell differentiation and recruitment of Th1 cells to the lungs of mice infected with *M. tuberculosis* [355]. Within the granuloma, IL-10 blocks phagosome maturation and IFN-γ mediated macrophage activation, resulting in impaired secretion of both ROIs and RNIs, required for mycobacterial killing [356]. In peripheral blood mononuclear cells isolated from TB patients, increased T-cell proliferation and IFN-γ production was observed following antibody neutralization of endogenous IL-10 [357]. These observations suggest that IL-10 favors mycobacterial infection by downregulating host Th1 immune responses.

1.13 Negative regulators of cell signaling

Several molecules negatively regulate cytokine signaling via various mechanisms to inhibit generation of excessive immune responses that result in host tissue pathology and autoimmunity. The key molecules involved in these signaling pathways are the primary

targets of negative regulators. Activation of the cytokine signaling pathways is blocked by the suppressor of cytokine signaling (SOCs) protein [358].

1.13.1 Suppressor of cytokine signaling (SOCs) and control of mycobacterial infection

Suppressor of cytokine signaling (SOCs) proteins are the negative regulator of cytokine signaling [358, 359]. Eight members of SOCs proteins (CIS and SOCs1-7) have been reported in mammals [358]. Overexpression studies have demonstrated that SOCs1 and 3 possess the same inhibitory role on various cytokine signaling [358]. Thus, SOCs1 and 3 have been reported as inhibitors of tyrosine phosphorylation and nuclear translocation of STAT1 following IFN-γ stimulation. In contrast to SOCs1, which show robust inhibitory activity to IFN-γ signaling due to its higher affinity to Jak2, SOCs 3 exhibit less inhibitory activity because of its higher affinity for Tyk2. Hence, this indicates that SOCs1 and 3 are the primary inhibitors of IFN-γ mediated Jak /STAT signaling pathways.

In addition to IFN-γ signaling, the initial step involved in TLR signaling is blocked by SOCs1 in macrophages [360]. SOCs protein has utilized several mechanisms to block TLR signaling pathways. For instance, SOCs inhibit TLR signaling by targeting IL-1 receptor-associated kinase 1 (IRAK1) via its SH2 domain [361]. Also, SOCs1 causes proteasomal degradation of Mal, leading to inhibition of TLR/Mal-dependent NF-*κ*B activation [362]. Furthermore, SOCs protein has been shown to regulate TLR-induced NF-*κ*B activation by causing the degradation of NF-κB p65 subunit, resulting in downregulation of p65 protein levels.

Bacterial pathogens exploit SOCs 1 and 3 proteins to manipulate cytokine receptor signaling and thereby influence infection outcomes as a strategy of evading host immune defenses [363]. This suggests that pathogens can induce SOCS1 and SOCS3 to evade deleterious host immune responses. Recent studies have reported that SOCs1 and 3 were elevated in active TB patients and contributed to the down-modulation of Th1-mediated IFN-γ responses [364]. Also, SOCs1 were reported to support mycobacterial growth in macrophages by inhibiting the secretion of IFN-γ production by T-cells in response to IL-12 [365]. Additionally, SOCS3 induced by PPE18 inhibited NF-κB activation by diminishing the phosphorylation of IκBα [366]. In general, the induction of SOCs1 and 3 by mycobacteria results in the blockage of the pro-inflammatory response required for effective TB control [367].

1.14 Rationale and major objective

Although numerous efforts have gone into studying the TB pathogenesis in the context of *M. tuberculosis*, it is still unclear the role and mechanism of *M. bovis* derive cell envelope antigens in bTB pathogenesis. From the above literature review, it is evident that the interaction between the *M. tuberculosis*-derived cell envelope antigens with the host innate immune cells is essential in determining the outcome of infection. However, there are gaps in knowledge, including how *M. bovis*-derived cell envelope antigens inhibit DCs maturation and how these antigens block the induction of protective Th1 immune response. Therefore, we sought to assess bovine dendritic (bDCs) activation upon treatment with *M. bovis*-derived cell surface protein, lipid and glycan antigens. We focused on bDCs because

they are key professional antigen-presenting cells that upon activation, can prime and direct the immune activity of naïve and memory T-cells essential for TB control [368, 369].

CHAPTER 2. Stimulation of bovine dendritic cells by *Mycobacterium bovis* cell surface sugar extract is associated with decreased pro-inflammatory cytokine response

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HYPOTHESIS

Distinct antigenic components of *M. bovis* cell envelope induces differential pro-inflammatory responses in bovine innate immune cells

OBJECTIVES

The primary objective is to use a functional biological assay to compare the stimulatory effects of bPPD, CWL and CSSE on bDCs *in vitro.*

1. Determine the production of pro-inflammatory cytokines (IL-12 & TNF-a) following treatment of bDCs with bPPD, CWL and CSSE using ELISA.
2. Assess for the phosphorylation of MAPKs (ERK) and NF-κB in bDCs following treatment of bDCs with bPPD, CWL and CSSE using Western blot.
3. Investigate the expression of SOCs protein following treatment with bPPD, CWL and CSSE on bDCs using Western blot

2.1 Introduction

While considerable effort is being spent in the research and development of vaccines for bTB disease prevention and control, a licensed vaccine for use in cattle has yet to emerge. The live attenuated *M. bovis* bacillus Calmette-Guerin (BCG) vaccine that is used to immunize people has been shown to confer protection in cattle [370, 371]. However, BCG is not used in cattle because the tuberculin skin test routinely used to diagnose bTB in the field does not distinguish BCG-vaccinated animals from *M. bovis*-infected animals [372]. Therefore, a better understanding of the effects of *M. bovis* cell surface antigens on the bovine immune system will help in the development of bTB vaccine.

Members of the *M.tb*-complex, which include *M. tuberculosis* - the human-adapted agent of TB and *M. bovis*, have on their cell surfaces a complex mixture of proteins, polar and apolar lipids, glycolipids and glycans with diverse biological properties [373-375]. These biomolecules play crucial roles in host-pathogen interactions and TB immunopathogenesis [61, 374-377]. Earlier studies reported that *M. bovis*-derived cell wall polar lipids compared to apolar lipids differentially stimulate proinflammatory responses in bovine monocyte-derived dendritic cells [52]. Thus, *M. bovis* antigens separated into fractions may be a useful approach to identify immunostimulatory antigens from non-stimulatory antigens. There is no information on the effects of distinct *M. bovis*-derived protein, lipid and glycan-enriched fractions on bovine immune cells to the best of our knowledge. In this study, the effects of three different types of *M. bovis* cell envelope fractions - purified protein derivative (bPPD) [378], total cell wall lipid (CWL) [379, 380], and cell surface sugar extract (CSSE) [65, 381] on bDCs activation was determined. We found that *M. bovis*

CSSE-treated bDCs exhibited little to no activation. The CWL fraction meanwhile induced more activation of bDCs than CSSE but less than bPPD and the lipopolysaccharide (LPS) control. Furthermore, the muted response of CSSE was found to be associated with decreased extracellular signal-regulated kinase (ERK) and nuclear factor kappa-B (NF-κB) activation, and increased expression of suppressors of cytokine signaling 1 and 3 (SOCS1 and 3). These observations taken together suggests that the *M. bovis* CSSE fraction contains immunomodulatory molecules that may aid in *M. bovis* pathogenesis.

2.2 Materials and Methods

2.2.1 Preparation or source of *M. bovis* cell envelope antigen fractions

2.2.1.1 Bovine purified protein derivative (bPPD) was purchased from Prionics AG (ThermoFisher Scientific Inc.). The production of bPPD, which is a purified mixture of proteins prepared from the culture filtrate of heat-killed *M. bovis* grown in a synthetic medium is described elsewhere [378]. bPPD was diluted in PBS before use and the same batch of bPPD was used for cell stimulation throughout the entire study.

2.2.1.2 Total cell wall lipid (CWL) from *M. bovis* was obtained commercially from BEI resources (https://www.beiresources.org/). The production of total CWL, which is a complex mixture of polar and apolar lipids and glycolipids has been described elsewhere [379, 380]. Briefly, *M. bovis* AF2122/97 was grown in glycerol-alanine-salts medium to late-log phase, pelleted and washed with phosphate-buffered saline (PBS), gamma-irradiated and lyophilized. Total lipids were then extracted from the dried cells with chloroform/methanol (2:1). Water-soluble molecules were then removed from the organic extract by biphasic partitioning in water. The organic phase enriched with total CWL was collected, dried and weighed. Before use, the CWL was resuspended in ethanol and diluted further in PBS before use. The same batch of CWL was used for cell stimulation throughout the entire study.

2.2.1.3 Cell surface sugar extract (CSSE), which has been shown to contain a complex mix of extracellular glycans and glycolipids, was enriched from the culture filtrates of *M. bovis* AF2122/97 grown in Sautons liquid media [65, 381, 382]. CSSE was extracted and

purified from *M. bovis* grown in our lab. Briefly, to 0.8 volumes of cell-free *M. bovis* culture filtrate, 2 volumes of methanol and 1 volume of chloroform were added to yield a homogenous single-phase mixture and incubated overnight at room temperature. To this mixture, water/chloroform (1:1) was added and after 30 minutes, the organic phase was allowed to partition from the aqueous phase and recovered. After washing with 0.9% NaCl, the organic phase containing CSSE was recovered, dried and weighed. Before use, the CSSE was resuspended in ethanol and diluted further in PBS before use. The same batch of CSSE was used for cell stimulation throughout the entire study.

2.2.2 Isolation of bovine CD14+ monocytes and culture of monocyte-derived mature dendritic cells (bDCs)

Blood was collected from healthy cattle in 60-mL syringes containing 7.5% ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMCs) were obtained from these blood samples using the Ficoll (GE Healthcare Bioscience) method of isolation[383]. $CD14^+$ monocytes were isolated from PBMCs by positive selection using MACs columns (Miltenyi Biotec Inc.). Mature bovine dendritic cells (bDCs) were subsequently derived from $CD14^+$ monocytes after culturing in a complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and HEPES (Hyclone Laboratories Inc.), 2-mercaptoethanol (Sigma-Aldrich), recombinant bovine GM-CSF at 100 ng/mL (Biorad) and recombinant bovine IL-4 at 50 ng/mL (Biorad) for 6 days as previously described[384]. bDCs were assessed by flow cytometry and the expression of DC-specific marker CD206 was found to be greater than 97%.

2.2.3 In vitro cell culture and treatment

Bovine dendritic cells were incubated in serum-free RPMI 1640 culture medium for 24 h. The serum-free medium was then replaced with 1 mL of complete medium containing either E.coli derived LPS (1 µg/mL; Sigma-Aldrich), bPPD (1 µg/mL), CWL (1 µg/mL), and CSSE (1 or 10 µg/mL as indicated). In all experiments, the treated cells were incubated overnight at 37^{o}C, 5% CO_2, before supernatant collection. Untreated cells (culture medium only) served as negative control. Culture supernatants were collected for analysis of cytokine using ELISA. In a different experiment, treated bDCs were lysed with RIPA lysis buffer containing protease inhibitors at 5, 15, 30, 60, 120 and 240 minutes following media removal. The resulting cell lysate was used for Western blot analysis.

2.2.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays were performed to determine the concentrations of IL-12 and TNF-α in bDC supernatants. Briefly, high-binding Immulon-2 (Thermofisher Scientific Inc.) ELISA plates and Maxisorp (Thermofisher Scientific Inc.) ELISA plates were respectively coated with bovine-specific TNF-α monoclonal antibodies produced in-house and bovine-specific IL-12 monoclonal antibodies (AbD Serotec)[385] in coating buffer (Sodium carbonate, sodium bicarbonate and distilled water at pH 9.6), and incubated overnight at 4^{o}C. Following incubation, the plates were washed 5 times with wash buffer (Tris buffered Saline containing 0.05% Tween-20 (TBST), pH 7.4) and blocked for 1 hr at room temperature. 200 µl/well of 0.1% gelatin (Sigma-Aldrich) and 0.1% casein (Sigma-Aldrich) was used for both TNF-α and IL-12 blocking respectively. The micro-well plates were rinsed 5 times with wash buffer, and appropriately diluted recombinant IL-12 and

TNF-α standards were titrated in a 2-fold dilution to generate the standard curve. Samples were added undiluted (100 µL/well) and incubated at room temperature for 2 hrs. Thereafter, the plates were washed 5 times with washing buffer and 100 µL of biotinylated detection antibody at 4 µg/mL was added to all wells. After 1 hr incubation at room temperature, the plates were washed 5 times and 100 µL of streptavidin at (1/5000 dilution) was added to the wells and also incubated for 1hr. Then, 100 µL of *p*-Nitrophenyl Phosphate (PNPP) (1 mg/mL) was added to all the wells and incubated for 1 hr at room temperature. Plates were read at 405 nm using Spectra Max plus microplate reader (Molecular Devices).

2.2.5 Western blot analysis.

Protein concentrations of lysates from bDCs treated with LPS, bPPD, CWL and CSSE were determined using Bicinchoninic acid assay (Thermofisher Scientific Inc.). 15 µg of total protein were loaded in each lane and resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the protein bands were transferred to a nitrocellulose membrane using the iBlot 2 dry blotting system (Thermofisher Scientific Inc.). The membranes were blocked with 5 % powdered skimmed milk in Tris Buffered Saline (TBS) and incubated for 1 hour at room temperature. Thereafter, the membranes were incubated overnight with primary antibodies diluted in TBS supplemented with 5% bovine serum albumin and 0.05% Tween 20 (TBST). Immunoblotting of total and phosphorylated ERK, total and phosphorylated NF-κB (p65 subunit), SOCS1 and 3, and β-actin was performed as described previously[386]. The antibodies used were: anti-p44/42 MAPK (ERK1/2) rabbit monoclonal antibody (clone 137F5) and anti-phospho-

p44/42 (ERK1/2) (Thr202/Tyr204) rabbit monoclonal antibody XP® (clone D13.14.4E) (Cell Signaling Technology), anti-NF-κB p65 rabbit mAb (clone C22B4) and anti-phospho-NF-κB p65 (Ser536) rabbit mAb (clone D13.14.4E) (Cell Signaling Technology), anti-SOCS1 (middle region) rabbit polyclonal antibody (Antibodies-online.com), anti-SOCS3 (Internal) rabbit polyclonal antibody (LSBio), and anti-β-actin mouse monoclonal antibody (clone mAbcam 8226) (Abcam). After washing with TBST, the membranes were incubated for 1 hr at room temperature with Alexa Fluor® conjugated secondary antibody, at a dilution of 1:10000 in antibody dilution buffer. Signals were acquired using LI-COR imaging system (LI-COR Biosciences). Densitometry was performed using the band analysis tools of the Image Lab software version (LI-COR Biosciences).

2.2.6 Statistical analysis.

Cytokine data are presented as means ± standard error of means while densitometry data are presented as means ± standard error of the mean. A one-way ANOVA was used to compare differences in cytokine production using GraphPad Prism software. Significance was considered if $p < 0.05$.

2.3 Results

2.3.1 Bovine dendritic cells treated with CSSE produce little IL-12 and TNF-α.

Antigen-presenting cells (APCs) like macrophages and dendritic cells produce the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12) in response to infection with members of the *M. tb*-complex[387, 388]. In turn, both TNF-α and IL-12 help trigger naïve T-cells to mediate a Th1 response critical for controlling intracellular pathogens like mycobacteria [369, 389]. Here we assessed the production of TNF-α and IL-12 by bovine dendritic cells (bDCs) upon treatment with 1 µg/mL each of *M. bovis*-derived bPPD, CWL and CSSE, with LPS serving as a positive control. Relative to no treatment, LPS at 1 µg/mL was found to induce the strongest response, followed by bPPD and CWL which were also at 1 µg/mL (Figure 2.1A and B). CSSE at 1 µg/mL induced very little to no production of TNF-α and IL-12 (data not shown). Strikingly, even when the concentration of CSSE was increased to 10 µg/mL, it failed to induce appreciably higher TNF-α and IL-12 compared to no treatment (Figure 2.1A and B). These results show that *M. bovis*-derived bPPD is best at stimulating bDCs followed by CWL, while CSSE is the poorest inducer of the pro-inflammatory response in these cells.

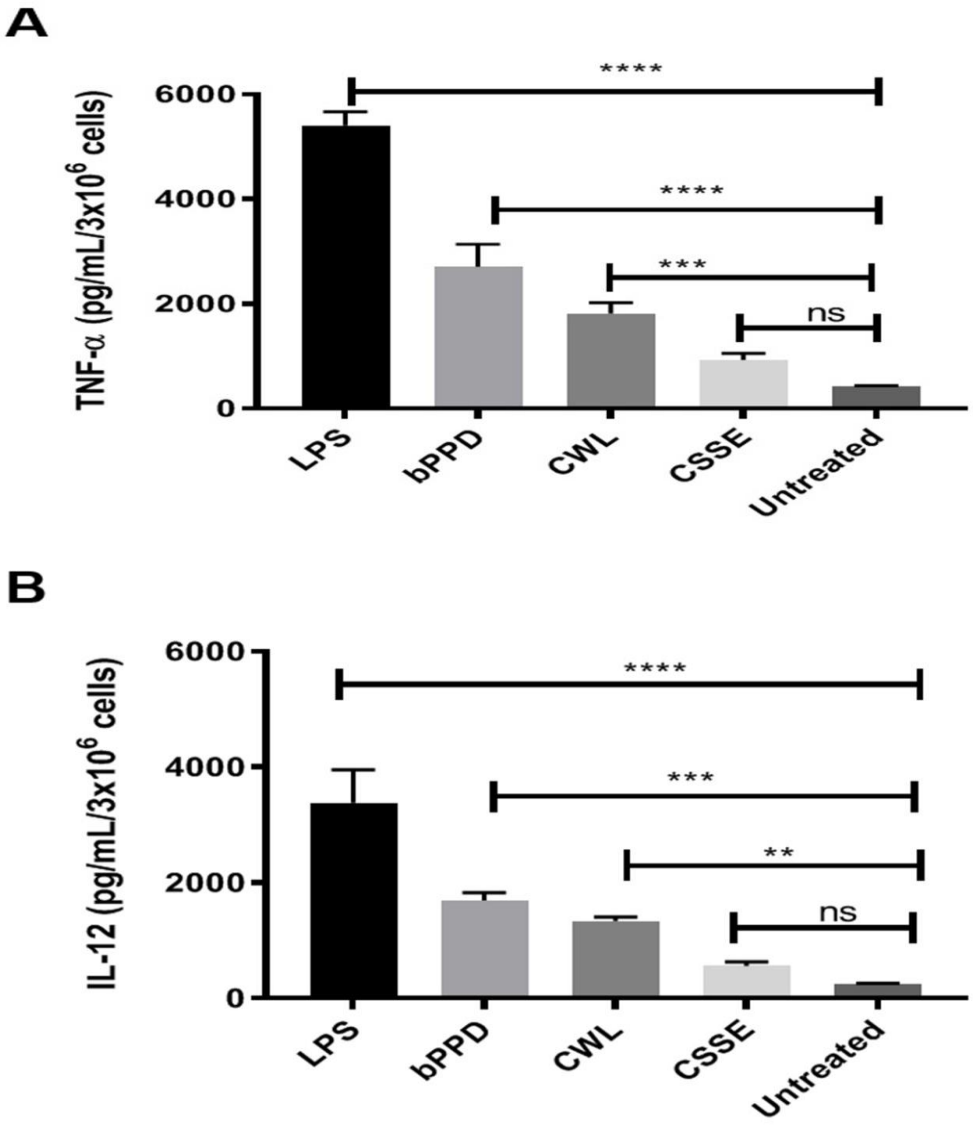
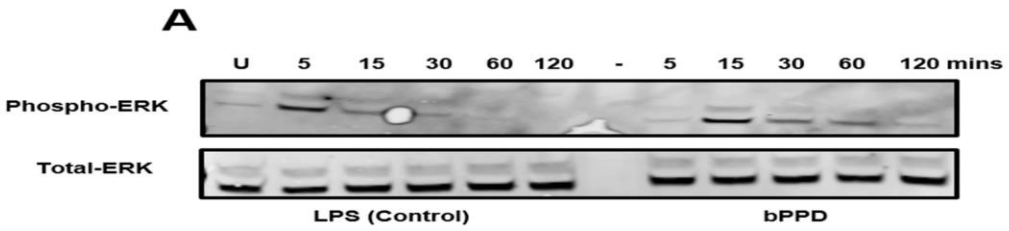


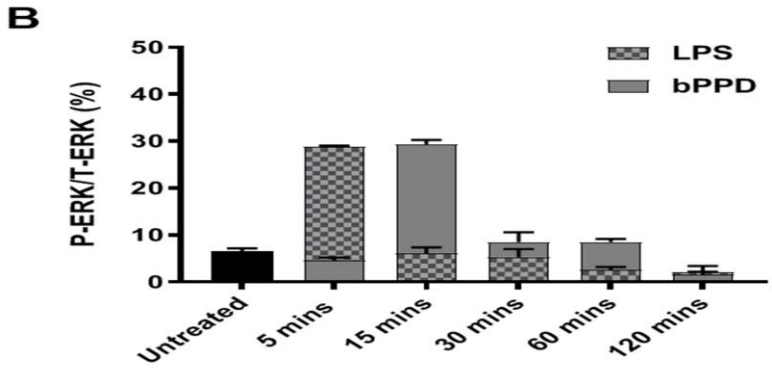
Figure 2.1: Low production of IL-12 and TNF-α in CSSE simulated bDCs.

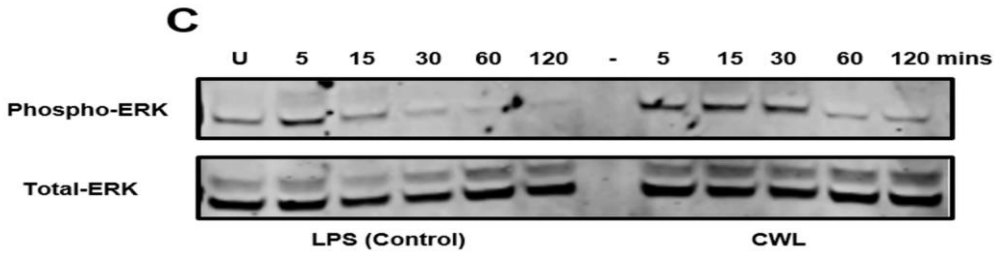
Bovine dendritic cells were stimulated *in vitro* with bPPD (1 µg/mL), CWL (1 µg/mL) and CSSE (10 µg/mL) for 24 hr and the production of TNF-α **(A)** and IL-12 **(B)** were determined by ELISA. In all experiments, LPS (1 µg/mL) and unstimulated cells served as positive and negative control, respectively. Results are presented as mean +/- SEM of replicate wells and are representative of 3 independent experiments (**, p <0.01; ***p <0.001; ****, p <0.0001).

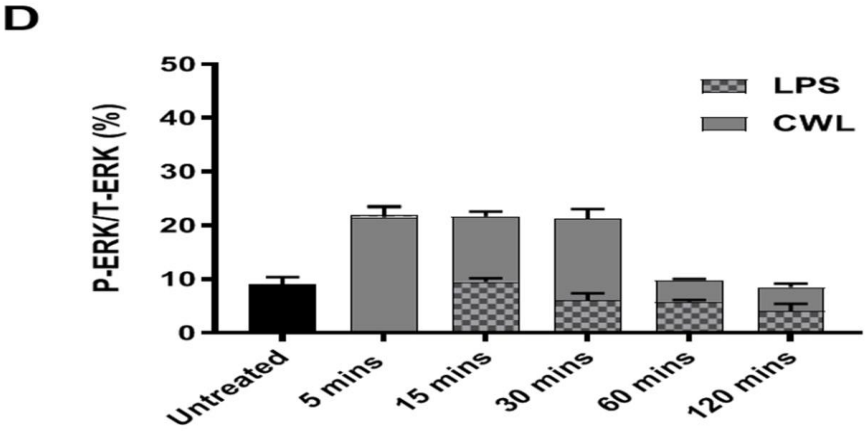
2.3.2 CSSE treated bDCs exhibit low phosphorylation ERK

The pro-inflammatory response in APCs is mediated by signal-transduction events involving multiple cytoplasmic proteins known as mitogen-activated protein kinases (MAPKs)[390]. Activation of a MAPK protein called extracellular signal-regulated kinase (ERK) via phosphorylation plays a key role in pro-inflammatory cytokine production in response to products of bacterial origin such as LPS[386, 390]. Therefore, we sought to examine the phosphorylation of ERK in bDCs treated with bPPD (at 1 µg/mL), CWL (at 1 µg/mL) and CSSE (at 10 µg/mL), with LPS (at 1 µg/mL) serving as positive control. LPS consistently induced phosphorylation of total ERK within 5 minutes post-addition, which decreased thereafter (Figure 2.2A to F). bPPD, on the other hand, induced phosphorylation of total ERK later at 15 minutes post-addition, and subsided shortly thereafter (Figure 2.2A and B). CWL treatment induced phosphorylation in ERK to the same level as LPS by 5 minutes post-addition but maintained the same rate of phosphorylation longer than LPS treatment which had waned after 5 minutes, before also decreasing (Figure 2.2C and D). In contrast, CSSE induced less phosphorylation of total ERK throughout the duration of the experiment (Figure 2.2E and F). These results show ERK in CSSE-treated bDCs is poorly phosphorylated and activated and is consistent with the poor induction of pro-inflammatory response in these cells.


A
U 5 15 30 60 120 - 5 15 30 60 120 mins
Phospho-ERK
Total-ERK
LPS (Control)
bPPD


B
P-ERK/T-ERK (%)
50
40
30
20
10
0
LPS
bPPD
Untreated
5 mins
15 mins
30 mins
60 mins
120 mins


C
U 5 15 30 60 120 - 5 15 30 60 120 mins
Phospho-ERK
Total-ERK
LPS (Control)
CWL


D
P-ERK/T-ERK (%)
50
40
30
20
10
0
LPS
CWL
Untreated
5 mins
15 mins
30 mins
60 mins
120 mins

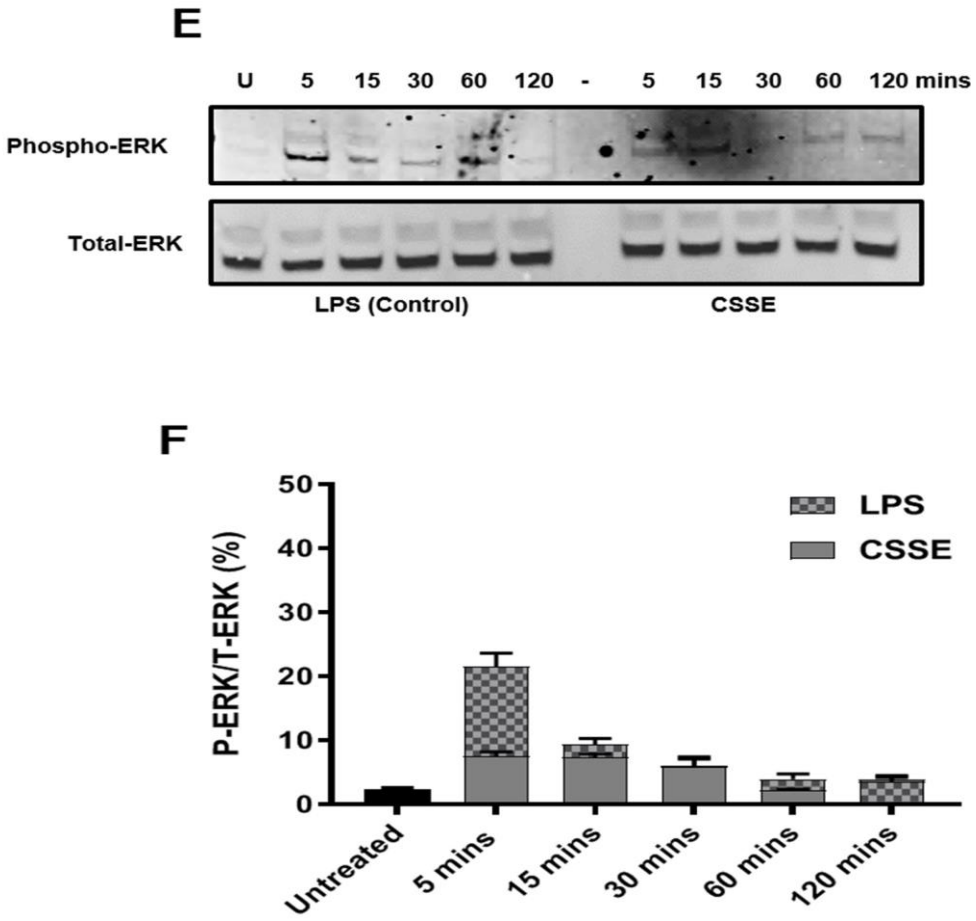
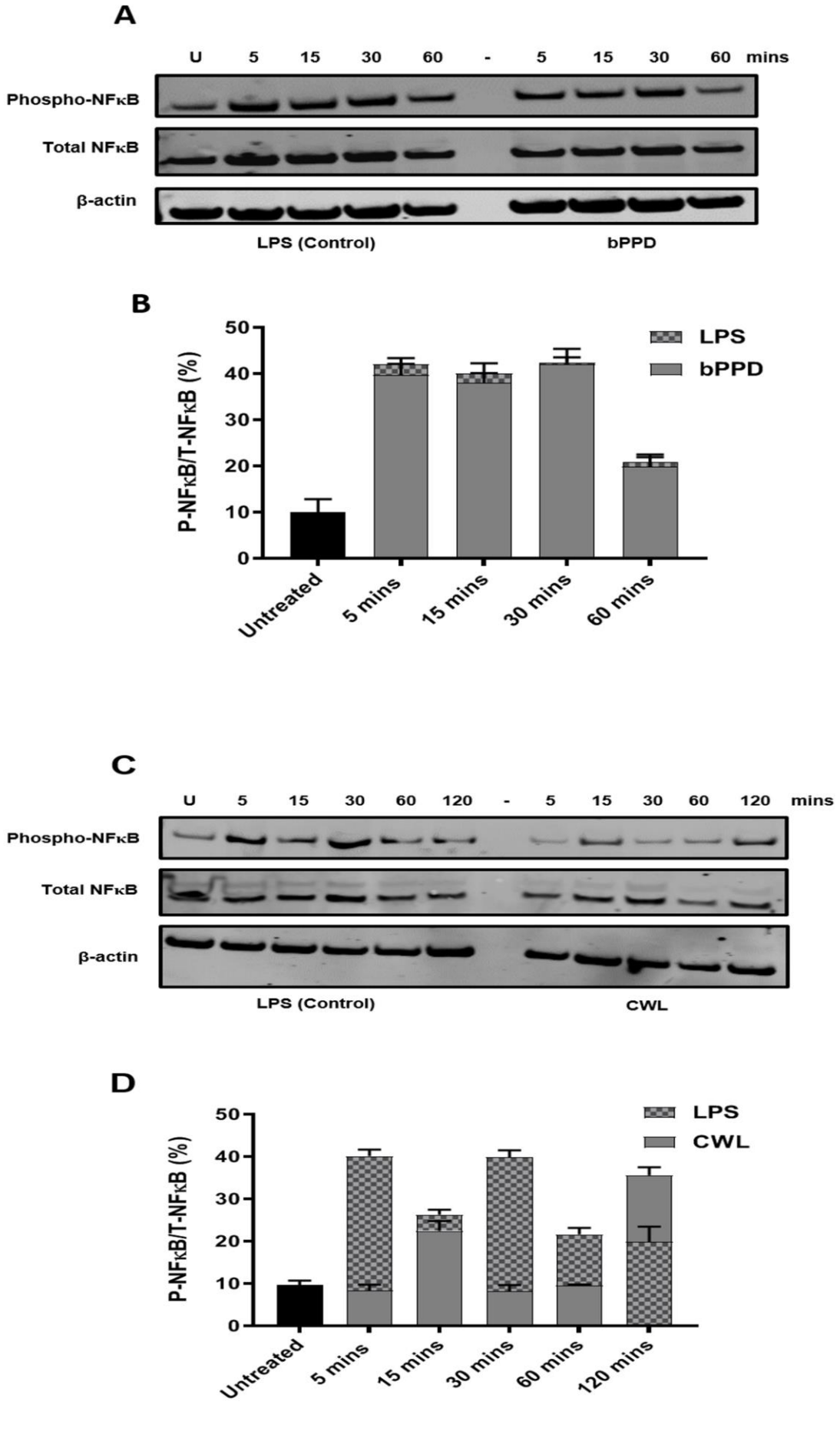


Figure 2.2: Decreased phosphorylation of ERK protein in CSSE stimulated bDCs. Bovine dendritic cells were stimulated *in vitro* with bPPD (1 µg/mL), CWL (1 µg/mL) and CSSE (10 µg/mL) and lysate were collected at different indicated times. Total cell lysate were assessed for phosphorylation ERK protein **(A, C & E)** by Western blot using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against β-actin, then used as loading controls. LPS (1 µg/mL) served as positive control. The ratios of phosphorylated ERK to their respective total proteins were calculated by densitometry and plotted as bar graphs **(B, D & F).** Results are presented as mean +/- SEM of replicate wells and are representative of 3 independent experiments.

2.3.3 CSSE treated bDCs exhibit low phosphorylation and transactivation of NF-κB p65 subunit

The multi-subunit NF-κB protein is a master transcriptional regulator that plays a vital role in immune activation[391, 392]. Moreover, transactivation of the p65 subunit of NF-κB by phosphorylation has been shown to upregulate pro-inflammatory gene expression in APCs[386, 391]. Therefore we also sought to assess the phosphorylation status of the NF-κB p65 subunit over time in bDCs upon treatment with bPPD (at 1 µg/mL), CWL (at 1 µg/mL) and CSSE (at 10 µg/mL), with LPS (at 1 µg/mL) treatment serving as positive control. LPS induced phosphorylation of total NF-κB p65 by 5 minutes post-addition and stayed at those levels for the entire duration of the experiment (Figure 2.3A to F). bPPD also induced phosphorylation of total NF-κB p65 within 5 minutes post-addition and with these levels remaining constant for the duration of the experiment (Figure 2.3A and B). Phosphorylation of NF-κB p65 upon treatment with CWL appeared to be more variable with total NF-κB p65 being phosphorylated within 15 minutes post-addition followed by a drop for the next 45 minutes before an increase of total NF-κB p65 by 120 minutes post-addition (Figure 2.3C and D). In striking contrast, CSSE treatment does not induce phosphorylation of total NF-κB p65 for the entire experimental duration (Figure 2.3E and F). These results clearly show that NF-κB p65 in CSSE-treated bDCs are poorly phosphorylated and activated. The results of these experiments are also consistent with the poor phosphorylation of ERK and the low production of pro-inflammatory cytokines by bDCs after CSSE treatment.


A
U 5 15 30 60 - 5 15 30 60 mins
Phospho-NFκB
Total NFκB
β-actin
LPS (Control)
bPPD
B
P-NFκB/T-NFκB (%)
50
40
30
20
10
0
LPS
bPPD
Untreated
5 mins
15 mins
30 mins
60 mins
C
U 5 15 30 60 120 - 5 15 30 60 120 mins
Phospho-NFκB
Total NFκB
β-actin
LPS (Control)
CWL
D
P-NFκB/T-NFκB (%)
50
40
30
20
10
0
LPS
CWL
Untreated
5 mins
15 mins
30 mins
60 mins
120 mins

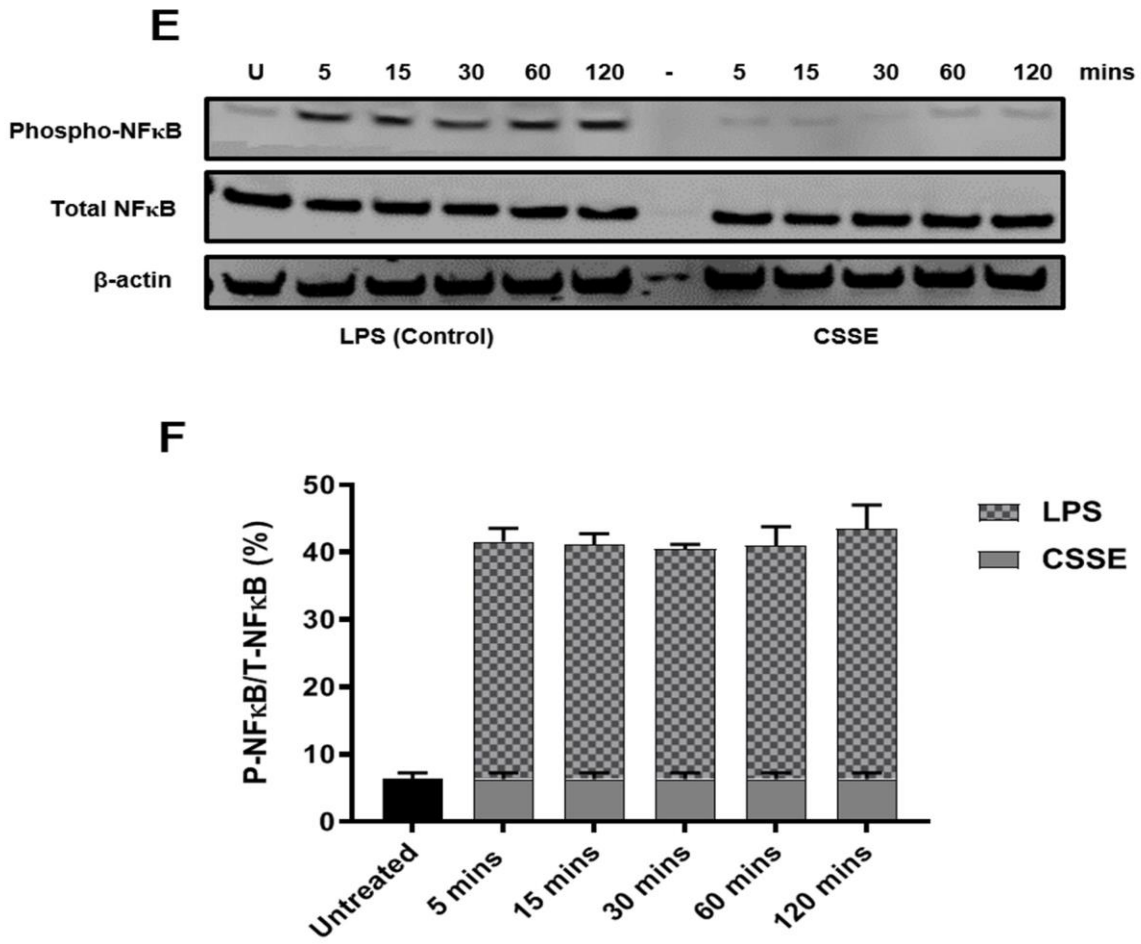
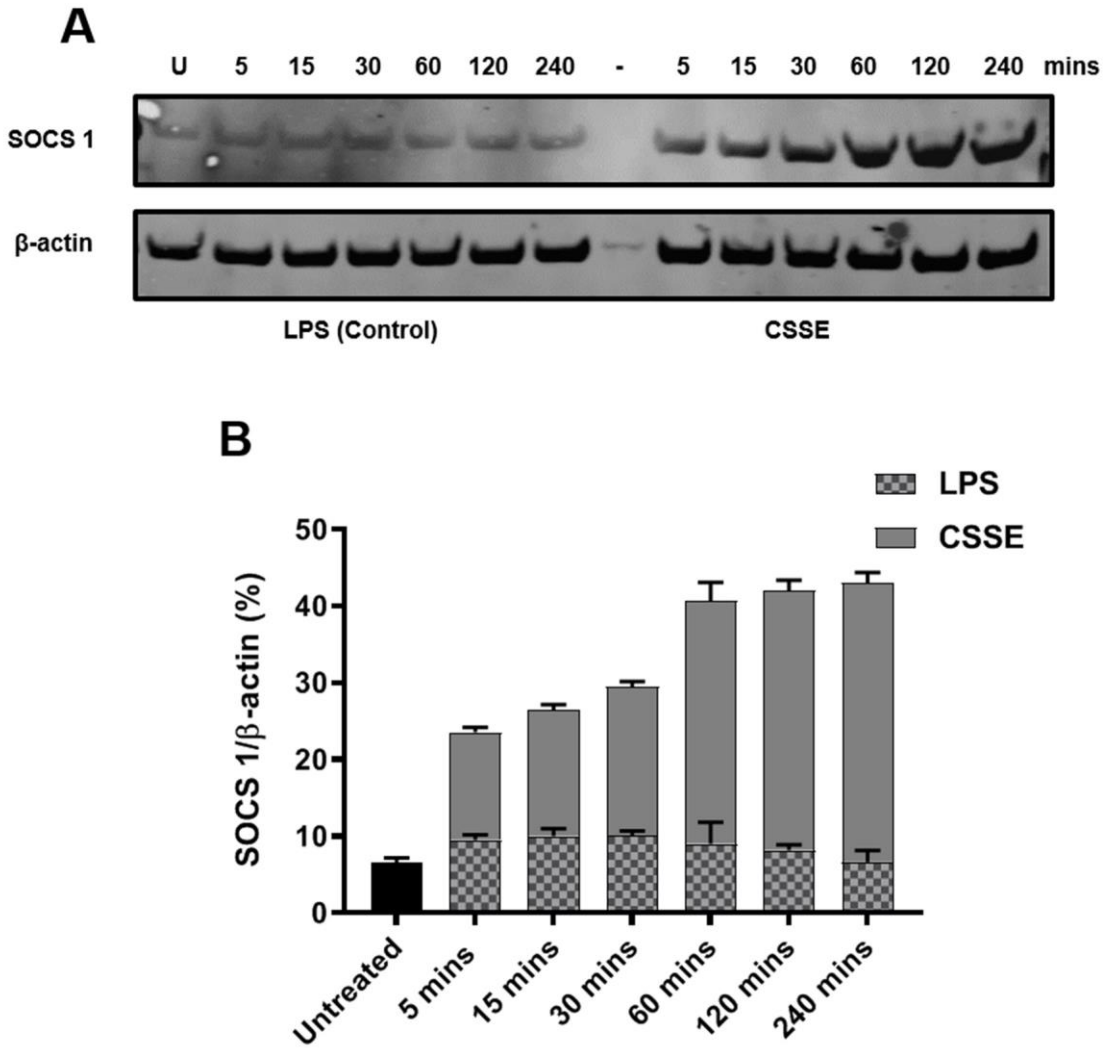


Figure 2.3: Reduced phosphorylation of NF-κB in CSSE stimulated bDCs.
Bovine dendritic cells were stimulated *in vitro* with bPPD (1 µg/mL), CWL (1 µg/mL) or CSSE (10 µg/mL) and the lysate was collected at different indicated times. The total cell lysate was assessed for phosphorylation NF-κB protein **(A, C & E)** by Western blot using appropriate primary and secondary antibodies. The same blots were stripped and re-probed with antibodies against β-actin, then used as loading controls. LPS (1 µg/mL) served as positive control. The ratios of phosphorylated NF-κB to their respective total proteins were calculated by densitometry and plotted as bar graphs **(B, D & F).** Results are presented as mean +/- SEM of replicate wells and are representative of 3 independent experiments.

2.3.4 Treatment of bDCs with CSSE up-regulates SOCS 1 and 3 expression.

Our results suggest CSSE is a poor inducer of inflammation in bDCs. However, to determine whether this fraction simply lacks sufficient amounts of the bacterial products needed to activate pro-inflammatory responses or if it contains immunomodulatory biomolecules that actually suppress activation of pro-inflammatory responses in bDCs, we decided to look at the status of negative regulators of inflammation like the suppressor of cytokine signaling (SOCS) proteins. SOCS proteins prevent hyper-inflammation following interaction with microbial products and thus play a key role in ensuring an appropriate and balanced immune response[393]. Moreover, SOCS proteins exert their regulatory effects by blocking pro-inflammatory signal transduction through a negative feedback loop to control the activation of immune cells as well as cytokine production[394]. Specifically, the initial steps of TLR signaling in macrophages are inhibited by SOCS 1, while other studies have implicated SOCS 1 and 3 in the blockage of NF-κB p65 activation [395, 396]. In light of the poor activation of NF-κB observed in CSSE-treated bDCs, we assessed the expression of SOCS 1 and 3 in bDCs treated with either LPS or CSSE. We found that by 5 minutes post-addition, CSSE caused an increase in the expression of SOCS 1 (Figure 2.4A and B). In contrast, the expression of SOCS 1 was not increased substantially in LPS-treated bDCs (Figure 2.4A and B). SOCS 3 expression was increased in CSSE-treated bDCs by 5 minutes post-addition (Figure 2.4C and D). However, LPS appeared to induce less SOCS 3 expression for the duration of the experiment (Figure 2.4C and D). These results clearly show that CSSE treatment increases SOCS 1 and 3 expression and may underlie the poor activation of NF-κB and pro-inflammatory cytokine production in bDCs.


A
U 5 15 30 60 120 240 - 5 15 30 60 120 240 mins
SOCS 1
β-actin
LPS (Control)
CSSE
B
LPS
CSSE
SOCS 1/β-actin (%)
50
40
30
20
10
0
Untreated
5 mins
15 mins
30 mins
60 mins
120 mins
240 mins

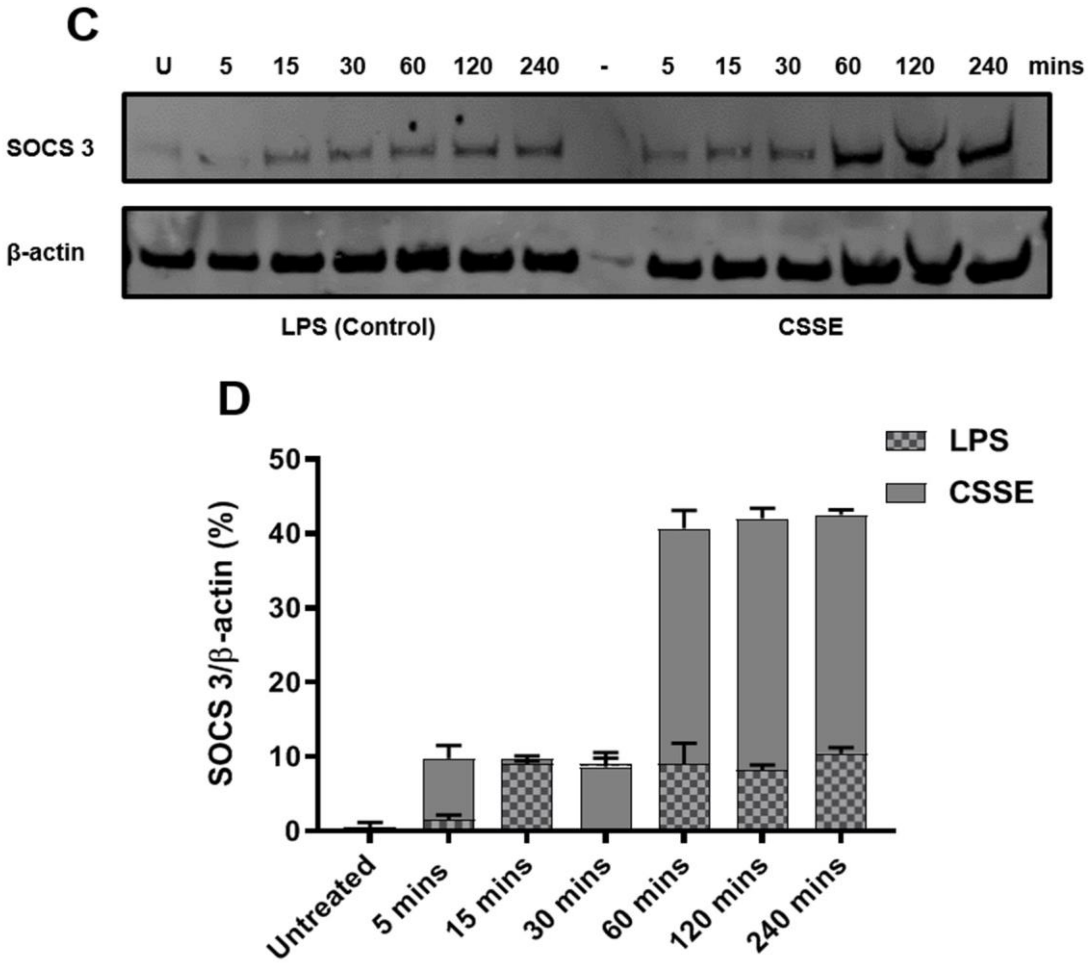


Figure 2.4: Increased expression of SOCS 1 & 3 protein in CSSE stimulated bDCs. Bovine dendritic cells were stimulated *in vitro* with bPPD (1 µg/mL), CWL (1 µg/mL) and CSSE (10 µg/mL) and lysate were collected at different indicated times. Total cell lysate was assessed for phosphorylation SOCS 1 and 3 proteins **(A & B)** by Western blot using appropriate primary and secondary antibodies. The same blots were stripped with stripping buffer and re-probed with antibodies against β-actin, then used as loading controls. LPS (1 µg/mL) served as a positive control. The ratios of expressed SOCS 1and 3 to their respective total proteins were calculated by densitometry and plotted as bar graphs **(C & D).** Results are presented as mean +/- SEM of replicate wells and are representative of 3 independent experiments.

2.4 Discussion & Conclusion

During mycobacterial infection, pro-inflammatory cytokines like TNF-α and IL-12 produced by DCs help drive the induction of Th1 immunity [368, 369]. However, numerous studies show that pathogenic mycobacteria can modulate pro-inflammatory signaling in macrophages and dendritic cells, thus enabling the bacterium to survive and persist within its host [61, 374-377]. Given that distinct lipidic components of the *M. bovis* cell surface appear to induce differential pro-inflammatory responses in bovine innate immune cells [52], the purpose of this study was to perform a direct head-to-head comparison of the effect of three different types of *M. bovis* cell envelope fractions on bDCs in order to help determine which of these might be suitable bTB vaccine components. We found that unlike bPPD, *M. bovis*-derived CWL and CSSE are relatively poor inducers of TNF-α and IL-12 production by bDCs, with the latter being the poorest of them all.

Further analysis revealed that the activation of ERK and NF-κB in CSSE-treated bDCs were significantly reduced, suggesting that the meagre production and secretion of TNF-α and IL-12 may be due to reduced activation of the ERK/NF-κB signaling pathway. Alternatively, it is conceivable that decreased phosphorylation of ERK protein directly or indirectly affects phosphorylation of NF-κB. It is also possible that CSSE inhibits phosphorylation of NF-κB by directly blocking the ability of the inhibitor of Kappa B kinase (IKK) complex to degrade the inhibitor of Kappa B (IkB) proteins, thus preventing the activation and translocation of NF-κB to the nucleus. Further studies to delineate the mechanisms by which CSSE affects NF-κB phosphorylation in dendritic cells are warranted.

Previous studies have shown that SOCS 1 suppresses dendritic cell maturation and

functions to prevent the development of systemic autoimmunity [397, 398]. Most importantly, pathogenic mycobacteria have been reported to induce the expression of SOCS 1 and 3 proteins as part of their virulence strategy [399, 400]. As such, we hypothesized that SOCS 1 and 3 might be involved in blocking IL-12 and TNF-α production in CSSE-treated bDCs. Indeed, we found CSSE treatment increased the expression of SOCS 1 and SOCS 3 proteins in bDCS. As such, the CSSE-mediated hypo-activation of ERK and NF-κB and concomitant lack of TNF-α and IL-12 production may also be due to increased expression of SOCs 1 and 3 proteins.

Components of CWL and CSSE fractions, especially lipids, glycolipids, and glycans, play prominent roles in the evasion of host immune responses by pathogenic mycobacteria [60, 61, 373-377]. While there is overwhelming evidence that many individual mycobacterial glycolipids and glycans exhibit immunomodulatory properties, their overall effect on the host immune system when immunostimulatory mycobacterial antigens are also present, remain to be ascertained and warrant further study. Nonetheless, based on our data, we speculate that the *M. bovis*-derived CSSE fraction and perhaps even the CWL fraction, albeit to a lesser extent, are enriched with immunosuppressive glycolipids and/or glycans that contribute to the overall survival and proliferation of the bacteria within its host. We further speculate that the mycobacterial products in these fractions would probably be detrimental to include or have present in any next-generation TB vaccine preparations.

In conclusion, our study shows that the *M. bovis*-derived CSSE fraction is a very poor immune stimulator. Furthermore, our data suggest that this fraction contains molecules that contribute to the virulence of *M. bovis* by inhibiting the generation of effective host immune responses.

Transition statement

In chapter 2, I showed the M. bovis culture supernatant and surface extract (CSSE) is poor at stimulating bDCs. Given that the CSSE is enriched in polysaccharides, phenolic glycolipids and glycans, I hypothesized that one or more of these molecules might actually be anti-inflammatory and contribute to the virulence of the TB bacillus. Indeed, previous studies have demonstrated that the M. tuberculosis-complex CSSE is particularly enriched in a family of phenolic glycans called para-hydroxybenzoic acid derivatives (p-HBADs), and these have been shown to regulate cytokine production by suppressing host inflammatory response [68, 69]. In addition, a recent report demonstrated that pure synthetic-HBADs affect acute macrophage activation by inhibition of pro-inflammatory response and reduction of bactericidal nitric oxide production following BCG vaccination [70]. This indicates that p-HBAD-1 and related molecules could be undermining the innate immune responses and explain why CSSE is a poor inducer of inflammation in bDCs. In this chapter, I sought to address this possibility and examine in detail the immunomodulatory effects of p-HBAD-1 on bDCs.

CHAPTER 3. *Mycobacterium bovis* parahydroxybenzoic acid derivatives (*p*-HBADs) exhibit a dual role in bTB pathogenesis by modulating pro-inflammatory responses in bDCs.

3.1 Introduction

It is known that *M. tuberculosis* -complex CSSE is enriched in *p*-HBADs, with *M. bovis* and BCG producing only *p*-HBAD-I, while *M. tuberculosis* generates both *p*-HBAD-I and II [46, 65]. In chapter 2, we observed that the *M. bovis*-derived cell surface sugar extract (CSSE) fraction exhibit a less immune-stimulatory effect on bDCs. Based on these results and given that the CSSE fraction of *M. tuberculosis* is enriched in *p*-HBADs which exhibit immunosuppressive properties in innate and adaptive immune cells [46]. In this chapter, I assessed the effect of *M. bovis* derived *p*-HBAD-I on bDCs stimulated with either lipopolysaccharide or bovine purified protein derivative. Result shows that the effect of *p*-HBAD-I in non-primed and IFN-γ primed bDCs varies. In non-primed bDCs, *p*-HBAD-I exhibits a tolerogenic response by blocking the production of pro-inflammatory cytokines. In contrast, the reversal effect was observed with IFN-γ primed bDCs, in which *p*-HBAD-I induces an immunogenic response resulting in increased pro-inflammatory cytokine production. These data suggest that *M. bovis*-derived *p*-HBAD-I is an immune-regulatory molecule that might have a dual function in bTB pathogenesis.

3.2 Materials and Methods

3.2.1 Preparation or source of *M. bovis* cell envelope antigen fractions

3.2.1.1 Bovine purified protein derivatives (bPPD) and total cell wall lipid (CWL)- As previously mentioned in the materials and methods section in chapter 2, bPPD was obtained from Prionics AG (Thermofisher Scientific Inc.). Similarly, CWL was acquired from BEI resources (https://www.beiresources.org/). The same batch of bPPD and CWL was used throughout the entire experiment

3.2.1.2 Parahydroxylbenzoic acid derivatives (*p*-HBAD-1)- were extracted from the culture filtrate of *M. bovis* cultured in Sautons liquid media using as previously described [382]. Specifically, 2 volumes of CH_3OH and 1 volume of $CHCl_3$ were used to extract pure *p*-HBAD-1. The chemical synthesis of *p*-HBAD-1 is described elsewhere [69]. The synthesized *p*-HBAD-1 was analyzed after resolving by thin-layer-chromatography (TLC) using $CHCl_3/CH_3OH/H_2O$ (90:10:1), and the structure of synthetic *p*-HBAD-I was confirmed using NMRI and mass spectrometer. For confirmation of *p*-HBAD-1 structure using mass spectrometry, it was performed using a Voyager DE-STR MALDI-TOF instrument equipped with a pulse nitrogen laser emitting at 337 nm. Briefly, samples were directly applied onto the sample plate and then allowed to crystallize at room temperature. Samples were analyzed in the Reflector mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive ion mode of 20 kV.

3.2.2 Assessment of *p*-HBAD-1 in *M. bovis* CSSE using Thin-layer chromatography (TLC)

M. bovis and M. bovis BCG-derived CSSE were extracted from the culture filtrate using a previously established protocol [382]. Determination of the presence of *p*-HBAD-1 was done by comparing extracted *M. bovis* CSSE with pure synthetic *p*-HBAD-I using Thin-layer chromatography [67]. The presence of *p*-HBAD-1 in *M. bovis* CSSE was identified by visual detection using a short wavelength UV lamp and alpha-naphthol spray [69].

3.2.3 Generation of monocyte-derived bDCs.

The generation of monocyte-derived bDCs has been previously described in the material and methods section of chapter 2. Briefly, isolation of $CD14^+$ monocytes from PBMCs was done via positive selection using MACs columns (Miltenyi Biotec Inc.) [401]. Immature bDCs were differentiated from $CD14^+$ monocytes using recombinant bovine GM-CSF (100 ng/mL; Biorad) and IL-4 as previously described (50 ng/mL; Biorad) [402].

3.2.4 *In vitro* cell culture and treatment

Monocyte-derived bDCs were cultured and treated as previously described in the material and methods section of chapter 2. Briefly, stimulation of differentiated bDCs suspended in complete RPMI medium was done either with LPS (1 µg/mL; Sigma Aldrich), bPPD (1 µg/mL), CWL (1 µg/mL) or one of several TLR agonists (Pam_3CSK_4 -10 µg/mL; HKLM-10^9 cells/mL; LPS-100 µg/mL; Flagellin -10 µg/mL; synthetic lipopeptide (FSL-1)-10 µg/mL; ODN-100 µg/mL; Poly I. C-500 µg/mL; Poly I. C [LMW]-500 µg/mL; Imiquimod-25 µg/mL; SSRNA-25 µg/mL (each from Invitrogen) in the presence or

absence of *p*-HBAD-I (200 μM; generously provided by Professor Eoin Scanlan, Trinity College Dublin, Dublin, Ireland). For IFN-γ primed bDCs, cells were first primed with recombinant bovine IFN-γ (rIFN-γ) (100 ng/mL; Sigma Aldrich Co.) for 2 h before stimulation in the presence and absence of *p*-HBAD-I. In all experiments, LPS-treated and untreated cells served as the positive and negative controls, respectively. The controls, as well as stimulated cells, were incubated overnight at 37^{o}C before supernatant collection. The concentrations of IL-12 and TNF-α (Sigma Aldrich) in the culture supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as briefly described below. Following removal of culture media, treated bDCs were lysed at 5, 15, 30, 60, 120 and 240 minutes with a combination of RIPA lysis buffer and protease inhibitors (Sigma- Aldrich). Cell lysate obtained was used for Western blot analysis.

3.2.5 Enzyme-linked immunosorbent assay (ELISA)

Assessment of the cytokine (IL-12 and TNF-α) concentration in bDCs supernatant was done using in-house antibodies by ELISA as was previously stated in the materials and methods section of chapter 2.

3.2.6 Western blotting

Activation of NF-κB p65, expression of TLR2/4 and SOCS1/3 was determined by Western blot as previously described in materials and method section of chapter 2, albeit with slight modification. Briefly, overnight incubation of membranes with primary antibodies diluted in antibody dilution buffer (TBS supplemented with 5% bovine serum albumin and 0.05% Tween 20 (TBST) were done. The antibodies used were: anti-NF-κB p65 rabbit mAb

(clone C22B4) and anti-phospho-NF-κB p65 (Ser536) rabbit mAb (clone D13.14.4E) (from Cell Signaling Technology), human anti-bovine TLR 2 and 4 (Clone 12542) (from Cedarlane), anti- SOCS1 (middle region) rabbit polyclonal antibody (from Antibodies-online.com), anti-SOCS3 (Internal) rabbit polyclonal antibody (from LSBio and anti-β-actin mouse monoclonal antibody (clone mAbcam 8226). After washing with TBST, the membranes were incubated for 1 h at room temperature with Alexa Fluor® conjugated secondary antibody, at a dilution of 1:10000 in antibody dilution buffer. Signals were acquired using LI-COR imaging system (LI-COR Biosciences). Densitometry was performed using the band analysis tools of the Image Lab software version (LI-COR Biosciences).

3.2.7 Statistical analysis.

Cytokine and densitometry data are presented as Mean ±SEM. Both one-way and two-way ANOVA was used to compare differences in cytokine production. Significance was considered if $p< 0.05$. All analyses were carried out via GraphPad Prism software.

3.3 Results

3.3.1 CSSE fraction of *M. bovis and* BCG contains *p*-HBAD-1

In chapter 2, we showed that *M. bovis*-derived cell CSSE fraction exhibit less immune-stimulatory effect on bovine dendritic cells (bDCs). We therefore speculated that the CSSE fraction might be enriched with molecules like *p*-HBAD-1 that possess immunosuppressive properties, and contribute to the survival and proliferation of the bacteria within its host. Indeed, analysis of *M. bovis* CSSE by thin-layer chromatography (TLC) confirmed the presence of *p*-HBAD-1 (Fig. 3.1).

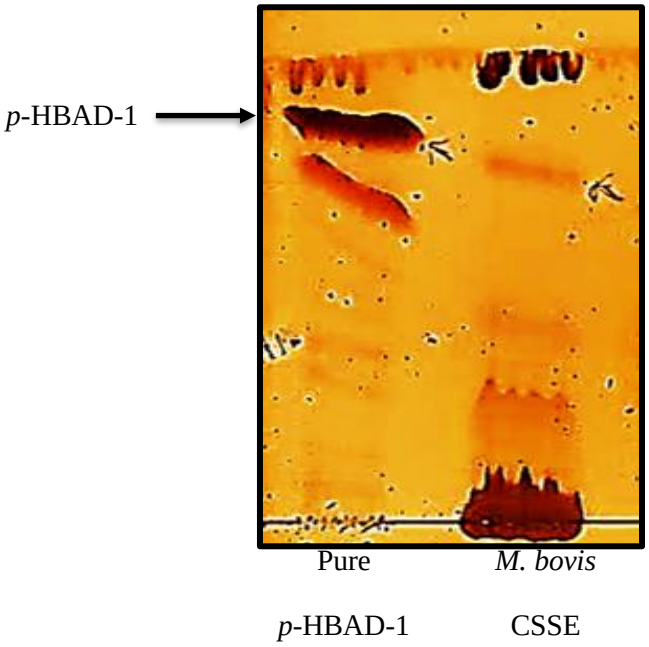


Figure 3.1: TLC showing the presence of *p*-HBAD-I in *M. bovis* derived CSSE. The arrow indicates the presence of *p*-HBAD-1 in *M. bovis* derived CSSE, which corresponds to the pure synthetic *p*-HBAD-1.

3.3.2 Pure *p*-HBAD-1 inhibits the production of TNF-α and IL-12 by LPS-, bPPD- and CWL-stimulated bovine bDCs in a dose-dependent manner

Various cytokines elicited by DCs during the innate phase of activation play a crucial role in deciding the outcome of mycobacterial infection. As reported by Hickman et al., IL-12 and TNF-α are pivotal for the induction of optimal Th1 responses against invading pathogens, including mycobacteria [403]. Earlier studies showed that pure synthetic *p*-HBADs suppressed the production of IL-12 and TNF-α in mouse macrophages stimulated with irradiated *M. tuberculosis* [69, 404]. However, the effect of *M. bovis* CSSE on bDCs had not been determined. To address this, the effect of different concentrations of synthetic *p*-HBAD-1 on IL-12 and TNF-α production was determined following overnight incubation with LPS, bPPD or CWL. The result obtained was compared to LPS positive control. Relative to no treatment, the induction of IL-12 and TNF-α was most potent in cells treated with 1 µg/mL of LPS (positive control). Likewise, treatment of bDCs with bPPD and CWL also led to the induction of these cytokines, albeit to a lesser extent than LPS (Figs. 3.2A to F). Interestingly, co-treatment of increasing concentrations of *p*-HBAD-1 with each of these three different antigen preparations significantly abrogated production of IL-12 (Figs. 3.2A to C) and TNF-α (Figs. 3.2D to F) in a dose-dependent manner. These results suggest that *M. bovis*-derived *p*-HBAD-I inhibits cytokine production by bDCs.

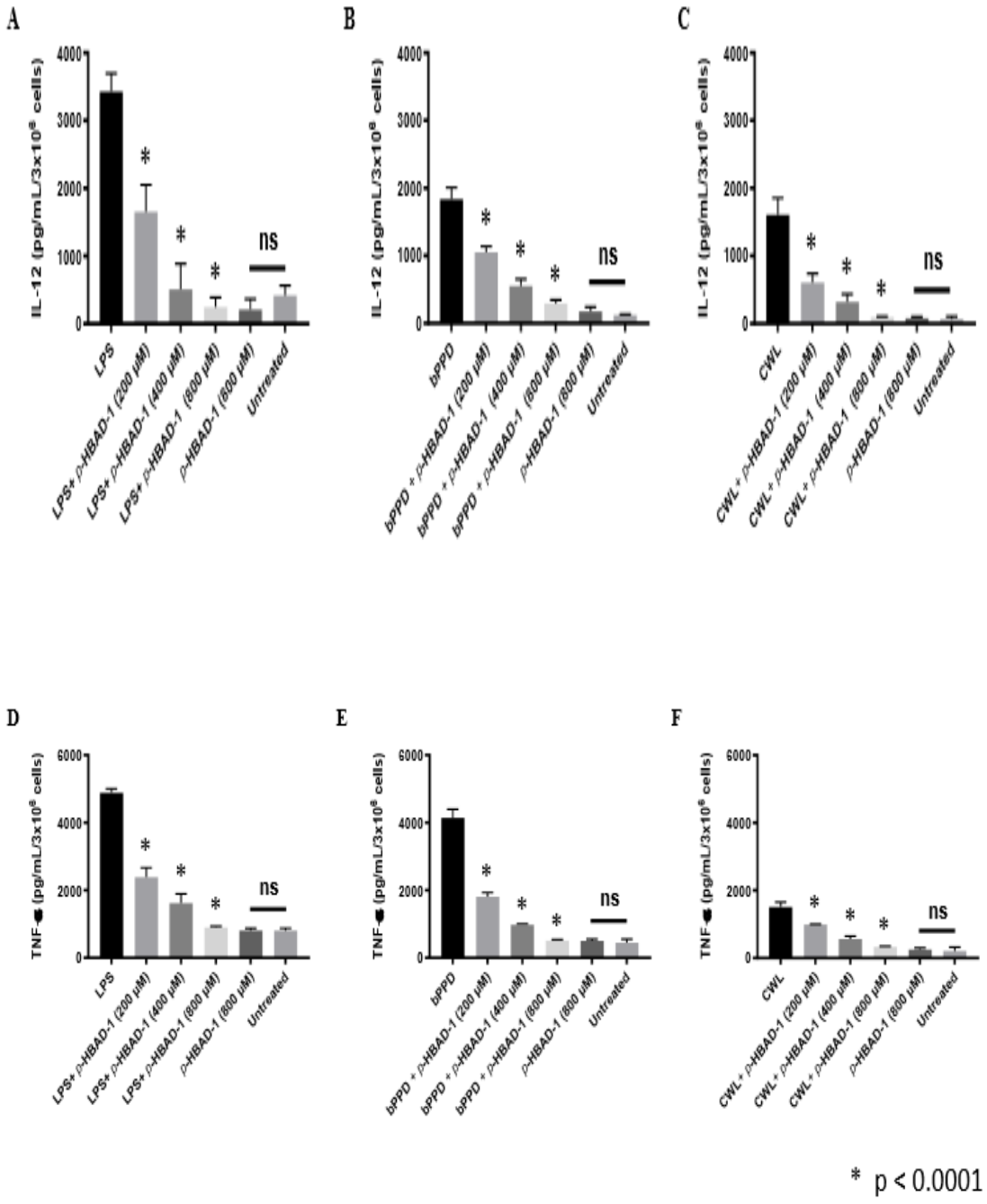


Figure 3.2: Production of IL-12 and TNF-α by non-primed bDCs stimulated with LPS and bPPD in the presence of various concentrations of *p*-HBAD-1. Bovine dendritic cells were stimulated with LPS (1 µg/mL), bPPD (1 µg/mL), CWL (1 µg/mL) in the presence of various concentrations of *p*-HBAD-1 (200, 400 & 800 µM/mL) overnight and the production of IL-12 **(A, B & C)** and TNF-α **(D, E & F)** were determined by ELISA. In all experiments, LPS (1 µg/mL) and untreated cells served as a positive and negative control, respectively. Results are presented as mean (+/- standard error of mean) of signals from replicate wells and represent 3 independent experiments (*p <0.0001).

3.3.3 Pure *p*-HBAD-1 inhibits cell surface TLR 1, 2, 4, 5 and 6-dependent signaling in naïve bovine bDCs stimulated with corresponding TLR agonists

Following the recognition of microbial components by TLRs, intracellular TLR signaling pathways get activated, resulting in cytokine production [405]. In addition, TLR agonists (a synthetic analog of microbial components) have been reported to initiate an intracellular signaling event that results in the activation of a variety of proinflammatory immune responses [239]. Therefore, to determine the precise TLRs signaling pathways that *p*-HBAD-1 targets to mediate its inhibitory effect, TLR-specific agonists were used to activate bDCs in the absence and presence of *p*-HBAD-I and cytokine production was assessed by ELISA.

Results showed that TLR agonists (Pam_3CSK_4, HKLM, LPS, Flagellin, synthetic lipopeptide-FSL-1) specific to surface membrane TLR (1, 2, 4, 5 and 6) exhibit their agonistic effects by inducing IL-12 and TNF-α production in bDCs. However, in the presence of *p*-HBAD-1, the production of these cytokines via surface membrane TLRs-dependent pathway was significantly suppressed (Figs. 3.3A and B). Given that binding of ligands to surface membrane TLR stimulates specific intracellular downstream signaling cascades that initiate host defense responses, this data suggests that *p*-HBAD-1 targets multiple surface membrane TLR signaling pathways to inhibit cytokine production. Alternatively, there might be a common and shared downstream point in surface TLR signaling pathway that is being targeted by *p*-HBAD-1.

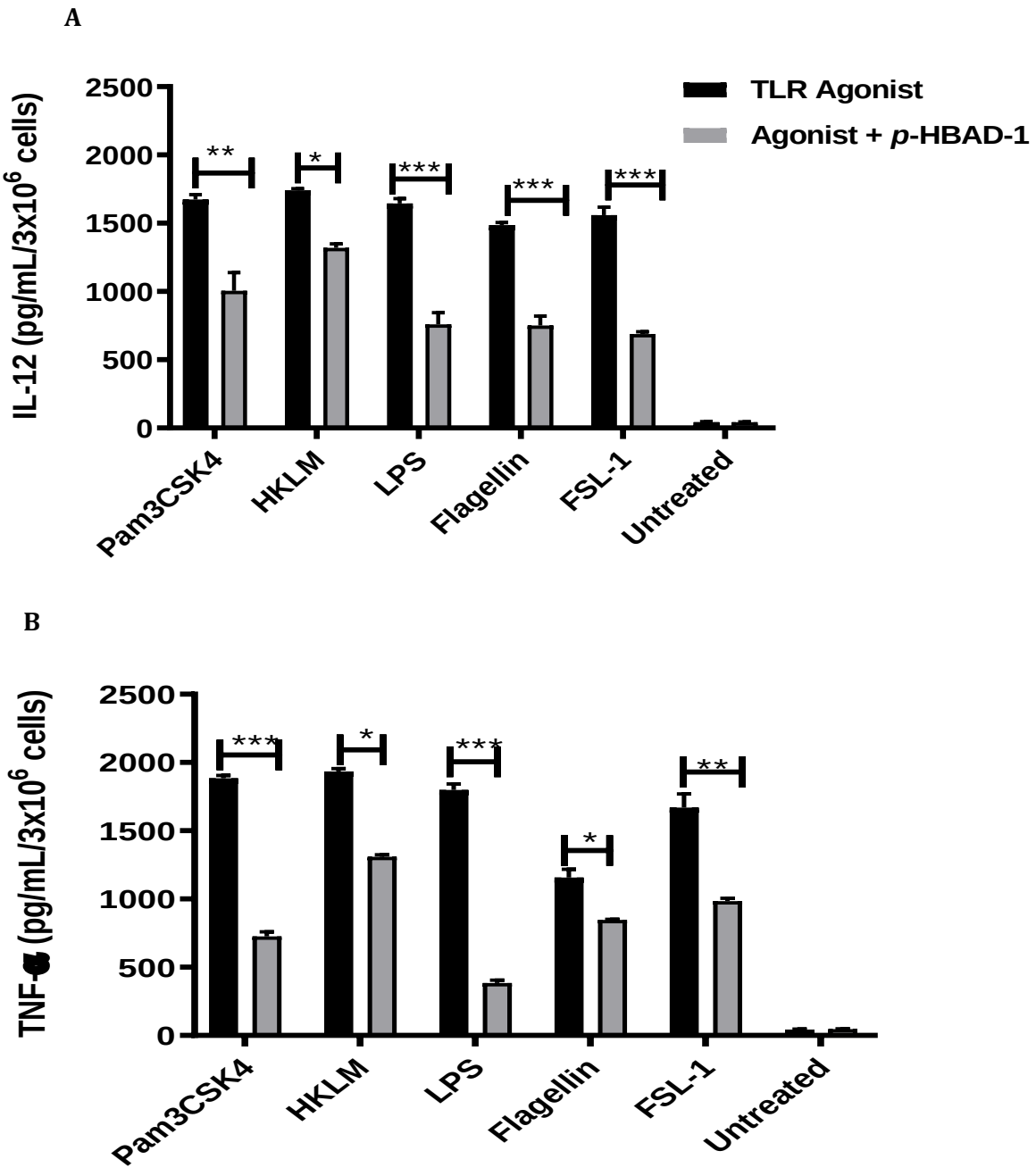


Figure 3.3: Production of IL-12 and TNF-α via surface membrane TLR signaling pathway in non-primed bDCs stimulated with TLR agonist in the presence of *p*-HBAD-1. Bovine dendritic cells were stimulated with surface membrane TLR agonist in the presence and absence of *p*-HBAD-1 (200 μM/mL), following overnight incubation, the production of IL-12 **(A)** and TNF-α **(B)** were determined by ELISA. In all experiments, untreated cells served as a negative control. Results are presented as mean (+/- SEM) of signal from replicate wells and represent three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$).

3.3.4 Pure *p*-HBAD-1 enhances endosomal TLR 3, 7, 8, and 9-dependent signaling in IFN-γ primed bDCs stimulated with corresponding TLR agonists

To determine if other TLRs signaling pathways are inhibited by *p*-HBAD-1, endosomal TLR agonists (ODN, Poly I. C, Imiquimod, ssRNA) were used to activate bDCs in the absence and presence of *p*-HBAD-1, and cytokine production was determined. However, IFN-γ priming is a requirement for endosomal TLR signaling [406]. Moreover, IFN-γ produced by T-cells has a combined effect with TLR ligation to enhance DCs activation and function [406]. Accordingly, bDCs were primed with rIFN-γ for 2 h before incubation with endosomal TLRs agonists in the absence and presence of *p*-HBAD-1, and cytokine production following overnight incubation was assessed and compared ELISA. In the absence of *p*-HBAD-I, endosomal TLR agonists stimulate IFN-γ primed bDCs to produce IL-12 and TNF-α. Interestingly, when *p*-HBAD-1 was added, it induced a robust increase of cytokine outputs over that triggered by endosomal TLR agonist alone (Figs. 3.4A and B).

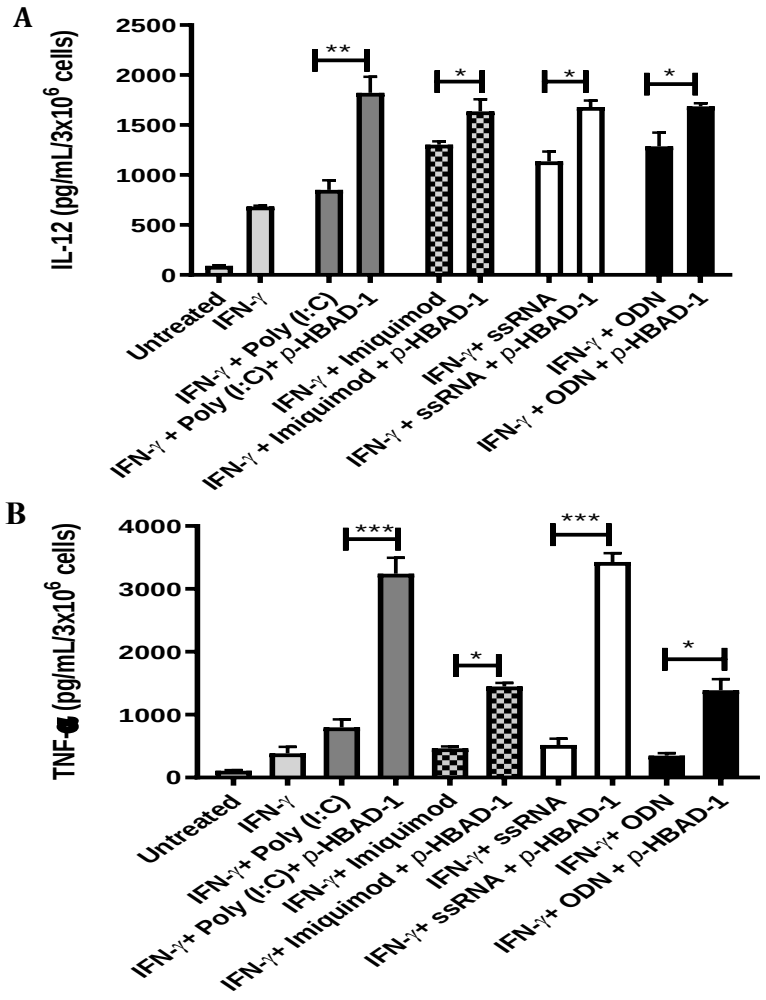


Figure 3.4: Production of IL-12 and TNF-α via endosomal TLR signaling pathway in IFN-γ-primed bDCs stimulated with TLR agonist in the presence and absence of *p*-HBAD-I. Bovine dendritic cells were primed with rIFN-γ (100 ng/mL) for 2 h before stimulation with endosomal TLR agonist in the presence and absence of *p*-HBAD-I (200 μM/mL), following overnight incubation, the production of IL-12 and TNF-α **(A & B)** were determined by ELISA. In all experiments, untreated cells served as a negative control. Results are presented as mean (+/- SEM) of signal from replicate wells and represent three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$).

3.3.5 Pure *p*-HBAD-1 promotes cell surface TLR 4, 5 and 6-dependent signaling in IFN-γ primed bDCs stimulated with corresponding TLR agonists

Based on the results with IFN-γ primed bDCs with respect to endosomal TLR signaling, in the effect of IFN-γ priming on surface membrane TLR signaling was also investigated. As was done with endosomal TLRs agonists, bDCs were primed with rIFN-γ for 2 h before incubating with surface membrane TLR agonists in the presence and absence of *p*-HBAD-1, and the production of IL-12 and TNF-α was assessed. Consistent with cytokine induction with endosomal dependent signaling pathway, result showed increased production of IL-12 and TNF-α in IFN-γ primed bDCs treated with surface membrane TLR agonist in the absence of *p*-HBAD-I. However, a slight increase in these cytokines was observed in the presence of *p*-HBAD-1 (Fig. 3.5A and B). Considering that IFN-γ priming promotes DCs response to TLR ligands, these data collectively suggest that with IFN-γ primed bDCs, the inhibitory effect of *p*-HBAD-1 on TLRs signaling pathway and associated cytokines is either reversed or at least somewhat mitigated.

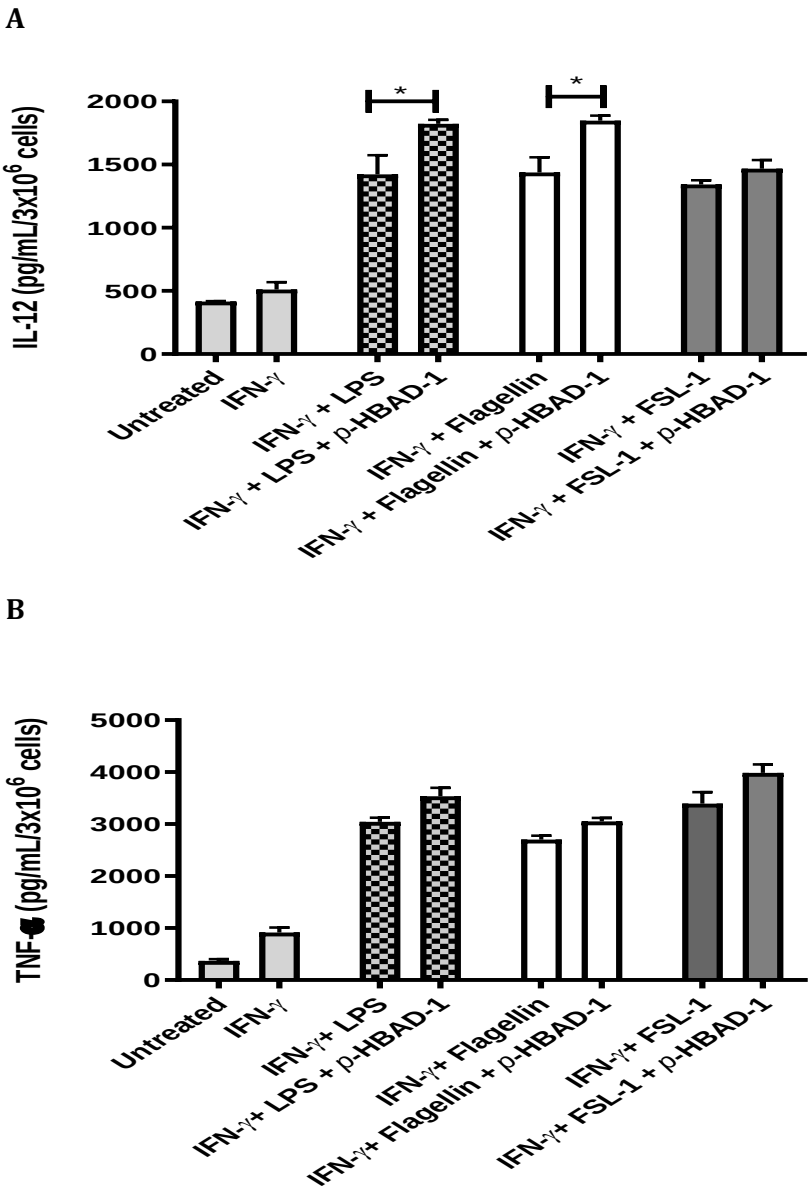


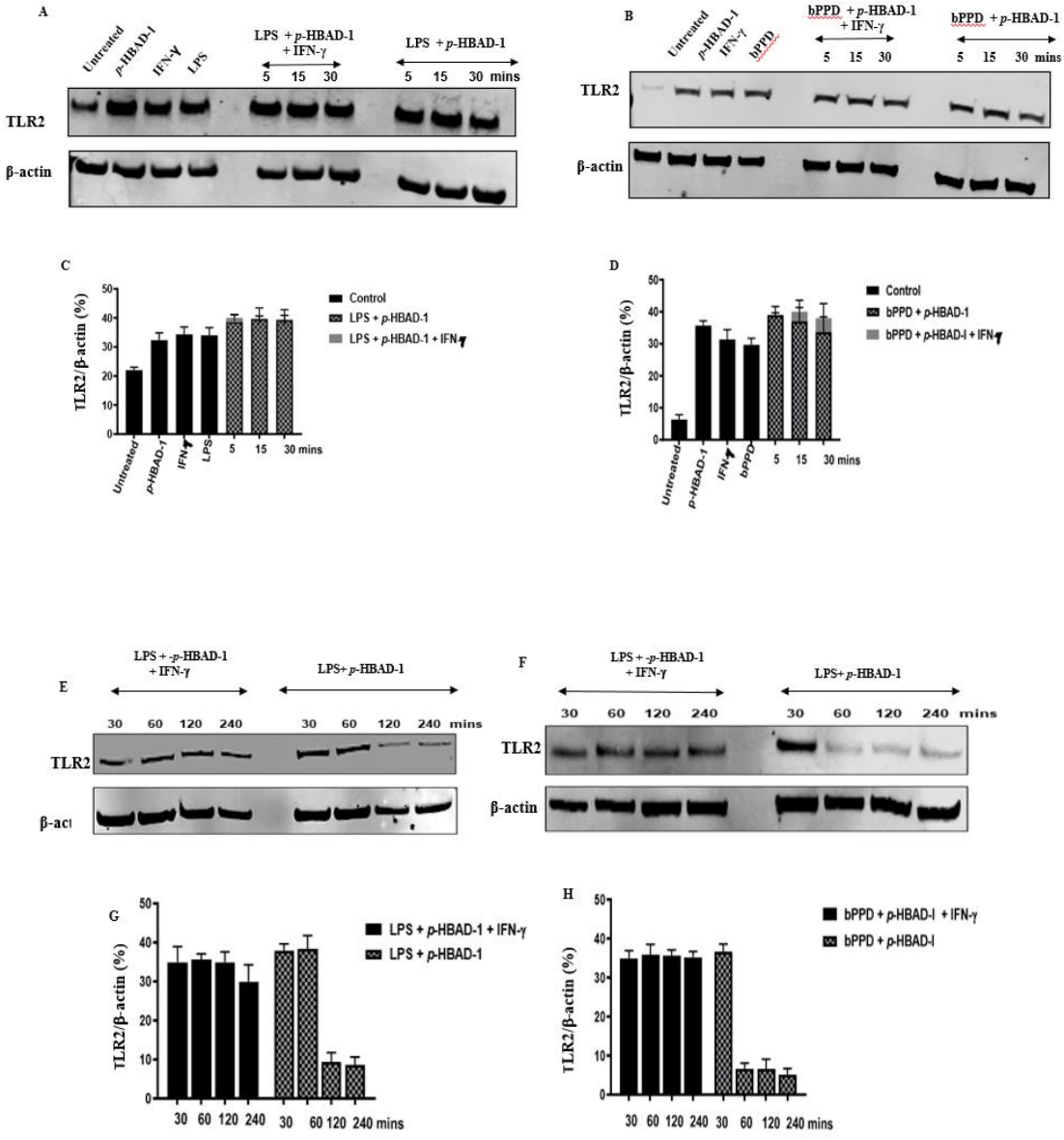
Figure 3.5: Production of IL-12 and TNF-α via surface membrane TLR signaling pathway in IFN-γ-primed bDCs stimulated with TLR agonist in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-γ (100 ng/mL) for 2 h before stimulation with surface membrane TLR agonist in the presence and absence of *p*-HBAD-I (200 μM/mL). Following overnight incubation, the production of IL-12 and TNF-α **(A & B)** were determined by ELISA. In all experiments, untreated cells served as a negative control. Results are presented as mean (+/- SEM) of signal from replicate wells and are representative of 3 independent experiments (*, $p < 0.05$).

3.3.6 Pure *p*-HBAD-1 decreases TLR-2 and 4 expression in LPS-stimulated bDCs but not IFN-γ primed and LPS-stimulated bDCs

Studies by others have shown that mycobacterial antigens can activate DCs maturation via ligation with TLRs, in particular TLR2 and TLR4 [407]. Given that priming of bDCs with IFN-γ appears to block the inhibitory effect of *p*-HBAD-1 on pro-inflammatory cytokine production via TLR signaling pathways, I decided to examine the expression of TLR2 and 4 in non-primed and IFN-γ primed bDCs stimulated with LPS or bPPD.

Indeed, exposure of both IFN-γ primed and non-primed bDCs to LPS or bPPD in the presence of *p*-HBAD-1 resulted in comparable induction of TLR2 expression at earlier time points (5-30 mins) (Figs. 3.6A to D). However, at later time points (30-240 mins), the up-regulation of TLR2 expression was suppressed by *p*-HBAD-1 in non-primed bDCs after 30 and 60 mins post-stimulation with LPS or bPPD, respectively (Figs. 3.6E to H). Interestingly, *p*-HBAD-1 potentiated LPS or bPPD-induced up-regulation of TLR2 expression in IFN-γ primed bDCs (Figs. 3.6 E to H).

Assessment of TLR4 revealed a similar pattern of receptor expression in IFN-γ primed and non-primed bDCs. LPS or bPPD-induced TLR4 expression level remains strongly up-regulated in IFN-γ primed bDCs during the later time point. However, in non-primed bDCs, *p*-HBAD-1 suppressed TLR4 expression post-stimulation with LPS or bPPD (Fig.3.6 I to L). Given that *M. tuberculosis* activated TLR2 and 4 promotes immunogenic response[408]. Therefore, the decreased production of pro-inflammatory cytokines in non-primed bDCs following LPS or bPPD stimulation is related to the reduced expression of these key receptors.


A
Untreated
p-HBAD-1
IFN-γ
LPS
LPS + p-HBAD-1 + IFN-γ
5 15 30
LPS + p-HBAD-1
5 15 30 mins
TLR2
β-actin
B
Untreated
p-HBAD-1
IFN-γ
bPPD
bPPD + p-HBAD-1 + IFN-γ
5 15 30
bPPD + p-HBAD-1
5 15 30 mins
TLR2
β-actin
C
TLR2/β-actin (%)
Control
LPS + p-HBAD-1
LPS + p-HBAD-1 + IFN-γ
Untreated
p-HBAD-1
IFN-γ
LPS
5 15 30 mins
D
TLR2/β-actin (%)
Control
bPPD + p-HBAD-1
bPPD + p-HBAD-I + IFN-γ
Untreated
p-HBAD-1
IFN-γ
bPPD
5 15 30 mins
E
LPS + -p-HBAD-1 + IFN-γ
LPS+ p-HBAD-1
30 60 120 240
30 60 120 240 mins
TLR2
β-act
F
LPS + -p-HBAD-1 + IFN-γ
LPS+ p-HBAD-1
30 60 120 240
30 60 120 240 mins
TLR2
β-actin
G
TLR2/β-actin (%)
LPS + p-HBAD-1 + IFN-γ
LPS + p-HBAD-1
30 60 120 240 30 60 120 240 mins
H
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bPPD + p-HBAD-I + IFN-γ
bPPD + p-HBAD-I
30 60 120 240 30 60 120 240 mins

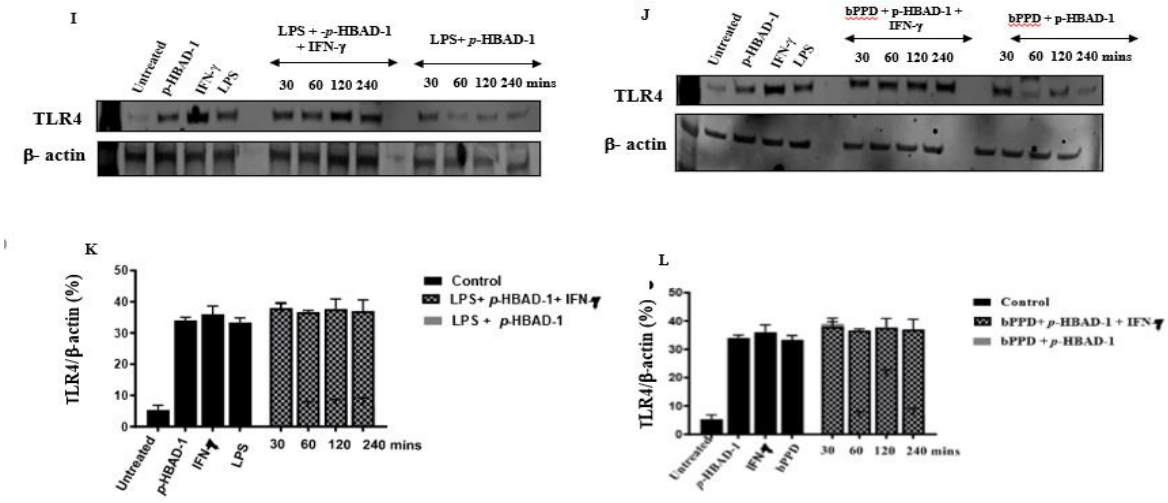


Figure 3.6: Expression of TLR2 & 4 protein by rIFN-γ primed and non-primed bDCs stimulated with LPS and bPPD in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-g for 2 h before stimulation with LPS (1 µg/mL) or bPPD (1 µg/mL) in the presence of *p*-HBAD-I (200 µM/mL). The cell lysate was collected at different time points and assessed for expression of TLR2 & 4 protein by Western blot, and the result obtained were compared with non-primed bDCs **(A, B, E, F, I & J)**. The same blots were stripped with stripping buffer and re-probed with antibodies against β-actin, then used as loading controls. The percentage of expressed TLR2 relative to β-actin was calculated by densitometry and plotted as bar graphs **(C D, G, H, K & L).** Results are presented as mean (± SEMs) of 3 different blots.

3.3.7 Pure *p*-HBAD-1 decreases LPS/bPPD-induced activation of NF-κB protein in non-primed bDCs unlike in IFN-γ primed bDCs.

NF-κB (p65 subunit) is a key transcription factor involved in the expression of pro-inflammatory genes, such as those encoding IL-12, TNF-α, IL-1β [409]. Previous studies demonstrated that *M. tuberculosis* induces proinflammatory cytokines in human DCs by activation of NF-*κ*B [302]. To examine the effects of *p*-HBAD-1 on the activation of NF-κB in unprimed and primed bDCs stimulated with either LPS or bPPD, its phosphorylation was assessed by Western blot. Consistent with cytokine analysis data, *p*-HBAD-I was found to dampen LPS or bPPD-induced NF-κB activation in non-primed bDCs. In contrast,

p-HBAD-I was found to augment LPS or bPPD mediated phosphorylation of NF-κB in IFN-γ primed cells (Fig. 3.7A and B). Given that NF-κB signaling plays a role in pro-inflammatory cytokine production, these results suggest that suppression of IL-12 and TNF-α by *p*-HBAD-1 in non-primed bDCs may be driven by its inhibition of NF-κB phosphorylation and consequently of its activation.

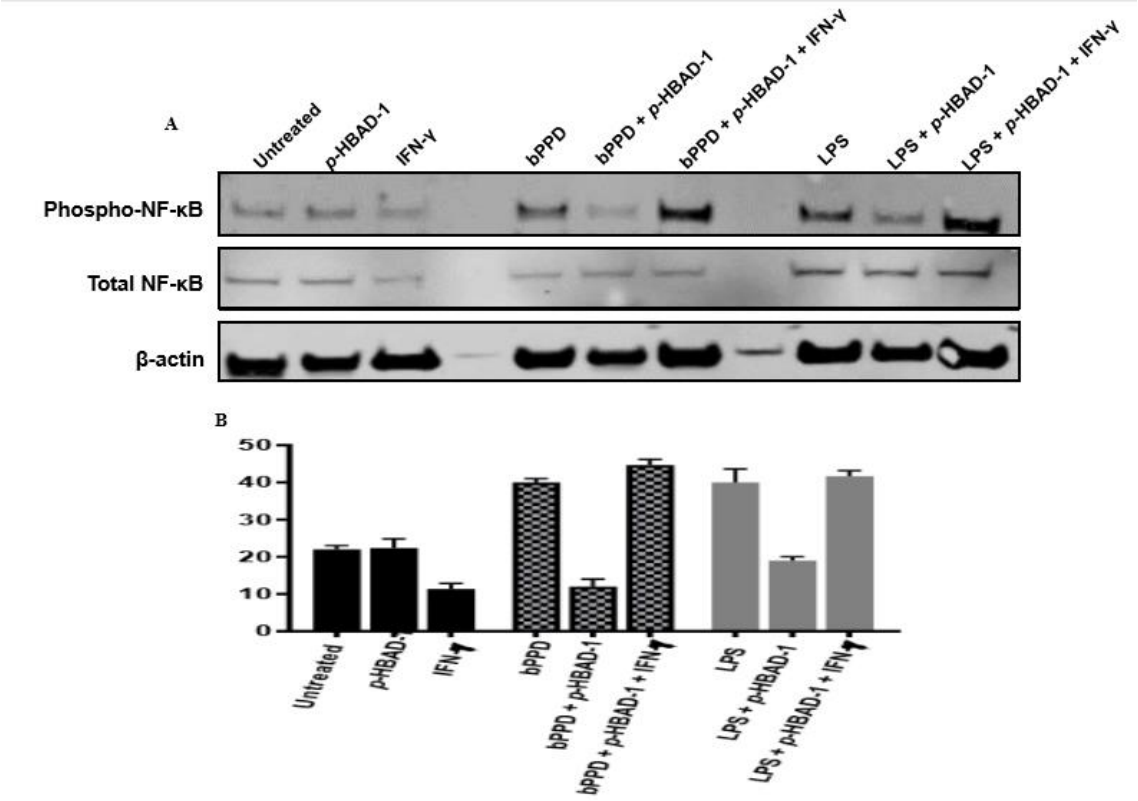


Figure 3.7: Phosphorylation of NFκB protein by rIFN-γ primed and non-primed bDCs stimulated with LPS or bPPD in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-γ (100 ng/mL) for 2 h before stimulation with LPS (1 µg/mL) or bPPD (1 µg/mL) in the presence of *p*-HBAD-1 (200 µM/mL). Cell lysate were collected after 1h and assessed for both phosphorylated and total NF-κB **(A)** by Western blot, and the result generated was compared with non-primed bDCs. The same blots were stripped with stripping buffer and re-probed with antibodies against β-actin, then used as loading controls. The percentage of phosphorylated NF-κB relative to β-actin was calculated by densitometry and plotted as bar graphs **(B).** Data are presented as mean ± SEMs of 3 different blot.

3.3.8 Pure *p*-HBAD-1 up-regulates SOCs1 and 3 expressions in non-primed bDCs stimulated with LPS or bPPD unlike in IFN-γ primed

To maintain a balanced inflammatory response following interaction with PAMPs, the TLR and IFN-γ signaling pathway is controlled by inhibitors of cytokine signaling known as SOCs proteins, which provides a negative-feedback loop to inhibit cytokine signal transduction [410, 411]. The first step involved in TLR signaling has been reported to be blocked by SOCs1 in macrophages [107]. Mycobacteria are known to survive in the host by up-regulating SOCs1and 3 protein expression, resulting in the manipulation of cytokine responses, especially IFN-γ that is required in the resolution of mycobacterial infections [399, 400]. Therefore, we investigated whether *p*-HBAD-1 affects the expression of SOCs1 and 3 in non-primed bDCs.

Results showed that in non-primed bDCs treated either with LPS or bPPD, *p*-HBAD-1 up-regulates SOCs1 expression during the early treatment period. However, the reverse was seen in IFN-γ primed bDCs (Fig. 3.8A to D). Increased SOCs3 expression by *p*-HBAD-1 in non-primed bDCs was maintained during the entire treatment period with LPS, but an increased expression of SOCs3 was only seen after 5 mins of stimulation of non-primed bDCs with bPPD. Interestingly, down-regulation of SOCs 1 and 3 in IFN-γ primed bDCs treated with LPS or bPPD during the entire treatment period were observed (Fig. 3.8A to H). Since SOCs 1 and 3 play a major role in cytokine inhibition, these results suggest that the suppressive effect of *p*-HBAD-1 in pro-inflammatory cytokine production may also be related to the induction of SOCS1 and 3, which block NF-κB activation.

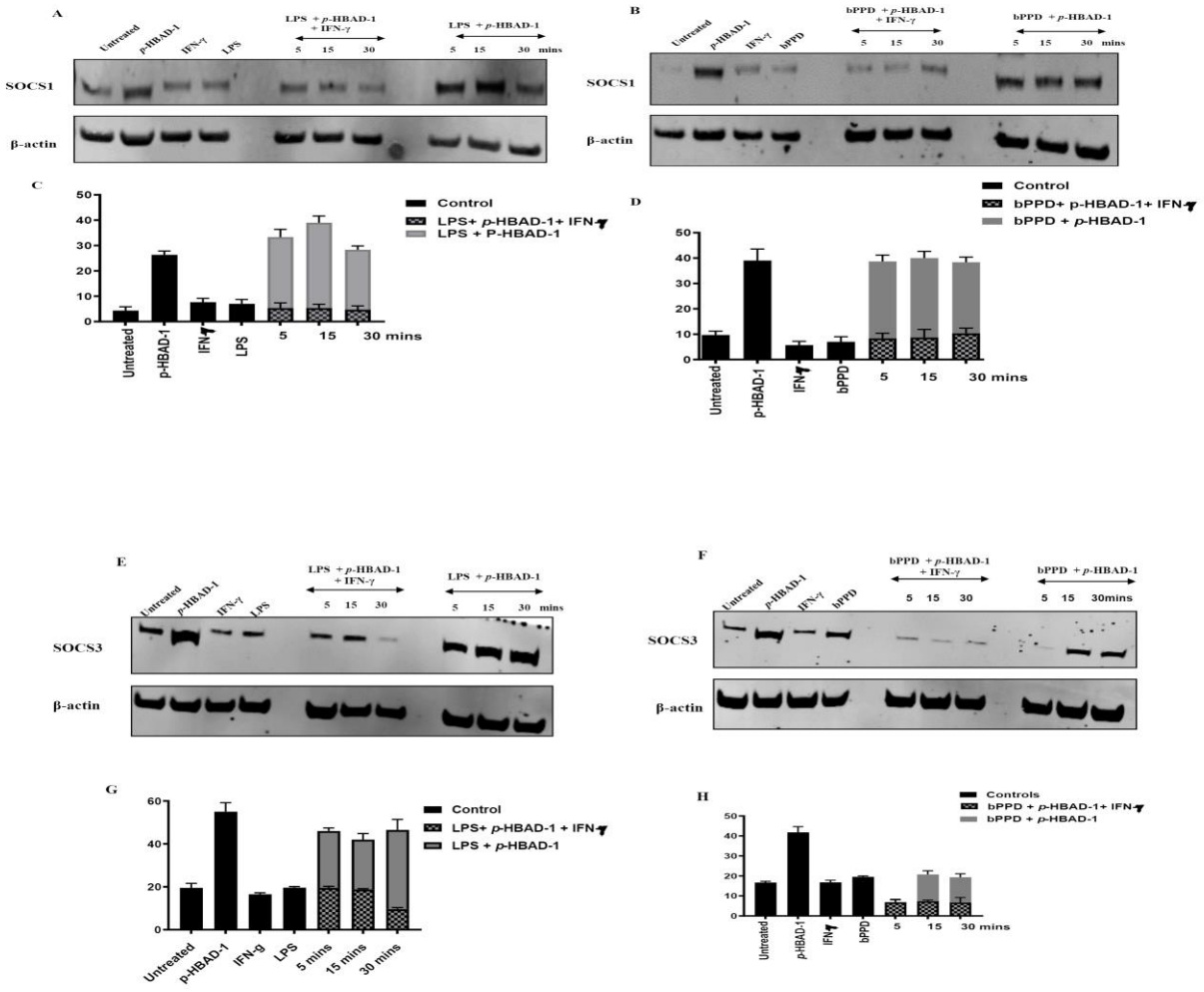


Figure 3.8: Expression of SOCS1 and 3 in non-primed and rIFN-γ primed bDCs stimulated with LPS or bPPD in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-γ(100 ng/mL) for 2 h before stimulation with LPS (1 µg/mL) and bPPD (1 µg/mL) in the presence of *p*-HBAD-1 (200 µM/mL). Cell lysate was collected at different time points and assessed for expression of SOCS1 **(A & B)** and SOCS3 **(E & F)** by Western blot, and result generated was compared with non-primed bDCs. The same blots were stripped with stripping buffer and re-probed with antibodies against β-actin, then used as loading controls. The percentage of expressed SOCs 1 and 3 relative to β-actin was calculated by densitometry and plotted as bar graphs **(C, D, G & H).** Results are presented as mean (± SEMs) of 3 different blots.

3.4 Conclusion

Since we have shown that *M. bovis*-derived CSSE is a poor immune stimulator of bDCs. In this chapter, we confirm the presence of *p*-HBAD-1 in CSSE and analyze the effect of *M. bovis* derived *p*-HBAD-1 on bDCs pro-inflammatory response. Using controlled conditions, we showed that the effect of *p*-HBAD-1 in non-primed and IFN-γ primed bDCs varies. In non-primed bDCs, we observed that *p*-HBAD-1 is capable of blocking the functional phenotype of bDCs by reducing the production of pro-inflammatory cytokines (IL-12 and TNF-α) induced by either LPS or bPPD. Furthermore, we propose that the molecular mechanism by which *p*-HBAD-1 mediates its immunosuppressive effect in non-primed bDCs depends on the regulatory effect of SOCs1/3 protein expression. Interestingly, these effects were reversed in IFN-γ primed bDCs, resulting in an increased pro-inflammatory response. These observations are consistent with previous studies, which showed that *M. tuberculosis*-derived *p*-HBADs suppress pro-inflammatory response in macrophages stimulated with LPS or irradiated *M. tuberculosis* [68, 70]. However, the only difference is that the effect of *p*-HBAD-1 in IFN-γ primed cells was not assessed in previous studies. In summary, our study suggests that *p*-HBAD-I plays a dual role in inducing immunosuppressive and immunostimulatory responses in non-primed and IFN-γ primed DCs, respectively.

Transition statement

We have shown in chapter 3 that the effect of p-HBAD-1 in non-primed and IFN-γ primed bDCs varies. In non-primed bDCs, p-HBAD-1 blocked the inflammatory response by decreasing pro-inflammatory cytokine production (IL-12 and TNF-α). Previous studies have shown that p-HBADs from M. tuberculosis can suppress host immune response in a mouse model and promote M.tb virulence [68, 69]. Furthermore, a recent report showed that M. tuberculosis derived p-HBAD-1 and related structures affect early macrophage activation by blocking pro-inflammatory response following BCG vaccination [70]. This suggests that the presence of p-HBAD-1 and related molecules could be undermining the host's protective innate immune response, perhaps by promoting a tolerogenic profile in DCs. In this chapter, we sought to investigate the tolerogenic effect of M. bovis derived p-HBAD-1 in non-primed and IFN-γ primed DCs.

CHAPTER 4. Pure *p*-HBAD-1 promotes a tolerogenic profile in non-primed bDCs treated with LPS or bPPD

4.1. Introduction

The immune system exists in equilibrium between tolerance and effective inflammatory responses. Recent findings suggest that innate immune cells (in particular DCs) are essential players in the induction of effective immunity and tolerance [412]. Immunogenic DCs mediate effective innate and adaptive immunity primarily due to their remarkable capacity to process and present antigens through major histocompatibility complexes (MHC) to naïve T-cells [316]. Unlike immunogenic DCs, regulatory DCs can induce tolerance resulting in T-cell anergy and deletion. Regulatory/tolerogenic DCs are characterized by the production of immunoregulatory factors (IL-10, IL-1β, TGF-β, IDO, arginase I and iNOS) and a decrease in the production of pro-inflammatory cytokines [163]. Considering the prolonged coevolution of tubercle bacilli with humans, the tubercle bacillus has evolved the capacity to persist in the host tissue in a dormant state. This ability primarily depends on cell wall glycolipids, which target antigen-presenting cells, thereby dampening effective T cell immunity [413]. A recent study demonstrated that mycobacterial glycolipid Di-*O*-acyl trehalose induces a tolerogenic phenotype in DCs by altering DCs maturation, leading to the expansion of regulatory T- cells [55]. In non-primed bDCs treated with cell wall antigens, we have shown that *M. bovis* derived *p*-HBAD-1 suppresses pro-inflammatory response, and the reverse was seen in IFN-γ primed bDCs. This inhibitory effect in non-primed bDCs could be related to IL- 10 production, which exerts negative feedback inhibition. Given that IL-10 is a major characteristic of

tolerogenic DCs, we hypothesize that *p*-HBAD-1 might be inducing a tolerogenic profile in non-primed bDCs by upregulating the production of IL-10. Thus, the tolerogenic effect of *p*-HBAD-1 on LPS or bPPD treated IFN-γ primed and non-primed bDCs was assessed by checking for production of IL-10, expression of DCs activation markers and DC-SIGN receptor. Results show that the effect of *p*-HBAD-1 in non-primed and IFN-γ primed bDCs varies. *p*-HBAD-1 induces a tolerogenic phenotype in non-primed bDCs activated with either LPS or bPPD by undermining DCs co-stimulatory molecules' expression and increasing the expression of DC-SIGN. In addition, the production of IL-10 was upregulated. However, in interferon-gamma primed bDCs, the tolerogenic phenotype was suppressed. These findings taken together suggest that *M. bovis*-derived *p*-HBAD-1 might promote a tolerogenic profile in non-primed bDCs.

4.2 Materials and methods

4.2.1 Generation of monocyte-derived bDCs

As previously described in materials and methods in chapter 2, generation of bDCs from $CD14^+$ monocytes was done using recombinant bovine GM-CSF at 100 ng/mL (Biorad) and IL-4 at 50 ng/mL (Biorad) [384]. bDCs were assessed by flow cytometry and the expression of DC-specific marker CD206 was found to be greater than 97%.

4.2.2 *In vitro* cell culture and treatments

Monocyte-derived bDCs were cultured as previously stated in the material and methods section of chapter 2, although with a slight alteration. Briefly, the stimulation of differentiated bDCs suspended in complete RPMI medium was done with LPS (1 µg/mL) in the absence or presence of *p*-HBAD-I (200 µM; Eoin Scanlan Lab, Trinity College Dublin). For IFN-γ primed bDCs, cells were first primed with recombinant IFN-γ (rIFN-γ) (100 ng/mL; Sigma Aldrich Co.) for 2 h before stimulation in the presence and absence of *p*-HBAD-1. The stimulated cells were incubated overnight at 37^{o}C before harvesting cells for flow cytometry staining. Following removal of culture media, treated bDCs were lysed at 5, 30 and 60 minutes with a combination of RIPA lysis buffer and protease inhibitors (Sigma- Aldrich). Cell lysate obtained was used for Western blot analysis.

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-10 in bDCs supernatant was determined by Enzyme-linked immunosorbent assays as previously described in the materials and methods section in chapter 2, but with slight modification. Briefly, high-binding Immulon-2 (Thermofisher

Scientific Inc.) ELISA plates and Maxisorp (Thermofisher Scientific Inc.) ELISA plates were respectively coated with bovine-specific IL-10 monoclonal antibodies produced in-house in coating buffer (Sodium carbonate, sodium bicarbonate and distilled water at pH 9.6), and incubated overnight at 4^{o}C. Following incubation, the plates were washed 5 times with wash buffer (Tris buffered Saline containing 0.05% Tween-20 (TBST), pH 7.4) and blocked for 1 hr at room temperature. 200 µl/well of 0.1% gelatin (Sigma-Aldrich) was used for IL-10 blocking. The micro-well plates were rinsed 5 times with wash buffer, and appropriately diluted recombinant IL-10 standards were titrated in a 2-fold dilution to generate the standard curve. Samples were added undiluted (100 µL/well) and incubated at room temperature for 2 hrs. Thereafter, the plates were washed 5 times with washing buffer and 100 µL of biotinylated detection antibody at 4 µg/mL was added to all wells. After 1 hr incubation at room temperature, the plates were washed 5 times and 100 µL of streptavidin at (1/5000 dilution) was added to the wells and also incubated for 1hr. Then, 100 µL of *p*-Nitrophenyl Phosphate (PNPP) (1 mg/mL) was added to all the wells and incubated for 1 hr at room temperature. Plates were read at 405 nm using Spectra Max plus microplate reader (Molecular Devices).

4.2.4 Western blotting

The expression of DC-SIGN was determined by Western blot as previously described in the materials and methods section in chapter 2, albeit with slight modification. Briefly, overnight incubation of membranes with primary antibodies diluted in antibody dilution buffer (TBS supplemented with 5% bovine serum albumin and 0.05% Tween 20 (TBST) were done. Also, immunoblotting of β-actin was performed. After washing with TBST,

the membranes were incubated for 1 h at room temperature with Alexa Fluor® conjugated secondary antibody, at a dilution of 1:10000 in antibody dilution buffer. Signals were acquired using LI-COR imaging system (LI-COR Biosciences). Densitometry was performed using the band analysis tools of the Image Lab software version (LI-COR Biosciences).

4.2.5 Flow cytometry

Bovine DCs were harvested using cold PBS (Thermofisher Scientific, USA) and centrifuged at 1200 rpm for 5 min. The supernatant was removed, pellet suspended at 5 $x10^5$ cells in100 µl of FACOLA and bovine serum for 15 mins at 4 degrees. Cells were washed with PBS at 400g for 3 mins and stained with fluorochrome-labeled antibodies against the following molecules: CD80, CD86, CD40, MHCI and MHCII (Biorad, Canada). After 30 mins incubation on ice, the cells were re-suspended and washed in 300 µl PBS and fixed in 2% paraformaldehyde. PE-labeled CD14 (marker of monocyte) monoclonal antibody (Biorad, Canada) was used to assess monocyte purity, while APC-labelled CD206 (marker of DCs) monoclonal antibody (Biolegend, USA) was used to assess the DCs markers. Flow cytometry analyses were carried out according to standard procedures. Briefly, stained cells were acquired using CyAn ADP (at least 100,000 events). To avoid false positive result, debris was first gated out, and a Near IR stain (Thermofisher Scientific) was used to remove dead cells. Thereafter, another gate was drawn to remove doublet cells. Subsequent gate was placed only on CD206 positive cells, and the geometric mean fluorescent intensity of the markers (CD80, CD86, CD40, MHCI and MHCII) were compared among the different treatment conditions in the overlay. The analyses were

performed using Kaluza analysis software.

4.2.6 Statistical analysis.

The difference in cell surface marker expression was compared using one-way analysis of variance (ANOVA). Significance was considered if $p < 0.05$. The data were further analyzed by GraphPad Prism software.

4.3 Results

4.3.1 Pure *p*-HBAD-1 increases IL-10 production in non-primed bDCs stimulated with LPS or bPPD compared to IFN-γ primed bDCs

In addition to pro-inflammatory cytokine production, DCs infected with mycobacteria also produce the anti-inflammatory cytokine interleukin 10 (IL-10) [153]. To determine whether *p*-HBAD-1 affects anti-inflammatory cytokine production, the production of IL-10 in unprimed and IFN-γ primed bDCs was assessed. The quantification of IL-10 revealed that LPS or bPPD induced IL-10 production in non-primed bDCs compared to non-treated cells. Interestingly, when *p*-HBAD-1 was added in non-primed bDCs treated with either LPS or bPPD, it stimulated a significant increase in IL-10 outputs over that induced by IFN-γ primed bDCs at 24 h of culture (Fig. 4.1). Considering that IL-10 production inhibits pro-inflammatory cytokine production. This suggests that the decreased pro-inflammatory response in non-primed bDCs could also be related to increased IL-10 production.

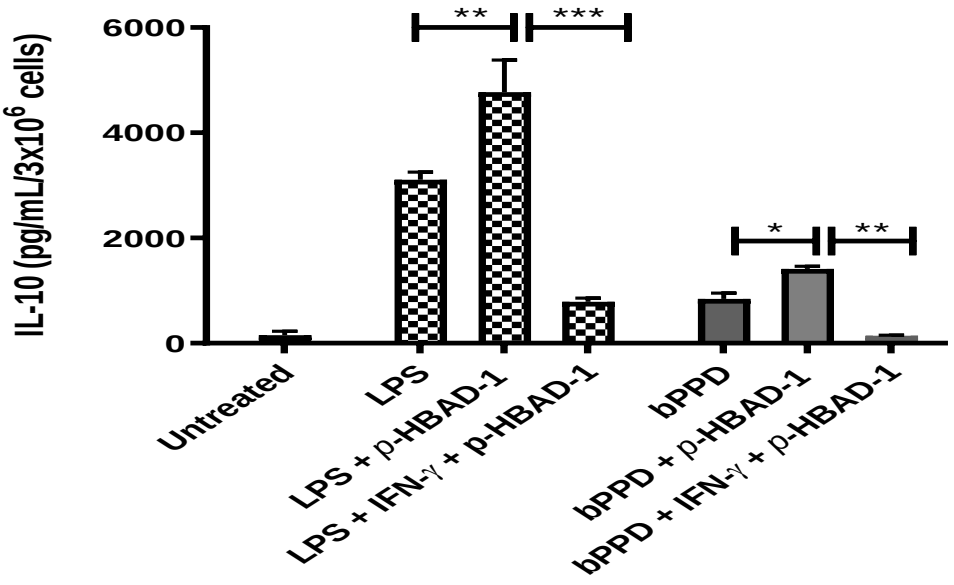


Figure 4.1: Production of IL-10 by IFN-γ primed and non-primed bDCs stimulated with LPS and bPPD in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-γ (100 ng/mL) for 2 h before stimulation with LPS (1 µg/mL), bPPD (1 µg/mL) in the presence of *p*-HBAD-1 (200 µM/mL) for 24 h. The production of IL-10 was determined by ELISA, and the result obtained was compared with non-primed bDCs. In all experiments, untreated cells served as a negative control. Results are presented as mean +/- SEM of signal from replicate wells and are representative of 3 independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

4.3.2 Pure *p*-HBAD-1 decreases the expression of co-stimulatory molecules in non-primed bDCs induced by LPS unlike in IFN-γ primed and similarly treated bDCs

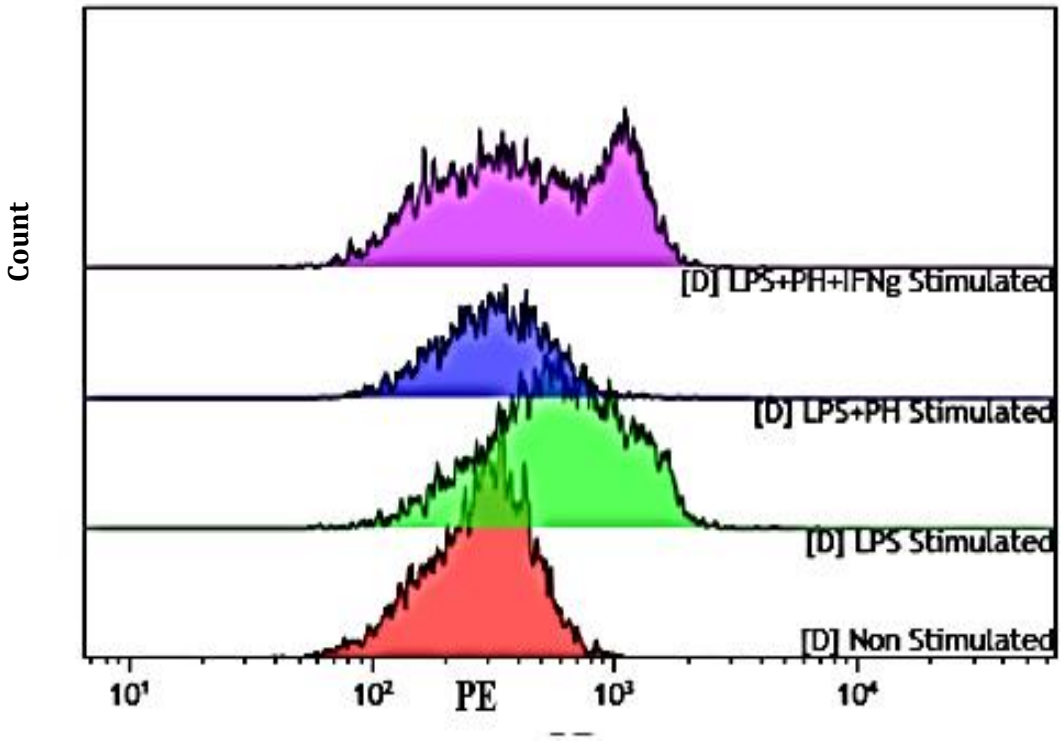
Antigen-presenting cells respond to stimulation by expressing co-stimulatory molecules, which provide signal two required for optimal T-cells activation [414]. The expression of these molecules is increased in response to inflammatory stimuli or by engagement of pattern recognition receptors [415]. During the chronic phase of *M. tuberculosis* infection, it is known that co-stimulatory molecules (CD40, CD80 and CD86) are downregulated in the lung, limiting the capacity of responding T-cells to escape anergy [128]. One

mechanism reported to drive the downregulation of these molecules is the presence of the surface lipid trehalose 6,6′-dimycolate (TDM) [151]. Similar studies showed that distinct macrophage populations that were infected with wild-type *M. tuberculosis* did not express high levels of co-stimulatory molecules, while strains that were devoid of TDM strongly induced the expression of these molecules [416].

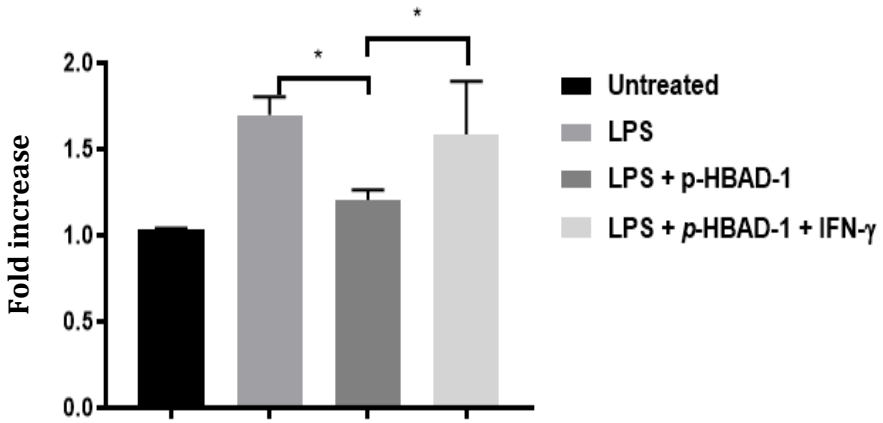
Given that these co-stimulatory molecules play a significant role in the initiation and optimal sustenance of Th1 immune response, I hypothesized that *p*-HBAD-1 also dampens immune responses or induces immune tolerance in non-primed bDCs by altering the expression of these molecules. To confirm this hypothesis, non-primed and IFN-γ primed bDCs were stimulated with LPS in the presence of *p*-HBAD-1, and the cell responses were quantified and compared by the changes in expression of co-stimulatory molecules (CD40, CD80 and CD86) on CD206 positive cells using flow cytometry. Relative to no treatment, non-primed bDCs exposed to LPS resulted in the maturation of bDCs as manifested by significant up-regulation of CD40, CD80 and CD86 markers during 24h culture. In non-primed bDCs exposed to a combination of LPS and *p*-HBAD-I, LPS-driven up-regulation of these co-stimulatory molecules was down-regulated by *p*-HBAD-I. However, in IFN-γ primed bDCs exposed to LPS, *p*-HBAD-1 augments LPS-induced significant expression of CD40 and CD80; however, CD86 expression was not statistically significant. The histogram plots (Figs 4.2A, C and E) and the corresponding bar chart (Figs 4.2 B, D and F) indicate the expression of co-stimulatory molecules by $CD206^{+}$ cells. Since the downregulation of co-stimulatory molecules is a characteristic marker of tolerogenic DCs, this suggests that *p*-HBAD-1 induces immune tolerance in non-prime bDCs.

CD40 Expression

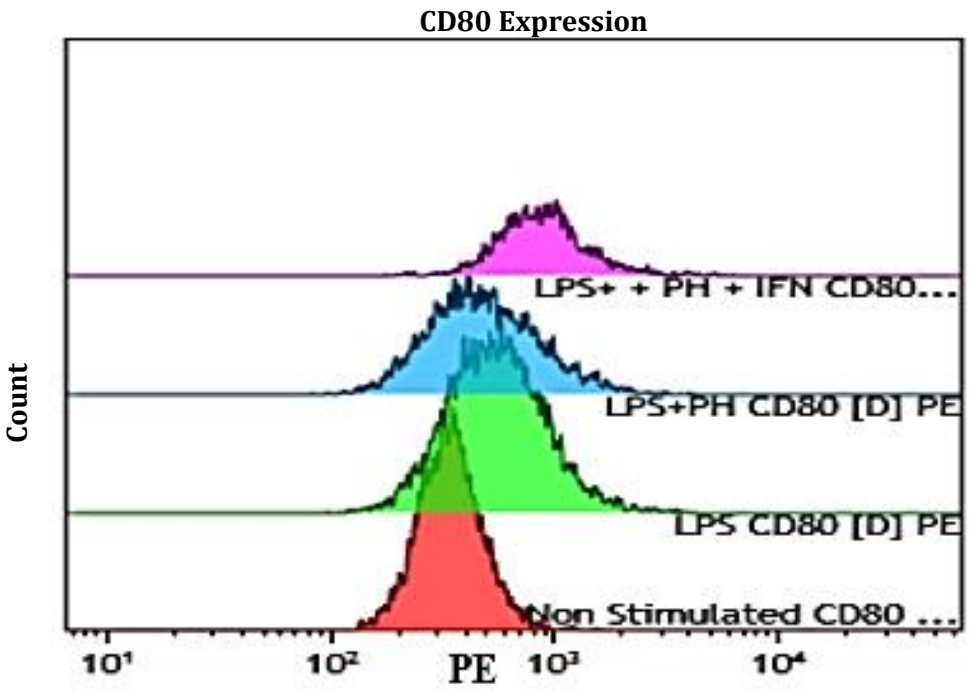
A



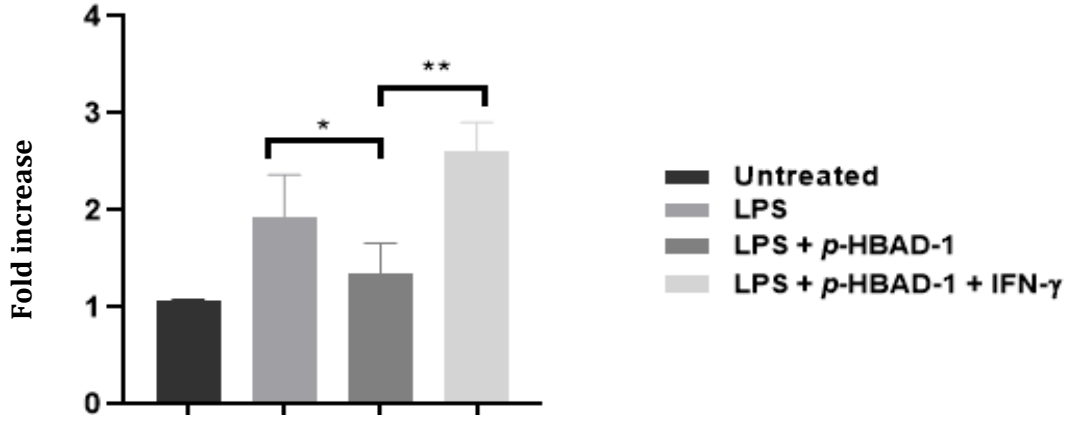
B



C


CD80 Expression
Count
LPS+ + PH + IFN CD80...
LPS+PH CD80 [D] PE
LPS CD80 [D] PE
Non Stimulated CD80 ...
10¹
10²
PE
10³
10⁴

D


Fold increase
4
3
2
1
0
*
**
Untreated
LPS
LPS + p-HBAD-1
LPS + p-HBAD-1 + IFN-γ

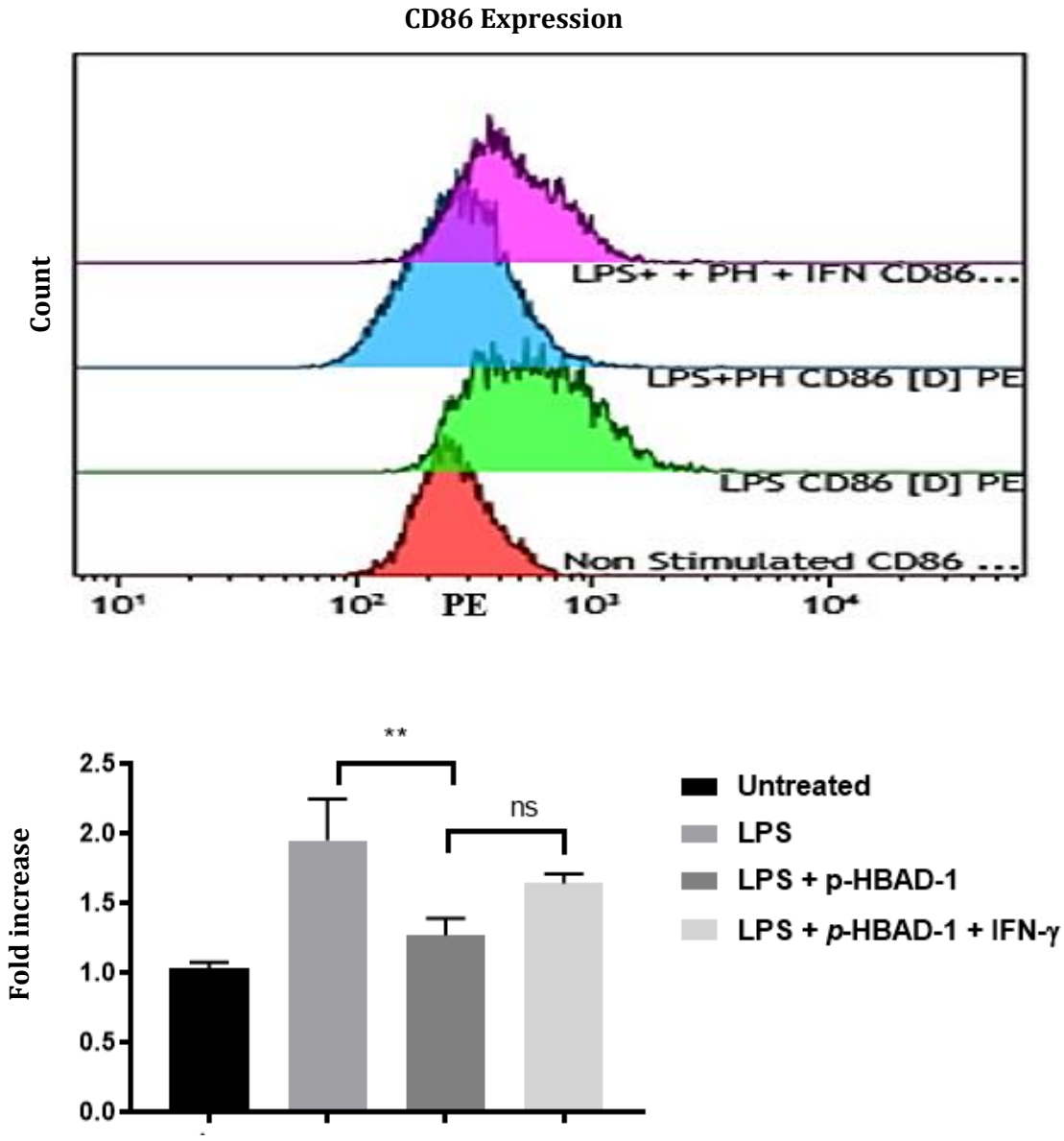
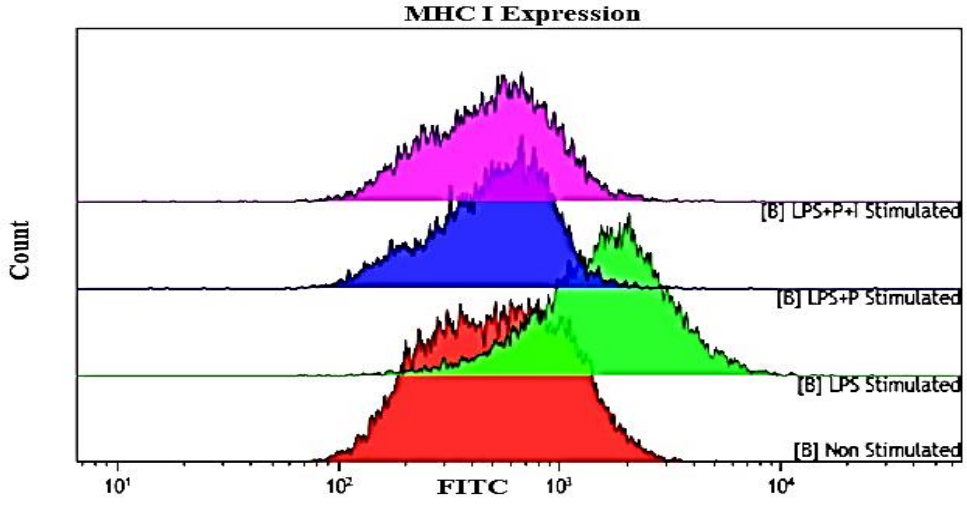


Figure 4.2: *p*-HBAD-1 antagonizes the effects of LPS on bDCs maturation by decreasing the expression of co-stimulatory molecules compared to IFN-γ primed bDCs. Monocyte-derived bDCs were stained with specific antibodies (materials and methods) and assessed for the expression of co-stimulatory molecules by flow cytometry. Shown are histogram plots **(A, C & E)** and the corresponding bar chart (**B, D & F**) indicating the expression of co-stimulatory molecule markers by $CD206^{+}$ cells. Results were expressed as means ± SEMs. Results are representative of 3 independent experiments (*, p <0.05; **, p <0.01).

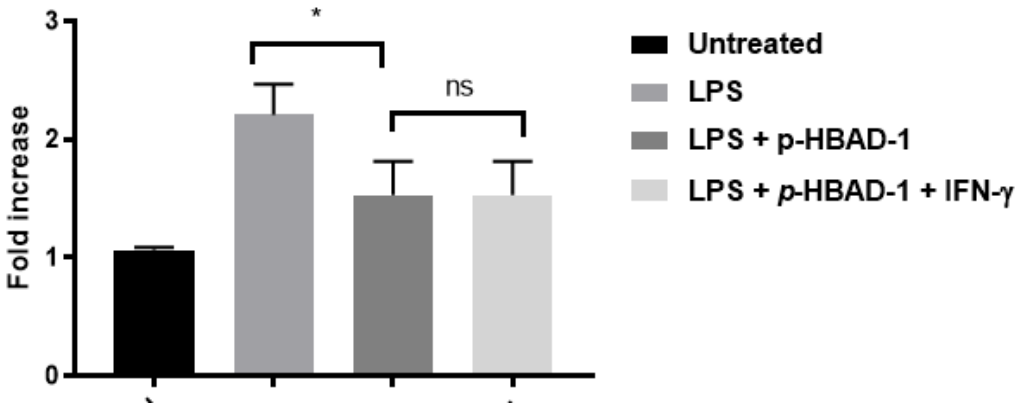
4.3.3 Pure *p*-HBAD-1 down-regulates the expression of antigen-presenting molecules in non-primed bDCs induced by LPS unlike IFN-γ primed and similarly treated bDCs

To further understand the mechanism by which *p*-HBAD-I induces tolerogenic DCs, its effect on antigen-presenting molecules was investigated. Regulatory DCs are characterized by normal levels of expression of antigen-presenting molecules. Thus, no difference in the expression of MHC molecules in both immunogenic and tolerogenic DCs [164]. To evaluate whether *p*-HBAD-I induces immune tolerance by affecting MHC molecule expression, I performed Flow cytometry to detect MHC I and II expression following stimulation of non-primed and IFN-γ primed DCs with LPS. Results showed that LPS promotes bDCs maturation by up-regulating MHC-I and II molecules in non-primed bDCs compared to the levels observed in untreated cells. Surprisingly, no difference was seen in the expression of these molecules in both non-primed and IFN-γ primed bDCs treated with LPS in the presence of *p*-HBAD-I. Shown are histogram plots **(**Figs. 4.3A and C) and the corresponding bar chart (Fig. 4.3B and D) indicating the expression of these MHC molecules by $CD206^{+}$ cells. Given that the expression of these molecules in both non-primed and IFN-γ primed bDCs remain the same, this suggests that MHC molecules do not contribute to tolerogenic phenotype induced by *p*-HBAD-I in non-primed bDCs.

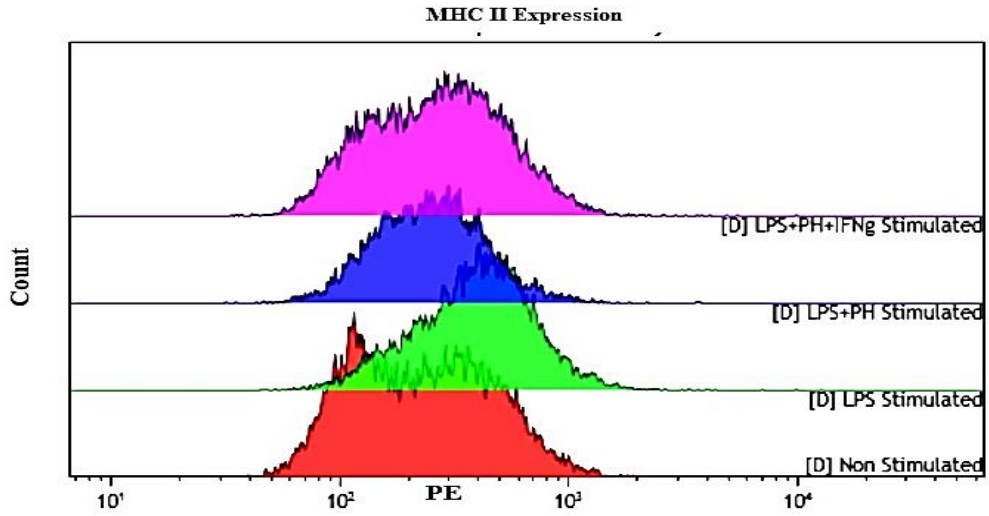
A


MHC I Expression
Count
[B] LPS+P+I Stimulated
[B] LPS+P Stimulated
[B] LPS Stimulated
[B] Non Stimulated
10¹
10²
FITC
10³
10⁴

B


Fold increase
3
2
1
0
*
ns
Untreated
LPS
LPS + p-HBAD-1
LPS + p-HBAD-1 + IFN-γ

C



D

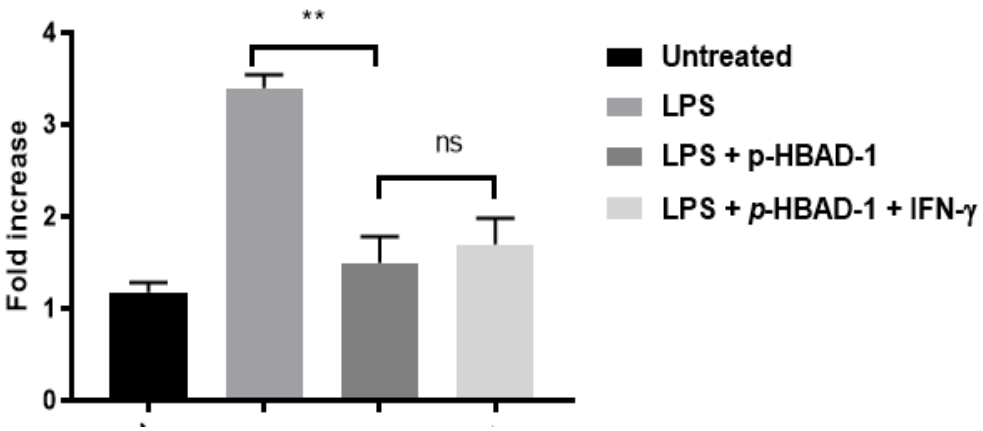


Figure 4.3: *p*-HBAD-1 antagonizes the effects of LPS on bDCs maturation by decreasing the expression of antigen-presenting molecules. Monocyte-derived bDCs were stained with specific antibodies (materials and methods) and assessed for the expression of antigen-presenting molecules by flow cytometry. Shown are histogram plots **(A & C**) and the corresponding bar chart (**B & D)** indicating the expression of MHC molecules by $CD206^{+}$ cells. Results were expressed as means ± SEMs. Results are representative of 3 independent experiments (*, p <0.05; **, p <0.01).

4.3.4 Pure *p*-HBAD-1 upregulates the expression of DC-SIGN in non-primed bDCs unlike IFN-γ primed

The interaction between mycobacteria and macrophages has been extensively investigated, and the mannose receptor has been demonstrated to act as receptors on macrophages for mycobacteria detection [417]. However, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) has been implicated in the interaction of mycobacteria with DCs, and this receptor has a high affinity for mannose-containing carbohydrates [418]. It has been reported that DCs activation by ManLAM via DC-SIGN impairs maturation and induces the production of interleukin 10, resulting in immune tolerance [160]. Based on its carbohydrate recognition profile and its ability to induce immune tolerance, I hypothesized that *p*-HBAD-1 targets DC-SIGN to induce immune tolerance in non-primed bDCs. Following stimulation of non-primed and IFN-γ primed bDCs with LPS in the presence of *p*-HBAD-1, the results showed that *p*-HBAD-1 up-regulates DC-SIGN expression in non-primed bDCs. However, the opposite effect was seen in IFN-γ primed bDCs, with *p*-HBAD-1 down-regulating DC-SIGN expression (Fig. 4.4). This result suggests that *p*-HBAD-1 targets DC-SIGN to induce immune tolerance.

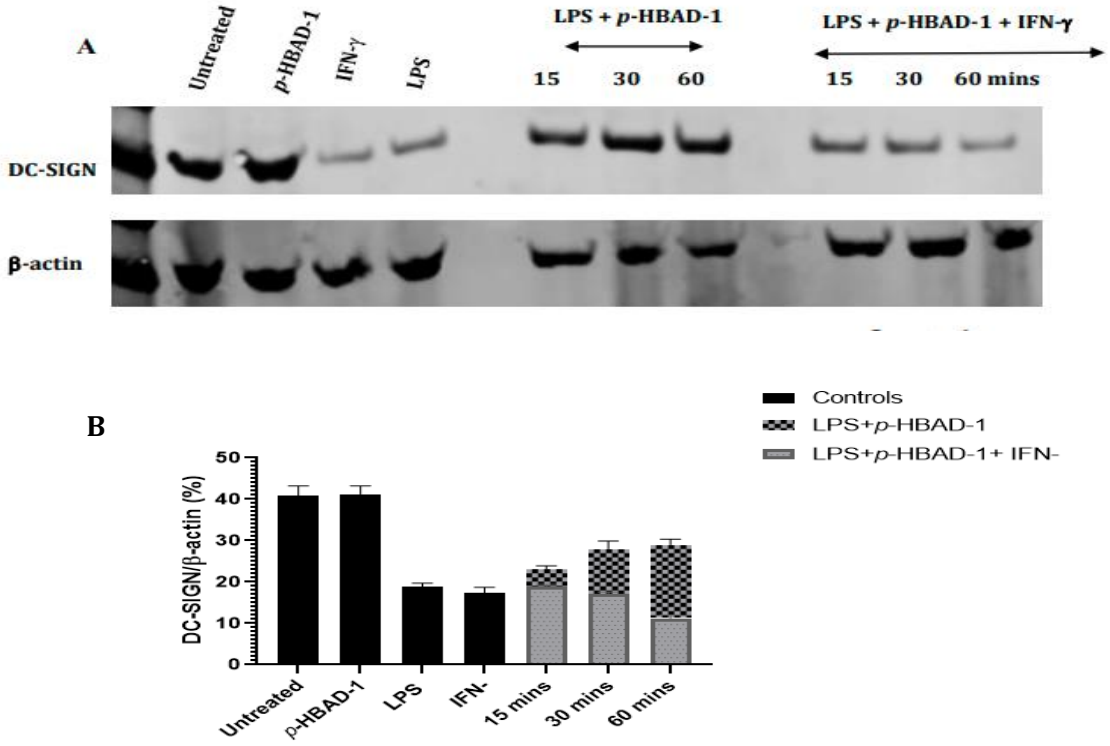


Figure 4.4: Expression of DC-SIGN in non-primed and rIFN-γ primed bDCS stimulated with LPS in the presence of *p*-HBAD-1. Bovine dendritic cells were primed with rIFN-γ (100 ng/mL) for 2 h before stimulation with LPS (1 µg/mL) in the presence of *p*-HBAD-I (200 µM/mL). Cell lysate was collected at different time points and assessed for expression of DC-SIGN **(A)** by Western blot, and the result generated was compared with non-primed bDCs. The same blots were stripped with stripping buffer and re-probed with antibodies against β-actin **(B),** then used as loading controls. Results were expressed as means ± SEMs.

4.4 Conclusion

Several factors, including DCs phenotype, antigen detection receptors, DCs maturation state, and exposure to microbial and soluble inflammatory factors, play a crucial role in the induction of regulatory DCs. Regulation of DCs activation makers by mycobacteria helps them evade host immune response and establish infection [419]. Several studies have shown that different mycobacterial components block antigen-presenting capacity by inhibiting the expression of antigen presentation molecules [420-422]. In addition, a recent study showed that Di-*O*-Acyl-trehalose promotes a tolerogenic profile in murine DCs activated with mycobacterial antigens, resulting in low expression of DCs activation markers [55].

We previously showed that *p*-HBAD-1 blocks the production of pro-inflammatory cytokines in non-prime bDCs, suggesting induction of immune tolerance. This chapter confirms immune tolerance by showing increased IL-10 production in non-primed bDCs, with the opposite effect seen in IFN-γ primed bDCs. Previous studies established that IL-10 blocks the protective pro-inflammatory response in *M. tuberculosis*-infected antigen-presenting cells [356, 423]. Hence, increased IL-10 production might be associated with the decreased production of pro-inflammatory cytokines in non-primed bDCs. Furthermore, we showed that *p*-HBAD-1 only suppresses the expression of co-stimulatory molecules in non-primed bDCs, resulting in impaired dendritic cell function. The inhibitory effect on co-stimulatory molecules was reversed in IFN-γ primed bDCs, leading to enhanced antigen presentation to naïve T-cells and increased immune response. However, MHC I and MHC II expression were similar in both non-primed and IFN-γ primed bDCs.

This observation is consistent with the earlier report demonstrating that tolerogenic DCs poorly express costimulatory molecules compared to immunogenic DCs, but the expression of MHC molecule is comparable in both immunogenic and tolerogenic DCs [424]. Thus, these results demonstrate that *p*-HBAD-1 might induce tolerance in non-primed bDCs by inhibiting secondary signals initiated by co-stimulatory molecules, thereby dampening DCs maturation and efficient antigen presentation required for optimal T-cells activation. Thus, by the lack of co-stimulatory molecules, non primed DCs are not to provide T cells with the necessary signal two required for optimal T cell activation. However, in IFN-γ primed bDCs, *p*-HBAD-1 does not induce a tolerogenic response. Further confirmation of tolerogenic DCs in non-primed bDCs was seen in increased expression of DC-SIGN known induction of immune tolerance following recognition of carbohydrate antigen. In conclusion, *p*-HBAD-1 released by *M. bovis* targets DC-SIGN in non-primed bDCs to induce immune tolerance.

Transition statement

In previously published studies of M. tuberculosis mutants deficient in the biosynthesis of p-HBADs, the mutants were found to grow similarly to the wild-type strain in both macrophages and C57BL/6 mice. However, the mutants induced a more severe and diffuse inflammation in the lungs compared to the wild-type strain [68]. Using in vitro studies, I have shown that pure p-HBAD-1 exhibits a dual role in non-primed and IFN-γ primed bDCs, with p-HBAD-1 inducing tolerogenic and immunogenic phenotypes, respectively. To further confirm the role of M. bovis derived p-HBAD-1 in bTB pathogenesis, it is important to assess the role of p-HBAD-1 by comparing M. bovis WT & mutant strains. To achieve this, I developed the genetic tools needed to generate M. bovis mutants devoid of p-HBAD-1 using a homologous recombination approach [425].

CHAPTER 5. Generation of *M. bovis* mutant

5.1 Introduction

Genetic manipulation of virulent mycobacteria is complicated due to its slow growth rate (>24 hour doubling time), high level of illegitimate recombination, pathogenicity (requires working in a biosafety level three laboratory), and low uptake of DNA [426]. Several techniques for generating mutants that were designed to prevent a high level of illegitimate recombination have been described [273]. Thus, the use of counter-selectable genetic markers and long linear dsDNA substrates inhibit illegitimate recombination [426, 427]. Here, I attempted generating *M. bovis* mutant (lacking *p*-HBAD-1) via chorismate pyruvate ligase (CPL) gene disruption by homologous recombination. The CPL gene (600 base-pair length) encodes the enzyme that produces *p*-HBAD-1 [67]. The synthesis of *p*-HBADs is thought to proceed from *p*-hydroxybenzoic acid [67]. CPL produces *p*-hydroxybenzoic acid from chorismate and it is the sole enzymatic source of this product [67]. The purpose of generating *M. bovis* mutant is to enable future studies to understand the role of *p*-HBAD-1 in bovine TB pathogenesis by comparing *M. bovis* WT & mutant strains.

5.1.1 Generation of *M. bovis* mutant via gene disruption by homologous recombination.

The ability to select genes to knock-out from the mycobacterial genome has greatly improved the understanding of mycobacteria. Here, the CPL gene is disrupted by the insertion of a hygromycin-resistance gene cassette in-frame [425]. A suicide delivery system (containing the mutated CPL gene and selection markers) was used to deliver the mutated CPL gene into *M. bovis* genomic DNA. The disrupted version of the CPL gene

replaces the wild-type version through a two-step homologous recombination process. Selection involves selecting a single cross over event (SCOs) followed by a double cross over (DCOs). Single cross-over events occur during the first recombination and involve integrating suicide delivery plasmid DNA bearing the mutated CPL gene into *M. bovis* chromosome. The SCOs contain both WT and mutated CPL genes. In contrast, DCOs (originating from the second recombination) occur when the mutated CPL gene replaces the *M. bovis* WT CPL gene. Thus the DCOs either contain WT or mutated CPL gene [425]. The four major steps involved in the generation of mutated CPL gene include:- generation and cloning of mutated CPL gene into p2NIL vector, construction of suicide delivery plasmid DNA, transformation of suicide delivery plasmid DNA bearing the mutated CPL gene to *M. bovis and* selection of the mutant strain using a 2-step process.

5.1.1.1 Generation and cloning of mutated CPL gene into p2NIL vector

The amplified CPL gene was first mutated by insertion of hygromycin-resistant gene cassettes before cloning into a p2NIL vector as outlined in the scheme below (Fig. 5.1).

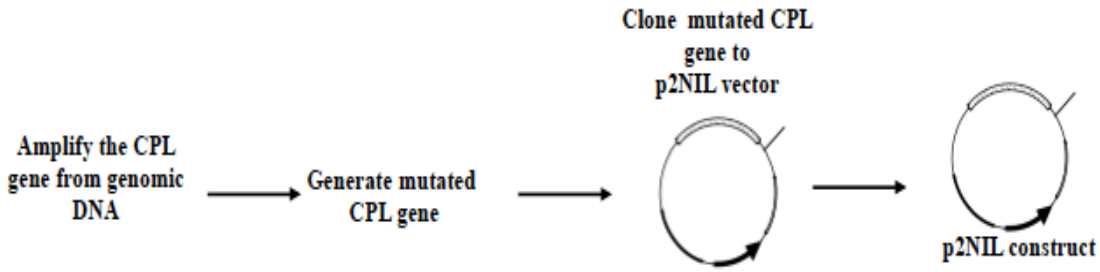


Figure 5.1 Steps in amplification of mutated CPL gene

5.1.1.2 Construction of suicide delivery plasmid DNA

A marker gene cassette (required for selection) obtained from the pGOAL17 vector following digestion with PacI enzyme was cloned into the p2NIL with mutated CPL gene to create a suicide delivery plasmid DNA (Fig. 5.2). The resultant suicide delivery plasmid, which is a non-replicative vector [428], contains a kanamycin-resistant gene (from p2NIL vector), hygromycin-resistant gene (from mutated CPL gene), and gene cassette (containing LacZ and sacB selection makers).

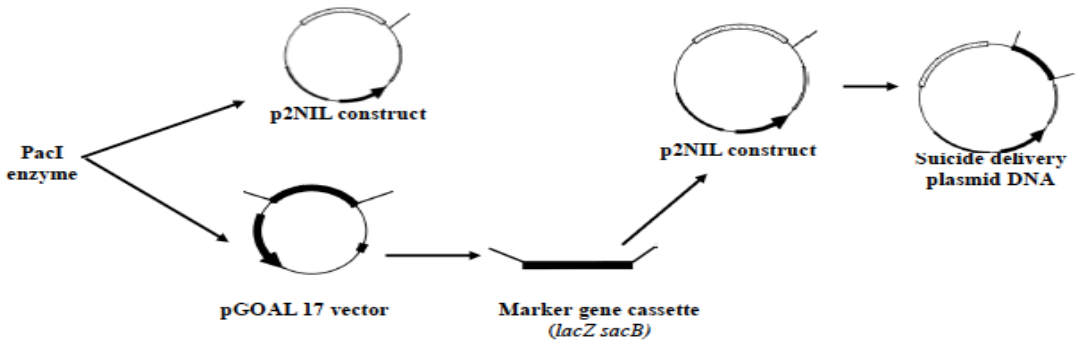


Figure 5.2 Steps in construction of suicide delivery plasmid DNA

5.1.1.3. Transformation of suicide delivery plasmid DNA bearing the mutated CPL gene to *M. bovis*

The suicide delivery plasmid DNA containing the mutated CPL gene was transformed or introduced to *M. bovis*, where the mutated version of the CPL gene replaces the wild-type version by a two-step homologous recombination process. Since the suicide delivery plasmid bears kanamycin and hygromycin resistant genes, as well as cassette (lacZ/SacB) gene, transformation into *M. bovis* was done in a 7H10 agar plate containing kanamycin,

hygromycin and X-gal

5.1.1.4 Selection of mutant strain using a 2-step process

5.1.1.4.1 Single cross over events (SCOs)

Since *M. bovis* cannot survive independently in hygromycin and kanamycin antibiotics, it has to integrate with suicide vector bearing these antibiotic-resistant cassette genes. Thus, suicide delivery plasmid DNA carrying the mutated CPL gene integrates into *M. bovis* chromosome following transformation. Hence, the first recombination event gave rise to SCOs containing two copies of the gene (both mutant and wild type) and the suicide vector integrated into the chromosome. The blue colonies, which are the SCOs containing mutant and WT gene, as well as the vector, were selected because they were hygromycin and kanamycin resistant (Fig. 5.3).

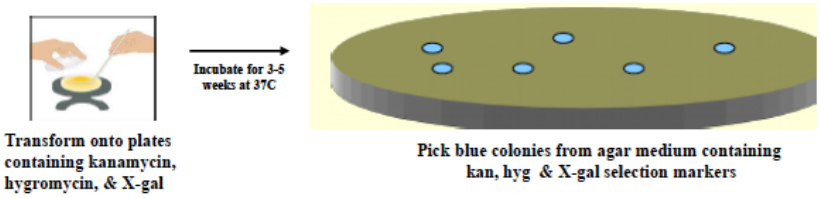


Figure 5.3 Steps involved in selecting single cross over events (SCOs)

5.1.1.4.2 Double cross over events (DCOs)

Here, the mutated CPL gene bearing the hygromycin-resistance gene replaces the WT CPL gene. Thus, a second recombination event gives rise to DCOs that are either WT or mutant genes. Briefly, the bluish SCOs were streaked on 7H10 agar media without antibiotics to allow for second recombination events (eliminate suicide vector bearing kanamycin and

lacZ/SacB gene). Thereafter the DCOs were selected by re-suspending SCOs to 7H9 media before plating it out on an agar plate containing X-gal with or without sucrose. Potential DCOs, which are white colonies (loss of *LacZ* gene) and sucrose resistant (Loss of *SacB* gene), were selected because they lost the selection markers. To confirm if the potential DCOs have lost kanamycin-resistant gene associated with the SCOs, DCOs were tested for kanamycin sensitivity by plating on agar plates with or without kanamycin. DCOs colonies that are sensitive to kanamycin were selected, and the mutant was further confirmed by doing colony PCR using kanamycin sensitive colonies (Fig. 5.4).

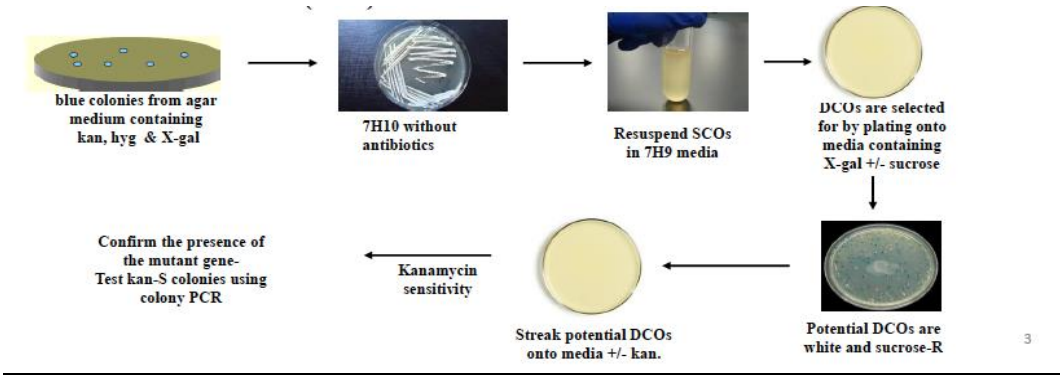


Figure 5.4 Steps involved in selecting double cross over events (SCOs)

5.2 Materials and Methods

5.2.1 PCR amplification of CPL gene

PCR amplification of the mutated CPL gene was performed in a total volume of 100 ul containing oligonucleotide primers, Phusion polymerase enzyme, dNTPs, DMSO, Phusion buffer, *M. bovis* AF2122 strain template, and sterile water. Following amplification, an aliquot was run in 0.8% agarose gel to confirm successful amplification. The amplified CPL gene was purified using an EZ-10 spin column PCR product purification kit, and the concentration of DNA was determined using NanoDrop® ND-1000 Spectrophotometer. Below is the PCR mixture setup and cycling program.

Reaction Mixture	**Concentration**
Sterile dH_20	
5 x phusion buffer	1 x phusion buffer
10mM dNTPs	200µM
100% DMSO	6%
100µM Forward primer	1µM
100µM Reverse primer	1µM
90ng/ml gDNA (*M . bovis* strain)	450ng
2000 U/ml Phusion polymerase	2 units
Total volume = 100 µl	

Table 5. 1: The reaction mixture for PCR setup

Step	Time	Temperature	Cycles
Initial denaturation	2 minutes	98 °C	1X
Denaturation	30 seconds	98 °C	30X
Annealing	1 minute	57 °C	30X
Extension	1.5 minutes	72 °C	30X
Final extension	7 minutes	72 °C	1X
Storage	Infinity	18 °C	Infinity

Table.5. 2: PCR cycling program used to amplify the CPL gene using Phusion polymeras**e**

5.2.2 Digestion of vector and insert

In a 50 µl volume, confirmation of successful cloning of mutated CPL gene to p2NIL was done using single and double digestion with *Bam*HI and HindIII. For the construction of suicide delivery plasmid DNA, P2NIL with mutated CPL gene and pGOAl17 was digested using PacI enzyme. Also, confirmation of suicide delivery plasmid DNA generation was done by digestion with Sal1 enzyme. Digestion reactions were gently mixed, spun down in microcentrifuge and incubated at 37°C overnight. 5µl volume of digested sample was run in agarose gel to confirm the success of digestion.

Reaction Mixture	p2NIL with Mutated CPL gene
Sterile dH_20	
10 X 2.1 NEBuffer	1X
DNA	4 µg
20,000 U/ml HindDIII	60 unit
20,000 U/ml *Bam*HI	100 unit
Total volume =50 µl	

Table 5. 3: Set up for double digestion of p2NILvector with mutated CPL gene using *Bam*HI and HindIII

Reaction Mixture	p2NIL with Mutated CPL gene
Sterile dH_20	
10X 2.1 NEBuffer	1X
DNA	4 µg
20,000 U/ml HindDIII	60 unit
Total volume = 50 µl	

Table 5. 4: Set up for single digestion of P2NILvector with mutated CPL gene using HindIII

Reaction Mixture	p2NIL with mutated CPL gene	pGOAL17
Sterile dH_20		
10 X CutSmart Buffer	1X	1X
Vector		4 µg
DNA Insert	4 µg	
10,000 U/ml PacI	100 unit	100 unit
Total volume = 50 µl		

Table 5. 5: Construction of suicide delivery plasmid DNA**.** Set up for single digestion of P2NILvector with mutated CPL gene and pGOAL17 using PacI

Reaction Mixture	Suicide delivery plasmid	p2NIL with mutated CPL gene
Sterile dH_20		
10 X CutSmart Buffer	1X	1X
Vector		4 µg
DNA Insert	4 µg	
20,000 U/ml SalI	100 unit	100 unit
Total volume = 50 µl		

Table 5. 6: Set up for single digestion of suicide delivery plasmid and P2NILvector with mutated CPL gene using Sal1enzyme

5.2.3 Ligation of the insert to vector using T4 DNA ligase enzyme

The mutated CPL gene was ligated into p2NIL at a ratio of 1:10 using T4 DNA ligase. For suicide delivery plasmid generation, p2NIL with mutated CPL was ligated to gene cassette using T4 DNA ligase at a ratio of 1:30. Ligation reactions were incubated overnight at 16 degrees and heat shock at 65 degrees for 10 minutes.

Reaction Mixture	**Volume**
Sterile dH_20	
10 x T4 buffer	1X
p2NIL vector	15ng
Mutated CPL gene	150ng
400,000 U/ml T4 DNA ligase	1 µl
Total Volume	20 µl

Table 5. 7: Set up for ligation reaction of mutated CPL gene with p2NIL

Reaction Mixture	**Volume**
Sterile dH_20	
10 x T4 buffer	1X
p2NIL with mutated CPL gene	15ng
Cassette gene	410ng
400,000 U/ml T4 DNA ligase	1 µl
Total Volume	20 µl

Table 5. 8: Set up for ligation reaction of p2NIL with mutated CPL gene with cassette gene

5.2.4 Transformation

Following ligation of mutated CPL gene to p2NIL, the ligation mixture was transformed to *E. coli* Top 10 cells. Same *E. coli* Top 10 cells were also used in transformation using a ligation mixture containing cassette gene and p2NIL with mutated CPL. Briefly, frozen competent *E. coli* Top 10 cells were removed from the –80 °C freezer and thawed on ice at room temperature. 50 µl of the cells and 10 µl of the ligation mixture were dispensed into sterile microcentrifuge tubes chilled on ice. The tube was incubated on ice for 35 minutes. The cells were heat-shocked at 42°C for 45 seconds in a water bath, then incubated in ice for 2 minutes. 500 µl of pre-warmed LB media were dispensed in the tube, and the mixture was incubated at 37°C in a shaking incubator (200 rpm) for 60 minutes. 250 µl was transferred to the center of an agar plate containing kanamycin (50 µg/ml), and a sterile spreader sealed in a flame was used to spread the solution over the entire surface of the plate. The plate was inverted and incubated overnight at 37 °C. After overnight incubation at 37 °C, the plate was examined for the presence of kanamycin-resistant colonies and stored at 4 °C.

5.2.5 UV irradiation of suicide delivery plasmid DNA and electroporation into electrocompetent *M. bovis*

Following plasmid purification of suicide delivery plasmid DNA, an aliquot of suicide delivery plasmid DNA was subjected to UV energy for a few minutes. UV treatment can cause the incorporation of point mutations on the treated DNA. Thereafter, electrocompetent *M. bovis* was prepared using an established protocol[429]. Briefly, *M. bovis* was grown in 30 mL of Middlebrook 7H9 liquid media to the mid-logarithmic phase

of growth (OD_{600nm} ~ 0.5 to 0.7). Then, 5ug of UV treated and non-treated suicide delivery plasmid DNA was electroporated into electrocompetent *M. bovis and* incubated for 24h. The entire transformation mix was plated out onto 7H10 plates containing kanamycin, hygromycin, and X-gal selection markers. Plates were incubated at 37 ℃ for 3 to 5weeks.

5.3 Results

5.3.1 Mutated CPL gene of fragment size 2.1kb amplified from pUC19 vector

PCR was used to amplify the mutated CPL gene bearing the hygromycin-resistant gene cassette from the pUC19 vector. PCR product was confirmed via gel electrophoresis. Result showed a correct fragment size of 2.1kb CPL gene (Fig. 5.5).

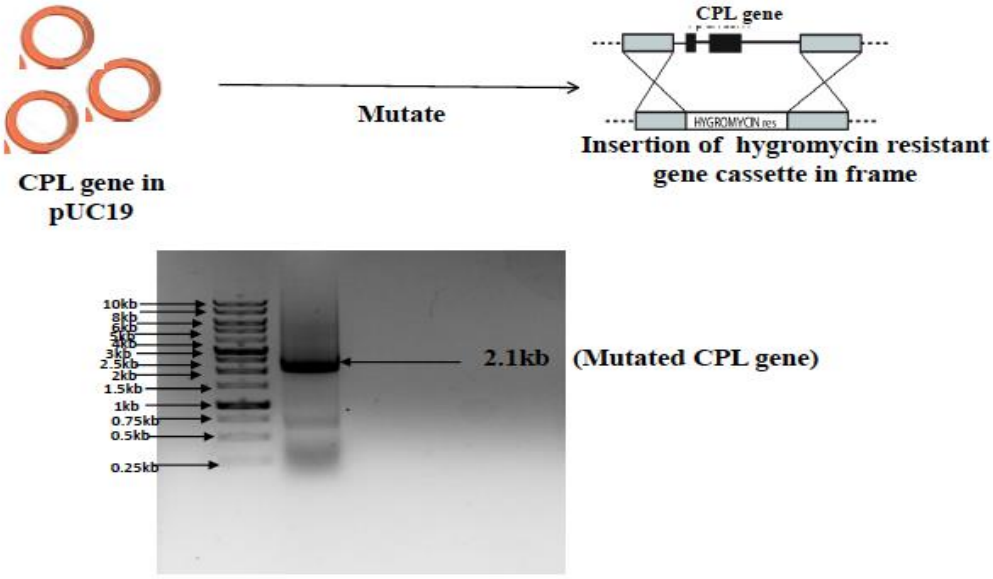


Figure 5.5: Amplified mutated CPL gene of fragment size 2.1kb

5.3.2 Mutated CPL gene of fragment size 2.1kb amplified from p2NIL vector following digestion with HindIII enzyme

Amplified mutated CPL gene and P2NIL vector were first digested and purified with HindIII (Shown in Tables 3 & 4). Thereafter mutated CPL gene was cloned into HindIII sites of p2NIL vector to generate p2NIL with mutated CPL. Following transformation into Top 10 *E. coli* competent cells, purified plasmid DNA from several clones were selected

for screening by PCR using CPL primers. PCR products were analyzed on a 0.8% agarose. Results showed 2 positive clones with a fragment size of 2.1kb, which correspond to the mutated CPL gene (Fig. 5.6).

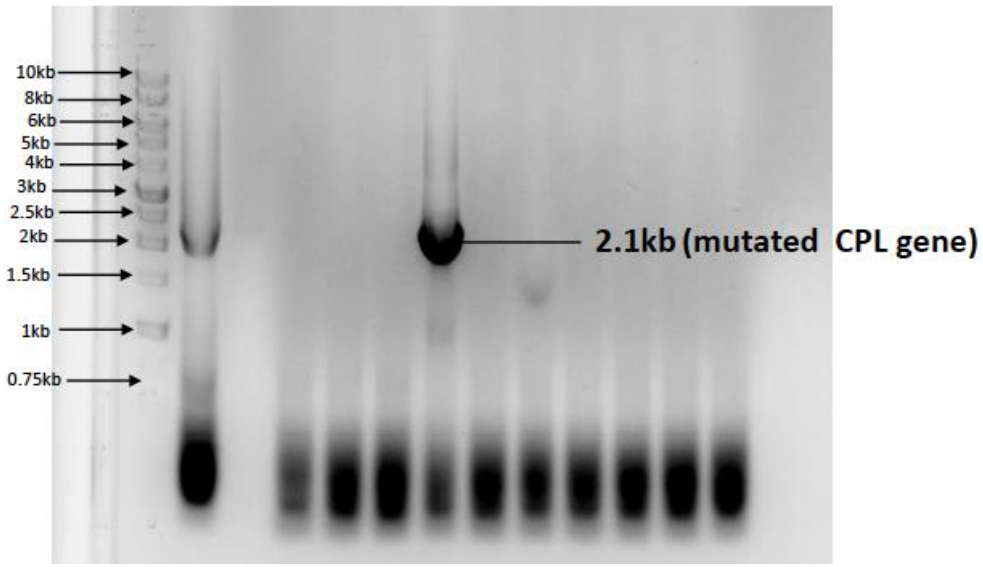


Figure 5.6: Amplified mutated CPL gene from 2 positive clones

5.3.3 Confirmation of p2NIL with mutated CPL gene from 2 positive clones using HindIII and BamHI enzymes

To further confirm the 2 positive clones obtained following transformation, single and double digestion using HindIII and BamHI was used to digest p2NIL with mutated CPL gene. For clone 1, uncut or single digestion of the p2NIL construct gave a correct size fragment of 6.8kb (correspond to p2NIL with mutated CPL gene). However, double digestion of the same construct gave a 2 sized fragment of 4.7kb and 2.1kb, which correspond to p2NIL and mutated CPL gene, respectively. A similar observation was seen

with clone 2 with double digestion resulting in 2 fragments compared to single digestion. This data showed successful cloning of the mutated CPL gene into p2NIL vector (Fig. 5.7).

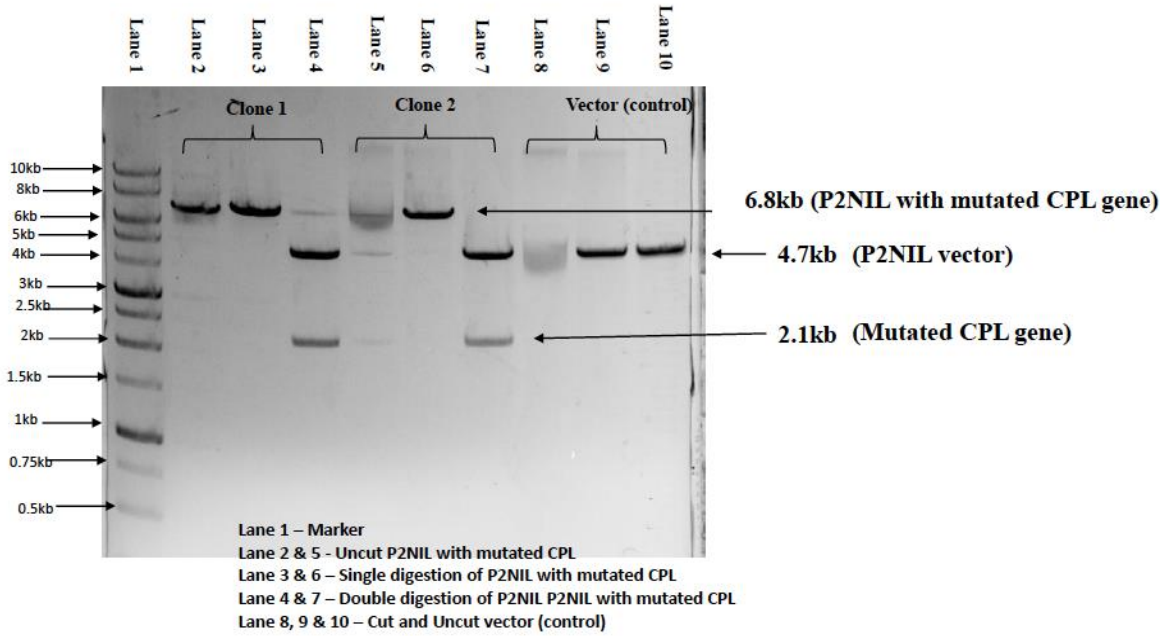


Figure 5.7: Confirmation of P2NIL with mutated CPL gene from 2 positive clones using HindIII and BamHI enzymes

5.3.4 Confirmation of pGOAL17 gene cassette of fragment size 6.3kb following digestion with PacI enzyme

To construct the suicide delivery plasmid DNA, PacI enzyme was used to digest pGOAL17 (to release the cassette gene) and p2NIL construct. The digested product was confirmed using gel electrophoresis. Result showed pGOAL17 gene cassette of fragment size 6.3kb as well as p2NIL with mutated CPL gene (6.8kb) (Fig. 5.8).

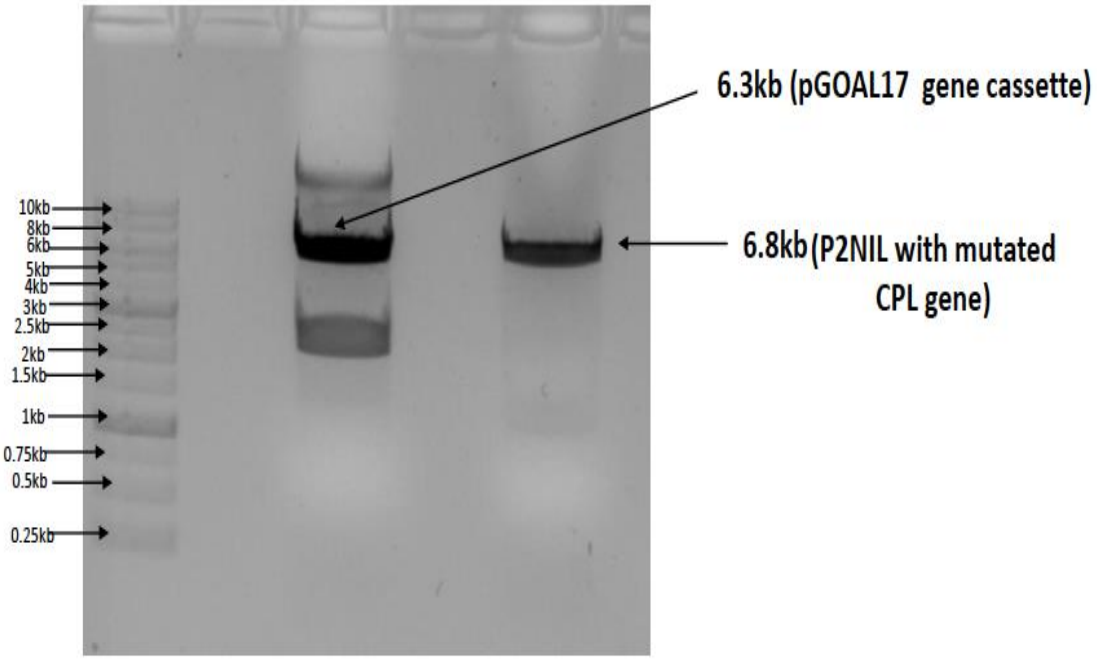


Figure 5.8: Digestion of pGOAL17 and p2NIL construct with Pacl enzyme

5.3.5 Confirmation of suicide delivery plasmid DNA by digestion with Sal-1 enzyme

The fragment containing the cassette gene (6.3kb size) from pGOAL-17 was gel purified and cloned into PacI site of the p2NIL construct. Following transformation in the Top 10 cells, one positive clone was obtained. The positive clone was confirmed by digesting with Sal-1 enzyme, and it gave the correct one-size fragment of 13.8kb of suicide delivery plasmid DNA. However, Sal-1 digestion of the p2NIL construct, which is a negative clone, gave a fragment size of 6.8kb. This suggests that the positive clone possesses suicide delivery plasmid DNA (Fig. 5.9).

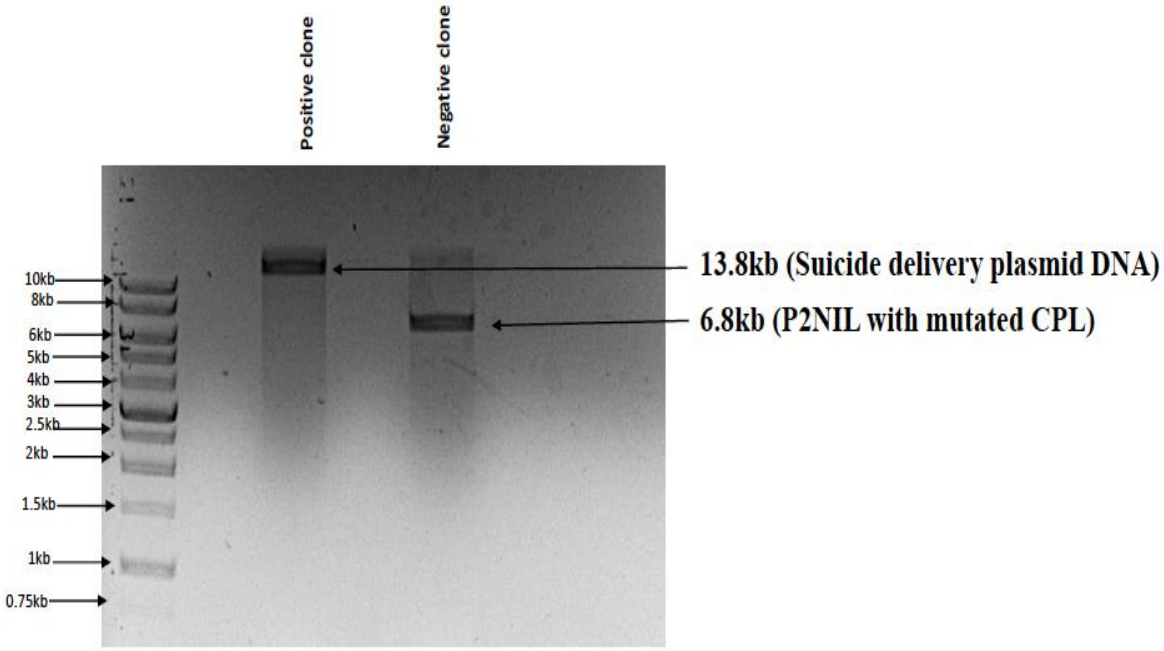


Figure 5.9: Confirmation of suicide delivery plasmid DNA by digestion with Sal-1 enzyme

5.3.6 Selection via single cross over events (SCOs)

The suicide delivery plasmid was transformed into *M. bovis*, plated in a 7H10 agar plate containing the ampicillin, hygromycin, and x-gal selective markers, followed by incubation at 37°C.*M. bovis* transformed with suicide delivery plasmid DNA was not successful. Thus far, no SCOs were observed in agar plates following extended periods of incubation.

5.4 Conclusion

Homologous recombination occurs between two copies of identical or highly-homologous sequences and is mediated by RecA [430]. Although homologous recombination is relatively straightforward in *Mycobacterium smegmatis*, it proved far more challenging to perform in slow-growing mycobacteria, and the reasons for this remain largely unknown. However, several strategies have been used to overcome this challenge. These include the use of long flanking regions [431, 432], UV-treated DNA substrates [433], counter-selectable markers [434], development of improved delivery strategies such as temperature-sensitive plasmids, and use of suicide (non-replicating) plasmids [434, 435]. In my attempt to generate *M. bovis* mutant using homologous recombination, some of the above strategies were used, and I successfully cloned and prepared plasmid constructs required to disrupt the gene encoding a key enzyme involved in *p*-HBAD synthesis in *M. bovis*. This will be used in future efforts to generate an *M. bovis* mutant deficient in *p*-HBAD-1 for detailed studies. Thus, naïve and BCG vaccinated mice will be challenged with *M. bovis* mutant lacking *p*-HBAD-1, and the pro-inflammatory response, bacterial burden, and host survival will be assessed.

CHAPTER 6: General discussion and conclusions

The overarching goal of this research work was to understand the pathogenesis of bTB by assessing the immunoregulatory role of *M. bovis* derived *p*-HBAD-1 on bDCs.

The cell envelope of all the members of *M. tuberculosis* complex is decorated with glycan and glycolipids that are involved in host-pathogen interaction and disease pathogenesis. Interaction of *M. t tuberculosis* cell envelope-derived glycolipids and glycan with DCs alter their cytokine production and antigen presentation efficiency [55]. However, there is limited information on the immunogenicity of *M. bovis* cell wall antigen. To fill in the knowledge gap, I hypothesize that *M. bovis* cell envelope distinct antigenic components induce differential pro-inflammatory responses in bDCs. This was tested by comparing the stimulatory effects of bPPD, CWL and CSSE on bDCs *in vitro*. Results showed that *M. bovis*-derived CSSE is a poor immune stimulator of bDCs [296]. Given that the CSSE fraction of *M. tuberculosis* has been found to be enriched in *p*-HBADs which suppress host immune response [46]. Therefore, to identify the particular molecules contributing to the low immune-stimulatory effect of CSSE, and we proposed that *M. bovis* CSSE fraction contains *p*-HBAD-1, which was confirmed via Thin-Layer Chromatography. Further assessment of the role of *p*-HBAD-1 on bDCs showed a varying effect in non-primed and IFN-γ primed bDCs.

In non-primed bDCs, *p*-HBAD-1 blocks the production of pro-inflammatory cytokines induced by LPS, bPPD and CWL. The reduction in IL-12 and TNF-α production in non-primed bDCs is consistent with the previous observation from *M. t tuberculosis* derived *p*-HBADs, which was reported to suppress cytokine production in bone marrow-derived

macrophages [404]. This suggests that activation machinery in non-primed bDCs has been altered by *M. bovis* derived *p*-HBAD-I. Thus, the attenuated production of IL-12 and TNF-α in non-primed bDCs would interfere with the activation of Th1 cell immunity (protective immune response), thereby tilting the immunological balance towards Th2 immune response [436].

On the contrary, *p*-HBAD-1 strongly promotes LPS/bPPD-stimulated IL-12 and TNF-α production in IFN-γ primed bDCs. This interesting observation could be related to the priming of DCs with rIFN-γ. A vital pathway for IL-12 maximal output is the requirement for priming of DCs and macrophages with IFN-γ [406]. During infection, NK cells, activated Th1 lymphocytes, and cytotoxic T lymphocytes (CTLs) are the principal source of IFN-γ, which serve as a positive feedback mechanism for robust IL-12 production, and DCs are responsive to IFN-γ as they express IFN-γR [437]. IFN-γ produced by these cells have a combined effect with TLR ligation to enhance DCs activation and function [406]. Another study reported that priming APCs with IFN-γ triggers subsequent TLRs inflammatory response to release high levels of pro-inflammatory cytokines [406].

In mycobacterial infection, it has been demonstrated that in the absence of IFN-γ, DCs infected with *M. tuberculosis* ligand produce high levels of IL-10 and IL-23, but a small amount of IL-12. However, priming DCs with IFN-γ restores their ability to produce IL-12 efficiently and block IL-10 production [438]. Given that IFN-γ priming of DCs promotes the maximal output of pro-inflammatory cytokines, this suggests that the increased pro-inflammatory response in IFN-γ primed bDCs is IFN-γ dependent, which

supplies a positive feedback signal to bDCs.

To reveal the molecular basis by which *p*-HBAD-1 mediated its effects on bDCs functions, we analyzed the expression of TLR 2 and 4, as well as its downstream signaling pathway. In non-primed bDCs, we observed that the decrease in pro-inflammatory cytokine production is associated with decreased expression of TLR2 and 4 and the downstream surface membrane signaling pathway. In contrast, increased cytokine production in IFN-γ primed bDCs is mediated via endosomal and surface membrane TLRs.

To further understand the molecular mechanism regulating cytokine production in non-primed and IFN-γ primed bDCs, the effect of *p*-HBAD-1 to NF-κB on activation was determined. Following initiation of the TLR signaling pathway, many signaling proteins are recruited, leading to the activation of a key NF-κB transcription factor. Previous studies showed that the anti-inflammatory effect of various mycobacterial components is due to their effect on NF-κB phosphorylation. For example, Liu et al. showed that *M. tb* recombinant leucine-responsive regulatory protein (rLrp) inhibits LPS-induced TNF-α, IL-6, and IL-12 production by blocking the nuclear translocation of NF-κB [439]. Furthermore, mycobacterial glycolipid (PGL-1 and PGL-tb) produced by *M. tb* clinical isolates were reported to block TLR2 agonist-driven activation of NF-κB and cytokine production [58, 440], as well as decreased TRIF-dependent TLR4 signaling in macrophages resulting in limited pro-inflammatory responses [441]. In line with this, we observed that *p*-HBAD-1 blocks the phosphorylation of NF-κB in non-primed bDCs unlike in IFN-γ primed. Given that NF-κB activation regulates pro-inflammatory cytokine

production, this suggests that the cytokine inhibitory effect of *p*-HBAD-1 in non-primed bDCs could be related to decrease activation of NF-κB.

Induction of the proinflammatory phenotype in DCs by mycobacterial infection is accompanied by the regulatory response, including SOCs protein expression. The SOCs family of inhibitors has received a lot of attention in relation to DCs. For instance, the expression of SOCs protein has been shown to be up-regulated in DCs infected with *M. tb* [442]. Specifically, DCs overexpressing SOCs3 exhibited a tolerogenic phenotype that showed a low level of expression of IL-12 but a high level of IL-10 [443].

It has been well established that SOCs proteins diminish the JAK/STAT signaling pathway and can inhibit various cytokines, resulting in the inhibition of DCs activation [444]. However, a more recent report shows that the function of SOCs1 and 3 goes beyond IFN-γ related JAK/STAT signaling. Thus, another report demonstrated that SOCs1 protein negatively regulates TLR-related NF-κB activation pathways by mediating the degradation of adaptor protein mal, which is involved in TLR 2 and 4 signaling [445]. Furthermore, various reports have demonstrated an interaction between SOCs1 with NF-κB p65 protein [446, 447]. The inhibitory effect of SOCs protein in NF-κB was reported to be due to specific translocation of SOCS1 protein into the nucleus, where it interacts with the NF-κB p65 subunit within the nucleus, resulting in ubiquitination and degradation, thus limiting NF-κB function as a transcription factor [448]. Thus, it is possible that in non-primed bDCs, *p*-HBAD-I inhibits activation of NF-κB by induction of SOCS1 and 3, which either block the initial steps in TLR signaling or translocate to the nucleus and cause its degradation. Further studies are vital to determine the mechanism through which *p*-HBAD-

1 regulates NF-κB phosphorylation in both IFN-γ primed and non-primed bDCs.

Another immunoregulatory feature of DCs is the secretion of IL-10 in the course of inflammatory responses [449]. Interleukin-10 is a potent anti-inflammatory cytokine that has been shown to directly or indirectly affect multiple cell types, including DCs, macrophages, and T-cells [450]. The dominant function of IL-10 is to deactivate DCs and macrophages, resulting in decreased Th1 cytokine production [451], which may have far-reaching consequences on both innate and adaptive immunity *in vivo*. Increased IL-10 production following infection with many intracellular pathogens is associated with decreased resistance to infection [449]. In mycobacterial infection, IL-10 inhibits the production of pro-inflammatory cytokines in *M. tb* infected macrophages, resulting in the prevention of reactive nitrogen/oxygen intermediates released by macrophages [452]. In this study, *p*-HBAD-1 induced higher levels of IL-10 production in non-primed bDCs stimulated with LPS and bPPD, compared to IFN-γ primed bDCs. Therefore, it could be that increased IL-10 released by non-primed bDCs is responsible for the downregulation of IL-12 and TNF-α production, thereby tilting Th1 immune response towards non-protective Th2 response.

Characteristic immunoregulatory factors associated with tolerogenic DCs include increased anti-inflammatory and decreased pro-inflammatory cytokines production. Since *p*-HBAD-1 promotes proinflammatory response in IFN-γ primed DCs, and the opposite effect was seen in non-primed bDCs. This suggests that *p*-HBAD-1 might be inducing a tolerogenic and immunogenic response in non-primed and IFN-γ primed bDCs, respectively. It has been established that immunogenic DCs are known for high expression

of costimulatory molecules, which promote optimal T-cell activation [453], while tolerogenic DCs weakly express costimulatory molecules that impair T-cell proliferation [453]. In addition, both immunogenic and tolerogenic DCs are known to express common markers, such as MHC Class I and II molecules [453]. Consistent with these studies, our results confirmed the tolerogenic effect of *p*-HBAD-1 in non-primed bDCs, with results depicting decreased expression of co-stimulatory, which was reversed in IFN-γ primed bDCs.

Recognition receptors expressed by DCs play a role in the induction of either tolerance or immunogenic response. While TLR 2/4 recognizes protein or lipid antigen and induces an immunogenic response, DC-SIGN only recognizes carbohydrate antigen and induces a tolerogenic response in DCs [454]. Therefore, further confirmation of tolerogenic phenotype in non-primed bDCs suggests that *p*-HBAD-1 might be targeting DC-SIGN to induce immune tolerance. This data is consistent with the earlier observation in DCs where ManLAM suppresses activation of DCs by targeting DC-SIGN [64].

In summary, this study showed that *p*-HBAD-1 might have a dual function. Based on these findings, I proposed a model for the dual role of *p*-HBAD-1 in bDCs and the mechanism through which it mediates its immune suppressive effect. In non-primed bDCs, *p*-HBAD-1 inhibits the pro-inflammatory response by blocking TLRs/NF-κB signaling pathways. This inhibitory effect is associated with the increased feedback inhibition exerted by IL-10 and SOCs proteins. This data suggest a tolerogenic response confirmed by reduced expression of co-stimulatory molecules and increased expression of DC-SIGN. Thus, in non-primed bDCs, *M. bovis* evade host immune response by releasing *p*-HBAD-1, which

suppresses host immune response, thereby increasing the chance of survival and pathogenesis.

On the contrary, *p*-HBAD-1 does the opposite in IFN-γ primed bDCs by promoting a robust pro-inflammatory response. The mechanism underlying IFN-γ mediated priming includes enhanced TLRs 2 and 4 expressions, which promote TLRs signaling and activation of NF-κB resulting in enhanced production of pro-inflammatory cytokines. IFN-γ achieved these by inactivating feedback inhibition induced by regulatory proteins (IL-10 and SOCS1/3) and increasing the expression of activation markers required for optimal T-cell activation. Thus in IFN-γ primed bDCs, *p*-HBAD-1 induces an excessive pro-inflammatory response, which can cause host tissue damage.

Conclusions

Theoretically, DCs found in various locations in and around the granuloma may be serving very different purposes. These immune cells exist at different maturation phases in the infected lung, with both matured and immature DCs responding differently to IFN-γ, resulting in IFN-γ and non-primed bDCs, respectively. The dual effect exerted by *M. bovis* derived *p*-HBAD-1 on IFN-γ primed and non-primed bDCs enable it to adapt within the host cell by balancing their ability to induce a protective immune response and immune tolerance mechanism. If the bacterium is not well adapted in the host, it can kill the host via its virulence factors, or the bacteria can be eliminated by the host response. Through *p*-HBAD-1, *M. bovis* maintain balance in host protective response and tolerance, thereby allowing *M. bovis* to adapt and survive in the dormant phase of chronic infection.

In the context of *M. tb* infection, during the early phase of infection, no IFN-γ is produced due to the lack of *M. bovis* specific T- cells resulting generation of non-primed bDCs that causes immune tolerance. Previous studies showed that delay in T- cell priming by bDCs leads to latent mycobacterial infection [455]. Thus, the non-primed DCs with reduced proinflammatory cytokine production (IL-12) caused a delay in T- cell priming, which promotes latency. Within the granuloma, following the onset of adaptive immunity, bDCs localized in T-cell and NK- cell zone transforms to IFN-γ primed bDCs while bDCs localized in non-T-cell zone converts to non-primed bDCs, which maintains granuloma by inducing an immunogenic and tolerogenic response, respectively. Collectively, this study points to a novel effect of *p*-HBAD-1 as an immunoregulatory molecule (Fig. 6.1).

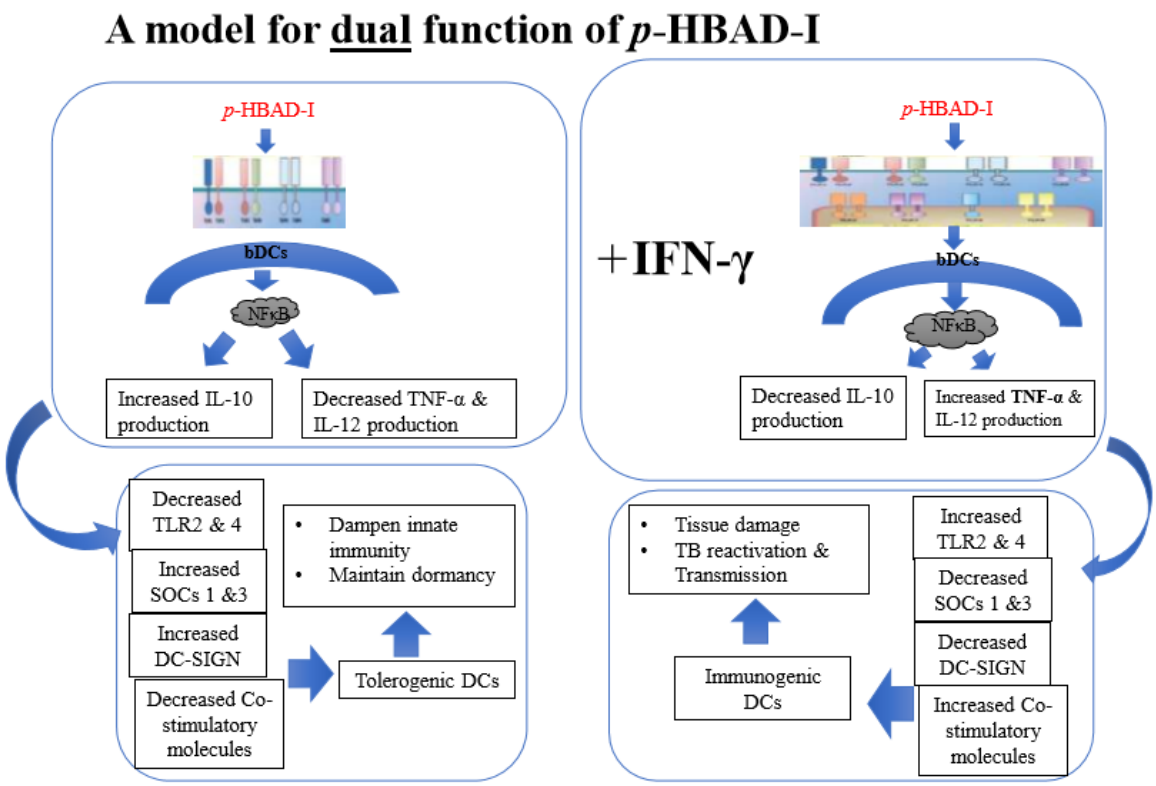


Figure 6.1: Proposed dual function of *p*-HBAD-1 in bovine TB pathogenesis

CHAPTER 7. Limitations

The utilization of an *in vitro* model is the major limitation of this study because some of the results generated *in vitro* might not always correlate with the *in vivo* results, leading to over-interpretation of the results and erroneous conclusions.

The growing field of immunometabolism has demonstrated how metabolic cellular reactions and processes not only provide a means to generate ATP and biosynthetic precursors, but are also a way of controlling immunity and inflammation. Metabolic reprogramming of immune cells is crucial for both inflammatory as well as anti-inflammatory responses. Activation of DCs or macrophages with a range of stimuli, including LPS [456] and TLR3 ligand poly (I:C) [457], induces a metabolic switch from OXPHOS to glycolytic pathway, in a phenomenon similar to the Warburg effect. Therefore, it would have been fascinating to determine in detail whether *p*-HBAD-1 affects the metabolic reprogramming of DCs following LPS/bPPD stimulation of non-primed and IFN-γ primed bDCs. Specifically, it would be necessary to determine whether *p*-HBAD-1 promotes Warburg metabolism effect on cytokine production by altering glycolytic pathway.

Furthermore, we showed that *p*-HBAD-1 treatment upregulates the expression of SOCs1 and 3 in bDCs treated with either LPS/bPPD. The data would have been strengthened if we knocked down SOCs1 and 3 by siRNA to definitively determine whether phosphorylation of MAPKs and NF-κB proteins and subsequent cytokine production in LPS/bPPD-treated cells following *p*-HBAD-1 treatment are rescued. Also, it would have been interesting to look for the expression of other intracellular regulators of cytokine production in DCS like

Toll interacting protein (TOLLIP) and whether their activation is upregulated upon *p*-HBAD-1 treatment.

This study also demonstrated that *p*-HBAD-1 promotes a tolerogenic phenotype in non-primed bDCs activated with either LPS/bPPD. Besides low expression of costimulatory molecules and upregulation of anti-inflammatory cytokines, Indoleamine 2, 3 dioxygenases (IDO), which is the immune-regulatory enzyme that participates in immunological tolerance, was not determined. In addition, the ability to expand regulatory T lymphocytes (a distinctive feature of tolerogenic DCs) was not determined. Thus, this study would have been strengthened by assessing whether *p*-HBAD-I induced non-primed bDCs to trigger activation of IDO and proliferation of regulatory T lymphocytes.

Granulomas limit bacterial growth in various ways, including oxygen and nutrient deprivation, acidic pH, and production of host factors such as nitric oxide. Dormancy survival regulator (DosR) is a transcription factor that plays a role in gene regulation and is strongly induced during hypoxia. Thus, DosR plays a key regulatory role in the adaptation of bacilli to survival under hypoxic conditions. It would have been interesting to determine the transcriptional behavior of genes involved in the *p*-HBAD-1 biosynthesis in a hypoxic condition. Specifically, it would be necessary to determine whether the *M. bovis* grown in hypoxic conditions induces the production of *p*-HBAD-1. This can be achieved by extracting *p*-HBAD-1 from the culture filtrate of *M. bovis* grown in hypoxic and non-hypoxic conditions and analyze results using TLC assay.

Future directions

Determine how p-HBAD-1 regulates SOCs1 and 3 protein expressions or enhance MAPKs/ NFkB phosphorylation

The expression of SOCs1 and 3 proteins were higher in non-primed compared to IFN-γ primed bDCs. This suggests that *p*-HBAD-1 might act as a negative regulator of SOCs proteins in IFN-γ primed bDCs. Also, this indicates a direct or indirect association between SOCs proteins and *p*-HBAD-1. Thus, it could be that IFN-γ priming of bDCs enables binding of *p*-HBAD-I to SOCs protein, thereby preventing their expression by directly or indirectly affecting the stability of SOCs proteins or enhancing their degradation. To determine whether *p*-HBAD-1 directly interacts with SOCs protein in bDCs, immunoprecipitation followed by western blotting or CHIP assays could be used.

Phosphorylation of intracellular signaling protein (MyD88) involved in TLR4 signaling in p-HBAD-1 treated non-primed and IFN-γ primed bDCs needs to be further investigated.

The response of bDC to LPS/bPPD in the presence of *p*-HBAD-1 is greatly influenced by the MyD88-dependent signaling pathway (Akira et al., 2004). The TIRAP–MyD88 dependent pathway plays a key role in regulating early NF-κB activation and pro-inflammatory cytokine production such as IL-12. Activation of MyD88 adaptor molecule results in recruitment and activation of the downstream proteins involved in LPS-TLR4 signaling. Thus, further investigation of MyD88 protein, which is the first protein recruited

during LPS/bPPD-TLR4 signaling, is necessary. This could be achieved using Western blotting technique.

Determine whether bDCs treated with p-HBAD-1 promote the expansion of regulatory T lymphocytes.

Tolerogenic DCs play essential roles in immune-related diseases and induce immune tolerance by shaping T-cell responses. The ability to expand regulatory T-cells is a distinctive feature of tolerogenic DCs [157]. Since the above findings are suggestive of a tolerogenic profile for non-primed bDCs treated with *p*-HBAD-1, it will be imperative to further investigate whether *p*-HBAD-1 treated non-primed bDCs triggered the proliferation of CD4+C25+FoxP3+ T lymphocytes. Thus, examining whether non-primed bDCs treated with *p*-HBAD-1 induce T-reg cell proliferation via mixed lymphocyte reaction (MLR) could be done using BrdU ELISA

Using CRISPR-CAS 9 to generate a mutant that lacks p-HBAD-1AD-1: Following mutation of the CPL gene, the absence of *p*-HBAD-1 will be determined using TLC. Thereafter mice (naïve and BCG immunized) will be challenged with *M. bovis* WT and mutant strains. Then the inflammatory response, bacterial burden, and host survival will be assessed and compared.

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