Host-directed targeting of IFN-γ induced long non-coding RNA-445 during *Mycobacterium tuberculosis* infection

Submitted by

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Declaration

I, Lwanda Abonga Buthelezi, hereby declare that the work on which this dissertation/thesis is based, is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

This thesis is dedicated to my husband, Lungelo, my parents, Lumka and Oscar, my grandmother, Zintle, and my siblings Mali and Camagu. Thank you for your infinite support.

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Publications

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Conferences

1. **Joint Allergy Society of South Africa (ALLSA) & South African Immunology Society(SAIS) 2023 Congress.** 29 September - 1 October 2023, Cape Town, Western Cape, South Africa. **Oral Presentation.**

Abbreviations

AEC	Animal Ethics Committee
AMPK	Adenose monophosphate-activated protein kinase
ASO	Antisense oligonucleotide
Bax-1	Bcl-2 associated X-protein
BCG	Bacille Calmette-Guérin
Bcl-2	B cell lymphoma-2
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
BSL	Biosafety level
CAGE	Cap analysis gene expression
cDNA	Complementary DNA
CFU	Colony forming unit
CO_2	Carbon dioxide
CpG	Cytosine-guanine
DMEM	Dublecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Erk-1/2	Extracellular signal-regulated protein kinases 1 and 2
ESAT	Early secreted antigen target
FACS	Fluorescence-activated cell sorting
FANTOM	Functional annotation of the mammalian genome
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FHS	Faculty of Health Sciences
GapmeR	Antisense LNA® GapmeR oligonucleotides
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hours
HDT	Host-directed therapy
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase 1

HERC	Human Ethics Research Committee
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LNA	Locked nucleic acid
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
LTBI	Latent TB infection
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MHCII	MHC class II
MOI	Multiplicity of infection
mRNA	Messenger RNA
Mtb	*Mycobacterium tuberculosis*
MYD88	Myeloid differentiation primary response protein 88
ncRNA	Non-coding RNA
NEAT1	Nuclear paraspeckle assembly transcript 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
p.i	Post-infection
p.s	Post-stimulation
p.t	Post-transfection
Pam_3CSK_4	$Pam_3CysSerLys_4$
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PRR	Pattern recognition receptor
RAF	Research Animal facility
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative PCR
SAVC	South African Veterinary Council

T cell	Thymus cell
TB	Tuberculosis
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TUNEL	TdT-mediated dUTP nick end labelling
UCT	University of Cape Town
WHO	World Health Organization
XDR	Extensively-drug resistant

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Abstract

Tuberculosis (Tb) continues to be the leading infectious disease due to a single infectious agent, *Mycobacterium tuberculosis* (*Mtb*). In 2022, there were an estimated 7.5 million new infections reported. Regardless of the availability of the BCG vaccine, tuberculosis infections continue to rise resulting in the current search for alternative effective treatment methods. An innovative concept that is promising and offers a different therapeutic approach is Host-directed Therapy (HDT), which is currently being investigated as a possible adjunctive therapy against tuberculosis. The ideal candidates for this type of therapeutic approach are long non-coding RNAs (lncRNAs) which can be further classified based on their location and function. These lncRNAs have been recently understood to play a crucial role in macrophage polarization and immune regulation, especially during infections and disease progression. For our study, cap analysis gene expression (CAGE) transcriptomics was previously performed on classically activated (IFN-γ stimulated) and alternatively activated (IL-4/IL-13 stimulated) murine macrophages. Upon *Mtb*-infection, 151 differentially expressed lncRNAs were discovered, 11 were validated, and one of these, lncRNA-445, was selected for this study. This project focused on the differentially expressed identified host lncRNA-445 which was subjected to loss-of-function, to further understand the functional role of lncRNA-445 during macrophage polarization and *Mtb*-infection. In this study, I demonstrate that lncRNA-445 is highly upregulated in IFN-γ stimulated macrophages and downregulated upon infection with *Mtb*. The expression of lncRNA-445 is mediated through the TLR9 and p38 signalling pathways. The knockdown of lncRNA-445 reduced the intracellular growth of *Mtb* and induced apoptosis, it further reduced pro-inflammatory cytokine release and increased nitrite production which correlates with nitric oxide release.

CHAPTER ONE

Literature Review

1.1 Introduction

Tuberculosis (TB) is a fatal airborne infectious disease that has caused an estimated 7.5 million new infections in 2022 and an estimated 1.3 million deaths (1). TB infection in humans is caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*), which was discovered in 1882 by Robert Koch (2). *Mtb* is primarily transmitted through the respiratory route by inhalation of infected aerosols, commonly causing pulmonary tuberculosis but can also result in extrapulmonary tuberculosis (3). Common symptoms of active TB include breathing difficulty, prolonged cough, coughing up blood, chest pain, weakness, fever, and weight loss (1). The immune response upon exposure determines whether *Mtb* is eliminated, or persists, resulting in active disease or latency. *Mtb* is a gram-positive, aerobic, rod-shaped, non-motile microbes measuring between 0.2-0.6 µm by 1-10 µm, lacking spores, toxins, or a capsule. Despite their simplicity, they feature a distinct three-layered, hydrophobic cell wall composed of glycolipids, peptidoglycans, arabinogalactan polysaccharides, long-chain mycolic acids, solvent-extractable lipids, outer membrane proteins and polysaccharides (4). This specialized structure acts as a permeability barrier crucial for bacterial survival and pathogenicity (4). The development of novel anti-TB therapies is imperative as research uncovers crucial factors in TB pathogenesis. Host-directed therapy (HDT) is gaining traction, aiming to target host responses exploited by *Mtb* for persistence rather than directly targeting the pathogen with antibiotics (5). HDT holds promise for reducing pathology, mycobacterial burden, and latency (5). Effective HDT targets should induce various protective host-pathogen mechanisms, but *Mtb* has evolved mechanisms to evade inhibition of crucial processes like phagosomal acidification, calcium signaling, and vesicular transport fusion. Promising host targets during *Mtb* infection are long non-coding RNAs (lncRNAs), due to the various regulatory roles they have been reported to be implicated in. Research on functionally validated lncRNAs in response to *Mtb* infection, particularly in polarized and/or *Mtb*-infected macrophages, is scarce. To address this gap, the study aimed to elucidate the functional role

of lncRNA-445 in polarized *Mtb*-infected macrophages and its potential prophylactic effect in *Mtb*-infected mice, as there is currently no scientific publication exploring these aspects.

1.2 Macrophages

Macrophages, a term introduced during the demonstration of innate immunity by Elie Metchnikoff (6), are monocytes that have differentiated based on their location which influences their morphology and phenotype (7, 8). Macrophages can reside in various tissues and are further classified based on those locations, such as alveolar macrophages which reside in the lungs (9, 10). Macrophages are renown for their ability to engulf pathogens, debris and dead cells by phagocytosis, including antigen presentation, and coordinating inflammatory responses, among other crucial roles (11). When exposed to various stimuli, macrophages can be polarized into a phenotype that is needed, either expressing a protective M1 phenotype or repairing M2 phenotype and functional responses. Macrophage polarization refers to how macrophages are activated as a result of various environmental stimuli together with the plasticity of macrophages, polarization is not a fixed state (12).

Classically polarized macrophages, known as M1 macrophages, are stimulated by bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), Th1 cytokines; interferon-γ (IFN-γ) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (13). They are associated with the secretion of pro-inflammatory cytokines; interleukin-6 (IL-6), IL-12, IL-1β, IL-1α, IL-23, and tumor necrosis factor-α (TNF-α) (13, 14). They have major histocompatibility complex class II (MHCII) with increased antigen presentation (13). Prolonged exposure to the M1 polarized state may contribute to the pathogenesis due to tissue injury (15). To counteract the classically activated state macrophages polarize to the alternatively activated state to prevent further injury to the host. Alternatively activated macrophages, M2 macrophages, are activated by Th2 cytokines IL-4 and IL-13, this less microbicidal state is associated with decreased MHCII expression, increased arginase 1 (ARG1) expression, along with the secretion of anti-inflammatory IL-10 and transforming growth factor-β (TGF-β) (13, 14).

Upon infection with *Mtb* via the aerosol route, alveolar macrophages are the first to encounter the pathogen (16). This is through the recognition of pathogen-associated molecular patterns (PAMPs), such as lipoproteins, carbohydrates, and glycolipids on the *Mtb* (17). These PAMPS are recognized by the macrophages' pathogen recognition receptors (PRRs), which include toll-like receptors (TLRs), NOD-like receptors (NLRs) and c-type lectin receptors (CLRs) (17). TLR2 identifies *Mtb* cell wall components, lipomannan (LM), lipoarabinomannan (LAM), and mannosyl-phosphatidylmyo-inositol-based glycolipids (PIM); while TLR4 detects bacterial lipopolysaccharide (LPS), and TLR9 recognizes unmethylated cytosine-guanine (CpG) motifs in the DNA of *Mtb* DNA (18).

Mtb uptake by the macrophages initiates a cascade of immune responses resulting activation of an antimycobacterial immune response. One of the significant roles of macrophages is pathogen antigen presentation. Antigen presentation is an essential function during *Mtb* infection, involving *Mtb* phagocytosis, degradation, and presentation of the antigens on the antigen-presenting MHC class molecules on the surface (19, 20). This alerts the immune system of the infection and thus initiating the necessary immune response. An antimycobacterial immune response in brief consists of but not limited to; phagolysosomal degradation of the pathogen, release of reactive oxygen- and nitrogen species, recruitment of other immune cells, pro-inflammatory cytokine expression, along with apoptosis of *Mtb* infected cells if infection cannot be contained (16, 21).

1.3 *Mycobacterium* evasion mechanisms in macrophages

Despite the array of antimycobacterial responses, *Mtb* can survive in the hosts macrophages by altering the environment making the conditions suitable for its proliferation. *Mtb* modifies the environment by disrupting maturation of the phagosome, inducing phagosomal escape into the cytosol, and manipulating host cell death (5). These mechanisms have further enhanced *Mtb* pathogenesis, despite the availability of treatment.

Mtb inhibits phagosome maturation for survival in macrophages. Phagosome maturation characterized by an acidification of pH 5.0 or lower, inhibiting bacterial growth and enhancing protease activity, vesicular trafficking, and phagosome-lysosome fusion for pathogen degradation. Vacuolar-adenosine triphosphatases (V-ATPases) facilitate the acidification by pumping hydrogen ions across the phagosome membrane. *Mtb* disrupts this process by producing tyrosine phosphatase (PtpA), which binds and prevents V-ATPases from reaching the phagosome and maintaining a pH between 6.2 and 6.5, favoring *Mtb* survival (22). Phagolysosome formation and function is disrupted by *Mtb*. This interference involves blocking the conversion of rat sarcoma virus (Ras)-associated binding (Rab) guanine triphosphatases (GTPases), Rab5 to Rab7, impeding phagosomal maturation, and recruiting Coronin1 to inhibit lysosome-phagosome fusion (23, 24). Furthermore, *Mtb* manipulates membrane trafficking by interfering with phosphatidylinositol 3-phosphate (PI3P), hindering phagosomes from obtaining lysosomal hydrolytic enzymes (25).

Mtb utilizes the ESX-1 secretion system, also known as the early secreted antigenic target (ESAT-6) secretion system, to perforate the phagosome membrane, thus allowing *Mtb* to

access the cytosol where it delivers most of its effectors (26). The eis gene of *Mtb* disrupts c-Jun N-terminal kinases (JNK) activation, hindering noncanonical autophagy through autophagy related gene 7 (ATG7) (27). Nicotinamide adenine dinucleotide hydrogen (NADH)-quinone oxidoreductase subunit G (NuoG), a virulence factor belonging to *Mtb*, neutralizes reactive oxygen species (ROS) suppressing apoptosis (28). ESX-1 secretion system enhances necrosis through TB necrotizing toxin (TNT) which hydrolyzes host NADH (29). Serine protease inhibitor, Rv3364c inhibits caspase-1 activity, deactivating inflammasome responses (30). Although many efforts have been put towards understanding the mechanisms used by *Mtb* for survival and persistence, further insight into the applied mechanisms of host-pathogen interactions is essential.

1.4 Long non-coding RNAs

Long non-coding RNAs (lncRNAs), are transcripts >200 nucleotides in length that do not encode proteins. Previously referred to as “Junk RNA” , extensive research has revealed the regulatory role of lncRNAs on gene expression from an epigenetic to a post-translational level. LncRNA can be further classified by their function and genomic location relative to protein-coding genes. The major roles of lncRNAs are involved in epigenetic regulation, modulating gene transcription, protein degradation, and enhancer activity within protein-coding genes. Due to their significant roles, dysregulation of lncRNAs have been linked to disease susceptibility or progression. The roles of lncRNAs during TB infection have not yet been extensively studied, especially in macrophages. Understanding the regulation of lncRNAs in this context could contribute towards their potential as diagnostic markers for TB.

1.5 LncRNAs mechanism of action

LncRNAs can act as cis- and trans-regulators of genes (31). Cis-regulating lncRNAs, influence neighboring genes, whereas trans-acting lncRNAs can remotely regulate gene expression from their transcription site (31, 32). The diverse functionalities of lncRNAs arise from their capacity to adopt various structures and engage in molecular interactions with proteins, RNA, and DNA (33). LncRNAs have been observed to interact with DNA, forming the RNA-DNA triplexes, which aid in the recognition of target genes by lncRNAs (34). Recent research highlights the importance of the DNA-mediated innate immune response in antibacterial defense, an example is the multi-subunit complex, including HEXIM1 and lncRNA NEAT1, along with DNA-PK subunits and paraspeckle proteins, has been identified as a central regulator in activating this immune response (35). The regulatory functions of long non-coding RNAs also depend on RNA-RNA interactions and this includes interactions with microRNAs (miRNAs), resulting in a lncRNA-miRNA-mRNA competing endogenous RNAs (ceRNAs), which results in a complex network that regulates gene expression post-transcriptionally (36). Additionally, lncRNAs have been reported to act as molecular sponges that sequester miRNAs thus preventing them from targeting other transcripts (37, 38). In a study done on pulmonary TB, the ceRNA network established using data from patients indicates that lncRNAs may modulate mRNA expression by functioning as miRNA sponges (39).

Additionally, LncRNAs have different mechanisms of action determined by their location. LncRNAs located in the nucleus are associated with modulating epigenetic processes thus impacting gene expression (40). In brief, they function as enhancers by promoting transcriptional activation, acting as guides to direct protein molecules to specific targets altering transcription factor availability, and acting as chromatin architects by recruiting chromatin modifying complexes (40). LncRNAs located in the cytoplasm operate at a post-transcriptional level, regulating the stability and translation of target mRNAs (41). They achieve this by directly interacting with target mRNAs or signaling, serving as miRNA sponges within their untranslated regions, and acting as precursors for shorter regulatory RNAs known as micropeptides (41).

1.6 Role of lncRNAs in macrophages

Various literature have highlighted the significance of lncRNA and immune function association, emphasizing their involvement in modulating immune responses by influencing the growth and specification of immune cells (42). LncRNAs have the capacity to modulate the polarization of macrophages towards classical (M1) or alternatively (M2) activated states through diverse mechanisms, thus contributing to disease progression (42). Recent studies have illustrated the involvement of numerous lncRNAs during TB infections, with a role in coordinating biological processes ranging from immune response to host-pathogen interactions (43). LncRNAs are involved in immune-inflammatory responses mediated by macrophages, crucial for antimicrobial defenses, where the inducible program of inflammatory gene expression is pivotal (43). Several examples of the roles of lncRNAs in macrophages are briefly discussed below.

A great example is lncRNA-Cox2 which can enhance the activation of macrophages, leading them towards the pro-inflammatory M1 phenotype, which is recognized for its effectiveness in combating *Mtb* infection (44). Long intergenic non-coding RNA (lincRNA)-cyclooxygenase 2 (Cox2), lincRNA-Cox2, regulates immune gene activation/repression, potentially influencing NF-κB and STAT3 activity to modulate inflammatory responses and enhance resistance against *Mtb* infection (45, 46). Moreover, lncRNAs are involved in the apoptosis and autophagy of macrophages induced by *Mtb* (47, 48). A study done on macrophages infected with Bacillus Calmette-Guerin (BCG) exhibit increased apoptosis, accompanied by elevated expression of lincRNA-Cox2; and the inhibition of Cox2

exacerbates reactive oxygen species (ROS) deposition and triggers apoptosis through activation of PERK-eIF-2α-CHOP signaling pathway (49). A similar mechanism is employed by lncRNA-EPS, which modulates both apoptosis and autophagy through the activation of JNK/MAPK signaling pathway (50). Lastly, the reduction of lncRNA-MEG3 in macrophages stimulates autophagy and improves the elimination of *Mycobacterium bovis* (M. bovis) (51).

1.7 LncRNAs validated in *Mycobacterium* infection

Due to the substantial evidence suggesting the involvement of lncRNAs in host immune responses to pathogens, lncRNAs provide a novel approach for investigating the host immune response to *Mtb* infection. Briefly discussed below are some of the essential roles of various lncRNAs during *Mtb* infection. During tuberculosis (TB) infection in human peripheral blood mononuclear cells (PBMCs), upregulated lncRNA CD244 results in TNF-α and IFN-γ suppression by CD244 in CD8+ T cells (52). Human macrophages infected with *M. smegmatis* resulted in the upregulation of lncRNA maternally expressed 3 (MEG3) expression and enhanced IFN-γ and TNF-α release, which is essential for effective bacterial clearance (53). Another study reported IFN-γ-facilitated autophagy induction resulted in lncRNA MEG3 downregulation that contributed towards *Mycobacterium bovis* (BCG) eradication in macrophages (51).

Homebox (HOX) transcript antisense intergenic RNA (HOTAIR), lncRNA HOTAIR, is associated with enhanced expression of NF-κB, IL-6 in addition to iNOS by deactivating the inhibitor of NF-κB (IKκB) protein in murine macrophages (54). Therefore, *Mtb* H37Rv decreases the expression of lncRNA HOTAIR in human macrophages by inhibiting special adenine-thymine-rich sequence binding protein 1 (SATB1) and dual-specificity MAPK phosphatase 4 (DUSP4) transcription, facilitating the upstream accumulation of restrictive H3K27me3 markers (55). Nuclear paraspeckle assembly transcript 1 (NEAT1), lncRNA NEAT1, has been implicated in inflammasome activation in murine macrophages by promoting the secretion of IL-1β through the stabilization of mature caspase-1 (56). In pulmonary alveolar macrophages, they found that NEAT1 regulates MiR-17-5p expression through TLR4, resulting in enhanced injury due to inflammation (57).

1.8 ASO targeting of lncRNAs

Antisense oligonucleotides (ASOs) are short single-stranded oligonucleotides, typically 18-30 base pairs long, chemically synthesized with a phosphorothioate backbone (58). Chemical modifications on their backbone such as locked nucleic acid (LNA) incorporation enhance the ASOs pharmacological functions including enhanced target binding, reduced toxicity, and resistance to nuclease degradation (59, 60). ASOs designed to specifically target lncRNAs are gapmeRs. GapmeRs are 16 nucleotides in length, restricted by LNAs including DNA in the LNA-free central gaps. ASOs have been used in several studies to comprehend the mechanisms of lncRNAs using a loss-of-function approach. For example, to understand lincRNA-MIR99AHG mode of action, an LNA-ASO gapmeR targeting lincRNA-MIR99AHG resulted in MIR99AHG knockout (61). The ASO mediated silencing of lincRNA-MIR99AHG decreased the intracellular proliferation of *Mtb* in murine and human macrophages, as well as suppressed pro-inflammatory cytokine production (61).

1.9 Research problem and focus

Mtb manipulates the hosts immune response by inducing a less effective bactericidal state ensuring its own survival. Our current research is focused on investigating host factors, specifically lncRNAs, including their various roles modulated by *Mtb*, leading to its proliferation and persistence. Since macrophages are the primary site for *Mtb* infection, targeting host factors within these cells is crucial. The primary objective of our research is to develop new host-directed drug therapies against *Mtb* infections, and this study aims to identify potential targets for recognition in that pursuit (5).

Our research group was part of the functional annotation of the mammalian genome 5 (FANTOM5) project, which investigated the transcriptional profile of mouse macrophages infected with *Mtb* HN878, a hypervirulent Beijing strain, and compared them to macrophages stimulated with IFN-γ and IL-4/IL-13, thus inducing a classical and alternative activation respectively (62). Utilizing deep cap analysis gene expression (CAGE) transcriptomics, these globally identify transcriptional starting sites and their expression levels, I analyzed the transcriptional landscape (63). Additionally, macrophages infected with *Mtb* after pre-stimulation with IFN-γ or IL-4/IL-13 were compared to the unstimulated *Mtb*-infected macrophages, identifying 151 differentially expressed lncRNAs. From this, 11 differentially expressed lncRNAs were validated and one candidate which was differentially expressed among the pre-stimulations and repressed after infection with *Mtb* HN878 ,was chosen for this study.

1.10 Aim and objectives

1.10.1 Aim

This project aims to functionally validate the candidate long non-coding RNA using a loss-of-function approach that utilizes ASO LNA GapmeRs in unstimulated and M1 (IFN-γ) polarized and/or *Mtb*-infected murine macrophages.

1.10.2 Objectives

a) Functionally validate the candidate lncRNA and its associated pathways in *Mtb*-infected mouse macrophages.

b) Investigate the role of the lncRNA in host immune responses against *Mtb* infection.

1.10.3 Hypothesis

Mtb targets lncRNA-445 to promote intracellular survival in macrophages.

CHAPTER TWO

Materials and Methods

2.1 Materials

The general laboratory consumables used in this project were purchased from the Sigma-Aldrich® brand, Merck Group (Darmstadt, Germany), B&M Scientific (Cape Town, South Africa) and the Lasec® Group (Cape Town, South Africa). The cell culture consumables used were purchased from Corning Incorporated (New York, United States), the CytOne® brand, USA Scientific Incorporated (Florida, United States), Greiner Bio-One International (Kremsmünster, Austria), and TPP Techno Plastic Products (Schaffhausen, Switzerland). Reagents used for cell culture were purchased from either Gibco™ Life Technologies brand, Thermo Fisher Scientific (Massachusetts, United States) or Lonza Group (Basel, Switzerland). Chemicals and reagents for general use were purchased from Merck Group (Darmstadt, Germany) or the VWR Chemical BDH® brand, VWR International Limited (Poole, United Kingdom).

2.2 Methods

2.2.1 Ethics

2.2.1.1 Animals

Male BALB/c mice, aged 8-11 weeks, were used for this study. The mice were accommodated in well-ventilated cages under specific pathogen-free conditions in accordance with the fundamental prerequisites for housing and husbandry as outlined by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) guidelines. The mice were allocated to cages based on their respective groups and age, with a maximum of three mice per cage. Each mouse was numbered using the method of ear clippings, and they were consistently monitored and provided with commercially available pellets and drinking water for their diet. The Research Animal Facility (RAF) of the University of Cape Town (UCT) is authorised to manage the welfare of the mice. The protocol number for the AEC-approved protocol used in this study is 022-024.

2.2.2 Generation of mouse bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were generated from the femur and tibia of mice, that were sacrificed using halothane and cervical dislocation for the confirmation of death (64). In brief, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin was used to flush the bone marrow cells. The cells were washed by centrifugation at 1200 rpm for 10 minutes (mins) at 4°C, the supernatant was discarded and the cell pellet was resuspended in PLUTZNIK media (a medium consisting of DMEM supplemented with 10% FBS, 5% horse serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-β-Mercaptoethanol, 30% L929 cell-conditioned medium, and 100 units/ml penicillin G and 100 μg/ml streptomycin (Pen-Strep)). L929 medium contains macrophages-stimulating factor (M-CSF), which is responsible for the differentiation of bone marrow cells into macrophages. To determine the cell concentration, staining with 4% trypan blue and counting on a heamocytometer was performed using light microscopy (Eclipse TS100 inverted microscope from Nikon, Tokyo, Japan). The cells were seeded in 100 mm CELLSTAR® culture dishes (Greiner Bio-One International, Kremsmünster, Austria) at a concentration ranging from $13x10^6$ - $25x10^6$ cells per culture dish. The cells were then incubated at 37°C under 5% carbon dioxide (CO_2) for a total of 7 days in PLUTZNIK medium with the addition of fresh PLUTZNIK medium on the third day. After the 7-day incubation period, the macrophage differentiation is observed which is characterized by an elongated morphological structure by using light microscopy. The supernatants of the cell culture were discarded, and the culture dish was washed with 1x PBS to remove non-adherent cells. Adherent cells were detached through incubation with a lidocaine solution (containing 0.25 mg/ml lidocaine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 1x PBS for 15 mins at 4°C or 5 mins at 37°C (5% CO2), followed by gentle scraping using a cell scraper. The collected cells were centrifuged at 1200 rpm for 10 mins at 4°C, and the pellet was resuspended in DMEM + 10% FBS + Pen-Strep. The cell concentration was determined once again through trypan blue staining, as previously described. Finally, the cells were seeded in various cell culture plates at predetermined concentrations (as indicated in Table 1) and incubated for 24 hrs at 37°C under 5% CO2, after which downstream experiments were conducted.

Table 1: Concentration of cells seeded in various cell culture plate formats.

Cells concentration (cells/well)	The volume of media per well	Plate format
2-3x10^6	2 ml	6-well
1.5-2x10^6	1.5 ml	12-well
1x10^6	1 ml	24-well
5x10^5	500 µl	48-well
1x10^5	200 µl	96-well

2.2.3 ASO GapmeR transfection of macrophages

Antisense LNA® GapmeR oligonucleotides (gapmeRs) (Exiqon™/Qiagen, Germany) were used to suppress the selected lncRNA. The gapmeRs were reconstituted according to the manufacturer's instructions and stored at -20°C until used. The gapmeRs were diluted in Opti-MEM™ medium (Life Technologies™, Thermo Fisher Scientific, Massachusetts, United States) to achieve a concentration of 50 nM. Subsequently, a solution containing 3% Lipofectamine™3000 reagent (Life Technologies™, Thermo Fisher Scientific, Massachusetts, United States) in OptiMEM™ medium was prepared. The diluted gapmeRs and the 3% Lipofectamine™3000 reagent were then combined in a 1:1 ratio. The cells were transfected with a final concentration of 25 nM gapmeR: lipofectamine solution per well and incubated at a temperature of 37°C for a duration of 48 hrs (5% CO_2).

2.2.4 Cytokine stimulation of macrophages

The stimulants were prepared in a cell culture medium prior to stimulating the macrophages. BMDMs were subjected to stimulation with 100 units/ml of recombinant mouse proteins, recombinant IFN-γ for the classical activation of macrophages and recombinant IL-4 and IL-13 for the alternative activation of macrophages (BD Biosciences, New Jersey, United States). The stimulated macrophages were incubated at 37°C (5% CO_2) for different time periods.

2.2.5 Mycobacterium culture

The *in vitro Mycobacterium* procedures were done in the UCT FHS BSLIII facility with the approval of the faculty's Health and Safety Committee. Stocks of *Mycobacterium* strains HN878, H37Rv, H37Ra, and BCG are prepared and stored in aliquots until used for *in vitro* infection. In brief, The various strains are grown at 37°C in Middlebrook 7H9 liquid medium (BD Biosciences, Franklin Lakes, New Jersey, United States) that is supplemented with 10%

oleic acid-albumin-dextrose catalase (OADC) enrichment medium (BD Biosciences, Franklin Lakes, New Jersey, United States)and 5% glycerol. This was done until a minimum optical density between 0.6-0.8 was obtained, and the Biowave Cell Density Meter CO8000 (Biochrom Limited, Cambridge, United Kingdom) was used at a wavelength of 600 nm to measure the optical density. The concentrations were confirmed by a colony-forming unit (CFU) assay, the stocks were prepared in 15% glycerol and stored in 1 ml aliquots at -80°C until further use.

2.2.6 *Mycobacterium tuberculosis* infections of macrophages

In preparation for infection, *Mycobacterium tuberculosis* (*Mtb*) HN878 stocks were thawed at room temperature and thereafter they were centrifuged for 5 mins at 10,000 rpm at room temperature (20°C to 25°C). The *Mtb* pellet was washed by resuspension in 1x PBS and centrifugation for 5 mins at 10,000 rpm at room temperature (RT). This wash step was done to remove any remaining glycerol. The pellet was resuspended in cell culture media (DMEM + 10% FBS) without penicillin G and streptomycin, and macrophages were infected at a multiplicity of infection (MOI) of 1 bacillus: 1 cell (1:1). After 4 hrs, the supernatant was removed, and fresh DMEM (with 10% FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin, 10 ug/ml Gentamicin, with or without stimulants) was added for the removal of extracellular bacteria, after 2 hrs the medium will be replaced with DMEM (containing 10% FBS with or without stimulants) in preparation for downstream experiments.

2.2.7 In vivo Mycobacterium and Listeria monocytogenes infection

For the *in vivo* infection of mice, *Mycobacterium* strains HN878, N0072, and CDC1551, were cultured as previously described (2.2.5). Infection of mice was done intranasally with 25 µl/nostril of Mycobacterium in 1x PBS at a dose of 100 CFU/mouse for 11 and 21 days. *Listeria monocytogenes* (*L. monocytogenes*) EGD strain cultures were grown in tryptic-soy broth following the method described for *Mycobacterium* cultures (2.2.5). Intraperitoneal infection of mice with 200 µl of *L. monocytogenes* in 1x PBS at a dose of 2 $x10^5$ CFU/mouse for 48 hrs. Thereafter, selected organs were harvested and homogenized in Qiazol for the extraction of RNA (2.2.8) using the Polytron PT 2500 E homogenizer (Kinematica, Malters, Switzerland).

2.2.8 RNA extraction

Cells were lysed in Qiazol (Qiagen, Hilden, Germany) for the different time points post-stimulation or infection, and the resulting lysates were stored at -80°C. The miRNeasy Mini

kit (Qiagen, Hilden, Germany) was used to extract total RNA from the lysates in accordance with the manufacturer's recommendations. The purity and concentration of the extracted RNA were measured using the ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

2.2.9 cDNA synthesis and RT-qPCR

Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) was used for the cDNA synthesis of the total RNA (100 ng) through reverse transcription according to the manufacturer's guidelines. Quantitative real-time PCR (RT-qPCR) was conducted for the protein-coding gene expression analysis, using the LightCycler® 480 II system combined with the LightCycler® 480 SYBR Green I Master (Roche, Manheim, Germany) as well as gene-specific primers (Integrated DNA Technologies, Coralville, Iowa, United States). The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to the internal control, Hprt1. The 0 hr timepoint (unstimulated and uninfected) was set as the reference sample where the $2^{-\Delta\Delta Ct}$ value is set to 1. Regarding lncRNAs, cDNA synthesis was conducted using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark) according to the manufacturer's recommendations. The total RNA was then diluted to 5 ng/µl and synthesized into cDNA using the Universal cDNA synthesis kit II. RT-qPCR was performed using the miRCURY LNA™ Universal RT microRNA PCR starter kit which consists of ExiLENT SYBR® Green master mix and MiRCURY LNA™ PCR primer sets for the specific lncRNA and HPRT as an internal control (Exiqon, Denmark) in a Roche 480 Lightcycler 656 (Roche, Germany).

2.2.10 Bacterial burden determination

Supernatants from the 24- and 48-hrs post-*Mtb*-infected macrophages were removed and stored at -80°C for downstream experiments. Cells were lysed at different time points post-infection in Triton X-100 and serial dilutions were plated on Middlebrook 7H10 agar (BD Biosciences, Franklin Lakes, New Jersey, United States) supplemented with 10% OADC enrichment medium and 5% glycerol. Incubation of plates was at 37°C (5% CO^2) for 15 days. After 15 days, the Colony-forming Units (CFUs) were enumerated to determine the bacterial burden.

2.2.11 Cell viability

Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay kit (Promega, Wisconsin, United States). A measured 20 µl CellTiter-Blue® Reagent was added per 100 µl culture medium to each sample and incubated for 4 hrs at 37°C (5% CO_2).

2.2.12 Cytokine production

The enzyme-linked immunosorbent assay (ELISA) was used for the measurement of various cytokine and chemokine production, using the ELISA development reagents (R&D systems, USA). The collected supernatants were diluted 2, 3, and 6 folds. The data was acquired using the Versama^{TM} Tumble microplate reader with Softmax Pro v6.3 (Avantor®, United States).

2.2.13 Griess reagent assay

Cell culture supernatants were collected for the measurement of protein levels of nitrite, which correlate to the production of nitric oxide (NO). Reagents for the Griess assay were prepared in the lab. Readout was acquired using Versama^{TM} Tumble microplate reader with Softmax Pro v6.3 (Avantor®, United States).

2.2.14 Arginase assay

Arginase activity was measured in cells lysed with Triton-X100 and reagents for the assay were prepared in the lab. The readout from the plates was acquired using Versama^{TM} Tumble microplate reader with Softmax Pro v6.3 (Avantor®, United States).

2.2.15 Apoptosis

The *in vitro* assessment of apoptosis in cells was done using the *in situ* TUNEL assay, this was done according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, Roche). Cells were incubated in 4% paraformaldehyde at RT for 1 hr, washed with 1X PBS and permeabilized with Triton X-100 on ice for 2 mins, and thereafter washed twice with 1X PBS. The BMDMs were labelled with the TUNEL reaction mixture for 1 hr at 37°C and thereafter analyzed by fluorescent microscopy. Apoptosis was also analyzed by flow cytometry using the FITC Annexin V Apoptosis Detection Kit II (BD Bioscience, Franklin Lakes, New Jersey, United States). Cells were resuspended in FACS buffer for acquisition with the BD LSRFortessa (BD Bioscience, New Jersey, United States), and data analysis was done on the FlowJo software (Treestar, Ashland, Oregon, United States).

2.2.16 TLR agonists stimulation

TLR agonists; $Pam_3CysSerLys_4$ [(Pam_3CSK_4), (Sigma-Aldrich®, Merck, Darmstadt, Germany)]; LPS. (InvivoGen, California, United States) or CpG ODN 1668 (Sigma-

Aldrich®, Merck, Darmstadt, Germany), were used at a concentration of 100 ng/ml to stimulate cells activating the TLR 2, TLR4, and TLR9 ligands, respectively. The cells were incubated at 37°C (5% CO_2) for stimulation at different time points.

2.2.17 Inhibition of innate signalling pathways

Pharmacological inhibitors of the innate immune signalling pathways; such as NF-κB inhibitor (Bay11-7082; 10 μM), c-JNK inhibitor (SP600125; 10 μM), extracellular signal-regulated protein kinases 1 and 2 (Erk-1/2) inhibitor (FR180204; 10 μM) (all three purchased from Sigma-Aldrich®, Merck, Darmstadt, Germany) and the p38 inhibitor (SB203580; 5 μM; Tocris Bioscience, Bristol, United Kingdom) were used to pre-treat the cells for 1 hr at 37°C (5% CO_2). Cells were stimulated with M1/M2 cytokines for 4 hrs at 37°C (5% CO_2) and then infected with *Mtb* HN878 (MOI 1:1) for various time points and incubated at the same conditions as above.

2.2.18 Statistical analysis

All data were analysed with the GraphPad Prism v8.0 software, using a Student's two-tailed t-test (with unequal variance) or unless stated otherwise in the Figure legends. Means are shown as SEM., $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$, respectively.

CHAPTER THREE

Host-directed targeting of IFN-γ induced lncRNA-445 suppresses intracellular growth of *Mycobacterium tuberculosis*

3.1 Introduction

Tuberculosis (Tb) is an airborne infectious disease that has caused an estimated 7.5 million new infections in 2022 and an estimated 1.3 million deaths (1). Discovered in 1882 by Robert Koch, *Mycobacterium Tuberculosis* (*Mtb*) is a bacterial pathogen responsible for Tb infection in humans (2). *Mtb* transmission is primarily through the respiratory route commonly causing pulmonary tuberculosis but can also result in extrapulmonary tuberculosis (3). *Mtb* persistently causes diseases in the host by targeting cellular host factors and utilizing these for its survival (5). Host factors have been researched for their host-pathogen interactions which result in either killing the pathogen or continued disease due to pathogen survival (65). Using this approach to target and employ host factors utilized by *Mtb* could result in decreased mycobacterial burden, reduced pathology, and possible latency (5). A cascade of immune responses is activated during *Mtb*-infection from the transcriptional and post-transcriptional level which allows for the rapid altering in gene expression, such as the activation of innate and adaptive immune cells which are critical for host defence (62). Recent literature has shown that long non-coding RNAs play a vital role in transcriptional programming resulting in the alteration of immune responses (66).

Long non-coding RNAs (lncRNAs) are non-protein coding RNAs characterized by their length of >200 nucleotides (67). The functional role of lncRNAs is in protein-coding gene expression where they are epigenetic regulators and they directly modulate the post-transcriptional expression of the gene (67). The functional role of lncRNAs in macrophages has been emerging (68), with lncRNAs such as lincRNA-Cox-2 (45) and Mirt2 (69), which regulate inflammatory gene expression in myeloid cells. The role of lncRNAs during various Mycobacterium infections has been recently reported. LncRNA-CD244 acts as an epigenetic regulator of IFN-γ and TNF-α production in $CD8^+$ T cells and impacts $CD8^+$ T-cell immunity against active *Mtb* infection (52). Despite the available literature, our knowledge regarding

the functional role of lncRNAs in polarized macrophages and during infection with a hypervirulent *Mtb* strain is still limited.

In this study, I identified lncRNA-445 which is abundantly expressed in IFN-γ stimulated murine macrophages and downregulated upon *Mtb* infection. The functional and regulatory role of lncRNA-445 was investigated using ASO-LNA gapmeR to knockout the expression of lncRNA-445. A study that employed similar methods found that lincRNA-MIR99AHG is a positive regulator of inflammation and macrophage polarization to promote *Mtb* growth (61). I found that lncRNA-445 knockdown reduces intracellular *Mtb* growth, increases nitrite production, and regulates protein cytokine expression in IFN-γ pre-stimulated murine macrophages that are infected with *Mtb*. I propose that the induction of lncRNA-445 by IFN-γ serves a host-protective mechanism in response to *Mtb* infection, aiming to suppress the intracellular survival and persistence of *Mtb* in macrophages.

3.2 Results

3.2.1 Long non-coding RNA-445 is induced by IFN-γ and repressed by *Mycobacterium* HN878 infection.

Deep Cap Analysis of Gene Expression (CAGE) transcriptomics has been utilized to define the transcriptome of bone marrow-derived macrophages (BMDMs) that are in an M1 (IFN-γ) or M2 (IL-4, IL-13, IL-4/IL-13) activated state (70) and during infection with the *Mtb* HN878 hypervirulent strain (62). Using this approach (Figure 1A), a list of 11 various differentially expressed lncRNAs were identified and from those, lncRNA-445 was selected. LncRNA-445 expression was significantly upregulated in IFN-γ stimulated macrophages compared to the unstimulated (non-stim) and IL4, IL-13, and IL-4/IL-13-stimulated macrophages (Figure 1B). At 2 hours (h) post-stimulation (p.s), IFN-γ significantly upregulated 11.23-fold the expression CAGE tags per million (TPM) of lncRNA-445 when compared to the unstimulated macrophages (Figure 1B). *Mtb* infection of pre-stimulated BMDMs resulted in a suppression of lncRNA-445 expression from 12h in IFN-γ stimulated BMDMs (Figure 1C). The CAGE TPM results were confirmed with RT-qPCR, which also showed a significantly high expression of lncRNA-445 in IFN-γ stimulated BMDMs from 2h compared to the other stimulations (Figure 1D) and downregulated upon *Mtb* infection from 4h (Figure 1E). The expression of arginase 1 (Arg1) is only upregulated at 4h in the unstimulated (*Mtb* only) macrophages and remains downregulated in all the other stimulations (Figure 1F). Nitric oxide synthase 2 (Nos2) is upregulated at 4h in all stimulations and remains highly upregulated in the IFN-γ stimulated macrophages (Figure 1G). This data shows that the expression of lncRNA-445 varies according to the macrophage activation state, with IFN-γ as the inducer and *Mtb* as the repressor. Understanding the importance of IFN-γ for the activation of macrophages during *Mtb* infection, the sudden suppression of IFN-γ induced lncRNA-445 was noted in this study.

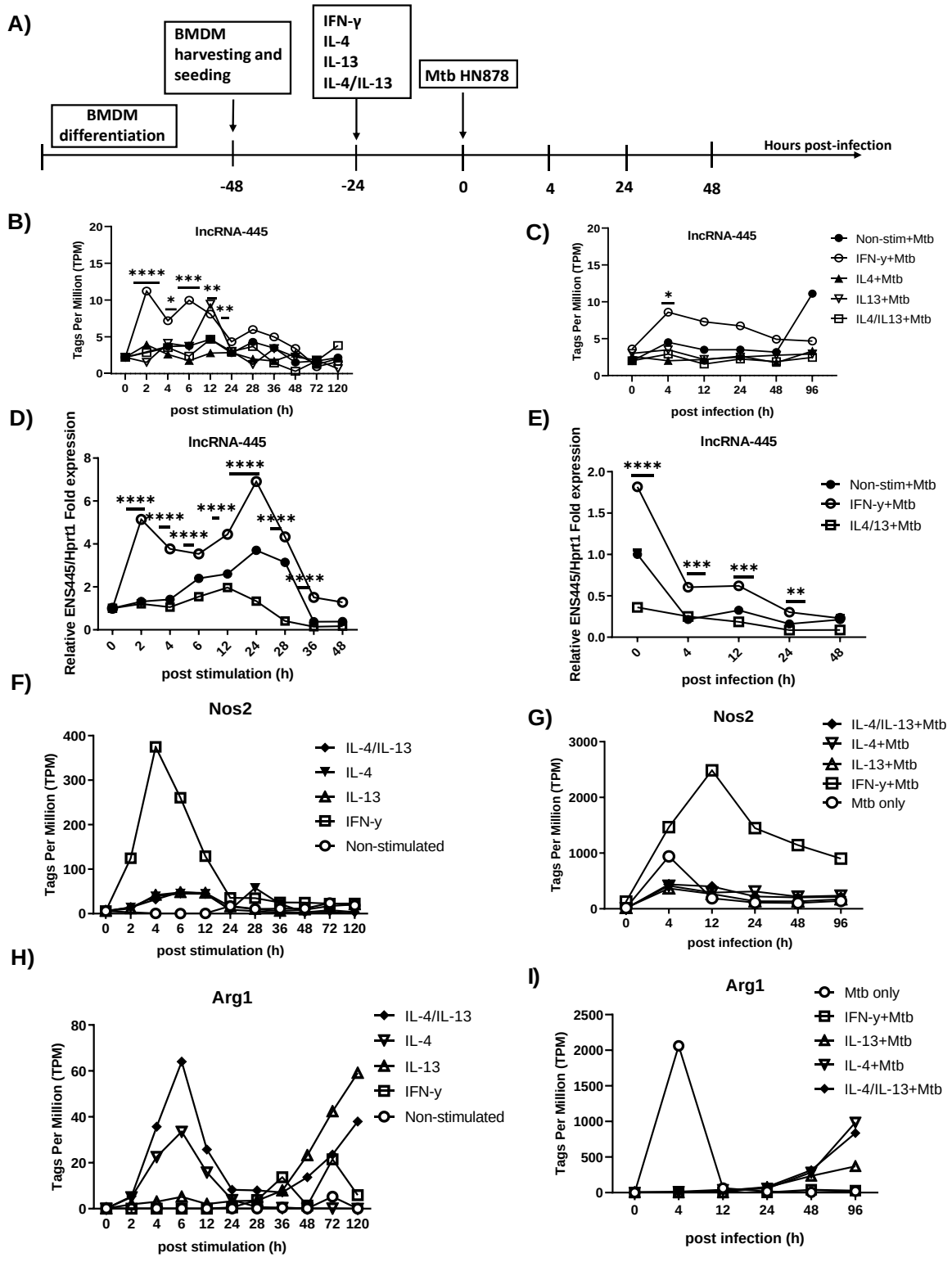

Figure 1: LncRNA-445 is upregulated in M1 (IFN-γ) activated macrophages but repressed upon *Mtb* HN878-infection. (A) Experimental timeline of differentiation, stimulation, and *Mtb* HN878 infection of bone marrow-derived macrophages (BMDMs). **(B, C)** Deep CAGE analysis of lncRNA-445 kinetic expression in unstimulated (Non-stim), IFN-

γ, IL4, IL13, and a combination of IL4 and IL13 pre-stimulated; uninfected and *Mtb*-infected BMDMs at various timepoints post-stimulation (p.s) and post-infection (p.i). **(D. E)** RT-qPCR of lncRNA-445 kinetic expression in unstimulated (Non-stim), pre-stimulated as well as uninfected, and *Mtb*-infected BMDMs at various time points post-stimulation (p.s) and post-infection (p.i) respectively. CAGE analysis of arginase 1(Arg1) **(F)** or nitric oxide synthase 2 (Nos2) **(G)** in unstimulated (*Mtb* only), IFN-γ, IL4, IL13, and a combination of IL4 and IL13 stimulated; *Mtb*-infected BMDMs. The fold change in gene expression was determined by RT-qPCR and normalised to HPRT expression. Three independent experiments were performed. Each of the data points represent the arithmetic mean of triplicates ± SD. Two-way ANOVA with Bonferroni post hoc test (B, C) and a Student's t-test (D, E) was used to evaluate statistical significance, P values represented as, *P<0.05, ** P<0.01, ***P<0.001, and ****P<0.0001.

Due to the increasing evidence that has shown that lncRNAs could potentially influence Tb susceptibility including disease progression (71), I determined the expression of lncRNA-445 in BMDMs infected with different *Mycobacterium* strains, *M. bovis* BCG, virulent H37rv and HN878, and avirulent H37ra (Figure 2A). I observed downregulation of lncRNA-445 with different *Mtb* infections at all time points, with a significant upregulation at 48h with H37ra when compared to the uninfected BMDMs (Figure 1A). The whole lung from mice infected with *Mtb* HN878 was processed and using RT-qPCR found that lncRNA-445 was downregulated at 11 days but highly upregulated at 21 days post *Mtb* infection (Figure 1B). To compare the expression of lncRNA-445 in other intracellular pathogens, mice were infected with *Listeria monocytogenes* (*L. monocytogenes*) for 48h (Figure 1C). LncRNA-445 was downregulated in both the whole liver and spleen of the *L. monocytogenes*-infected mice (Figure 1C). Altogether this data shows that irrespective of the strain and virulence, lncRNA-445 expression is repressed during *Mycobacterium* infection and other intracellular pathogens.

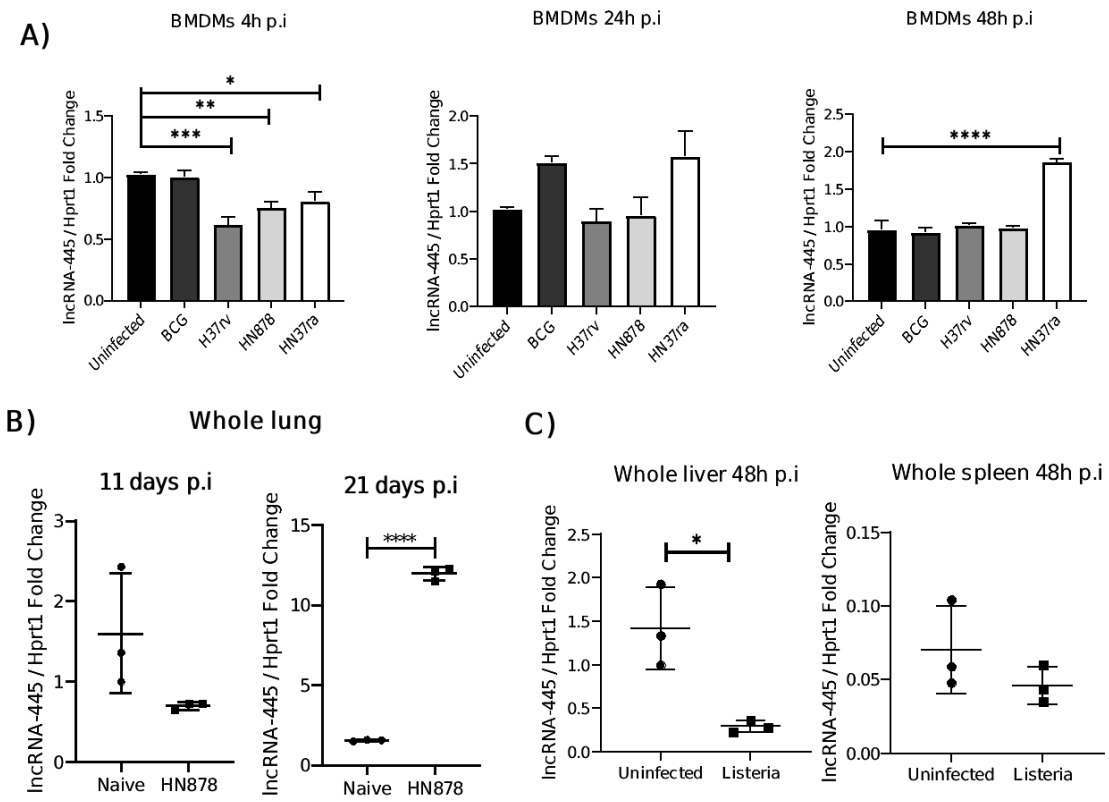

Figure 2: **Expression of lncRNA-445 is repressed during infection with various Mycobacterium and Listeria monocytogenes.** RT-qPCR analysis of lncRNA-445 expression in **(A)** BMDMs infected with BCG, H37rv, HN878, and H37ra for 4, 24, and 48h p.i; **(B)** the lung of mice infected with *Mtb* HN878 for 11 and 21 days (n=3 mice); **(C)** the whole liver and whole spleen of mice infected with Listeria monocytogenes for 48h (n=3 mice). Data is represented as mean ± SEM. One-way ANOVA with Bonferroni post hoc test (A) and a student's t-test (B, C) was used to evaluate statistical significance, P values represented as, *P<0.05, ** P<0.01, ***P<0.001, and ****P<0.0001.

3.2.2 Expression of LncRNA-445 is mediated through the TLR9 and p38 signaling transduction pathways.

Toll-like receptors (TLRs) are an imperative part of the signalling pathway where they are pattern recognition receptors (PRRs), with each TLR recognizing specific pathogen-associated molecular patterns (PAMPs) of *Mtb* (72). BMDMs were stimulated with TLR agonists to activate respective TLRs, Pam3CSK4 (TLR2), LPS (TLR4), and CpG ODN (TLR9) for up to 24h (Figure 3A). The expression of lncRNA-445 was significantly upregulated in BMDMs exposed to CpG at 2 and 4h, as well at 4h in BMDMs exposed to Pam3CSK4 and LPS. Sub-cellular fractionation was performed to determine the location of lncRNA-445 in M1 polarized macrophages. RT-qPCR analysis of lncRNA-445 expression was shown to be higher in the nucleic RNA fraction at 4h, but at 24h its expression is significantly higher in the cytoplasmic RNA fraction (Figure 3B). BMDMs were pre-treated with selective pharmacological inhibitors to understand the functional relevance of this signalling pathway in the regulation of lncRNA-445 expression. Pharmacological inhibitors were used for the NF-κB (Bay11-7082), JNK (SP600125), p38 (SB203580) or Erk1/2 (FR180204) pathways, and the BMDMs were *Mtb* infected (Figure 3C) or LPS stimulated (Figure 3D). The p38 inhibitor (SB203580) restored the *Mtb*-induced downregulation of lncRNA-445 (Figure 3C), this was also observed in the LPS-stimulated BMDMs (Figure 3D). LncRNA-445 expression remained significantly repressed in the NF-κB, Erk1/2, and JNK pathways. Altogether this data suggests that the TLR9 and p38 signalling pathways are responsible for the *Mtb*-mediated downregulation of lncRNA-445.

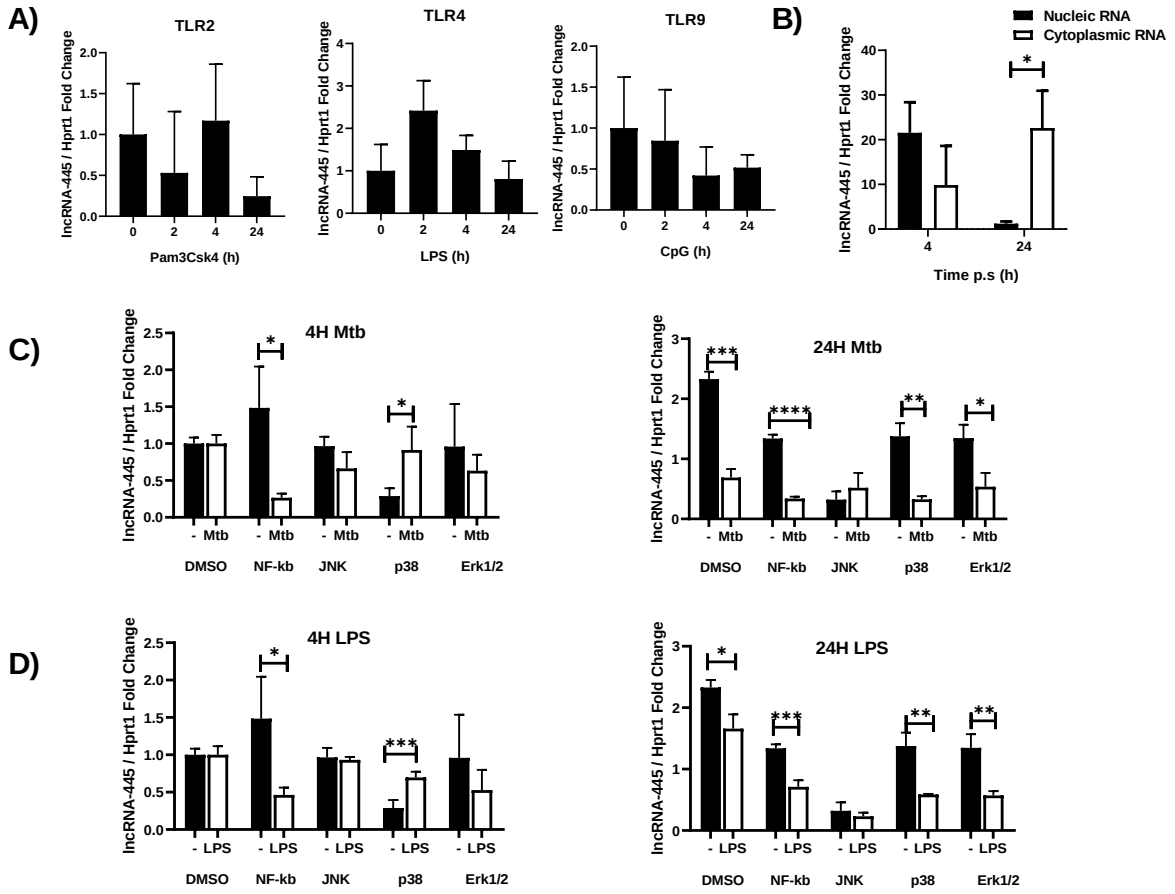

Figure 3: LncRNA-445 expression is regulated through TLR9 and the p38 signaling transduction pathway during *Mtb* infection. LncRNA-445 expression obtained by RT-qPCR in **(A)** BMDMs stimulated with TLR agonists; Pam3CSK4 (TLR2), LPS (TLR4), and CpG ODN (TLR9) for 2, 4, and 24h, as well as BMDMs pre-treated with selective pharmacological inhibitors for NF-κB (Bay11-7082), JNK (SP600125), p38 (SB203580) or Erk1/2 (FR180204) and infected with *Mtb* HN878 **(C)** or stimulated with LPS **(D)** for 4 and 24h. RT-qPCR of lncRNA-445 expression in nucleic and cytoplasmic RNA **(B)** in IFN-γ stimulated macrophages at 4 and 24h.The data shown is representative of three independent experiments, each data point is represented as mean ± SEM (n=3 technical triplicates). Statistical significance is symbolized by *P<0.05, ** P<0.01, ***P<0.001, and ****P<0.0001 determined by a one-way (A) and two-way (B, C) ANOVA with Bonferroni *post hoc* test.

3.2.3 Knockdown of lncRNA-445 expression reduces intracellular *Mtb* growth and inflammatory cytokines.

To further understand the functional role of lncRNA-445, a loss-of-function experiment using antisense oligonucleotide (ASO) locked nucleic acid (LNA) gapmeRs was performed according to the experimental outline (Figure 4A). The transfection efficiency was observed in uninfected and *Mtb* HN878 infected BMDMs for 48 hours using RT-qPCR. The ASO knockdown of lncRNA-445 was measured in IFN-γ stimulated only and IFN-γ pre-stimulated and *Mtb*-infected macrophages using RT-qPCR, with the knockdown efficiencies calculated as the percentage (%) difference between the gapmeR control and gapmeR lncRNA-445. BMDMs were transfected with LNA gapmeRs Knockout of lncRNA-445 was successful, I observed a 98 and 78% downregulation at 24 and 48h in M1 polarized macrophages and downregulation of 65, 59, and 91% at 4, 24, and 48h in the *Mtb*-infected macrophages respectively (Figure 4C). Treatment of the macrophages with gapmeR control or gapmeR lncRNA-445 did not cause any toxicity to the macrophages, as seen with the stable cell viability in pre-stimulated and infected BMDMs (Figure 4D). The functional role of lncRNA-445 during infection with *Mtb* was investigated using the colony-forming units (CFU) assay, here the effects of lncRNA-445 knockout resulted in significantly reduced intracellular growth of *Mtb* in macrophages (Figure 4E).

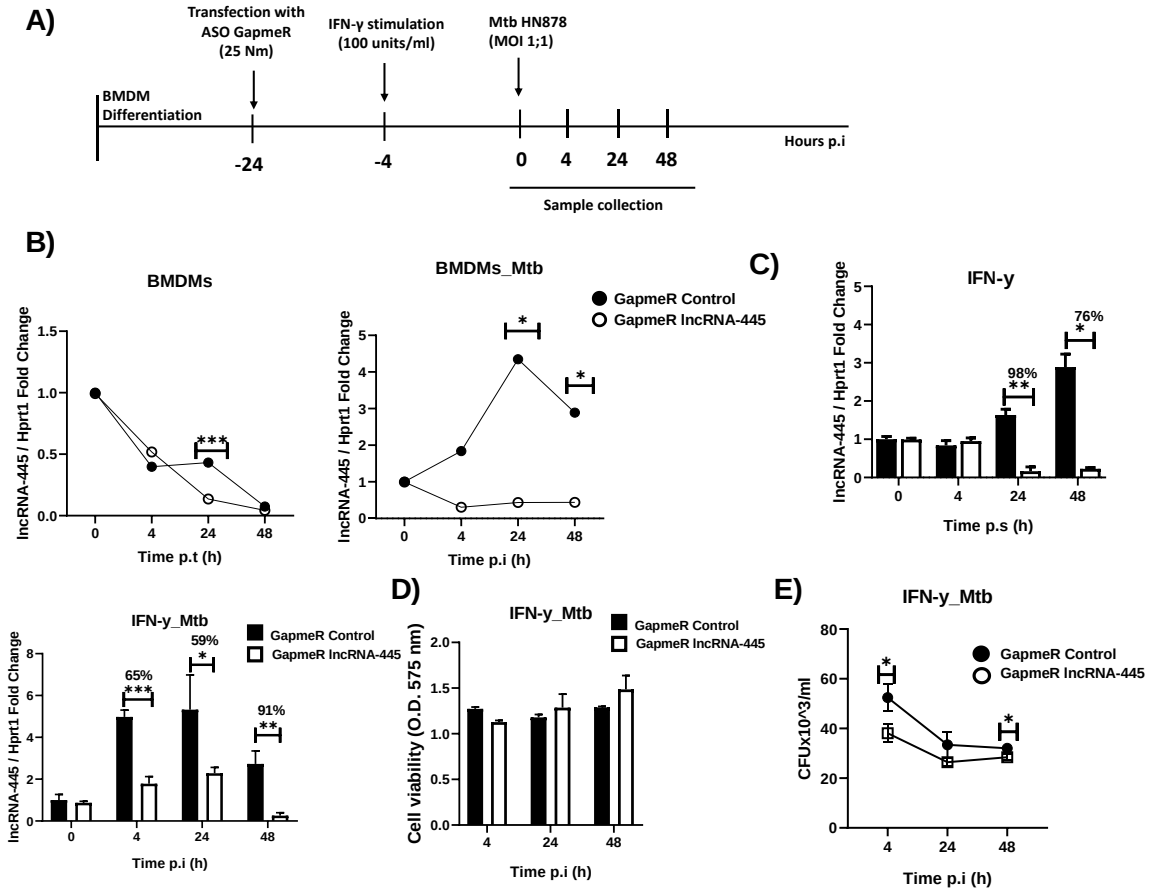

Figure 4: ASO knockdown of lncRNA-445 reduces intracellular growth of *Mtb*. GapmeR knockdown of lncRNA-445 **(A)** experimental outline. Transfection efficacy of LNA-gapmeR measured using RT-qPCR in **(B)** uninfected and *Mtb* HN878 infected BMDMs. ASO knockdown efficiency measured by RT-qPCR in **(C)** IFN-γ stimulated and pre-stimulated BMDMs infected with *Mtb* HN878 for 0, 4, 24, and 48h. LncRNA-445 knockdown effects on **(D)** cell viability determined by the CellTiter-Blue® assay and on intracellular *Mtb* growth **(E)** determined by the CFU assay. Data shown is representative of three independent experiments, each data point is represented as mean ± SEM (n=3 technical triplicates). Statistical significance is symbolized by *P<0.05, ** P<0.01, and ***P<0.001 determined by a student's t-test.

Further characterization of lncRNA-445 functional role in the modulation of pro-inflammatory cytokines was assessed using Caspase-1 assay, ELISA and RT-qPCR. Caspase-1 activity is critical due to it being a direct marker of the activation of the caspase-1/IL-1β inflammasome which results in improved mycobacterial clearance by macrophages (73). The detected caspase-1 activity was significantly reduced in the ASO-lncRNA-445 treated

macrophages (Figure 5A). Using ELISA, a significant decrease in the protein pro-inflammatory cytokine levels was noted in IL-1β and IL-1α, at 24 and 48h post-*Mtb* infection (Figure 5B). RT-qPCR was performed to measure the mRNA levels of TNFα which was significantly increased (Figure 5C). IL-10 cytokine release was significantly increased at 24 and 48h post-*Mtb* infection. Overall, the data shows that *Mtb* regulates inflammatory responses, such as inhibiting pro-inflammatory cytokines and inducing anti-inflammatory cytokine production, by targeting lncRNA-445 to promote its intracellular growth in macrophages.

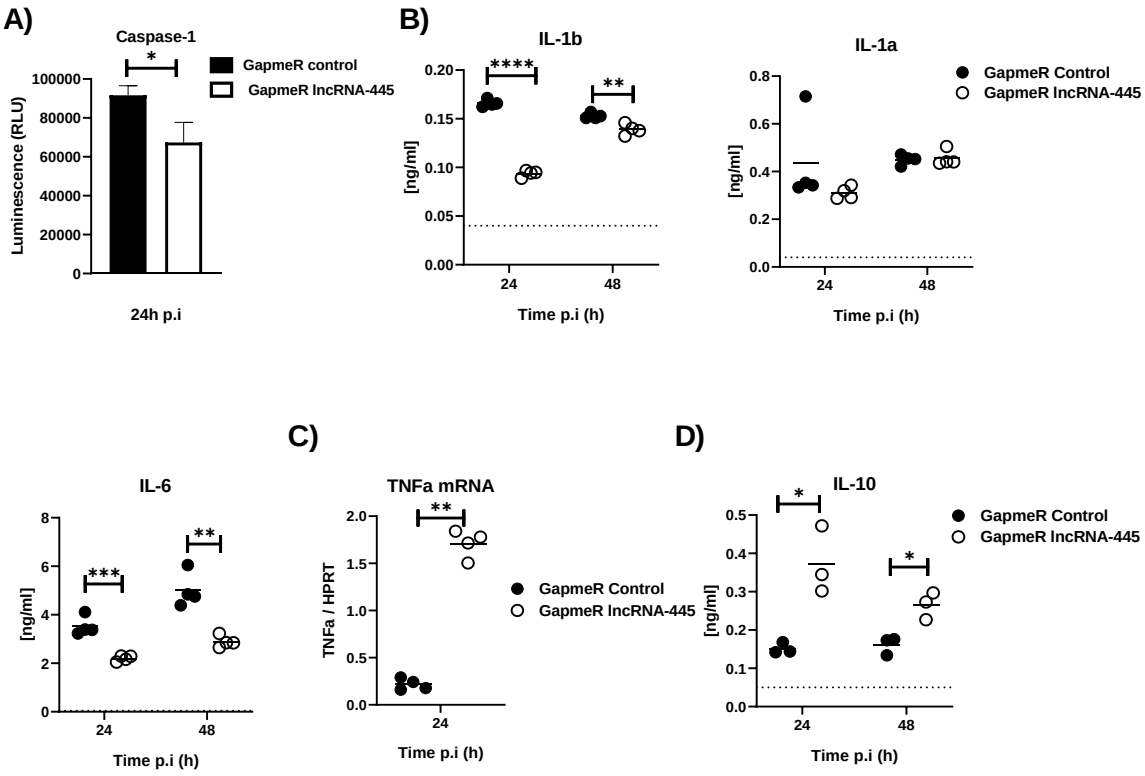

Figure 5: Knockdown of lncRNA-445 results in reduced pro-inflammatory cytokine expression. Detection of **(A)** caspase-1 activity and protein pro-inflammatory cytokine levels **(B)** measured by ELISA, and mRNA cytokine levels **(C)** measured by RT-qPCR, along with anti-inflammatory IL-10 protein cytokine release measured by ELISA in IFN-γ pre-stimulated BMDMs infected with *Mtb* for 24 and 48h. Data shown is representative of three independent experiments, each data point is represented as mean ± SEM (n=3 technical triplicates). Statistical significance is symbolized by *P<0.05, ** P<0.01, ***P<0.001, and ****P<0.0001 determined by a two-way ANOVA with Bonferroni post hoc test.

3.2.4 Knockdown of lncRNA-445 expression increases apoptosis and nitrite production in IFN-γ pre-stimulated BMDMs infected with *Mtb*.

Apoptosis is an important part of host defence against *Mtb* as it prevents the pathogens escape thus limiting *Mtb* infection and can further activate the innate and adaptive immune responses (74). The effects of lncRNA-445 knockdown were assessed using; 7'AAD and Annexin V flow cytometry and fluorescence-based TUNEL assay. Knockdown of lncRNA-445 resulted in a significant increase in the percentage of early and late apoptotic cells, but a significant decrease in necrotic cells (Figure 6A), similar results were observed with the significantly increased number of live cells along with early and late apoptotic cells but a decrease in the number of necrotic cells (Figure 6B). Furthermore, lncRNA-445 knockdown increased the number of TUNEL positive apoptotic cells (Figure 6C). Overall, the knockdown of lncRNA-445 significantly increases apoptotic activity in M1-polarized macrophages and could be responsible for the reduced intracellular *Mtb* growth (Figure 4D).

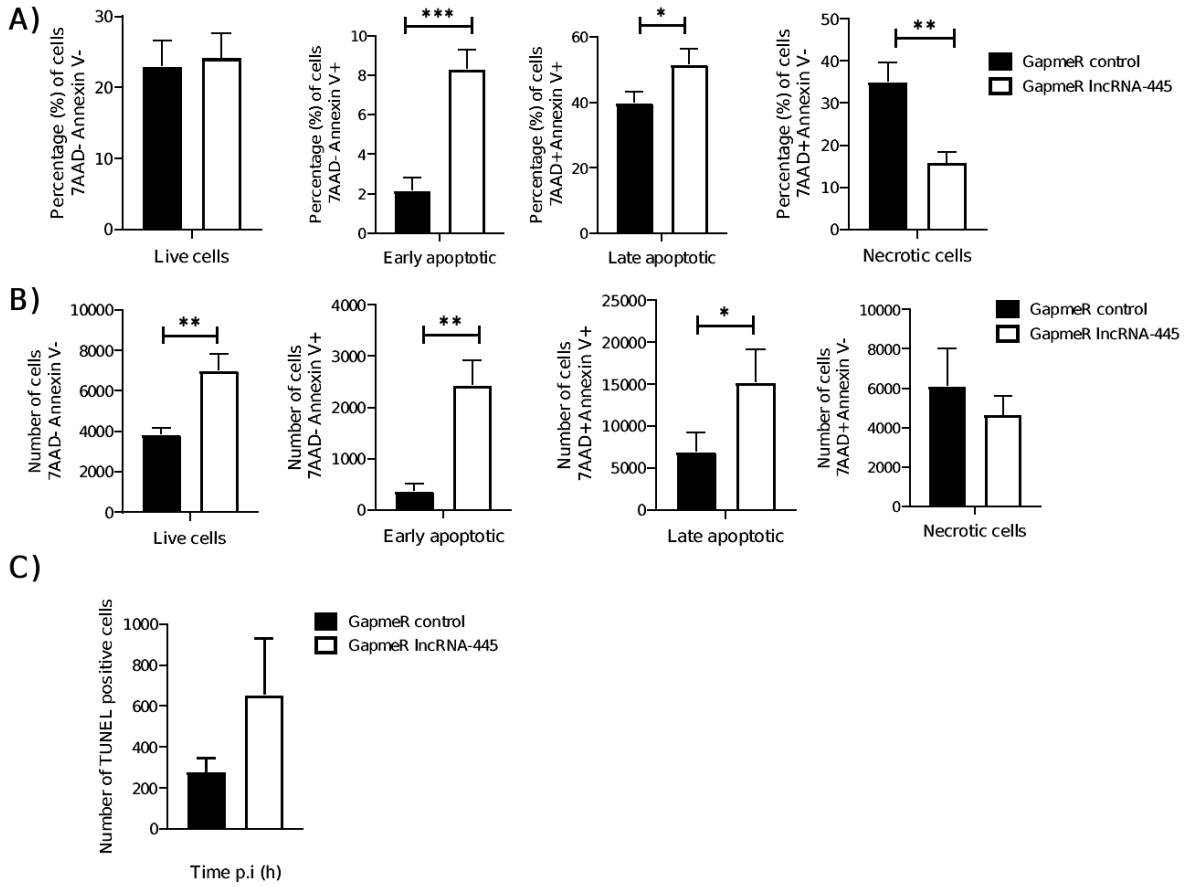

Figure 6: Knockdown of lncRNA-445 increases apoptosis in IFN-γ pre-stimulated *Mtb*-infected BMDMs. Flow cytometry quantification using 7' AAD and Annexin V staining indicating **(A)** number and **(B)** percentage of live, early apoptotic, late apoptotic, and necrotic cell populations, and **(C)** TUNEL assay fluorescent microscopy quantification. Data shown is representative of one (C) and two (A, B) experiments, each data point is represented as mean ± SEM (n=3 technical triplicates). Statistical significance is symbolized by *P<0.05, ** P<0.01, and ***P<0.001 determined by a student's t-test.

The effects of lncRNA-445 knockdown on apoptosis were further assessed using RT-qPCR of pro-apoptotic (Bax-1) and anti-apoptotic (Bcl-2) gene expression. The knockdown of lncRNA-445 increased the expression of Bax-1 and reduced Bcl-2 expression (Figure 7A). Nitrite production was measured in the unstimulated and IFN-γ pre-stimulated macrophages infected with *Mtb* and found that nitrite production is significantly upregulated in M1-polarized macrophages at 24 and 48h post-infection (Figure 7B). Altogether this data shows that lncRNA-445 knockout increases expression of pro-apoptotic genes as well as nitrite production in IFN-γ pre-stimulated and *Mtb*-infected macrophages.

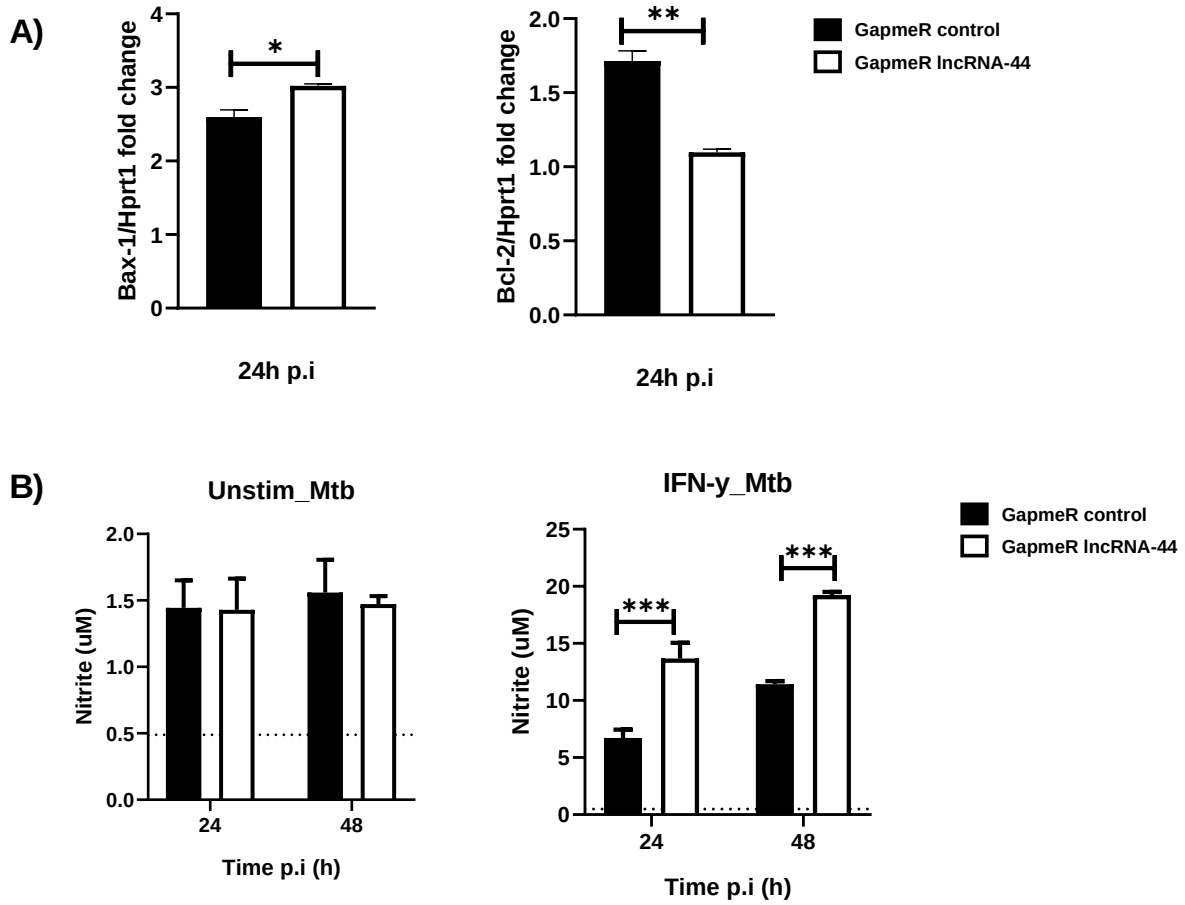

Figure 7: Knockdown of lncRNA-445 increases nitrite production in IFN-γ pre-stimulated *Mtb*-infected BMDMs. (A) RT-qPCR of the effects of lncRNA-445 knockdown on Bax-1 and Bcl-2 expression, in IFN-γ pre-stimulated BMDMs infected with *Mtb* for 24h p.i. **(B)** Nitrite production measured by Griess reagent at 24 and 48h post-*Mtb*-infection. Data shown is representative of three independent experiments, each data point is represented as mean ± SEM (n=3 technical triplicates). Statistical significance is symbolized by *P<0.05, ** P<0.01, and ***P<0.001 determined by a student's t-test.

CHAPTER FOUR

Discussion

Mycobacterium tuberculosis (*Mtb*) is the causative agent for tuberculosis (Tb), a highly infectious airborne disease (75). Even with the different treatment regimens, the Tb burden remains a persistent global health challenge with the constantly elevated number of infections annually. Treatment of Tb has been a challenge with the various drug-resistant *Mtb* strains, re-infections, and latent Tb infections that may become active (76, 77). *Mtb* thrives due to its ability to subvert the hosts' immune response to favour its survival and persistence in macrophages by implementing mechanisms, such as inhibiting the formation of the phagolysosome, which prevents critical antimycobacterial immune responses (78).

Macrophages are the first line of defence found in major tissues such as the lungs. Upon *Mtb* infection, alveolar macrophages are one of the primary immune cells to identify the pathogen-associated molecular patterns (PAMPs) of *Mtb* resulting in phagocytosis of the pathogen into the phagosome of the cell (16). Infection of macrophages activates an M1 (IFN-γ stimulated) anti-mycobacterial state which initiates a cascade of pro-inflammatory and bactericidal immune responses to eliminate *Mtb* (79), but *Mtb* can evade these responses and activate macrophages to an M2 (IL-4/IL-13 stimulated) anti-inflammatory state which is less microbicidal for its survival and persistence in macrophages (80). *Mtb* has been shown to successfully inhibit maturation and acidification of the phagolysosome and inhibit reactive oxygen/ nitrogen species as well as apoptosis and autophagy (80-82). With all these immune evasion techniques and different drug-resistant *Mtb* strains, new and improved therapeutic options are needed. An innovative approach that targets host-pathogen interactions is host-directed therapy (HDT) which focuses on immune augmentation or modulation as therapeutic options (5).

LncRNAs have recently been characterized as possible host-directed targets due to the various roles they play in host immune regulation (5). LncRNAs are key regulators of the differentiation and activation of immune cells (83). Microarray analysis of lncRNA expression has shown that the expression of lncRNAs varies based on the *Mtb* strains macrophages are infected with (84). Several lncRNAs have been associated with mediating macrophages to an M1 polarization state and inducing the associated inflammatory response, such as MIR155HG (85) and MALAT1 (86). Due to the various roles of lncRNAs, it has

been shown that *Mtb* targets and modulates host lncRNA expression for its survival in macrophages (61), therefore lncRNAs can be employed as potential indicators and therapeutic targets during TB infection. (71).

Antisense oligonucleotides (ASOs) are chemically engineered single-stranded RNA nucleotides that influence gene expression by gene silencing and other similar mechanisms (87). These modified oligonucleotides selectively bind to complementary target pre-mRNA or mRNA (88). Enhancing the effectiveness of ASOs involves incorporating chemical modifications into the structure of the molecule, further customizing ASOs for various clinical applications (59, 89). An example of these modifications is the locked nucleic acid (LNA), which increases binding affinity and potency of the ASO (90). Previous literature has stated that ASOs are highly effective due to their target specificity and with their use less side effects, such as off-target effects have been reported (59). This was also observed in our study as the use of ASO-LNA gapmeRs successfully reduced the expression of the target lncRNA-445, while leaving the cells viable suggesting that ASO-gapmeR treatment did not cause toxicity to the BMDMs.

The current study revealed that lncRNA-445 is highly upregulated in IFN-γ stimulated macrophages and downregulated upon infection with *Mtb*. To further understand the role of lncRNA-445 in macrophages, I used a loss-of-function approach and studied the role of lncRNA-445 at a cellular level. The expression of lncRNA-445 is mediated through the TLR9 and p38 signalling pathway. The knockdown of lncRNA-445 reduced intracellular burden of *Mtb* and induced apoptosis. The knockdown further reduced pro-inflammatory cytokine release and increased nitrite production which correlates with nitric oxide release.

Deep CAGE analysis and RT-qPCR validation have shown that lncRNA-445 is upregulated in IFN-γ stimulated mouse macrophages (BMDMs) and repressed upon infection with *Mtb*. This formed the basis of our study as I investigated the role of lncRNA-445 in M1-polarized BMDMs and its potential as a target for HDT. The expression of lncRNA-445 was also repressed in BMDMs infected with various *Mycobacterium* strains, suggesting that lncRNA-445 downregulation is not strain or virulence-specific. Additionally, lncRNA-445's expression was investigated in whole lungs from mice infected with *Mtb* HN878. The significantly increased expression could be a result of the recruitment of other immune cells in the lungs that may express lncRNA-445. Suppression of lncRNA-445 was also observed

during infection with *L. monocytogenes* in murine macrophages suggesting infection with intracellular pathogens may regulate the expression of lncRNA-445.

The initial phase of macrophage polarization occurs when the macrophage's pathogen recognition receptors (PRRs), including toll-like receptors (TLRs), recognize *Mtb* pathogen-associated molecular patterns (PAMPs) which initiates an appropriate immune response (17). TLRs play a vital part in both the innate and adaptive immune responses. (72). The expression of lncRNA-445 is regulated through the TLR9 and p38 signaling pathways during *Mtb* infection in macrophages. The TLR9 and p38-mediated signaling pathway has been associated with coordinating antimycobacterial responses (91, 92). An interesting factor to investigate would be the expression of lncRNA-445 in myeloid differentiation response 88 (MYD88) deficient mice compared to wild-type mice, as MYD88 plays a central role by linking the signaling pathways necessary for an immune response. The role of lncRNAs varies based on their location in the cell. Purified RNA from the cytoplasm and nucleus compartments demonstrated that there is a lncRNA-445 localization in the cytoplasm upon IFN-γ stimulation. LncRNAs are essential in the post-transcriptional regulation of gene expression in the cytoplasm, such as modulating mRNA stability and translation of target mRNAs (93).

Functional validation of lncRNA-445 in M1 polarized murine macrophages was achieved by using a loss-of-function approach where the expression of lncRNA-445 would be suppressed using ASO LNA gapmeRs. LncRNA-445 was successfully suppressed in IFN-γ stimulated and *Mtb*-infected macrophages. I further investigated the effects of lncRNA-445 knockdown in the various immune responses to infection with *Mtb* HN878. LncRNA-445 knockdown decreased the intracellular growth of *Mtb* in M1 polarized macrophages. *Mtb* inhibits the caspase-1/IL-1β inflammasome activation which is a crucial response due to its antimycobacterial functions (73). The activation of this inflammasome results in an enhanced secretion of IL-1β, phagolysosome formation, *Mtb* clearance and reduced bacterial burden in macrophages (73). The suppression Of lncRNA-445 resulted in a decrease in the inflammasome activation and IL-1β cytokine release in M1-polarized *Mtb*-infected macrophages. Secretion of pro-inflammatory cytokines such as IL-`` 6, TNF-α, IL-1β, is crucial for defence against *Mtb* infection, these are necessary to maintain control of infection (94). There was an overall decrease in pro-inflammatory cytokines IL-1β, IL-1α and IL-6, suggesting that lncRNA-445 might be a positive regulator of inflammation. An upregulation in TNF-α mRNA expression was observed and anti-inflammatory IL-10 protein release was

also increased in the M1 polarized macrophages. This could be due to *Mtb* inducing an M2 immune response by activating macrophages to a M2 polarized through IL-10 stimulation, resulting in an anti-inflammatory immune response which promotes *Mtb* pathogenesis in the macrophages (95, 96).These findings identified lncRNA-445 as a possible regulator in macrophage polarization and inflammation. In addition, *Mtb* manipulates the hosts immune response by targeting and suppressing lncRNA-445 to support its proliferation in macrophages.

Apoptosis is an innate immune response against *Mtb* infection in which there is programmed cell death of infected cells resulting in diminished *Mtb* viability (47). The effects of lncRNA-445 knockdown on apoptosis were investigated in IFN-γ pre-stimulated and *Mtb* infected macrophages. Knockdown of lncRNA-445 in IFN-γ pre-stimulated and *Mtb* infected macrophages resulted in increased apoptotic activity which is noted by the significantly high number of apoptotic cells in the 7'AAD and Annexin V flow cytometry, along with increased expression of the damaged DNA observed using TUNEL assay, and high expression of pro-apoptotic Bax-1 and low expression of anti-apoptotic Bcl-2. Nitric oxide (NO), which is converted by NOS from L-arginine, can be directly used by IFN-γ stimulated macrophages to kill *Mtb* or it can be converted to nitrite which can induce bactericidal killing (97). I further assessed the levels of nitrite produced in the macrophages and found that nitrite was significantly increased in BMDMs stimulated with IFN-γ and infected with *Mtb*. Collectively the results show that the knockout of lncRNA-445 increases apoptosis and nitrite production, these results further suggest that apoptosis is used as a host defense mechanism to reduce intracellular *Mtb* burden seen in the M1 polarized *Mtb*-infected macrophages.

A few lncRNAs have been functionally validated and extensively studied with regards to their roles during Tb infection, examples include lncRNA CD244 (52), HOTAIR (55), and MEG3 (51). In this study I sought to understand the role of lncRNA-445 in *Mtb*-infected macrophages and the main findings were that knockdown of lncRNA-445 reduced intracellular *Mtb* growth, decreased pro-inflammatory cytokine response, promoted apoptosis, and nitric oxide production. LncRNA-cox 2 was reported to enhance intracellular killing of mycobacterial tuberculosis by upregulating M1 macrophage polarization and production of nitric oxide (98). In a similar study, lincRNA-MIR99AHG knockdown using ASOs resulted in significantly reduced intracellular growth of *Mtb* and reduced pro-inflammatory cytokine production in murine macrophages (61). Similar findings were also reported with the knockdown of lincRNA-Cox2 in macrophages infected with *Mtb* H37Ra

where inflammatory responses were inhibited, apoptosis promoted (46), and significantly upregulated by TLR-2 and TLR-4 which are associated with apoptosis induction (99).

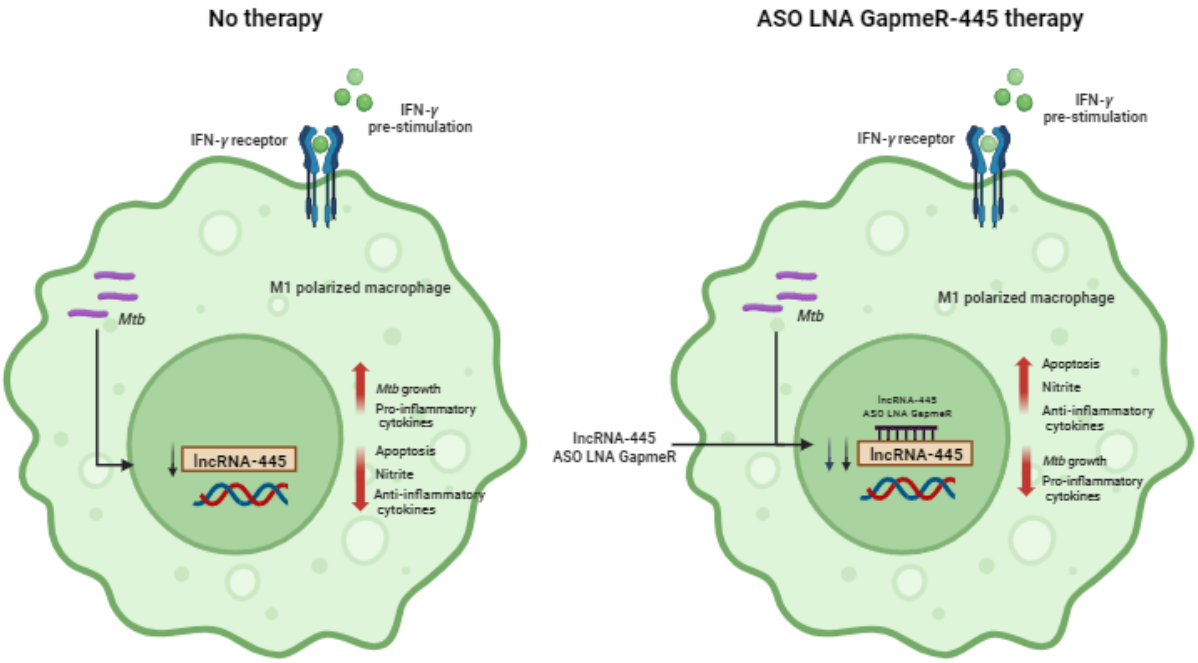

Figure 8: The role of lncRNA-445 in M1 polarized and *Mtb*-infected macrophages. The expression of lncRNA-445 is downregulated upon *Mtb* infection of the macrophage, resulting in increased *Mtb* survival. Transfection with ASO LNA gapmeR-445 suppressing the expression of lncRNA-445 resulting in reduced *Mtb* growth and increased apoptosis and nitrite production.

CHAPTER FIVE

Conclusion

Mycobacterium tuberculosis (*Mtb*) is the pathogen responsible for tuberculosis (TB), a highly infectious airborne disease (75). Even with the different treatment regimens, the TB burden remains a pressing issue in global health with the constantly elevated number of infections annually. Treatment of TB has been a challenge with the various drug resistant *Mtb* strains, strain mutations, re-infections, and latent TB infections that may become active. *Mtb* thrives due to its ability to evade the host's immune response to favour its survival and persistence in macrophages by implementing mechanisms that prevent critical antimycobacterial immune responses (78). An innovative approach that is being investigated for its possible application against TB is host-directed therapy (HDT). HDT targets the clinically relevant biological pathways equipping the host with an improved immune (5). Incorporating HDT alongside current therapies may enhance treatment efficacy by decreasing the duration of TB treatment, mitigating antibiotic resistance, and lessening lung damage. (5, 100). Promising candidates for HDT are long non-coding RNAs (lncRNAs) because of their pivotal role in immune responses to infections.

LncRNAs are transcripts that do not encode proteins are > 200 nucleotides in length and their roles have been described as essential regulators of gene expression at various levels, ranging from epigenetic to post-translational (101). Due to their crucial functions, any disruption in the expression of lncRNAs has been linked to the onset of numerous diseases, such as neurodegenerative diseases and cancer (102). LncRNAs have been recently implicated to have a role in the regulation of immune responses during the progression of an infectious or inflammatory disease (103). Multiple lncRNAs have been identified to be associated with Tb infection but only several lncRNAs have been functionally validated with regard to immune responses against *Mtb* infection, such as MEG3 (51), and CD244 (52).

Macrophages are pivotal in the development of TB since they are the initial immune cells to react to infection. Due to their functional and phenotypic plasticity, they can be activated to a specific phenotype depending on the stimuli (14). Macrophage polarization entails classically activated macrophages, prompted by IFN-γ stimulation, assuming an M1 pro-inflammatory phenotype, which is vital for host defense against pathogens. Conversely, IL-4/IL-13 alternatively activated macrophages are directed toward an M2 anti-inflammatory phenotype, which is crucial for tissue regeneration and repair (15). In the FANTOM 5 project, our group used deep CAGE analysis to investigate the transcriptional landscape of murine macrophages

infected with a hypervirulent Beijing *Mtb* strain HN878 comparing the classically activated (IFN-γ stimulated) plus the alternatively (IL-4/IL-13 stimulated) macrophages, as well as the IFN-γ or IL-4/IL-13 pre-stimulated and *Mtb*-infected macrophages (62). Among the 151 identified differentially expressed lncRNAs, lncRNA-445 was chosen for this study. This study aimed to functionally validate lncRNA-445 in classically activated (IFN-γ stimulated) and IFN-γ pre-stimulated and *Mtb*-infected murine macrophages (BMDMs).

The functional validation of the identified lncRNA required a loss-of-function approach which required targeting lncRNA-445 and suppressing its expression using antisense oligonucleotide-locked nucleic acid gapmeRs (ASO-LNA gapmeRs). ASOs are engineered single stranded RNA sequences that bind and modulate the complementary target mRNA, with the LNA modification for increased binding affinity and potency (87, 90). A similar approach has been used by targeting lincRNA-MIR99AHG and inhibiting its expression to understand its functional role in macrophages during *Mtb* infection (61). Extensive research has also been done on the use of ASOs to target various host factors during viral infections, such as the ACE2-ASO during SARS-CoV-2 infection (59).

This study functionally validated the role of lncRNA-445 in M1 polarized murine *Mtb* HN878-infected macrophages. Overall, host-directed targeting of lncRNA-445 with the use of ASO-LNA gapmeRs successfully reduced the intracellular growth of *Mtb* in IFN-γ stimulated macrophages. ASO gapmeR treatment did not result in any toxicities as the cells remained stable. The reduced *Mtb* growth may be due to the increased production of nitrite, which correlates to nitric oxide release, and induced apoptosis which I noted by increased apoptotic cells, the number of TUNEL positive cells, and an increase in the expression of Bax-1 in infected macrophages. Additionally, the expression of lncRNA-445 was induced by the TLR9/p38 signaling transduction pathway. LncRNA-445 was localized in the cytoplasm in IFN-γ stimulated macrophages, suggesting that its role may be in the post-transcriptional level. One of the many roles of lncRNA-445 may include macrophage polarization due to its high expression in IFN-γ stimulated macrophages. LncRNA-445 was further understood to be a positive regulator of inflammation because when lncRNA-445 knockout occurred there was suppressed expression of pro-inflammatory cytokines IL-1β, IL-1α, IL-6, and TNFα, and the decrease in caspase-1 activity which is associated with the activation of the caspase-1/IL-1β inflammasome. LncRNA-445 knockout did result in increased anti-inflammatory IL-10 production, further validating the role of lncRNA-445.

The expression of lncRNA-445 was investigated in various *Mtb* strains and noted high expression in the avirulent H37ra strain but suppressed in the BCG, and virulent HN878 and H37rv strains. LncRNA-445 expression in *Mtb*-infected whole lung is downregulated at 11 days but significantly upregulated at 21 days. Infection with listeria also suppresses lncRNA-445 expression in the liver and spleen. Therefore, *Mtb* represseslncRNA-445 expression to promote *Mtb* survival in macrophages and regulate the host's innate immune response to a less microbicidal state. Based on our results I have achieved the objectives of our study and can conclude that lncRNA-445 plays a crucial role in the innate immune response with its expression being regulated through the TLR9/p38 signaling transduction pathway, and its role is implicated in regulating macrophages to an M1 state and inducing the release of pro-inflammatory cytokines, thus creating a bactericidal environment unfavourable for *Mtb* survival.

Future studies are needed to further understand the regulatory role of lncRNA-445 such as cis-regulatory effects on neighbouring genes as this could further improve our understanding of its role in immune response. Furthermore, the mechanism and interaction of the lncRNA in the regulation of its respective immune-related neighbouring genes. Additional beneficial readouts include assessing the effects of lncRNA-445 knockout on the expression of CD86 (classically activated) and CD206 (alternatively activated) surface receptors using flow cytometry. Furthermore, the expression of lncRNA-445 in myeloid differentiation response 88 (MYD88) deficient mice will be compared to wild-type mice using RT-qPCR to elucidate its role in the context of innate immune signaling. The next step involves investigating the expression of lncRNA-445 in human monocyte-derived macrophages (hMDMs) by identifying a human homologue of lncRNA-445. Subsequently, RT-qPCR will be utilized to examine its expression in M1 and M2 polarized macrophages. Similar to the experimental layout used in BMDMs, a loss-of-function approach using human gapmeRs will be implemented to investigate the functional role of lncRNA-445 in *Mtb*-infected human macrophages. Finally, our objective is to apply this research in mouse models by administering ASO-gapmeRs as a preventive or therapeutic measure for *Mtb* infection. This would be significant for future work toward the development of a suitable lncRNA-targeted HDT in Tb clinical trials.

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Appendix A

Table 1: RT-qPCR mouse gene-specific primers and amplification temperatures.

1. **HPRT**

Forward	5’- GTT GGA TAT GCC CTT GAC -3’
Reverse	5’- AGG ACT AGA ACA CCT GCT -3’

2. **LncRNA-445**

Forward		5’- CCT GTT GTG GAG GCT GGA AA -3’					
Reverse		5’- TGT GAT AAA GCC GGA CCA CC -3’					
Denaturation	95°C	**Annealing**	60°C	**Extension**	72°C	**Acquisition**	74°C

3. **Bax-1**

Forward		5’-GCT CAA GGC CCT GTG CAC TAA A-3’					
Reverse		5’-TCT TGG ATC CAG ACA AGC AGC CG-3’					
Denaturation	95°C	**Annealing**	63°C	**Extension**	72°C	**Acquisition**	75°C

4. **Bcl-2**

Forward		5’-TGA CTT CTC TCG TCG CTA CCG-3’					
Reverse		5’-GTG AAG GGC GTC AGG TGC AG-3’					
Denaturation	95°C	**Annealing**	63°C	**Extension**	72°C	**Acquisition**	75°C

5. **TNF-α**

Forward		5’-TCT CAT CAG TTC TAT GGC CC-3’					
Reverse		5’ -GGG ACT AGA CAA GGT ACA AC-3’					
Denaturation	95°C	**Annealing**	63°C	**Extension**	72°C	**Acquisition**	75°C