AN ABSTRACT OF THE THESIS OF

Robert F. Ndzeidze for the degree of Master of Science in Comparative Health Sciences presented on March 16, 2021.

Title: Virulent *Mycobacterium avium* subspecies *Hominissuis* Subverts Macrophages during Early Stages of Infection

Abstract approved:

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Interference with the host post-translational mechanisms, such as protein phosphorylation, is a key strategy used by many intracellular bacterial pathogens to subvert host immune cell function. Virulent non-tuberculous Mycobacteria (NTMs) unlike attenuated or non-pathogenic NTMs, successfully reside and multiply within the phagosomes of phagocytic cells such as monocytes and macrophages. Several studies have suggested that virulent NTMs alter host protein activation to enhance their survival. Macrophages play a very important role in the innate clearance of intracellular pathogens including NTMs. Low virulence NTMs such as *Mycobacterium avium* subsp. *hominissuis* 100 (MAH 100), and *Mycobacterium abscessus* subsp. *abscessus* are cleared from mice, whereas the highly virulent strains such as MAH 104 and MAH 101 disseminates and replicates in mice organs such as lungs, spleen, and liver. In this study we compared the macrophage early response to *M. avium* subsp. *hominissuis* (MAH) strains, *M. abscessus*, and a non-pathogenic *Mycobacterium smegmatis*. Our

findings indicate that infection of the macrophage with MAH 100, *M. abscessus*, and M. *smegmatis* favors the development of M1 macrophage phenotype, while infection of the macrophage with MAH 104 inhibits M1 phenotype and favors the development of M2 macrophage, an anti-inflammatory phenotype associated with the healing process. By comparing protein phosphorylation patterns of infected macrophages, we observed that uptake of both MAH 100 and *M. smegmatis* resulted in MARCKS-related protein phosphorylation, which has been associated with macrophage activation. In contrast, in macrophages infected with MAH 104, methionine adenosyltransferase IIβ, an enzyme which catalyzes the biosynthesis of S-adenosylmethionine, was phosphorylated 15 min post-infection. S-adenosylmethionine is a methyl donor for DNA methylation. Inhibition of DNA methylation with 5-aza-2 deoxycytidine, significantly impaired the survival of MAH 104 in macrophages. Our findings suggest that the virulent MAH 104 enhance its survival in the macrophage possibly through interference with the epigenome responses (DNA methylation) while inhibiting the activation of MARCKS-related protein.

Virulent *Mycobacterium avium* subspecies *Hominissuis* Subverts Macrophages during Early Stages of Infection

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Robert F. Ndzeidze, Author

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CONTRIBUTION OF AUTHORS

Chapter 1: Robert Ndzeidze wrote the general introduction. Dr. Luiz Bermudez contributed to the editing process.

Chapter 2: Dr. Bermudez designed the experiments, guided the ideas, provided important review of the manuscript, and funded the project. Robert Ndzeidze helped with the design of some experiments. Robert Ndzeidze performed the assays, analyzed the data, and wrote the manuscript. Dr. Bermudez helped with the editing process, data analysis.

Chapter 3: Robert Ndzeidze wrote the discussion and conclusions.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

Figures Page

LIST OF TABLES

Chapter 1: Introduction

Non-tuberculous Mycobacteria

Non-tuberculous mycobacteria (NTMs) refer to mycobacteria other than the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. NTMs are ubiquitous in the environment from where they occasionally infect humans (1). NTMs are a health concern, especially in immunocompromised individuals with underlying lung conditions (2,3). NTMs are also associated with a range of pathologies of different organs including skin, joint, bone, pulmonary and disseminated diseases (4). Regarding pulmonary infection, majority of cases occur as a complication of pre-existing lung diseases, such as bronchiectasis, cystic fibrosis, or chronic obstructive pulmonary disease. Disseminated disease is most common in immunocompromised patients such as in HIV-1 patients (3). There are at least 180 species of NTMs (2). NTMs range from slow growing to fast-growing mycobacteria. For example, *Mycobacterium avium* is a slow-growing mycobacterium with a doubling time of 10 – 12 hours in culture (5) while *Mycobacterium abscessus* (4 – 5 hours doubling time in culture) and *Mycobacterium smegmatis* (3 – 4 hours doubling time in culture) are rapidly growing NTMs (6,7). In the United States, *M. avium* is the most common lung mycobacterial infection, while *M, abscessus* is responsible for approximately 80% of lung disease due to rapidly growing NTMs and is associated with significant morbidity and mortality. *M. smegmatis* is a non-pathogenic mycobacterium that does not cause disease even in immunocompromised individuals (8). The main routes of NTM infection are the gastrointestinal and respiratory routes (3).

Innate Immune Response to NTM infection

NTMs mainly infect the bronchial airways by colonizing and invading epithelial mucosal cells as well as mononuclear phagocytes such as monocytes and macrophages. NTMs effectively enter macrophages by binding to receptors on the cell surface. *M. avium* interact with macrophages by binding to the complement receptors 3 and 4, mannose receptors, CD14, or fibronectin, vitronectin receptors and Toll-like receptors (TLR2) (9,10,11). *M. abscessus* gain entry by binding to dectin-1 and TLR2 (12). After entering the macrophage, NTMs reside within phagosomes (13). Non-virulent mycobacteria are effectively killed by macrophages whereas the virulent NTMs generally survive and grow within the phagosomes. Phagosome maturation, a process involving the fusion of the phagosome with the lysosome is important in the killing of NTMs (14). Virulent NTM, unlike the non-virulent NTM, successfully reside and multiply within non-mature macrophage phagosomes by inhibiting phagosome maturation and modulating the macrophage defense mechanisms (13).

NTM modulates the Mitogen Activated Protein kinases (MAPKs) and NF-κB signaling pathways (9). MAPKs, specifically p38 and the signal regulated kinases 1 and 2 (ERK 1/2), and NF-κB are activated upon TLR2 stimulation. The activation of the MAPKs and NF-κB results in the secretion of pro-inflammatory cytokines which increase the mycobactericidal activity of the macrophage (15). It has shown that *M. avium* slowly inhibits the activation of the MAPKs and NF-κB (9). Within the first 30 minutes of macrophage infection *M. avium* activates the MAPKs and NF-κB which slightly hinders the progression of the infection. However, *M. avium*

downregulates MAPK and NF-κB activation significantly after one hour of infection, and by 24 h *M. avium* has complete control of the host cell (9, 16). The inhibition of MAPKs and NF-κB activation by *M. avium* leads to a marked decrease in pro-inflammatory cytokines, especially TNF-α, IL-12 and IL-1β, which impairs the host's ability to fight off the infection. Prolonged activation of p38 and ERK1/2 and increased TNF-α is observed when macrophages are infected with the non-virulent mycobacterium, *M. smegmatis*. (16). It is possible that other components such as phosphatases that lead to inactivation of MAPKs and NF-κB may be more prominently triggered by *M. avium* after TRL2 stimulation. *M. abscess*us, like *M. avium*, also activate the MAPKs and NF-κB signaling pathways through the TLR2 stimulation (15). However, *M. abscessus* induces higher production of proinflammatory cytokines and chemokines than *M. avium*. These distinct cytokine responses between *M. abscessus* and *M. avium* are seen early after infection, with *M. abscessus* causing higher responses than *M. avium* as early as 4 hours after stimulation (15).

Both proinflammatory cytokines (TNF-α, IL-12, IL-1β) and anti-inflammatory cytokines (IL-10 and transforming growth factor-beta) are produced during virulent NTM infection (9). IL-10 negatively regulates NF-κB activation which increases the chances of virulent NTM surviving within the macrophage (9, 15). The activation of the p38 and ERK1/2 MAPKs is sufficient to build an immune response. The slow inhibition of the MAPKs by *M. avium*, however, leads to a marked decrease in pro-inflammatory cytokines, thus impairing the host's ability to fight off the infection (9).

Adaptive immune response to NTM infection

Helper T cells such as T helper 1, T helper 2 and T helper 17 have been shown to play a significant role in adaptive immune response during mycobacterial disease (17). Macrophages and dendritic cells are important antigen presenting cells (APC) and they display Class I and II major histocompatibility complex (MHC) molecules that present antigens to the adaptive immune system. Infected macrophages and dendritic cells initiate the adaptive T cell immunity by antigen presentation (18). In general, intracellular bacteria are degraded in the phagolysosome and the resulting microbial products (antigens) are further processed into peptides (antigens) and presented through MHC II molecules to naïve T cells in the secondary lymphoid organs. Antigenic stimulation induces a network of downstream signaling pathways, that eventually lead to naïve T cell proliferation and differentiation into specific effector cells (T helper 1, T helper 2 or T helper 17 cells) (19). Resistance to non-tuberculosis mycobacterial infections is primarily mediated by T helper 1 responses; immune responses dominated by T helper 2 or T helper 17 cells correlate with disease susceptibility and the virulence of the non-tuberculosis mycobacterial strain. (19). T helper 1 cells secret IFN-γ which activates macrophages inducing strong antimicrobial activity. (20). Virulent *M. avium* has been shown to interfere with the JAK/STAT signaling pathways (or the interferon-gamma signaling pathway). *M. avium* downregulate the expression of the IFN-γ receptor making the macrophage unresponsive to IFN-γ (21). Genetic defects in the IFNγ-IL-12 signaling pathways, has been shown to predispose an individual to mycobacterial infections (22). T helper 2 cells secret cytokines such as IL-4 and IL-10 which act

on macrophages and downregulate the production of macrophage antimicrobial molecules. T helper 2 cytokines also reduce the responsiveness to macrophage-activating cytokines such as IFN-γ and TNF-α (23). Th17 cells secret IL-17 which have been shown to have a protective role during the early stages of mycobacterial infection. The role of IL-17 is still unclear in mycobacterial infections. However, it is thought that hypersecretion of IL-17 may be responsible for disease aggravation (24,25).

Macrophage Phenotype and function in NTM disease

Macrophages are diverse and can be divided into two main activation or polarized states that correlate with the signals from the surrounding microenvironment and are strongly regulated by T helper 1 and T helper 2 cells. The two main macrophage phagocytes are M1 and M2 macrophages. M1 macrophage differentiation is induced by IFN-γ or TNF-α (26). The phagocyte has high antimicrobial capacity and is proinflammatory, producing cytokines such as TNFα, IL-1β, IL-6, IL-12 and IL-23 and is more efficient in the production of antimicrobial molecules such as nitric oxide and reactive oxygen intermediates. In contrast, M2 macrophages are anti-inflammatory. M2 macrophages can be divided into three groups: M2a, M2b, and M2c macrophages, based on their specific functions. M2a are induced by IL-4/IL-13, M2b are induced by immune complexes and TLR agonists, and M2c are induced by IL-10 and glucocorticoid hormones (27,28). All the three M2 subtypes have anti-inflammatory properties. However, M2a and M2b macrophages are considered immunoregulatory and are known for

mediating the T helper 2 response, whereas M2c cells are mainly immunosuppressive (29).

The manipulation of macrophage polarization states has been shown to be an important pathogenesis mechanism of some intracellular bacteria (30). Bacterial pathogens including *Salmonella* Typhimurium, and *Mycobacterium tuberculosis* have been shown to drive polarization to an M2 phenotype to enhance their survival in the host (30). *M. tuberculosis* virulence strains secrete the effector protein ESAT-6 that has been demonstrated to drive a strong M2 polarization (31). S*almonella* Typhimurium has also been shown to manipulate macrophage polarization to enhance its survival (32). After uptake by macrophages, *Salmonella* reprograms macrophages by secreting effectors such as *Salmonella* anti-inflammatory response activator (SarA) through the *Salmonella* pathogenicity island 2 Type III secretion system. These effectors dampened proinflammatory innate immune responses and induced anti-inflammatory macrophage polarization allowing *Salmonella* cells to survive for extended periods in their host (32). Several studies have shown that effectors secreted by intracellular bacteria modulate macrophage polarization by subverting host-signaling pathways associated with macrophage polarization (30). The secretion of molecules by bacterial pathogens that subvert host responses often requires specialized secretion systems to deliver effector proteins into host cells. The type VII secretion system has been identified in mycobacteria and this system may be responsible for subverting host signaling pathways and modulating macrophage polarization during NTM infection.

Mycobacterial secreted effector proteins interfere with normal macrophage function.

The type VII secretion system in mycobacteria has been shown to secret effector proteins which interfere with the normal macrophage function. The type VII secretion system is encoded by gene clusters (ESX-1 through ESX-5). ESX-1 and ESX-5 have been shown to play a major role in virulence (33,34). ESX-1 in *M. tuberculosis* and the non-tuberculosis mycobacterium, *M. marinum,* has been shown to be involved in the secretion of ESAT 6, a protein which modulates TLR2 signaling and cytokine inhibition. *M. avium* lacks the ESX-1 gene locus. *M. avium* contains four ESX loci, ESX-2 through ESX-5. *M. abscessus* 19977 carries only ESX-3 and ESX-4 (35). Variation in the number of ESX loci among NTMs highlights the important differences in virulence determinants among NTMs. Abdallah M. et al (2008) characterized the effect of *M. marinium* on macrophage function and the results showed that ESX-5 effector molecules clearly manipulate the induction of different macrophage cytokines; wild-type *M. marinum* but not the ESX-5 mutant, were able to suppress the production of IL-12p40, IL-6, and TNF-α (33). The ESX-4 locus of *M. abscessus* have been shown to play a role in inhibiting phagosomal acidification and promoting phagosomal escape in macrophages. When inside macrophages, the *esx-4* mutant is less efficient at blocking phagosomal acidification when compared to the wild-type bacteria. The mutant strain also showed decreased of expression of the ESX-4 effector proteins, EsxT and EsxU (36,35). A few *M. avium* secreted proteins have been shown to interact with host proteins interfering with macrophage function. The *M. avium*

protein MAV_2941 have been shown to interact with the vesicle trafficking proteins syntaxin-8 (STX8), adaptor-related protein complex 3 (AP-3) complex subunit beta-1 (AP3B1) and Archain 1 (ARCN1) in mononuclear phagocytic cells. These trafficking proteins play a role in the maturation of phagosomes. The MAV_2941 protein mimics Phosphoinositol-3-Kinase (P13K) to interfere with macrophage phagosome maturation. PI3K play a role in the regulation of vesicle trafficking proteins (37). The *M. avium* protein MAV_4644 have also been shown to interact with the host cathepsin Z protein. This interaction protects *M. avium* from rapid macrophage killing. Cathepsin is Z a key regulator of cell signaling and inflammation (38). Knocking-down the cathepsin Z in human macrophages enhanced the growth of the attenuated phenotype of *MAV* 4644:Tn clone (38). Danelishvili et al (2018) showed that the *M. avium* protein MAVA5_06970 effector interacts with the host protein, Phosphoprotein 1 in THP-1 cells and this interaction enhances the THP-1 cell apoptosis (39). Although macrophage apoptosis is an innate defense and a strictly regulated mechanism *M. avium* escapes apoptotic killing. *M. avium* has been shown to use apoptosis as one of mechanisms to spread from cell-to-cell and for dissemination (39). More research is needed to be conducted to determine which mycobacterial effector molecules that are involved in modulating the macrophage defense mechanisms or macrophage polarization, and the host targets of these secreted effector molecules.

Mechanism of bacterial interference with cellular signaling pathways.

Hijacking the host post-translational mechanisms such as protein phosphorylation is a key strategy for bacterial pathogens to subvert host cell function and several studies have shown that this may be the case for virulent NTM (9,16). The most common post translational modification of proteins is phosphorylation. Protein phosphorylation is catalyzed by kinases which reversibly transfer a phosphate group from ATP to a residue of a target protein usually the tyrosine, serine, and threonine residues (40). Protein activity or function is regulated by phosphorylation either directly by modulating protein activity or indirectly by providing a docking site for intra- and intermolecular interactions among proteins and other molecules (41). Protein phosphorylation can transmit a signal from the cell surface receptor through a series of phosphorylation and de-phosphorylation events to the cell nucleus. Several biological processes including key processes of the immune system, such as differentiation, cytokine/chemokine production, inflammation, and bacterial killing, are controlled by protein phosphorylation (42). Infection by pathogens usually stimulate pattern recognition receptors (PPRs) such as Toll like receptors (TLRs) resulting in the consecutive activation or phosphorylation of many kinases, including interleukin-1 receptor-associated kinases (IRAK1, 2 and 4), mitogen-activated protein kinases such as MAP3K7/TAK1, p38-alpha and JNK and IκB kinase (IKK) and the subsequent phosphorylation of downstream targets, such as activator protein 1 (AP-1) and nuclear factor κB (NF-κb), which are transcriptional regulators of proinflammatory cytokine secretion (43). Several studies have shown that bacterial pathogens including NTMs exploit host signaling pathways associated with host defense mechanisms such as

phagosome maturation, apoptosis, macrophage polarization and inflammation to enhance their survival in the host. Bacterial pathogens interfere with the kinase-mediated phosphorylation of host signaling pathways (30). For example, *Salmonella* Typhimurium has been shown to drive M2 polarization via activation of the STAT3 pathway. Once *Salmonella* translocates the effector SarA (*Salmonella* anti-inflammatory response activator) into the host cell, it interacts with the host-pleiotropic serine/threonine kinase, GSK-3 (Glycogen synthase kinase-3). This interaction leads to the phosphorylation of SarA, and the GSK3 in the resulting complex is driven to interact with and phosphorylate the substrate transducer and activator of transcription 3 (STAT3) on tyrosine-705. STAT3 can then translocate to the nucleus and drive the anti-inflammatory macrophage polarization program (44). Roland et al. (2015) carried out a quantitative proteomics approach to globally map alterations in the host phosphoproteome during EPEC infection, and one of the most prominent events identified in the study was the increased phosphorylation of SEPT9 during EPEC infection, regulated by the EPEC type III secretion system. siRNA knockdown of SEPT9 decreased bacterial adherence and EPEC-mediated cell death. Increased SEPT9 Ser-30 phosphorylation has also been detected in phosphoproteomic studies of *Shigella*- and *Salmonella*-infected epithelial cells (45,46). It has been shown that SEPT9 is recruited to the actin-enriched site of bacterial host cell entry during *Shigella* infection (45). Comprehensive quantitative phosphoproteomics carried out by Ernesto et al. (2013) to evaluated cell signaling stimulated by the *Francisella novicida* lpcC (hyper-cytotoxic) strain in comparison to its parent wild-type strain revealed that *Francisella novicida* may downregulate tristetraprolin phosphorylation, a key

component of post-transcriptional regulation in innate immune response. Tristetrapolin. Phosphorylation has been shown to inhibit RNA degradation. Tristetrapolin is a key player in the regulation of cytokine production. Downregulation of tristetraprolin by *Francisella novicida* may be a mechanism to shut down the translation of important factors that control its infection (47).

Scope of the master's thesis

The driving questions investigated in this master's thesis is, "what pathways are activated or suppressed by virulent mycobacteria that benefit the pathogens survival in macrophages". To identify the pathways that are activated or suppressed by virulent mycobacteria in macrophages, we compared protein phosphorylation in macrophages upon ingestion of *M. avium*, *M. abscessus* and a non-virulent *M. smegmatis*. We hypothesized that pathways activated or suppress by virulent mycobacteria may also be responsible for modulating macrophage polarization. Mass spectrometry is a powerful tool for identification, and characterization of post-translational modifications such as phosphorylation.

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Chapter 2

Virulent *Mycobacterium avium* subspecies *Hominissuis* Subverts Macrophages during Early Stages of Infection

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Introduction

Infections caused by non-tuberculous Mycobacteria (NTMs) are a health concern, especially in immunocompromised individuals and in patients with underlying lung conditions such as bronchiectasis, cystic fibrosis, and chronic obstructive pulmonary disease (1,2). NTM is associated with a range of infections like pulmonary infection in individuals with chronic lung disease and disseminated disease in immunocompromised patients such as HIV-1 patients (2,3).

More than 180 species of NTM have been identified, and they are ubiquitous in the environment from where they occasionally infect humans (4). In the United States, *Mycobacterium avium* is the most common lung NTM infection, while *Mycobacterium abscessus* is associated with significant morbidity and mortality due to its inherent antibiotic resistance. (5,6). NTMs can infect the host through both the gastrointestinal (eating/drinking) and respiratory (breathing in aerosols) routes (7).

In the lung environment, NTMs mainly infect and reside in both epithelial mucosal cells and macrophages. NTMs effectively enter macrophages by binding to receptors on the cell surface such as the complement receptors 3 and 4, mannose receptors, CD14, fibronectin, vitronectin receptors and Toll-like receptors (TLR2) (8,9,10). *M. abscessus* gain entry by binding to dectin-1 and TLR2 (11). NTMs reside within cytoplasmic phagosomes (12). Non-virulent mycobacteria are effectively killed by macrophages whereas the virulent NTMs generally survive and grow within the phagosomes (12).

Macrophages are diverse and can be divided into two main activation or polarized states that correlate with the signals from the surrounding microenvironment and are strongly regulated by T helper 1 and T helper 2 cells (13). The two main macrophage phagocytes include M1 and M2 macrophages. M1 macrophage phenotype is induced by IFN-γ (produced by T helper 1 cells) or TNF-α (mainly produced by natural killer cells). The phenotype express high antimicrobial capacity and are pro-inflammatory. M1 macrophages produce high levels of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-12, and IL-23, and are efficient in the production of antimicrobial molecules such as nitric oxide and reactive oxygen intermediates. M2 macrophages are anti-inflammatory. M2 macrophages can be divided into three groups: M2a, M2b, and M2c macrophages, based on their specific functions. M2a are induced by IL-4/IL-13 (produced by T helper 2 cells), M2b are induced by immune complexes and TLR agonists, and M2c are induced by IL-10 and glucocorticoid hormones (14,15). All the three M2 subtypes are anti-inflammatory. M2a and M2b macrophages however are considered immunoregulatory and are known for mediating the T helper 2 response, whereas M2c cells are mainly immunosuppressive (16).

The interference with macrophage polarization or activation states has been shown to be an important pathogenesis-related mechanism of some intracellular bacteria. For instance, *Salmonella* Typhimurium, and *Mycobacterium tuberculosis* have been shown to drive polarization to an M2 phenotype to enhance their survival in the host (17). Virulent strains of *Mycobacterium tuberculosis* have been demonstrated to drive a stronger M2 polarization (18). Virulent strains of *Mycobacterium tuberculosis* express ESAT-6, which induces a stronger M2 polarization (18).

S*almonella* Typhimurium has also been shown to manipulate macrophage polarization to enhance its survival. After uptake by macrophages *Salmonella* reprogram macrophages by secreting effectors such as *Salmonella* anti-inflammatory response activator (SarA) through the *Salmonella* pathogenicity island 2 type III secretion system (T3SS) (19). These effectors dampened proinflammatory innate immune responses and induced anti-inflammatory macrophage polarization allowing *Salmonella* cells to survive for extended periods in their host (19). The secretion of molecules by bacterial pathogens that subvert host responses often requires specialized secretion systems to deliver effector proteins into host cells. These secreted effector proteins may modify host protein post-translational modifications such as protein phosphorylation which can affect the normal anti-bacterial macrophage response (20). The type VII secretion system (T7SS) has been identified in mycobacteria and this system may be responsible for subverting host signaling pathways and modulating macrophage polarization during NTM infection (21). In this study we compared protein phosphorylation in macrophages during early infection with either MAH 100, MAH 104, *M. abscessus* or non-virulent *M. smegmatis.* It was determined that for both MAH 100, the attenuated strain of *M. avium* and *M. smegmatis,* MARCKS-related protein is phosphorylated in RAW 264.7 macrophages following infection. MARCKS-related protein (MRP) is a protein kinase C substrate which has been shown to partake in the response of activated macrophages. MARCKS-related protein upon phosphorylation by protein kinase C is involved in actin turnover and finally phagosomal maturation and lysosomal fusion (22). MARCKS-related protein also plays an important role in macrophage transmigration and modulation of

inflammation (23). In contrast, macrophages infected with MAH 104, the virulent strain of *M. avium*, did not activate the MARCKS-related protein like the non-virulent strains. Instead, phosphorylation of methionine adenosyltransferase IIβ occurred 15 min post-infection. Methionine adenosyltransferase IIβ is an enzyme which catalyzes the biosynthesis of S-adenosylmethionine, a unique methyl donor for DNA methylation (24). Our results suggest that upon infection, the virulent MAH 104 modulate the epigenome response to enhance its survival in the host cell. Macrophages infected with *M. abscessus* activated RecQ mediated genome instability 1, a helicase responsible for maintaining genome stability (25).

Materials and Method

Bacterial Strains and infection

Four strains of mycobacteria were used: *Mycobacterium avium* subsp. *hominissuis* 104 (MAH 104), MAH 100, *Mycobacterium abscessus* subsp. *abscessus* strain 19977 and *Mycobacterium smegmatis* mc^2155. MAH 104 and MAH 100 were originally isolated from the blood of AIDS patients (42). MAH 104 causes disseminated disease in immunocompetent mice, while MAH 100 is cleared by the immune response of immunocompetent mice. *Mycobacterium abscessus* subsp. *abscessus* strain 19977 is a virulent clinical isolate that was isolated from a patient with skin disease. *M. abscessus* 19977 is cleared by the immune response of immunocompetent mice but can infect immunodeficient mice (5). *M. smegmatis* mc^2155 is a non-virulent strain of mycobacteria. All four mycobacterial strains were grown at 37^0C on Middlebrook 7H10 agar supplemented with 10% w/v oleic acid-

albumin-dextrose-catalase (OADC; Hardy Diagnostics; Santa Maria, Ca); MAH 104 and MAH 100 were grown for approximately 12 days where as *M. abscessus* and *M. smegmatis* were grown for 4 days. Bacterial suspensions were prepared in Hanks Balanced Salt Solution (HBSS, Cellgro, Manassas, VA). The bacterial suspensions were passaged 10 times through a 22-gauge needle to break bacterial aggregates before quantifying the bacterial concentrations using a spectrophotometer; *M. smegmatis* was passaged 20 times. Before infection, the quantified bacterial suspensions were properly diluted in fresh complete media depending on the MOI needed to prepare the inoculum for infection. Inoculums were diluted, plated, and incubated at 37°C to determine the CFUs.

Host Cell culture: RAW 264.7 Peritoneal murine macrophages (American Type Culture Collection; Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C and maintained in 5 % CO_2 incubator. Before infection and cytokine treatment, RAW 264.7 cells were seeded in a 48 or 6-well tissue culture plate for 24 hours to establish monolayers. Prior to seeding cells were counted with a hemocytometer.

Macrophage activation and infection with Mycobacterial species

RAW 264.7 cells (1 x 10^5 cells/0.5ml) were cultured for 24 hours in a 48-well tissue culture plate in DMEM supplemented with 10% FBS. Monolayers were then treated for 2 hours with either 10 ng/ml of mouse IFN-γ (Rockland antibodies and assays), TNF-α or IL-4 (Novus Biologicals). Control wells were left untreated. For infection, bacterial suspensions were prepared and quantified using the

spectrophotometer as described above. The bacteria suspensions were diluted in fresh complete media to achieve an MOI of 5:1 for 500 µL per well of the inoculum. The infection was centrifuged for 10 minutes to synchronize infection of macrophages, and then incubated for 1 h at 37^0C. After 1 h of infection media was removed, cells were washed 2 times with HBSS and then fresh complete media containing 200 µg/mL of amikacin was added for 1 hour at 37°C to kill remaining extracellular bacteria. After antibiotic treatment, cells were washed once more with HBSS, and some of the wells were immediately lysed by adding 500 µl of 0.1% triton X-100 (Sigma Aldrich, St. Louis, MO) in H_20 for 10 minutes and then serially diluted and plated for CFU determination. The CFUs after 1 h infection was considered day zero of experiments. On day two post infection, cytokines were added to MAH 100, MAH 104 and *M. smegmatis* infected macrophage monolayers that were not pretreated before infection; cytokines were added after 4 hours post-infection to *M. abscessus* infected monolayers. On day four post infection, the monolayers were again washed, host cells lysed, and then serially diluted and plated for CFU determination. *M. abscessus* monolayers were lysed and plated on day 2 for CFU determination. *M. abscessus* was treated differently from the other strains because it escapes from the macrophage (43) and is a rapidly growing mycobacterium.

To determine the effect of the DNA methyltransferase inhibitor, 5-aza-2 deoxycytidine on the growth of MAH 104 and MAH 100 in macrophages, macrophages were first pretreated for 24 hours with the inhibitor (2.5 µM). The monolayers were then infected with MAH 104 and MAH 100. The monolayers were washed, host cells lysed, at 2 hours, 72 hours and 96 hours post infection for CFU determination.

Macrophage differentiation into M1 or M2 phenotype by cytokine stimulation

RAW 264.7 cells (2 x 10^6 cells/2.5ml) were seeded in a 6-well tissue culture plate in DMEM supplemented with 10% FBS. Monolayers were established and treated for 2 hours with either 10 ng/ml of mouse IFN-γ (Rockland antibodies and assays) or IL-4 (Novus Biologicals). Control wells were left untreated. RAW cells were infected at MOI of 5:1 for 2 h, 24 h, and 72 h. After 1 h post-infection, monolayers were treated for an additional 1 h with 200 µg/ml amikacin to kill extracellular bacteria, washed once, and fresh media containing cytokine was added. On day 2, cytokines were added to monolayers that were not pretreated before infection. At 2 h, 24 h, and 72 h post infection, monolayers were prepared for flow cytometry. Media was removed and monolayers were washed 2 times with HBSS and then incubated with 0.25% of 600 µl TrypLE for 10 minutes. 750 µl warm growth media (DMEM) was added to quench the trypsin after which the monolayers were scraped gently, and the cell suspension transferred into new tubes. Samples were centrifuged at 100 x g for 4 minutes at room temperature, then the supernatant was discarded, and the cell pellet resuspended in fresh 0.1% BSA/HBSS (500 µl). Samples were again centrifuged at 100 x g for 4 minutes at room temperature to wash off any remaining cell debris; the supernatant was discarded, and the pellet resuspended in 1 ml of DMEM, and 100 µl were transferred into new tubes. The cells were centrifuged, the supernatant was discarded, and the cells were then resuspended and incubated with 100 µl of 1% BSA/HBSS for 30 mins to block non-specific antibody binding. The cells were again centrifuged, and the

supernatant discarded. Mouse APC-conjugated MHCII, mouse APC-conjugated IgG antibodies (M5/144.15.2) (Thermofisher scientific) at 0.03 µg/test (1:666 dilution), and mouse PE-conjugated CD206, mouse PE-conjugated IgG2a,k antibodies (MR6F3) (Thermofisher scientific) at 0.125 µg/test (1:160 dilution) and a combination of mouse APC-conjugated MHCII and mouse PE-conjugated CD206 antibodies were added to each sample and incubated for 1 h at 37 ^{0}C. After antibody staining, cells were washed with 100 µl of 0.1 % BSA/HBSS. Cells were then fixed in 500 µl of 2% paraformaldehyde in PBS and incubated at room temperature for 10 mins. The cells were centrifuged and washed once with 100 µl of 0.1 % BSA/HBSS; data was then acquired by flow cytometry. Samples were run in the BD Accuri C6 flow cytometer and the BD Accuri C6 software was used to analyze the samples. Forward and side scatter gating was used to gate out dead cells. Mouse APC-conjugated MHCII and mouse PE-conjugated CD206 antibodies were used to detect the percentages of M1 and M2 macrophage population, respectively. Mouse APC-conjugated IgG and mouse PE-conjugated IgG2a,k are isotype control antibodies.

Fluorescence Microscopy to determine the percentage of infected macrophages at MOI of 5 and MOI of 100

RAW 264.7 cells (1 x 10^5 cells/0.5ml) were seeded in a 48-well plate in DMEM supplemented with 10% FBS. When monolayers were established RAW 264.7 cells were treated for 2 hours with either 10 ng/ml mouse IFN-γ or IL-4 and then infected with MAH 104 containing the pCMV-tdTomato plasmid, expressing a red fluorescent protein. Control wells were not treated with cytokines. The bacterial

suspension was prepared in HBSS and then diluted in fresh media to obtain a MOI of 5:1 or 100:1 for 500 µl per well of the bacterial inoculum prepared. Cells were infected as described previously. The percentage of infected cells was then determined with the use of the fluorescence microscope. The number of infected cells per field of view were counted; five fields of view were counted per monolayer. The percentage of infected cells per field of view was calculated as a percentage of the number of infected cells counted. The total number of cells were established from the images acquired. The percentage of infected cells per monolayer was determined from the average of the percentages of infected cells per field of view. The percentage of infected cells for each monolayer were determined from 5 fields of view per monolayer.

Preparation of whole cell lysates from infected RAW 264.7 cells for western blot

RAW 246.7 macrophages (2 x 10^6) were seeded in a 6-well tissue culture plate and infected with either MAH 104, *M. smegmatis*, MAH 100 or *M. abscessus* for 15 min, 30 min, 1 h and 4 h at MOI of 100:1. At each timepoint monolayers were washed 3x and lysates were prepared by lysis for 30 min at 4^0 C in 1% Triton X-100 containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1% protease phosphatase inhibitor cocktail (Thermofisher Scientific). After incubating the monolayers with the lysis buffer for 30 minutes, lysates were transferred into tubes and centrifuged for 10 min at 15000 x g at 4^0C. The supernatant was transferred into new tubes and the protein concentration was measured using the Nanodrop. Samples were kept at -80^0C for SDS PAGE and Western blot. Aliquots of cell lysates were resolved on the Bolt Bis-Tris Plus gels (Thermofisher scientific) and blotted on to nitrocellulose membranes using

the iBlot 2 Gel transfer device (life technologies). The nitrocellulose membranes were blocked for 1 h with 5% BSA in 1X Tris-Buffered Saline (TBS) at pH 7.5, containing 0.1 % Tween 20. After blocking the membranes were incubated with anti-phosphotyrosine primary antibody (pY20) (Thermofisher scientific) or anti-Phosphoserine, clone 4A4 (Millipore Sigma) overnight at 1:500 dilution in 0.5% BSA in 1X TBST (1X Tris-Buffered Saline plus 0.1 % Tween 20). Membranes were wash 3x with 1x TBST and incubated with the secondary antibodies IRDye 680LT goat anti-mouse (LI-COR Biosciences) at 1:5000 dilution in 0.5% BSA in 1x TBST for 1h. The membranes were washed 3x with 1x TBST and then once with water and analyzed using the Odyssey infrared imager (LI-COR Biosciences) and LI-COR Bioscience Software.

On-Membrane Trypsin Digestion of proteins for Mass Spectrometry sequencing

Blotting of proteins onto nitrocellulose membranes as described previously was followed by adding 10 mL of Ponceau-S solution on to the membrane. This was agitated at room temperature on a platform shaker until proteins appeared as red bands. The Ponceau-S solution was removed, and the membrane was washed with water several times until the background staining was completely removed. Bands of interest were then excised with a clean scalpel and transferred on to 1.5 ml Eppendorf tubes. The membranes were washed 4x with 1 ml of distilled water with agitation (1000 rpm) on a thermomixer rotating platform at room temperature to completely remove the stain. Water was removed from the tubes and 1 ml of PVP-40 solution (100 mM acetic acid, 0.5% poly-vinylpyrrolidone) was added to each tube to block nonspecific protein

(trypsin) binding sites on the nitrocellulose. This was incubated at 37°C for 30 mins with gentle agitation using the thermomixer at 300 rpm. The membrane was then washed 5 times with 1 ml of water to remove excess PVP-40. Membranes were transferred to 200 µl Eppendorf tubes and 20 µL of trypsin at 12.5 ng/µL in 50 mM ammonium bicarbonate buffer and 20 µl digestion buffer (50 mM ammonium bicarbonate, pH 7.8) was added to each tube. The tubes were caped tightly and covered with parafilm to avoid evaporation and then incubated at 37°C for 5 h using a thermomixer at 300 rpm. After completing the digestion, the membranes and the trypsin solution were transferred into new tubes. The samples were dried under vacuum and 90 µl of acetone was added to each tube. The tubes were vortexed and incubated for 30 mins at room temperature to allow complete dissolution of the nitrocellulose and precipitation of the tryptic peptides adsorbed onto it. The tubes were centrifuged for 10 mins at 14,000 × *g* and acetone containing the dissolved nitrocellulose was carefully removed. The precipitated peptides were air-dried and resuspended in sample solution (2% acetonitrile, 0.1% formic acid) followed by sonication for 10 mins. Samples were stored at -80ºC until mass spectrometry analysis.

Real-Time PCR of proinflammatory and anti-inflammatory genes

Macrophages were infected at MOI of 5:1 with MAH 104, MAH 100, *M. abscessus*, and *M. smegmatis*. Total RNA was isolated for real-time PCR analysis at 1h, 6 h and 24 h post infection to measure the mRNA levels of TNF-α, IL6, IL1β, IL10, and CCL3. RNA was purified using the RNeasy Mini kit (Qiagen, USA) following the manufacturer' instructions. RNA samples were then DNase-treated and cDNA was

synthesized using the Biorad iScript. cDNA was then used for real time qPCR. The SYBR Green qPCR mix (Biorad, USA) was used to perform the RT-qPCR on the CFX Real-Time PCR detection system. For each gene, three sets of qPCR analysis were performed. The data obtained show the relative abundance of mRNA normalized to that of GAPDH, a housekeeping gene.

Statistical Analysis

Statistical analysis was performed using the software package GraphPad Prism 8. When comparing two groups, data were analyzed by the ANOVA test. Significance was considered at $P \leq 0.05$.

Results

Effect of macrophage activation on the growth of mycobacteria

The capacity of macrophages treated with three cytokines to inhibit the growth of mycobacteria was determined. Figure 1 shows the antimicrobial capacity of macrophage induced by the cytokines (IFN-γ, IL-4 and TNF-α) to interfere with the growth of mycobacteria. The results show that the growth of MAH 104 was inhibited when macrophages were activated before infection with IFN-γ and TNF-α ($P < 0.05$) (Figure 1B). Such inhibition was not observed when macrophages were pre-treated with IL-4 and in control macrophages. There was no significant inhibition of growth ($P > 0.05$) when macrophages were activated and infected with *M. abscessus* (Figure 1C). There was significant inhibition of *M. smegmatis* growth by all pre-treated and control macrophages.

Figure 1

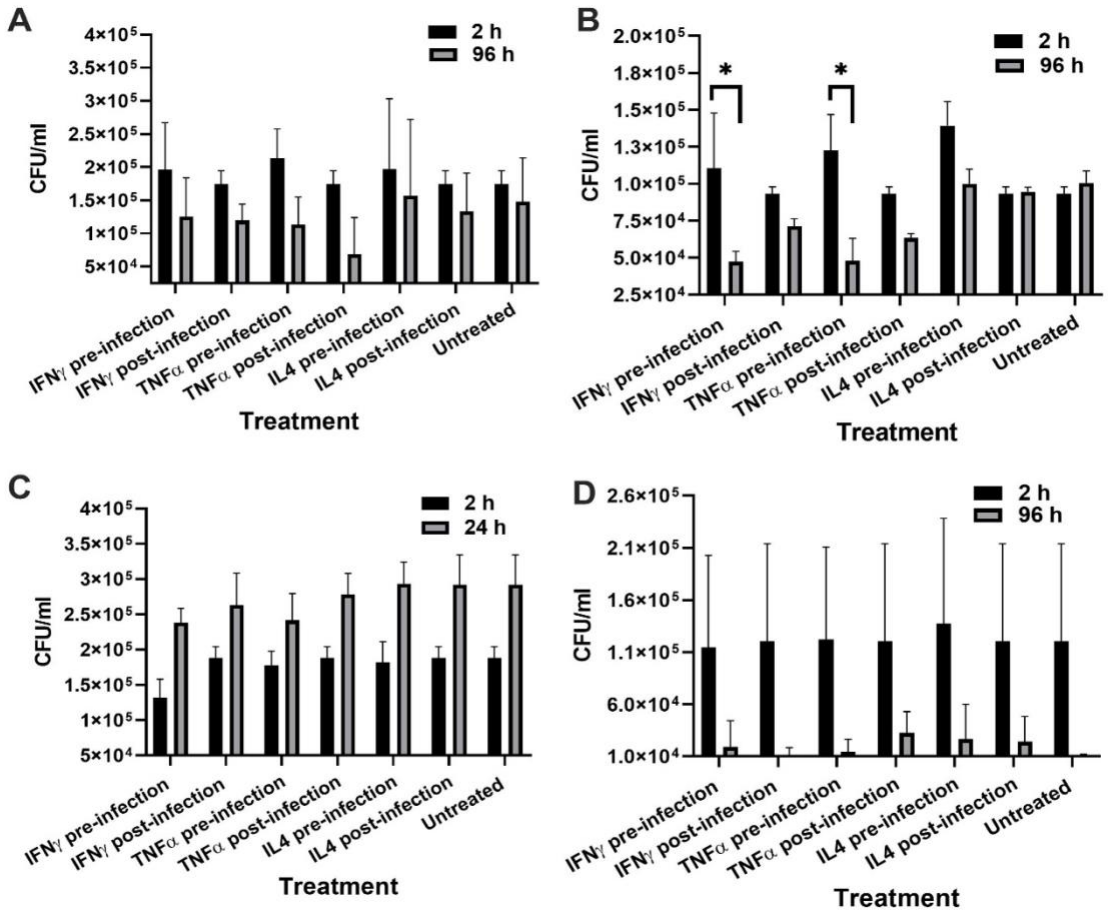

Figure 1. Effect of macrophage activation by cytokines on the growth of mycobacteria. Indicated cytokines were added to monolayers (10 ng/ml) before infection and on day 2 after infection for all mycobacteria except M. abscessus. Cytokines were added after 4 hours post-infection to *M. abscessus* infected monolayers. A) MAH 100 infection. B) MAH 104 infection. C) *M. abscessus* infection. D) *M. smegmatis* infection. The experiments were repeated two times. * $P < 0.05$.

Differences between the percentages of infected macrophages at different Multiplicity of infection

The percentage of infected cells at MOI 5:1 and 100:1 was determined. RAW 264.7 cells were pretreated with cytokines and infected with MAH 104 which contains

the pCMV-tdTomato plasmid that expresses a red fluorescent protein. Figure 2A shows an example field of view for infected macrophage monolayers. Figure 2B shows the percentage of infected macrophages at MOI 5 and 100.

Figure 2

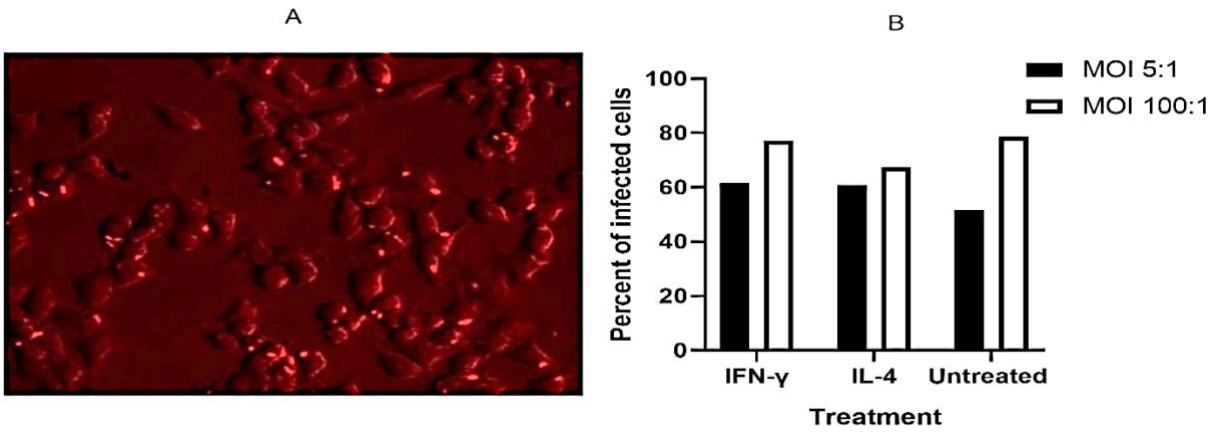

Figure 2: Percentage of infected cells at MOI of 5:1 and 100:1. RAW 264.7 cells were treated for 2 hours with 10ng/ml mouse IFN-γ or IL-4 and then infected with MAH 104 which contains the pCMV-tdTomato plasmid. A) Example of field of view for infected macrophages for percentage calculations. B) Calculated percent of infection at MOI 5:1 and MOI 100:1.

RAW 246.7 cell polarization into M1 or M2 phenotype

The flow cytometry results for macrophages that were treated with cytokines are shown in Figure 3. Figure 3 shows percentage of CD 206 positive or MHC class II positive cells, representing M1 and M2 macrophages, respectively, at 2 hours (A), 24 hours (B) and 72 hours (C). Flow cytometry histograms of CD 206 (an M2 marker) and MHC class II (an M1 marker) are shown. PE-CD206-A and APC-MHCII-A on the x-axis of the histograms represent M1 and M2 population, respectively. The black, red, and blue lines in the histogram represent the untreated, IFN-γ treated or IL-4 treated

macrophages, respectively. RAW 246.7 macrophages were treated with 10ng/ml of either IFN-γ or IL-4, and the percentage CD206 positive or MHC class II positive were determined at 2 h, 24 h and 72 h post cytokine treatment by flow cytometry. No significant M1 or M2 population was induce at 2 h for either cytokine. However, at 24 h, 81.16% of the macrophages was M1 and at 72 h the population of M1 macrophages was 76.24% when treated with IFN-γ (Table 1, Figure 3). Table 1 represents a summary for the percentage of CD206 positive and MHC class II positive cells from flow cytometry at 2 h, 24 h, and 72 h.

Figure 3

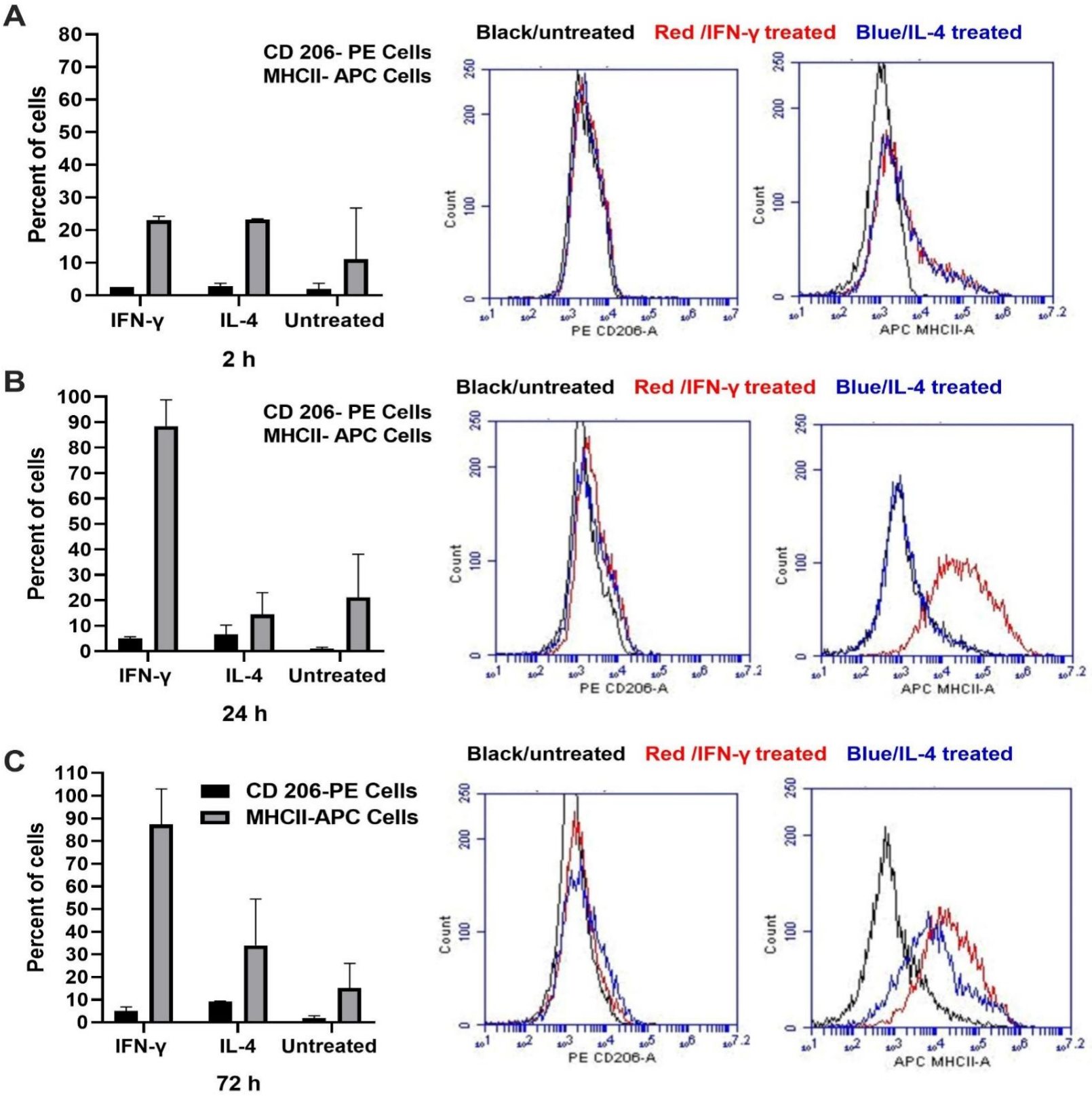
A
Percent of cells
CD 206- PE Cells
MHCII- APC Cells
IFN-γ
IL-4
Untreated
2 h
Black/untreated
Red /IFN-γ treated
Blue/IL-4 treated
Count
PE CD206-A
APC MHCII-A
B
Percent of cells
CD 206- PE Cells
MHCII- APC Cells
IFN-γ
IL-4
Untreated
24 h
Black/untreated
Red /IFN-γ treated
Blue/IL-4 treated
Count
PE CD206-A
APC MHCII-A
C
Percent of cells
CD 206-PE Cells
MHCII-APC Cells
IFN-γ
IL-4
Untreated
72 h
Black/untreated
Red /IFN-γ treated
Blue/IL-4 treated
Count
PE CD206-A
APC MHCII-A

Figure 3. Macrophage differentiation into M1 or M2 phenotypes by cytokine stimulation. CD 206 positive or MHC class II positive cells, representing M1 and M2 macrophages, respectively at A) 2 h, B) 24 h, C) 72 h post cytokine treatment; Percentages were determined in two experiments. Representative histograms show cell population shifts.

Table 1: Percenage of CD 206 (M2) and MHC II (M1) positive cells

Macrophage Treatment Groups	% of positive	
	CD 206-PE (M2)	MHCII-APC (M1)
2h Untreated	0.73	0.02
2h IFN-γ	2.50	23.90
2h IL-4	2.20	23.37
24h Untreated	0.60	9.19
24h IFN-γ	4.67	81.16
24h IL-4	4.01	8.51
72h Untreated	1.12	7.58
72h IFN-γ	3.90	76.24
72h IL-4	9.36	48.40

Flow cytometry with mycobacterial infected macrophages pretreated with cytokines

Figure 4 shows the flow cytometry results for mycobacterial infected macrophages (MOI 5:1). The statistical results are presented in the bar charts. CD206-PE and MHCII-APC on the x-axis of the bar charts represent M2 and M1 population, respectively. Figure 4 shows the flow cytometry results of the percentage of CD 206 and MHC class II positive macrophages when macrophages were infected with MAH 104 (Figure 4A), *M. smegmatis* (Figure 4B), MAH 100 (Figure 4C), *M. abscessus* (Figure 4D). Figures 4Ai, 4Bi, 4Ci and 4Di show the flow cytometry results when macrophages were not pre-treated with cytokines before infection. Figures 4Aii, 4Bii,

4Cii and 4Dii show the flow cytometry results when macrophages were pre-treated with IFN-γ and then infected with MAH 104 (Figure 4Aii), *M. smegmatis* (Figure 4Bii), MAH100 (Figure 4Cii), and *M. abscessus* (Figure 4Dii). MAH104 trigger the development of significant M2 population ($P < 0.05$) of about 40 – 60 % at 72 hours timepoint (Figure 4Aii). *M. smegmatis* (Figure 4Bii), MAH100 (Figure 4Cii) and *M. abscessus* (Figure 4Dii) unlike MAH104 induce significant ($P < 0.05$) M1 population at 24 hours timepoint. MAH100 (Figure 4Cii) and *M. abscessus* (Figure 4Dii) did not induce significant M2 population ($P > 0.05$) at 24 hours timepoint. Figures 4Aiii, 4Biii, 4Ciii and 4Diii show the flow cytometry results when macrophages were pretreated with IL-4 and then infected with MAH 104 (Figure 4Aiii), *M. smegmatis* (Figure 4Biii), MAH100 (Figure 4Ciii) and *M. abscessus* (Figure4Diii). MAH104 (Figure 4Aiii) and *M. smegmatis* (Figure 4Biii) induced significant M2 population ($P <0.05$) at 72 hours timepoint.

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Figure 4

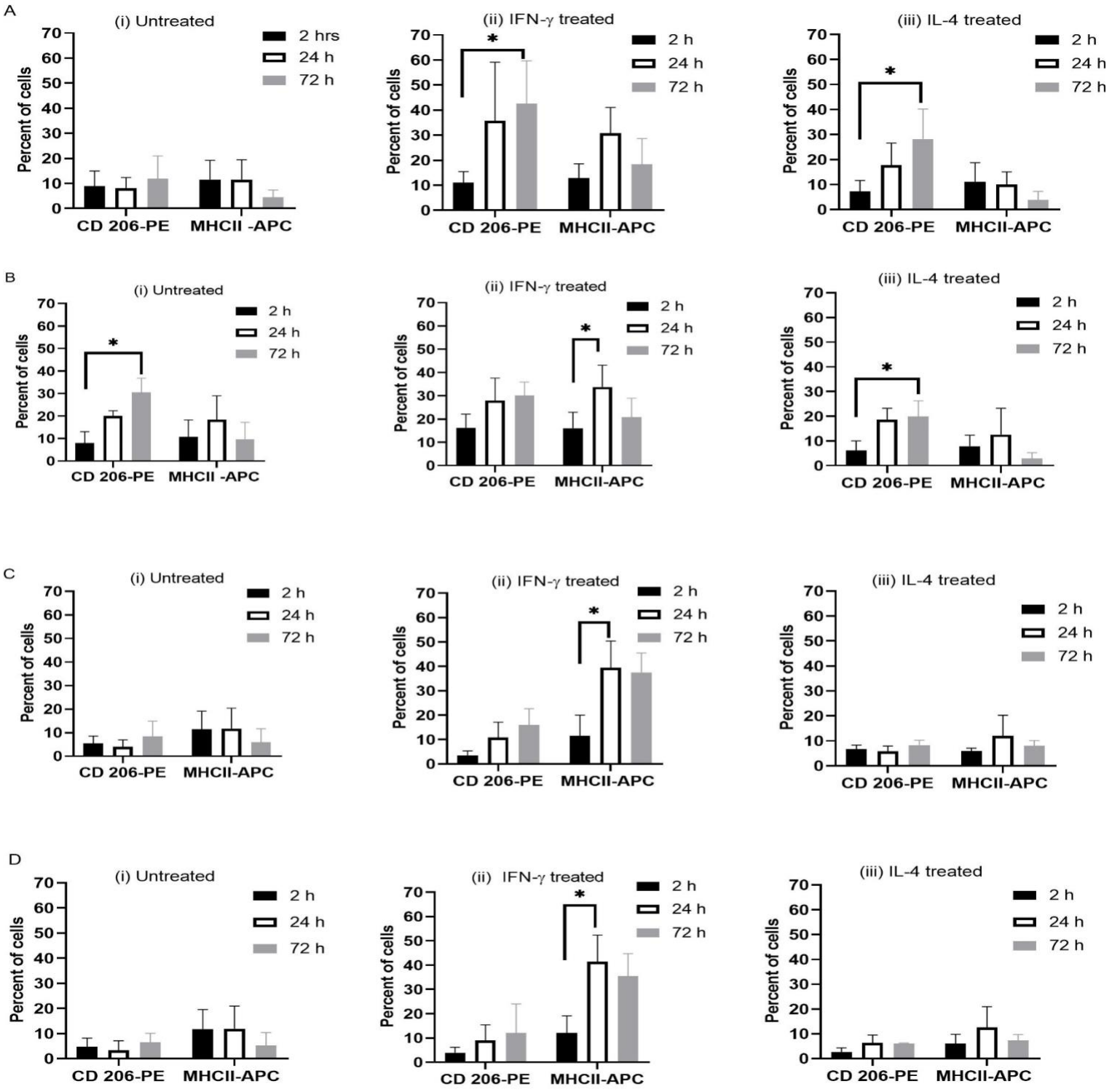

Figure 4: Graphic representation of the flow cytometry for mycobacterial infected macrophages pretreated with cytokines. Percentage of CD 206 and MHC class II

positive macrophages for cells pre-treated with either IFN-γ, IL-4, or untreated macrophages infected with MAH 104 (Figure 4Ai-iii), *M. smegmatis* (Figure 4Bi-iii), MAH100 (Figure 4Ci-iii) and *M. abscessus* (Figure 4Di-iii) at 2 h, 24 h, 72 h post-treatment. The experiments were repeated two times. *P < 0.05.

Flow cytometry with mycobacterial infected macrophages treated with cytokines post-infection

The role of mycobacterial infections in influencing or manipulating the signaling effect of IFN-γ and IL-4 on macrophages was determined by flow cytometry. Macrophages were infected with mycobacteria at MOI of 5 and on day 2 IFN-γ or IL-4 were added to the monolayers. After 48 hours, the population of CD 206 positive or MHC class II positive cells were then visualized by flow cytometry. Figure 5 shows the percentage of M1 or M2 macrophages after treating with cytokines post-infection; MAH 104 (Figure 5Ai-ii), *M. smegmatis* (Figure 5Bi-ii), MAH 100 (Figure 5Ci-ii) and *Mycobacterium abscessus* (Figure 5Di-ii) did not induce significant M1 or M2 populations when IFN-γ or IL-4 was added to the monolayers post-infection ($P < 0.05$).

Figure 5

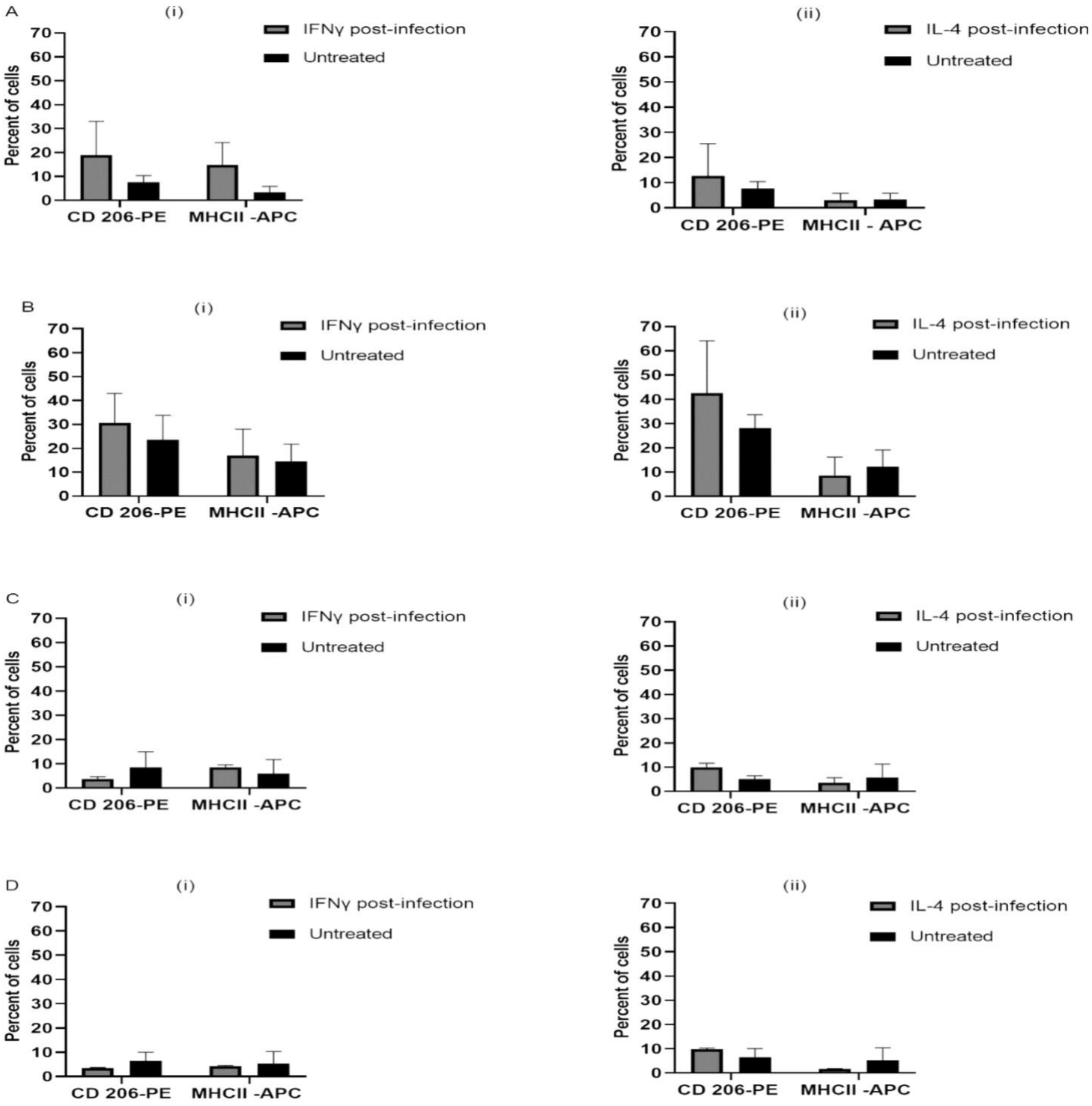

Figure 5: Graphic representation of the flow cytometry for mycobacterial infected macrophages treated with cytokines post-infection. Percentage of CD 206 and MHC class II positive macrophages for cells treated with either IFN-γ, IL-4, or untreated

macrophages infected with MAH 104 (Figure 4Ai-ii), *M. smegmatis* (Figure 4Bi-ii), MAH100 (Figure 4Ci-ii) and *M. abscessus* (Figure 4Di-ii). The population of CD 206 positive or MHC class II positive cells were visualized by flow cytometry 48 h after cytokine treatment. The experiments were repeated two times. *P < 0.05.

Mycobacterial infection of RAW 264.7 cells induced phosphorylation of host proteins.

Protein phosphorylation in macrophages upon macrophage infection by MAH, *M. abscessus* and the non-virulent *M. smegmatis* were examined. MAH, *M. abscessus* and *M. smegmatis* induced phosphorylation of host proteins upon macrophage uptake as shown on the western blots in Figures 6A, B and C. The host protein with approximate size of 40 KDa was phosphorylated upon mycobacterial uptake for up to 1 h, and by 4 h the phosphorylation decreased.

Figure 6

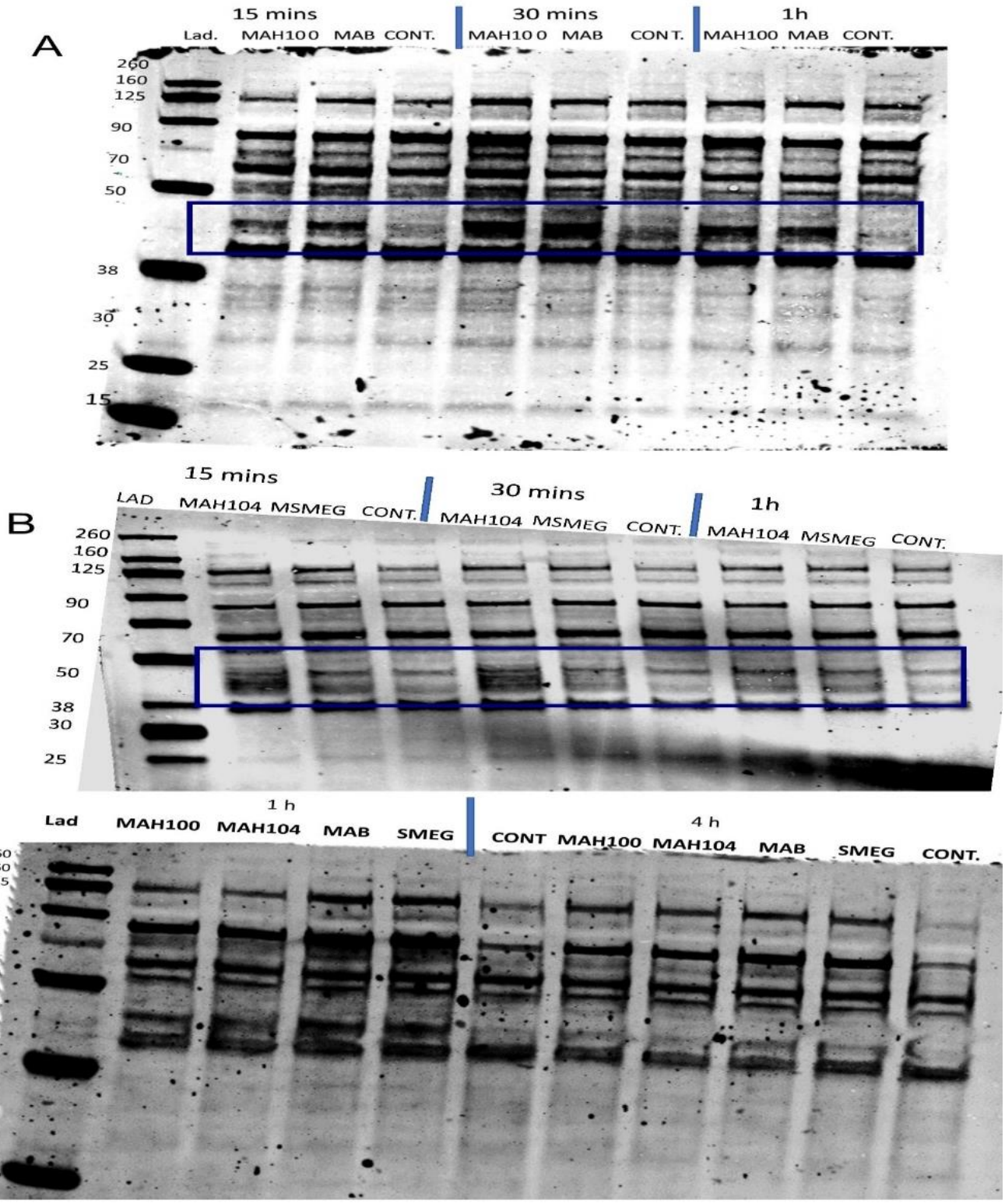
A
15 mins
30 mins
1h
Lad.
MAH100
MAB
CONT.
MAH100
MAB
CONT.
MAH100
MAB
CONT.
260
160
125
90
70
50
38
30
25
15
B
15 mins
30 mins
1h
LAD
MAH104
MSMEG
CONT.
MAH104
MSMEG
CONT.
MAH104
MSMEG
CONT.
260
160
125
90
70
50
38
30
25
C
1 h
4 h
Lad
MAH100
MAH104
MAB
SMEG
CONT
MAH100
MAH104
MAB
SMEG
CONT.
260
160
125
90
70
50
38
30
25

Figure 6: Western blots showing host protein phosphorylation upon mycobacterial uptake by macrophages. MAH 100, *M. abscessus* (Figure 6A) and MAH 104, *M. smegmatis* (Figure 6B) induced phosphorylation of host proteins (indicated by the rectangle) 15 min and 30 min post infection. Figure 6C shows that the phosphorylation decreased by 4 h.

Phosphorylated proteins

Detection of phosphorylated proteins post-infection by western blot was followed by on-membrane trypsin digestion of the phosphorylated proteins electroblotted onto nitrocellulose membrane and liquid chromatography- mass spectrometry (LC-MS) to determine these phosphorylated proteins. Table 2 shows the phosphorylated proteins determined by LC-MS. After running a western blot, the ~40 KDa bands were cut, processed, and sent to the LC-MS laboratory to determine phosphorylated proteins induced post-infection. For MAH 100 and *M. smegmatis*, MARCKS-related protein (MRP) is phosphorylated in RAW 264.7 macrophages post-infection whereas infection of macrophages with MAH 104 phosphorylated methionine adenosyltransferase IIβ (MAT2B) 15 min post-infection. *M. abscessus* infection phosphorylated RecQ mediated genome instability 1.

Table 2: Differential Phosphorylated proteins determined by LC-MS

Timepoint/strain	15 minutes	30 minutes
MAH 100	MARCKS-related protein	
MAH 104	methionine adenosyltransferase II, beta	
M. abscessus	RecQ mediated genome instability 1	
M. smegmatis		MARCKS-related protein

Effect of 5-aza 2-deoxycytidine (methyltransferase inhibitor) on the growth of MAH 104 and MAH 100 in RAW 264.7 macrophages

Since MAH 104 activates methionine adenosyltransferase IIβ, an enzyme which catalyzes the biosynthesis of the DNA methyl donor, s-adenosylmethionine, we determined the effect of inhibition of DNA methylation on the growth of MAH 100 and MAH 104 in macrophages. As shown in Figure 7, the inhibitor of DNA methylation, 5-aza 2-deoxycytidine (AZA) inhibited the growth of MAH 104 (Figure 7A) in RAW 264.7 macrophages ($P < 0.05$). Incubation of MAH 104 and MAH 100 broth culture for 2 – 48 h in 7H9 broth containing 2µM of 5-aza 2-deoxycytidine did not inhibit the growth of MAH 104 (Figure 7C) and MAH 100 (Figure 7D).

Figure 7

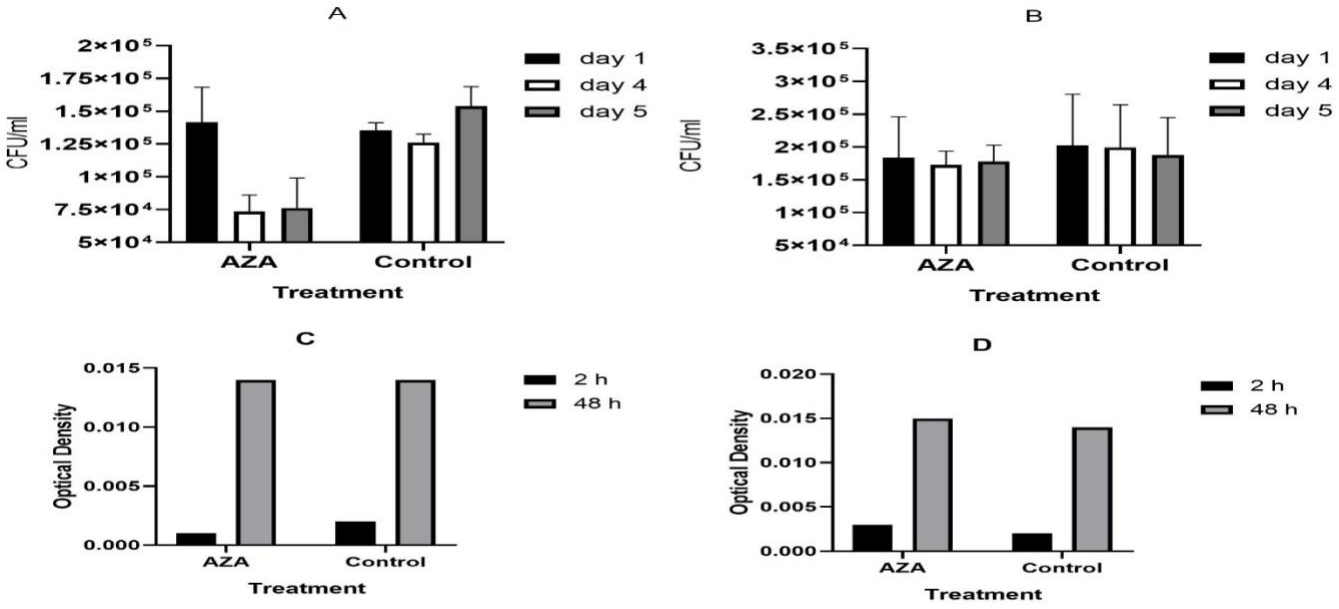

Figure 7: The effect of 5-aza-2 deoxycytidine on the growth of A) MAH 104 infection and B) MAH 100 infection in macrophages. The experiment was repeated two times. * $P < 0.05$. Figures C and D show that the drug (2µM) did not have any effect on the growth of MAH 104 (Figure C) and MAH 100 (Figure D) in 7H9 broth culture.

Real-time PCR

Since SAM has been shown to play a role in anti-inflammatory response while the MARCKS-related protein plays a role in proinflammatory response, we compared the relative mRNA expression of proinflammatory (TNF-α, IL1β and IL6) and anti-inflammatory cytokines (IL10) and the chemokine CCL3 in macrophages infected with MAH 104, MAH 100, *M. abscessus*, and *M. smegmatis*. MAH 100, MAH 104, *M. smegmatis, M. abscessus* induced different mRNA expression levels of proinflammatory and the anti-inflammatory cytokine and the chemokine (CCL3). MAH 100 infection induced significantly higher levels of expression of IL1β mRNA 6 h post infection (Figure 8D) compared to MAH 104 ($P < 0.05$). Also, MAH 104 infection induced significantly higher levels of expression of IL10 mRNA at 6 h post infection (Figure 8 C) compared to MAH 100 ($P < 0.05$). MAH 100 and MAH 104 induced higher levels of expression of TNF-α mRNA compared to *M. abscessus* and *M. smegmatis* (Figure 8A) ($P < 0.05$) 6 h post infection.

Figure 8

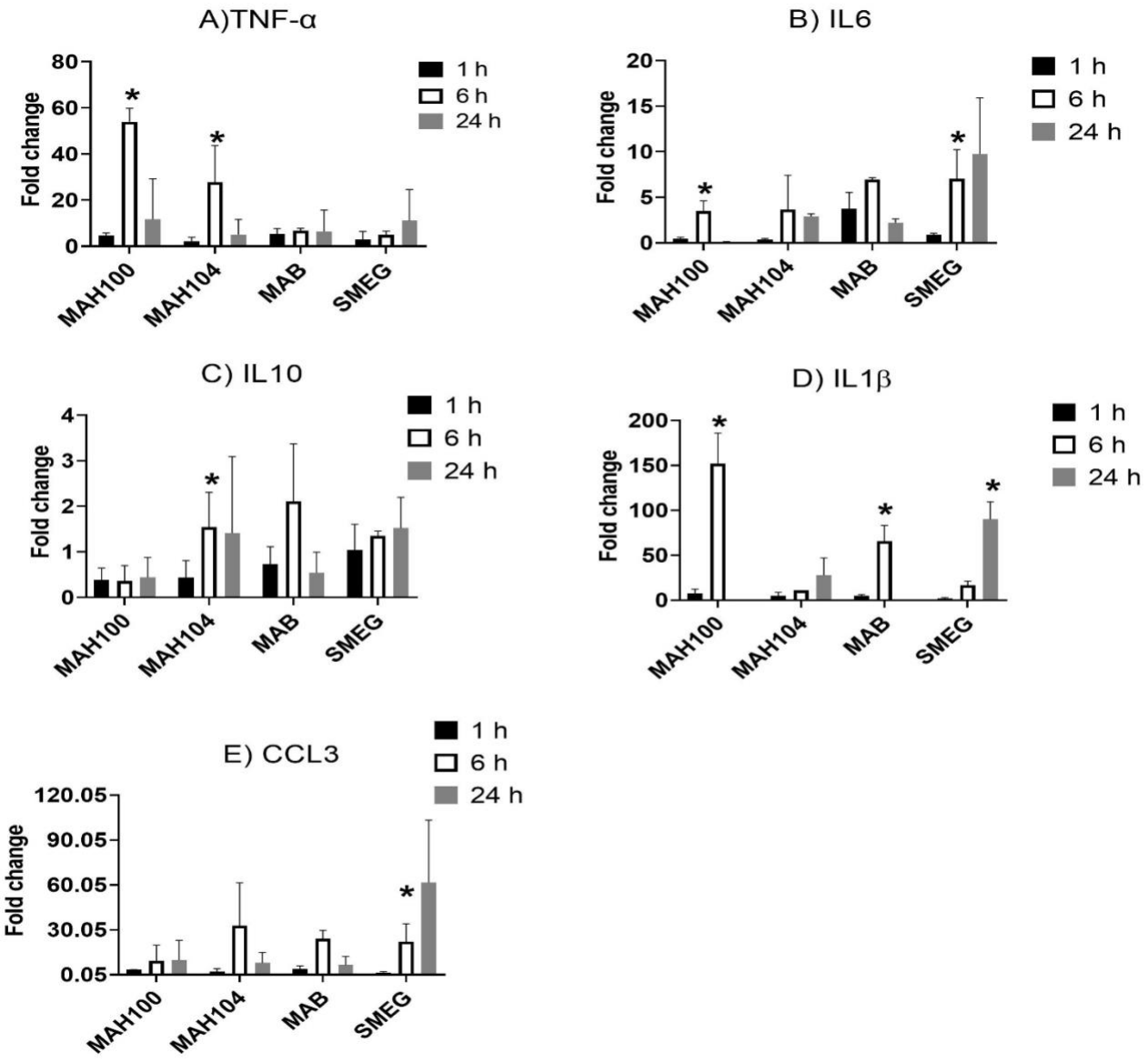

Figure 8: Real time qPCR gene expression of proinflammatory and anti-inflammatory genes and CCL3 (chemokines) in RAW 246.7 cells infected with MAH 104, MAH 100, *M. abscessus*, and *M. smegmatis*. The relative mRNA expression levels of proinflammatory and anti-inflammatory cytokines and CCL3 (chemokine) vary among the mycobacterial strains. * $P < 0.05$.

Visual representation of results

Figure 9 show the visual representation of results. Figure 9 show pathway activated by MAH 104 (Figure 9A), MAH 100 and *M. smegmatis* (Figure 9B).

Figure 9

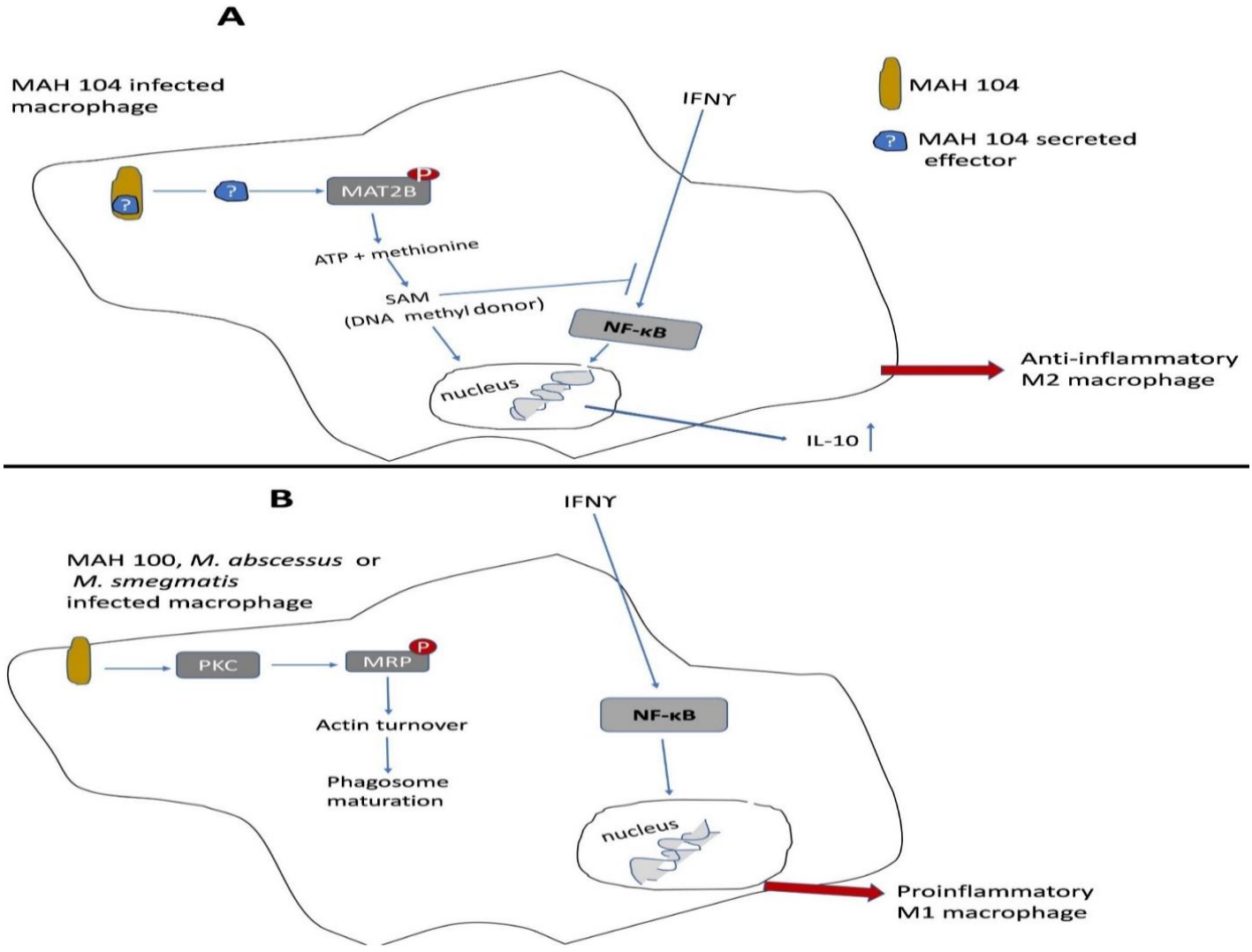

Figure 9: Depiction of pathways activated by virulent MAH 104, attenuated MAH100, non-virulent *M. smegmatis* and *M. abscessus*.

Discussion

Many bacterial pathogens interfere with the host post-translational mechanisms such as phosphorylation to subvert phagocytic function, and several studies have suggested that this may be the case for NTMs (8,26). Manipulation of the host phagocytic response such as inhibition of the phagosome maturation has been shown to be an important mechanism used by virulent NTMs to enhance intracellular survival (12). Virulent NTMs may also be responsible for the inhibition of the antimycobacterial macrophage activation by IFN-ϒ produced by T helper 1 cells (27). Virulent NTMs modulates the Mitogen Activated Protein kinases (MAPKs) and NF-κB signaling pathways to enhance intracellular survival (26). Within the first 30 minutes of macrophage infection, *M. avium* infection activates the MAPKs and NF-κB which slightly hinders the progression of *M. avium*. However, virulent *M. avium* downregulates MAPKs and NF-κB activation significantly after an hour of infection, and by 24 h virulent *M. avium* has complete control of the host (8,26). The maturation of the phagosome can be affected by NF-κB and the consequence of NF-κB inhibition can be the indirect inhibition of phagosome maturation (29). The inhibition of MAPKs and NF-κB activation by *M. avium* leads to a marked decrease in pro-inflammatory cytokines, especially TNF-α, IL-12 and IL-1β, which impairs the host's ability to fight off the infection. Prolonged activation of p38 and ERK1/2 and increased TNF-α is observed when macrophages are infected with the non-virulent mycobacterium, *M. smegmatis*. (26). Our results showed that the non-virulent *M. smegmatis* and the attenuated MAH 100 induced significantly higher levels of expression of the proinflammatory cytokine IL1β mRNA 6 h post infection compared to the virulent

MAH 104. Also, MAH 104 infection induced significantly higher levels of expression of the anti-inflammatory cytokine IL10 mRNA at 6 h post infection compared to MAH 100.

Infection with different NTMs strains have been associated with diverse host immune responses in mice. For example, MAH 100 and *Mycobacterium abscessus* infections are cleared from mice, while MAH 104 and MAH 101 disseminates and replicates in mice organs such as lungs, spleen, and liver (30,31,32). To understand the difference in virulence and host immune response among mycobacteria, we investigated the differences in early macrophage response to four different NTMs: MAH 104 (virulent strain), MAH 100 (attenuated strain), *Mycobacterium abscessus*, and the non-virulent *M. smegmatis*.

Resistance to NTMs infections is primarily mediated by T helper 1 responses. Immune responses dominated by T helper 2 cells correlate with disease susceptibility and the virulence of the NTM strain (33). M1 macrophages are induced by T helper 1 responses and these macrophages constitute the first line of defense against NTM (13). In this study we confirmed the importance of T helper 1 cytokines, IFN-γ, and TNF-α (mostly produced by natural killer cells) in inhibiting the growth of mycobacteria in macrophages. T helper 1 cells secret IFN-γ which activates macrophages inducing strong antimicrobial activity (13,33). M2 macrophages are induced by T helper 2 responses. T helper 2 cells secret cytokines such as IL-4 and IL-10 which act on macrophages and downregulate the production of macrophage antimicrobial molecules (14,15). T helper 2 cytokines also reduce the responsiveness to macrophage-activating cytokines such as IFN-γ and TNF-α (33). Virulent NTMs have been shown to interfere

with the JAK/STAT signaling pathways (or the interferon-gamma signaling pathway). *M. avium* downregulates the expression of the IFN-γ receptor making the macrophage unresponsive to IFN-γ (27). The manipulation of macrophage polarization or activation states into M1 or M2 have been shown to be an important pathogenesis mechanism of some intracellular bacteria (17,18,19). Bacterial pathogens have been shown to trigger polarization to an M2 or M1 phenotype to enhance their survival in the host. (18,19). We investigated whether the virulent NTMs could manipulate macrophage polarization. Our findings demonstrated that infection of macrophages by the virulent NTM (MAH 104) triggered the development of M2 phenotype while infection with the non-virulent or attenuated strain triggered the development of M1 phonotype. These findings suggest a very important process in the ability of virulent mycobacteria to modify the host immune response to their benefit.

To understand how virulent NTMs affect the host immune response for their benefit, we compared protein activation in macrophages upon ingestion of MAH, *M. abscessus* and a non-virulent *M. smegmatis***.** Hijacking the host post-translational mechanisms such as protein phosphorylation is a key strategy for many bacterial pathogens to subvert macrophage function and several studies have shown that this may be the case for virulent NTMs (8,26). Protein phosphorylation is catalyzed by kinases which reversibly transfer a phosphate group from ATP to a residue of a target protein usually the tyrosine, serine, and threonine residues (34). Protein activity or function is regulated by phosphorylation (35). Protein phosphorylation can transmit a signal from the cell surface receptor through a series of phosphorylation and de-phosphorylation events to the cell nucleus. Several biological processes including key

processes of the immune system, such as differentiation, cytokine/chemokine production, inflammation, and bacterial killing, are controlled by protein phosphorylation (36). Bacterial pathogens have been shown interfere with the kinase-mediated phosphorylation of host signaling pathways (20,37,38). For example, *Salmonella typhimurium* has been shown to drive M2 polarization via activation of the STAT3 pathway. Once *Salmonella* translocates the effector SarA (*Salmonella* anti-inflammatory response activator) into the host cell, it interacts with the host-pleiotropic serine/threonine kinase, GSK-3 (Glycogen synthase kinase-3). This interaction leads to the phosphorylation of SarA, and the GSK3 in the resulting complex is driven to interact with and phosphorylate the substrate transducer and activator of transcription 3 (STAT3) on tyrosine-705. STAT3 can then translocate to the nucleus and drive the anti-inflammatory macrophage polarization program (20). By examining phosphorylation of proteins in macrophages infected with mycobacteria strains, our results demonstrate that in macrophages infected with both MAH 100 and *M. smegmatis,* MARCKS-related protein is phosphorylated while infection with MAH 104, induced methionine adenosyltransferase IIβ phosphorylation 15 min post-infection. MARCKS-related protein is a protein kinase C substrate which has been shown to play a role in activated macrophages. MARCKS-related protein upon phosphorylation by protein kinase C is involved in actin turnover and finally phagosomal maturation and lysosomal fusion (22). MARCKS-related protein has also been shown to play an important role in macrophage transmigration and modulation of inflammation (23). Methionine adenosyltransferase IIβ (MAT2B) is an enzyme which catalyzes the biosynthesis of S-adenosylmethionine (SAM) from methionine and ATP,

a unique methyl donor in DNA methylation (24). Treatment of macrophages with SAM has been shown to mediate the inhibition of inflammatory response and changes in DNA methylation (39). Our results suggest that upon infection, the virulent MAH 104 may modulate the epigenome response to enhance its survival in the host. Several studies have shown that some pathogens modulate epigenetic processes such as DNA methylation to tackle host immune response and cause chronic disease (40).

DNA methylation is catalyzed by DNA methyltransferases. Inhibition of DNA methyltransferases with 5-aza-2 deoxycytidine (DNA methyltransferase inhibitor) inhibited the growth of MAH 104 and not MAH 100 in RAW macrophages These results show that MAH 104 may be upregulating and using DNA methyltransferases to methylate DNA unlike MAH 100. Yang et al. (2016) have shown that 5-aza-deoxycytidine inhibits the growth of *Mycobacterium tuberculosis* in RAW macrophages (41).

Since SAM has been shown to play role in anti-inflammatory response while the MARCKS-related protein plays a role in proinflammatory response, we compared the relative mRNA expression of proinflammatory (TNF-α, IL1β and IL6) and anti-inflammatory cytokines (IL10) and the chemokine CCL3 in macrophages infected with MAH 104, MAH 100, *M. abscessus*, and *M. smegmatis*. MAH 100 infection induced significantly higher levels of expression of IL1β mRNA at 24 h post infection compared to MAH 104. Also, MAH 104 infection induced significantly higher levels of expression of IL10 at 6 h post infection compared to MAH 100.

In conclusion, our results suggest that the virulent MAH 104 may be secreting effectors and activating MAT2B. Activated MAT2B then synthesizes SAM from

methionine and ATP; SAM methylates DNA preventing the expression of genes important in inhibiting the growth of MAH 104 in macrophages. The methylation of DNA by SAM also interferes with the IFN-γ signaling effect. It is possible that MAH 104 modulated the epigenome response by DNA methylation to enhance its survival in the macrophage. This modulation may also be responsible for the triggering of M2 phenotype observed during MAH 104 infection of macrophages. More work is needed to determine what MAH 104 effectors may phosphorylate MAT2B and the epigenetic enzymes that may be involved in the modulation of the epigenome response.

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Chapter 3: Discussion and Conclusion

Overview

NTM infects the host through both the gastrointestinal and respiratory routes. NTM mainly infect the airways by colonizing and invading epithelial mucosal cells as well as mononuclear phagocytes such as monocytes and macrophages. Manipulation of the host phagocytic response such as inhibition of the phagosome maturation has been shown to be an important mechanism used by virulent NTM to enhance intracellular survival. Virulent NTM may also be responsible for the inhibition of the antimycobacterial macrophage activation by IFN-ϒ produced by T helper 1 cells. Many bacterial pathogens interfere with the host post-translational mechanisms such as phosphorylation to subvert phagocytic function, and several studies have suggested that this may be the case for NTM. To identify pathways activated or suppressed by virulent NTM to enhance survival, we compared protein phosphorylation in macrophages upon ingestion of MAH 100 (attenuated strain), MAH 104, *M. abscessus.* and a non-virulent *M. smegmatis.* Our study showed that virulent MAH 104 activates methionine adenosyltranferase II B (MAT2B) while suppressing MARCKS-related protein (MRP) activation. MAT2B is an enzyme which catalyzes the biosynthesis of S-adenosylmethionine, a unique methyl donor in DNA methylation. MRP has been shown to play a role in activated macrophages. Our findings suggest that the virulent MAH104 enhance their survival in the macrophage possibly through interference with the epigenome responses (DNA methylation) while inhibiting the activation of MARCKS-related protein.

Optimizing Protocols

Performing cell culture while keeping a sterile technique was the first thing I had to learn and accustom myself to. It was my first experience with Raw 246.7 cells. I needed to learn how to seed plates, flasks, passage cells and I also had to be aware of the doubling time of Raw 246.7 cells. When performing survival assays, I needed to learn how to generate consistent MOIs. I used MOI of 5 for survival assays.

To generate consistent MOI, I passaged bacterial suspensions through a 22-gauge needle to break bacterial aggregates or clumps before quantifying the bacterial concentrations using a spectrophotometer; *M. smegmatis* suspension was passaged 20 times because it was very clumpy. Consistent MOIs will give close CFU readouts between replicates.

Before performing flow cytometry experiments, I had to first understand how the flow cytometer works. To select fluorophores for flow cytometry it was important to know the number, types, and excitation capabilities of the lasers present in the flow cytometer available. The first M1 macrophage marker I chose to determine the population of M1 macrophages after treating Raw 267.4 cells with IFN-γ was CCR7. IFN-γ did not induce significant surface expression of CCR7 in RAW 267.4 macrophages. Mouse APC-conjugated CCR7 antibodies were employed. Later I switched to another M1 marker, MHCII, to determine M1 population using Mouse APC-conjugated MHCII antibodies. IFN-γ induced significant surface expression of MHCII. This was my first experience with flow cytometry.

Before performing Western blot experiments, I had to make sure that 70 to 100% of the cells are infected. To infect 70 to 100% of the monolayer, I had to infect

at MOI of 100. To maintain the ionic strength and stability of the proteins, especially phosphorylated proteins, in the lysate, I had to use lysis buffer with pH within the physiological range (pH 7.5). Protein samples for LC-MS analysis were also kept at pH within the physiological range. Another important skill that required optimization was real-time qPCR. Before performing qPCR, I needed to have a strong sense of the primer design (GC content, melting temperature, and length of primers), annealing temperature and PCR cycles. This was key to amplifying only the band of interest and generating consistent Cq values between technical replicates.

Experimental Process

The main goal of my project was to identify pathways that are activated or suppressed by virulent mycobacteria that could benefit the pathogens survival. To achieve this goal, I compared the differences in early macrophage response to four strains of NTM: MAH 104 (virulent strain), MAH 100 (attenuated strain), *M. abscessus*, and a non-virulent *M. smegmatis*. I compared protein activation in macrophages upon ingestion of MAH 104, MAH 100, *M. abscessus*, and *M. smegmatis*.

The first step in my research was to confirm the importance macrophage activating cytokines, IFN-γ, and TNF-α, in inhibiting the growth of mycobacteria in macrophages. Macrophages were treated with these cytokines and then infected with mycobacteria. IFN-γ, and TNF-α inhibited the growth of *Mycobacterium avium.*

The second step in my research was to determine if virulent mycobacteria interfere with the IFN-γ signaling effect and trigger the development of M2 macrophage phenotype. I treated macrophages with IFN-γ and IL-4 and then in infected the macrophages with

mycobacteria. MAH 104, unlike the non- virulent and attenuated strain, triggered the development of significant M2 macrophage population. This finding suggested that the pathways activated by virulent mycobacteria to enhance survival in the macrophage may also interfere with the IFN-γ signaling effect.

To identify pathways suppressed or activated by virulent mycobacteria that could benefit the pathogens survival, I examined protein phosphorylation in macrophages infected with mycobacteria. To compare protein phosphorylation between the mycobacterial strains, I performed western blot with whole cell lysate from infected Raw 264.7 macrophages using the phospho-tyrosine and phosphoserine primary antibodies to detect phosphorylated proteins followed by on-membrane trypsin digestion of the phosphorylated proteins electroblotted onto nitrocellulose membrane and liquid chromatography- mass spectrometry (LC-MS) to determine these phosphorylated proteins. For both MAH 100 and *M. smegmatis,* MARCKS-related protein is phosphorylated in Raw 264.7 macrophages post-infection. MARCKS-related protein is a protein kinase C substrate which has been shown to play a role in activated macrophages. Macrophages infected with MAH 104, evidenced methionine adenosyltransferase II, beta phosphorylation 15 min post-infection. Methionine adenosyltransferase II, beta is an enzyme which catalyzes the biosynthesis of S-adenosylmethionine, a unique methyl donor in DNA methylation. S-adenosylmethiomine has been shown to mediate the inhibition of inflammatory responses, possible through DNA methylation. My findings suggest that the highly virulent MAH104 may be activating methionine adenosyltransferase II, beta upon infection to enhance their survival in the macrophage possibly through interference

with the epigenome responses (DNA methylation) while inhibiting the activation of MARCKS-related protein. The results also suggest that SAM may be responsible for interfering with the IFN-γ signaling effect. Since SAM has been shown to play role in anti-inflammatory response while the MARCKS-related protein plays a role in proinflammatory response, I compared the relative mRNA expression of proinflammatory (TNF-α, IL1β and IL6) and anti-inflammatory cytokines (IL10) and the chemokine CCL3 in macrophages infected with MAH104, MAH100, *M. abscessus*, and *M. smegmatis.*

Overall, the results suggest that upon infection of macrophages, virulent MAH 104 may be secreting effectors and activating MAT2B. MAT2B then synthesizes SAM from ATP and methionine. SAM therefore methylates DNA preventing the expression of genes important in inhibiting the growth of MAH 104 in macrophages. The methylation of DNA by SAM also interferes with the IFN-γ signaling effect. It is possible that MAH 104 modulate the epigenome response by DNA methylation to enhance its survival in the macrophage. This modulation may also be responsible for the triggering of M2 phenotype observed during MAH104 infection of macrophages. More work is needed to determine the MAH104 effectors that phosphorylate MAT2B and the epigenetic enzymes that may be involved in the modulation of the epigenome response.