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Channing Lin March 11, 2020

Non-Tuberculous Mycobacteria Infection Changes Macrophage Nitric Oxide Levels and Mitochondrial Bioenergetics: Improvement by mitoTEMPO

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Abstract

Non-Tuberculous Mycobacteria Infection Changes Macrophage Nitric Oxide Levels and Mitochondrial Bioenergetics: Improvement by mitoTEMPO

By Channing Lin

Non-tuberculous mycobacteria (NTM) are ubiquitous bacilli that cause lung infections in patients with a weakened immune system or an underlying lung disease such as cystic fibrosis, COPD, or prior tuberculosis. While it is well-established that NTM cause lung disease, there are currently no established treatment regimens, and the host response to NTM infection has not been well-characterized. The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by innate immune cells is considered a major defense mechanism against mycobacteria. Nitric oxide (NO), a bactericidal and anti-inflammatory product of inducible nitric oxide synthase (iNOS) in macrophages, plays an essential role in M. tuberculosis (MTb) infection. Therefore, we hypothesized that NTM infection may upregulate iNOS expression, which in turn may contribute to the host response to NTM infection. RAW 264.7 cells, virus-transformed murine macrophages, MH-S alveolar macrophages, and bone marrow-derived macrophages (BMDM) were exposed to NTM, specifically M. avium-intracellulare (MAI) and M. abscessus (MAB), in our studies. NO levels decreased following 4 hours of NTM exposure and remained decreased for 48 hours. NTM infection increased the expression of iNOS ($P<0.05$). siiNOS2-transfected cells and L-NNA-inhibited cells infected with MAI had greater levels of intracellular MAI compared to control. Disrupted mitochondrial biology and decelerated bioenergetics were observed in MAI infection ($P<0.05$). Our data are the first to demonstrate that NTM increases NOS2 expression while decreasing NO levels. We hypothesize that ROS reduce NO availability and that mitochondrial antioxidant such as mitoTEMPO can alleviate infections. We found that the increase in ROS levels during MAI infection could be reduced by mitoTEMPO and that mitoTEMPO restores cellular bioenergetics and improves NO levels and bacterial killing. Therefore, antioxidant treatments targeting ROS can be developed as an alternative to conventional antibiotic treatments.

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CHAPTER 1

Introduction

Non-Tuberculous Mycobacteria

Non-tuberculous mycobacteria (NTM) are Gram-positive, acid-fast, rod-shaped bacilli that are commonly found in the environment. Humans are frequently exposed to NTM, as the bacteria live in natural waters, engineered water systems, and soil[1]. NTM are coated with a lipid-rich cell wall that not only serves as a hydrophobic biofilm, but also enables the bacteria to adhere to hard surfaces, like pipes, drains, and faucets. Biofilms inside pipes can remain attached even when the flow rate of water is high. Because of their ability to form biofilms, NTM are notably resistant to decontamination with common disinfectants and antiseptics, including chlorhexidine and glutaraldehyde[2, 3].

NTM are usually acquired through environmental exposure and not transmitted from human to human, except for immunocompromised or cystic fibrosis patients[4]. In adults, NTM most frequently infect the lung, although they can also infect the skin and soft tissue[5]. In children, lymphadenitis is the most common symptom[6]. While NTM do not cause tuberculosis or leprosy, NTM are associated with the development of NTM pulmonary disease (NTM-PD).

NTM-PD has become increasingly prevalent worldwide in the past two to three decades, particularly in Western countries, where most clinical cases of NTM are located[7]. The increased emergence of NTM-PD may be the result of declining incidence of tuberculosis (TB) in developed countries, aging population with chronic lung diseases, and improved identification of pulmonary abnormalities with advances in radiological diagnostics[8, 9]. Symptoms of NTM-PD are variable and may be indistinguishable from TB, lung cancer, and other respiratory

diseases[10, 11]. However, almost all patients present with chronic or recurring cough[11]. Other symptoms may consist of sputum production, fatigue, malaise, dyspnea, fever, hemoptysis, chest pain, and weight loss[11].

While some NTM species are pathogenic, most NTM species do not cause disease in humans[10]. NTM species can be categorized into rapid and slow growers. Several examples of the rapid growers include *Mycobacterium abscessus* (MAB), *M. fortuitum*, *M. chelonae*, and *M. smegmatis*, while those of slow growers include *M. avium* complex (MAC; consists of *M. avium*, *M. intracellulare* {MAI} and *M. chimaera*), *M. kansasii*, *M. marinum*, and *M. xenopi*. The most virulent strains of NTM include MAB, MAC, and *M. kansasii*, and they are most commonly isolated from NTM-PD patients[11].

Known risk factors for the development of NTM-PD are abnormal lung anatomy, e.g. bronchiectasis from unrelated prior infections, pneumoconiosis, and emphysema; genetic disorders that lead to bronchiectasis and/or lung infections, e.g. cystic fibrosis, alpha-1-antitrypsin deficiency, and primary ciliary dyskinesia; and immunodeficiency disorders, e.g. HIV/AIDS. [11, 12]. Infection in patients with cystic fibrosis and primary ciliary disease has been investigated extensively. Abnormally low ciliary beat frequency, abnormal response to bacterial toll-like receptor (TLR) agonists, and decreased nasal nitric oxide (NO) are associated with deceased ability to clear bacteria in the respiratory tract[13]. Interestingly, numerous cases of NTM-PD are correlated to immunocompetent, slender, postmenopausal women, and this is probably due to abnormal expression of adipokines, sex hormones, and/or TGF- β[14].

Antibiotic resistance is common in NTM-PD and can greatly contribute to poor clinical outcomes for patients. Due to their good microbicidal activity against NTM, tolerability in long-term use, and oral availability, macrolide antibiotics, e.g. clarithromycin and azithromycin, are critical drugs for treating NTM-PD[11, 15]. Consequently, the development of acquired macrolide resistance can exacerbate patient outcomes and cause treatment-resistant NTM-PD[16, 17]. Furthermore, MAB exhibits both acquired and intrinsic resistance to macrolide, making NTM-PD caused by MAB more difficult to treat. Unlike treatment regimens for *M. tuberculosis*, those for NTM have not been extensively investigated clinically. As a result, treatment decisions are usually made based on case studies and expert opinion in published guidelines[11, 18, 19].

Macrophages in NTM Immune Response

Despite all the information stated above, little is known about the pathogenesis of NTM on the molecular level. In innate immunity, macrophages are the first line of defense against intracellular infections such as NTM. Macrophages can eliminate NTM through the expression of pattern recognition receptors (PRR), production of cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS), and maturation of phagolysosome, including other killing mechanisms such as autophagy and cell death. The following is an introduction to what is known about the macrophages' role in NTM infection.

The entry of NTM into host cells begins with NTM's recognition of extracellular PRRs, including TLRs and C-type lectins (CTLs), which facilitate the internalization of microbes via

phagocytosis. As pathogen-associated molecular patterns (PAMPs) on NTM's surface bind to PRRs on host cell surface, inflammatory signaling is triggered, and NTM are phagocytosed. Phagocytosis of NTM leads to the development of phagosomes, which mature and merge with lysosomes to ultimately become phagolysosomes that can achieve killing of intracellular bacteria (Figure 1.1)[20]. PAMP binding to cytosolic receptors – e.g. leucine-rich repeat (LRR)-containing nucleotide-binding and oligomerization domain (NOD) proteins, and NOD-like receptors (NLRs) – can lead to pro-inflammatory cytokine production, autophagy activation, and cell death[21].

Although macrophages are important effector cells for immunity against intracellular bacteria, they are exploited as host cells by some microorganisms such as *Mycobacterium tuberculosis* (MTb) and some NTM species[22, 23]. Mycobacteria can evade antimicrobial mechanisms to survive and replicate within macrophages through various processes. The development of phagosomes into phagolysosomes requires acidification accomplished by proton pumps and fusion with lysosomes in addition to activation of degradative enzymes and other microbicidal effectors[20]. Some mycobacteria, such as MTb and *M. avium*, can manipulate phagosomal environments to make it less damaging by excluding proton pumps and preventing lysosome fusion[24-26]. The arrest of lysosomal fusion is achieved by maintaining tight apposition between mycobacterial surface lipids and the phagosome membrane[27].

Through ESX secretion system (also known as type VII secretion system), pathogenic mycobacteria can escape from phagosomes into the cytosol or communicate with the extra-

phagosomal environment by secreting virulence factors[28]. MTb can also block phagosome maturation in macrophages with the cell wall component, lipoarabinomannan (LAM). When LAM is incorporated into the membrane rafts of macrophages, it can block calcium signaling and inhibit enzymes responsible for fusion and fission of vesicles necessary for phagosome maturation. However, possibly through induction of and intersection with the autophagic pathway, IFN-γ activated macrophages can overcome blockage of phagosome maturation[23].

Nitric Oxide and Reactive Oxygen Species

As mycobacteria are in the phagolysosome, they are deprived of essential nutrients and are exposed to microbicidal effectors – antimicrobial peptides/proteins, such as cathelicidin, ROS, and RNS. Production of ROS and RNS is a rapid process that can happen minutes after phagocytosis of pathogens and is carried out by inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX2), respectively[20, 29].

NO is an important signaling molecule for maintaining cellular homeostasis. Produced endogenously, NO is also critical in host defense against infections[30]. NO is produced by one of the nitric oxide synthase (NOS) isoforms – neuronal NOS, iNOS, and endothelial NOS, also known as NOS1, NOS2, and NOS3, respectively. While NO produced by nNOS and eNOS mainly functions in intracellular signaling[31], NO produced, in notably higher amounts than other isoforms, by iNOS functions is a critical microbicidal molecule in the innate immune system[30]. Compared to other isoforms, iNOS is less affected by NO feedback inhibition[32, 33]. This allows sustained production of NO for immunity against infection.

NO exert its antimicrobial property in two ways. At low concentration, NO is a signaling molecule that supports growth and activity of the immune system[34]. At high concentration, NO covalently binds DNA, proteins, and lipids, thereby inhibiting and killing pathogens[35]. RNS, formed from autoxidation of NO[36], can directly react with DNA and result in DNA damage, which includes DNA strand breaks and deamination of cytosine, guanine, and adenine[30, 37]. In addition to reaction with DNA, NO can also inhibit DNA repair and increase generation of genotoxic molecules, alkylating agents and hydrogen peroxide[36]. NO is an integral and highly conserved part of host immune response, and, expectedly, bacteria are rarely able to evade its antimicrobial effects.

In the phagosome, NO and ROS react spontaneously to generate toxic and highly reactive intermediates that can disable pathogens by oxidizing DNA, membrane lipids, and thiol- and tyrosine- protein residues[38-40]. The majority of ROS in a cell is generated as a byproduct of ATP synthesis through oxidative phosphorylation in the mitochondria[41]. ROS plays a crucial role in many physiological signaling pathways, including innate immunity, proliferation, and cell death[41, 42]. Despite being an important inflammatory signaling molecule, overproduction of ROS can be detrimental to the host. High ROS production is associated with oxidative damage of mitochondrial DNA, proteins, and membranes[43]. This is because ROS is generated in the mitochondrial matrix, where mtDNA is located. ROS can also impact mitochondrial ATP synthesis and other metabolic pathways that happen in the mitochondria, such as fatty acid oxidation, amino acid metabolism, and the TCA cycle[44]. The Keap1-Nrf2 system functions as a stress sensor that regulates cytoprotective gene expression to oxidative stress[45]. However, it has been shown that Keap1-Nrf2 system can enable

intracellular growth of MAI by downregulating the necessary inflammatory signaling for containing MAI infection[46].

Because of the low amounts of NO produced by macrophages, the role of NO during mycobacterial infection of macrophages was not well understood until recently. In multiple studies, it has been shown that iNOS and RNS play an important role in MTb infection of human macrophages[47-49]. Particularly, NO has been demonstrated to protect mice by repressing interleukin-1- and 12/15-lipoxygenase-dependent neutrophil recruitment cascade that promotes bacterial replication[49]. NO not only possesses microbicidal characteristics, but it can also limit damaging inflammation. Therefore, disruptions of nitric oxide synthase may result in a weakened immune response against mycobacterial infection.

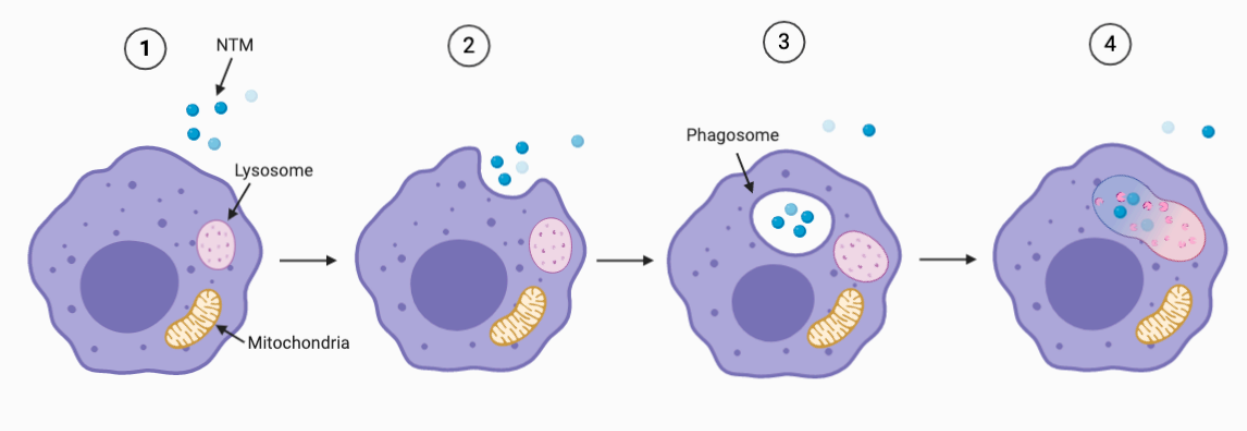


Figure 1.1 Phagosome Maturation and Bacterial Killing. **1)** Macrophage's PRRs recognize NTM's molecular patterns. **2)** Phagocytosis and Inflammatory signals triggered. **3)** Phagosome forms. **4)** Phagosome merge with lysosome and mature, becoming phagolysosome. NTM are exposed to microbicidal effectors such as ROS, RNS, and degradative enzymes.

Mitochondria

Mitochondria are autonomous cellular organelles responsible for many functions that are important for cell homeostasis, such as metabolism, energy production, and calcium buffering[50-53]. Apoptosis is an evolutionarily preserved programmed cell death important in many physiological processes, such as aging, embryonic development, and immune function[54]. Apoptosis is one of the innate defense mechanisms that is initiated during stress caused by pathogens. As a key regulator of innate immune response and cell stress, mitochondria play an important role in controlling apoptotic cell death[55-59].

Pathogenic bacteria have developed numerous ways to manipulate host cells for their survival by either inhibiting or promoting apoptosis. As observed in MTb infection, mycobacteria can inhibit apoptosis in macrophages by depletion of cytochrome c in mitochondria and upregulating antiapoptotic proteins, such as B-cell lymphoma (Bcl)-2[60-62]. Similarly, rough morphotype of MAB's cell surface glycopeptidolipids can target mitochondria and suppress apoptotic response by reducing ROS production and inhibit cytochrome c release[63].

How NTM affect macrophage energy metabolism for their own survival is poorly understood. Many diseases – such as diabetes, neurodegeneration, cancer, and cardiac disease – are correlated to abnormal cellular bioenergetic profiles. The aberrant bioenergetics in these diseases has been successfully characterized using metabolic extracellular flux (MXF) analysis[64-67]. Furthermore, MTb decreases the rate of glycolysis and oxidative phosphorylation, thereby slowing down mitochondrial ATP production. In MTb infection,

decreased mitochondrial dependency on glucose and increased dependency on exogenous fatty acids are observed indicating mitochondrial stress[68]. Disrupted mitochondrial bioenergetic function are associated with macrophage damage, increased inflammatory signaling, and impaired host defense. Such dysfunction has been observed in MTb infection of human macrophages, in which tuberculosis necrotizing toxin triggers the production of ROS in mitochondria, promoting cell death and mycobacterial replication[69, 70].

Summary and Hypothesis

The incidence and prevalence Non-Tuberculous Mycobacteria Pulmonary Disease (NTM-PD) is rising worldwide. While humans are exposed to NTM constantly, people with risk factors such as underlying lung disease and immunodeficiency are more susceptible. There are currently no established treatment regimens for NTM-PD, and NTM is notorious for its bacterial resistance, which often contributes to poor treatment outcomes.

Macrophages are important effector cells in innate immunity against intracellular infections such as NTM. Macrophages ingest mycobacteria via phagocytosis and expose them to microbicidal effectors, such as nitric oxide (NO) and reactive oxygen species (ROS), in addition to other degradative enzymes, in the phagolysosome. However, NTM have evolved ways to persist and replicate in macrophages, e.g. blocking phagosome maturation, escaping into the cytosol, and altering the phagosomal environment.

NO is an important microbicidal molecule that also functions in cell signaling to promote the growth of immune cells at low concentrations. In response to intracellular pathogens, inducible nitric oxide synthase (iNOS) is expressed and produces NO to fight off the infection. In multiple studies, it has been shown that NO plays an important role in MTb infection of human macrophages.

Like many other diseases, mycobacterial infections can impact mitochondrial function. Mitochondria are a key regulator of apoptosis, programmed cell death critical for innate immune function. However, pathogenic bacteria manipulate apoptotic processes, either by inhibiting or promoting apoptosis, for their survival and replication. Mycobacterial infection can also lead to aberrant mitochondrial bioenergetics, contributing to increased mitochondrial ROS, which promotes inflammation, cell death, and bacterial replication. Therefore, we hypothesize that NTM infection upregulates iNOS expression, which may, in turn, contribute to host defense. We also hypothesize that mitochondrial ROS, whose production is correlated with disrupted mitochondrial function, scavenges NO to form toxic radicals, thereby inhibiting endogenous NO's bactericidal and anti-inflammatory effects. We propose that approaches to restore mitochondrial function and/or inhibit mitochondrial ROS can enhance macrophage host defense against mycobacteria.

CHAPTER 2

Methods

Cell Culture

RAW 264.7 cell line, a macrophage-like cell line that was derived from tumors induced in male BALB/c mice, was obtained from American Tissue Type Culture Collection (Manassas, VA) and grown in Dulbecco modified Eagle medium (DMEM) – high glucose culture medium with 10% fetal bovine serum (FBS), 50 IU of penicillin per mL, and 50 mg of streptomycin per mL. Cells were cultured in a 5% CO_2 humidified incubator at 37°C.

The mouse alveolar macrophage cell line, MH-S was obtained from American Tissue Type Culture Collection (ATCC) and grown in RPMI 1640 medium (HyClone, Logan, UT) with 10% FBS, 0.05 mM 2-mercaptoethanol, 11.9 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (40 µg/ml). Cells were cultured in a 5% CO_2 incubator with humidity in 37°C.

All cells were counted using Countess II Automated Cell Counter and Trypan Blue Stain (Thermo Fisher Scientific, Waltham, MA).

BMDMs

Bone marrow-derived macrophages (BMDMs) were derived from C57BL/6J mice. Mice were euthanized by CO_2 asphyxiation and their cellular material was aspirated from the femur and centrifuged at 400 X g at 4°C for 5 minutes. The cells were then resuspended in DMEM containing 10% FBS and 10% L929 cell-conditioned medium with macrophage colony-stimulating factor. The cells were incubated for 5 days in the presence of L929 cell-conditioned

medium, which allows them to mature into phenotypic macrophages. The purity of macrophages was confirmed by flow cytometry (>90% $CD11b^+$; $F4/80^+$).

NTM Stocks

NTM (*M. abscessus* and *M. avium-intracellulare*) cells were provided by the Emory clinic following isolation from patients and grown on Lowenstein-Jensen slants at 35°C in 5% CO_2. NTM were suspended in phosphate-buffered saline (PBS). The optical density (OD) of the resulting mixture was determined at a wavelength of 600 nm to estimate the bacterial density, which was confirmed by plating the mixture on Middlebrook 7H11 Agar (BD Bioscience, San Jose, CA) and calculating colony forming unit (CFU) per milliliter. In our studies, OD of 0.090 at a wavelength of 600 nm corresponded to 6.85 X 10^8 CFU/mL, approximately.

Reagents

S-Nitrosoglutathione (GSNO), Mercury (II) chloride ($HgCl_2$), Sulfanilamide, N-1-naphthylethylene-diamine dihydrochloride, lipopolysaccharide (LPS) and L-N^G-Nitroarginine (L-NNA) were obtained from Sigma-Aldrich (St. Louis, MO). MitoTEMPO and clarithromycin were obtained from Cayman Chemical (Ann Arbor, MI). For cell treatments, reagents are diluted with or dissolved in PBS.

Real-Time Quantitative Reverse Transcription PCR (RT-qRT-PCR)

Total cellular RNA was extracted from MH-S and RAW 264.7 cells using an RNeasy RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Briefly, the cells were lysed with RLT lysis buffer with 2% β-mercaptoethanol. A volume of 70% ethanol equal to RLT buffer was added to the lysate, and the resulting mixtures were centrifuged for 15 seconds at 8000 x g in the supplied RNeasy Mini spin columns. After the flow-through was discarded, buffer RW1 was added, and samples were spun for 15 seconds at 8000 x g. Then, the flow-through was discarded, and the samples were centrifuged after buffer PRE was added. The samples were then eluted with RNase-free water into collection tubes by centrifugation for 2 minutes at 3000 x g. The concentrations of RNA were determined with Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized from 500 or 250 ng of total RNA using the SuperScript first-strand synthesis system (Fermentas, Pittsburgh, PA). Real-time PCR was performed with SYBR Green (Applied Biosystems, Hercules, CA) to analyze the expression of NOS isoforms, Mitochondrial Transcriptional Factor A (TFAM), and Mitofusin 2 (MFN2) in cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control for normalization. PCR was conducted using Applied Biosystems (ABI Prism, 7900HT). Data were analyzed using the $2^{-\Delta\Delta CT}$ method[71]. Mouse primer sequences for nNOS (Qiagen, PPM05114E), eNOS (Qiagen, PPM03801A), and MFN2 (Taqman, Mm00500120_m1) are not listed below because they are proprietary.

Gene	Species	Sequence (5’ -> 3’)
GAPDH	Mouse	Forward: GCC CAA TAC GAC CAA ATC C Reverse: AGC CAC ATC GCT CAG ACA C
iNOS	Mouse	Forward: GAT CTT CCC AGG CAA CCA Reverse: CCA CAA CTC GCT CCA AGA TT
TFAM	Mouse	Forward: GAA CAA CTA CCC ATA TTT AAA GCT CA Reverse: GAA TCA GGA AGT TCC CTC CA

Table 2.1 Sequences of primers used for RT-qRT-PCR

NTM Infection and Nitric Oxide Quantification Assay

RAW 264.7 cells were exposed to MAI and MAB at the multiplicity of infection (MOI) of 25 for 1, 4, 8, 11, 24, and 48 hours. MH-S cells were also exposed to the same strains of NTM but were exposed for 6, 24, and 48 hours. The nitric oxide (NO) concentration is determined using the nitric quantification assay, a colorimetric assay involving Griess reagents, with GSNO as standard. 200μL of Solution C (1 volume of 5% $HgCl_2$ in dH_2O and 10 volumes of 6.8% Sulfanilamide in 0.4N HCl) was added to 400 μL of standards, which contained diluted GSNO, or cell media collected after infection. To this mixture, 20 μL of Solution D (4% N-1-naphthylethylene-diamine dihydrochloride in 0.4N HCl) was added. The mixtures were incubated in room temperature for 10 minutes, and their absorbance was measured at 550 nm.

Absorbance values obtained were compared to a linear standard curve for GSNO that ranged from 0.055 to 0.30, approximately.

Transfection and NTM Infection

Gene silencing in MH-S cells was achieved through the second small interfering RNA (siRNA) in the Trifecta RNAiKit (Integrated DNA Technologies, Coralville, IA) targeting iNOS mRNA and scrambled control (Sc) siRNA (Life Technologies) as the negative control. Transfection was performed using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells were incubated with transfection complexes for 6 hours, after which fresh media and NTM were added. 48 hours after transfection, RNA was extracted, and cells were lysed for bacterial killing assay.

Bactericidal Killing Assay

MHS cells were plated on 24-well plates at a concentration of 50,000 cells/well and were treated with 300μM L-NNA, which is an inhibitor of NOS isoforms, or 100μM mitoTEMPO overnight. In the following morning, NTM were added at an MOI of 50 and incubated for 2 hours at 37°C with 5% CO_2. To remove extracellular bacteria, the cells were washed with PBS and incubated in the growth medium containing 20 μg/mL clarithromycin in addition to 300 μM L-NNA or 100μM mitoTEMPO. The cells were further incubated for 4, 24, and 48 hours. After each incubation time, cells were counted with an automated cell counter. The cells were also

washed with PBS and lysed by incubation in PBS containing 0.1% triton X-100 (Thermo Fisher Scientific) for 5 minutes at 37°C. The lysed cell samples were serially diluted in sterile PBS and cultured on 7H11 agar plates. The plates were incubated at 37°C with 5% CO_2 and humidity for 5 days before quantifying CFUs.

Mitochondrial Bioenergetics

The Seahorse XF analyzer Cell Energy Phenotype assay (Agilent Technologies, Santa Clara, CA) was performed on RAW 264.7 cells treated for 24 hours with NTM supernatants. Bacterial supernatants were used because we found that cells treated with bacteria have unusual ECAR and OCR levels and do not respond to mitochondrial inhibitors. NTM supernatants were made by incubating NTM with RAW 264.7 cells at MOI of 25 for 24 hours in the DMEM. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed before and after treatment of cells with mitochondrial toxins, which included oligomycin (1 µM), an ATP synthase inhibitor, and Carbonyl cyanide-4-phenylhydrazone (FCCP, 0.25 µM), an uncoupling agent, to determine baseline and stressed levels of oxidative phosphorylation and glycolysis, respectively. The Seahorse XF analyzer Mito Stress Test assay (Agilent Technologies) was also performed on RAW 264.7 cells treated for 24 hours with NTM supernatants. OCR is plotted over time in response to a series of treatments with inhibitors of the electron transport chain: oligomycin (1 µM), FCCP (0.25 µM), and rotenone and antimycin A (0.75 µM). OCR and ECAR levels were normalized to cell number.

Reactive Oxygen Species Measurement

RAW 264.7 cells were detached from cell culture plates by scraping with blunt pipette tips. The cells were then collected by centrifugation and resuspended in media. MitoSOX™ Red (Invitrogen) was added at a final concentration of 5 mM for 20 mins at 37 °C. The cells were washed once in warm PBS immediately before fluorescence-activated cell sorting (FACS) analysis. Cells were acquired using CytoFLEX Flow Cytometer (Beckman, Indianapolis, IN) and analyzed using CytExpert for CytoFLEX Acquisition and Analysis Software. The level of intracellular ROS corresponded to an increase in fluorescence. Cells expressing fluorescence above a defined threshold were considered positive and were evaluated based on the population defined by double discrimination gating and the unstained and untreated controls.

Mitochondrial DNA Damage

Mitochondrial DNA damage was determined as described previously[72]. Briefly, genomic DNA was extracted using DNeasy kit (Qiagen), and quantitative PCR was performed on genomic DNA using SYBR Green PCR genomic cDNA synthesis kit (Thermo Fisher Scientific). DNA damage was assessed by measuring the relative content of the copy number of short mtDNA-79 bp fragments, which are damaged DNA, to the copy number of the long mtDNA-230 fragments, which are intact DNA, of the 16S-RNA gene. The sequence for the forward primer (both, mtDNA79 and mtDNA230) was 5’-CAGCCGCTATTAAAGGTTCG-3’, and the reverse primers were 5’-CCTGGATTACTCCGGTCTGA-3’(mtDNA79) and 5’-GGGCTCTGCCATCTTAACAA-3’ (mtDNA230).

CHAPTER 3

Results

Effects of NTM Infection on Expression of Nitric Oxide Synthase Isoforms

To determine if NTM infection affects the expression of NOS isoforms, RNA was extracted from MH-S macrophages treated with two strains of NTM (MAI or MAB). RT-qRT-PCR and mRNA analysis on NOS isoforms – nNOS, iNOS, and eNOS – were performed. Cells were treated with NTM at an MOI of 25 for 6, 24, and 48 hours. After exposure to both strains of NTM, iNOS mRNA expression increased, and the increase was significant at 48 hours of exposure (Figure 3.1). There was no significant change in the expression of nNOS and eNOS mRNA after NTM treatment.

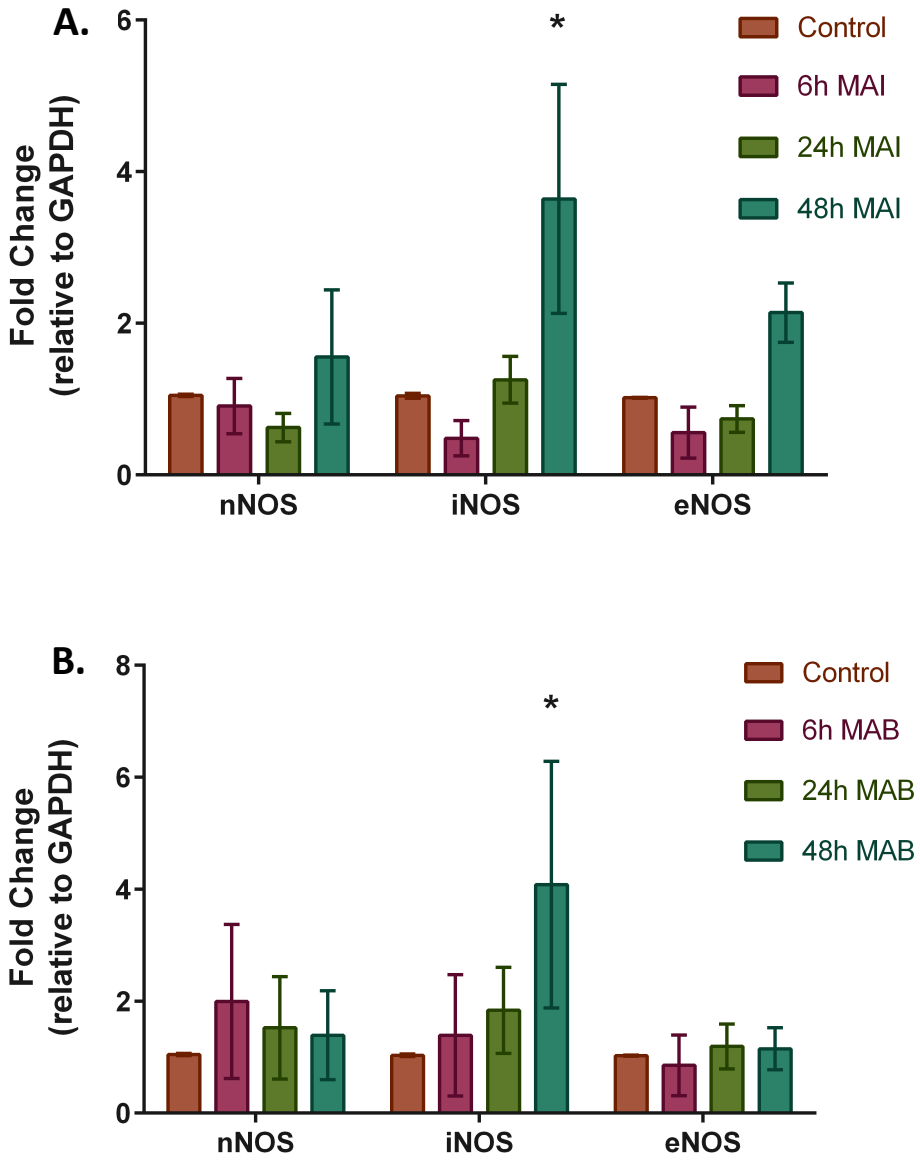


Figure 3.1. Effects of NTM exposure on NOS isoform expression. **A)** Expression of NOS isoforms measured in MH-S cells by RT-qRT-PCR following 6, 24, and 48 hours of treatment with *M. avium-intracellulare* at an MOI of 25 (n=4, 2 replicates). **B)** Expression of NOS isoforms measured in MH-S cells by RT-qRT-PCR following 6, 24, and 48 hours of treatment with *M. abscessus* at an MOI of 25 (n=4, 2 replicates). *p<0.05; multiple comparison by two-way ANOVA. Error bars represent mean ± SEM.

Effects of NTM Exposure on Nitric Oxide Levels

To determine the effects of NTM infection on macrophage NO production, we quantified NO in cell media of cells post-infection. We infected RAW 264.7 cells with both strains of NTM at an MOI of 25 for various time intervals and collected the cell media for NO quantification. In MAI infection, NO levels decreased at 1 hour of infection and remained decreased up to 11 hours of infection (Figure 3.2A). In MAB infection, the NO concentration decreased at 1 hour of infection and remained decreased for 48 hours (Figure 3.2B).

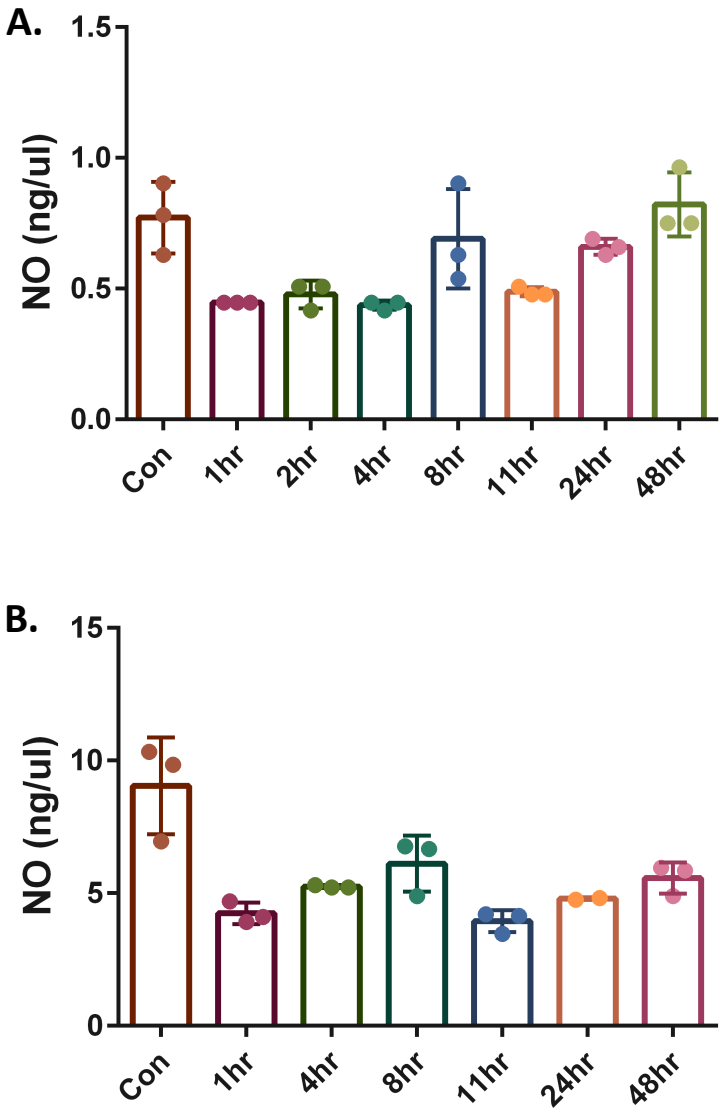


Figure 3.2. Effects of NTM exposure on NO levels. **A)** NO concentration in cell media measured in RAW 264.7 cells by NO quantification assay following 1, 2, 4, 6, 8, 11, 24, and 48 hours of treatment with *M. avium-intracellulare* at an MOI of 25 (n=1, 3 replicates). **B)** NO concentration in cell media measured in RAW 264.7 cells by NO quantification assay following 1, 4, 8, 11, 24, and 48 hours of treatment with *M. abscessus* at an MOI of 25 (n=1, 3 replicates). Error bars represent mean ± SEM.

Bacterial Killing

To study the effects of knocking down iNOS on intracellular bacterial killing, we conducted transfection on MH-S cells using iNOS siRNA and scrambled control before infecting with NTM and determined bacterial killing. Following the transfection, cells were infected with MAI at an MOI of 25 for 2 hours. After clearing all the extracellular bacteria, we incubated the cells in growth media for 0.5, 4 and 24 hours. Then, the cells lysates were plated on 7H11 agar plates. For 4- and 24-hour incubation times, the cells with silenced iNOS have higher intracellular CFU/cell compared to their respective scrambled control (Figure 3.3A).

Using another way to suppress iNOS function, we treated MH-S cells with 300 µM L-NNA before infecting them with MAI at an MOI of 50. After 4- and 24-hour incubation times, cell lysates were plated on 7H11 agar plates. After 24 hours of incubation, cells treated with L-NNA have higher intracellular CFU per milliliter of lysate compared to its control (Figure3.3B).

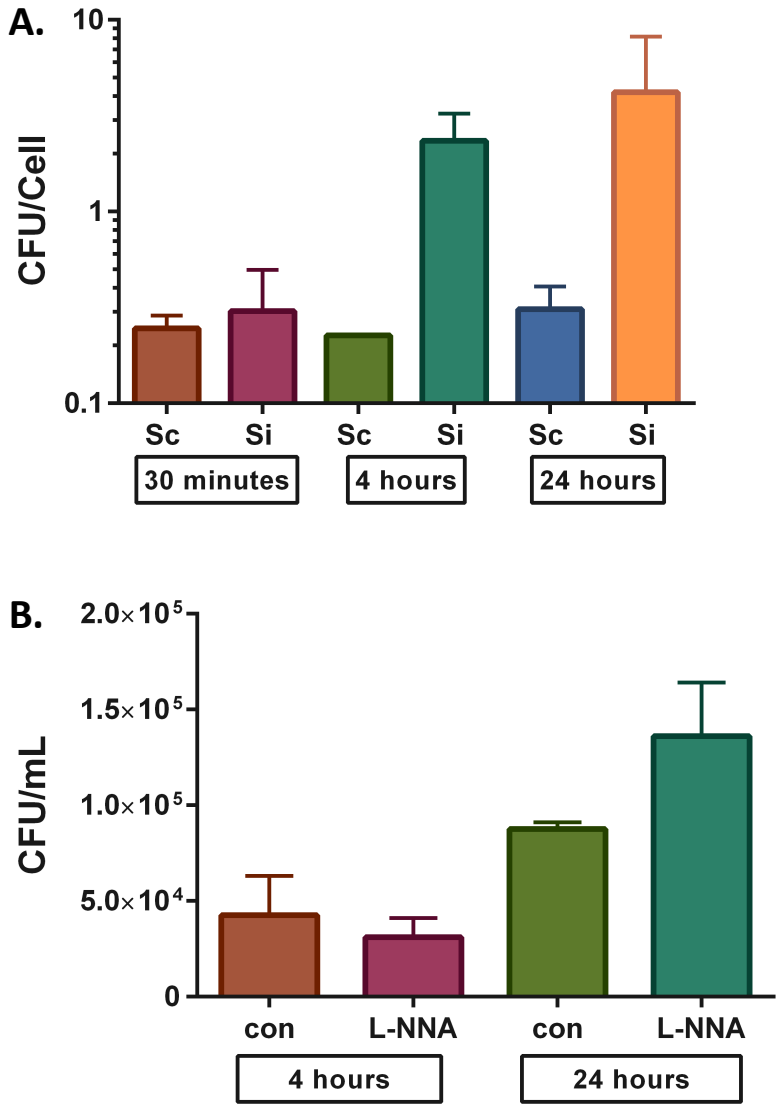


Figure 3.3. Effect of iNOS inhibition on NTM killing. **A)** Intracellular CFU determined in scrambled control (sc) and iNOS siRNA transfected (si) MH-S cells by using a bacterial killing assay following 2 hours of infection with *M. avium-intracellulare* (MOI of 25) and 0.5, 4, and 24 hours of incubation (n=1, 2 replicates). **B)** Intracellular CFU determined in MH-S cells treated with 300 µM L-NNA using a bacterial killing assay following 2 hours of infection with *M. avium-intracellulare* (MOI of 50) and 4 and 24 hours of incubation (n=1, 2 replicates). Error bars represent mean ± SEM.

Mitochondrial Biology

To determine how MAI infection can impact mitochondria of macrophages, we examine mitochondrial DNA damage and changes in expression of two important mitochondrial transcriptional regulators – TFAM and MFN2. Mitochondrial DNA damage was assessed by determining the mtDNA 79bp:230bp ratio after we infected BMDMs with MAI at an MOI of 25 for 6 and 24 hours. At 24 hours, higher 79bp:230bp ratio was observed in MAI infected cells compared to its control (Figure 3.4). TFAM and MFN2 mRNA expression was determined after BMDMs were infected with MAI for 6 and 24 hours at MOIs of 25 and 50. Both genes showed a decrease in expression after 6 hours of infection (MOI = 50) and 24 hours of infection (MOI = 50 & 25; Figure 3.5A & B).

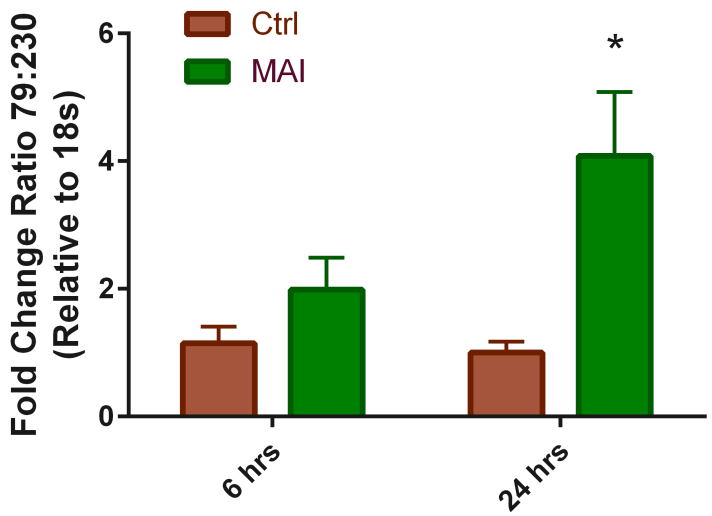


Figure 3.4. Effects of NTM on Mitochondrial Damage. Mitochondrial DNA 79:230 ratio in BMDMs was determined by quantitative PCR following 6 and 24 hours of treatment with *M. avium-intracellulare* at an MOI of 25 (n=3, 2 replicates). *p<0.05; multiple comparison by two-way ANOVA. Error bars represent mean± SEM.

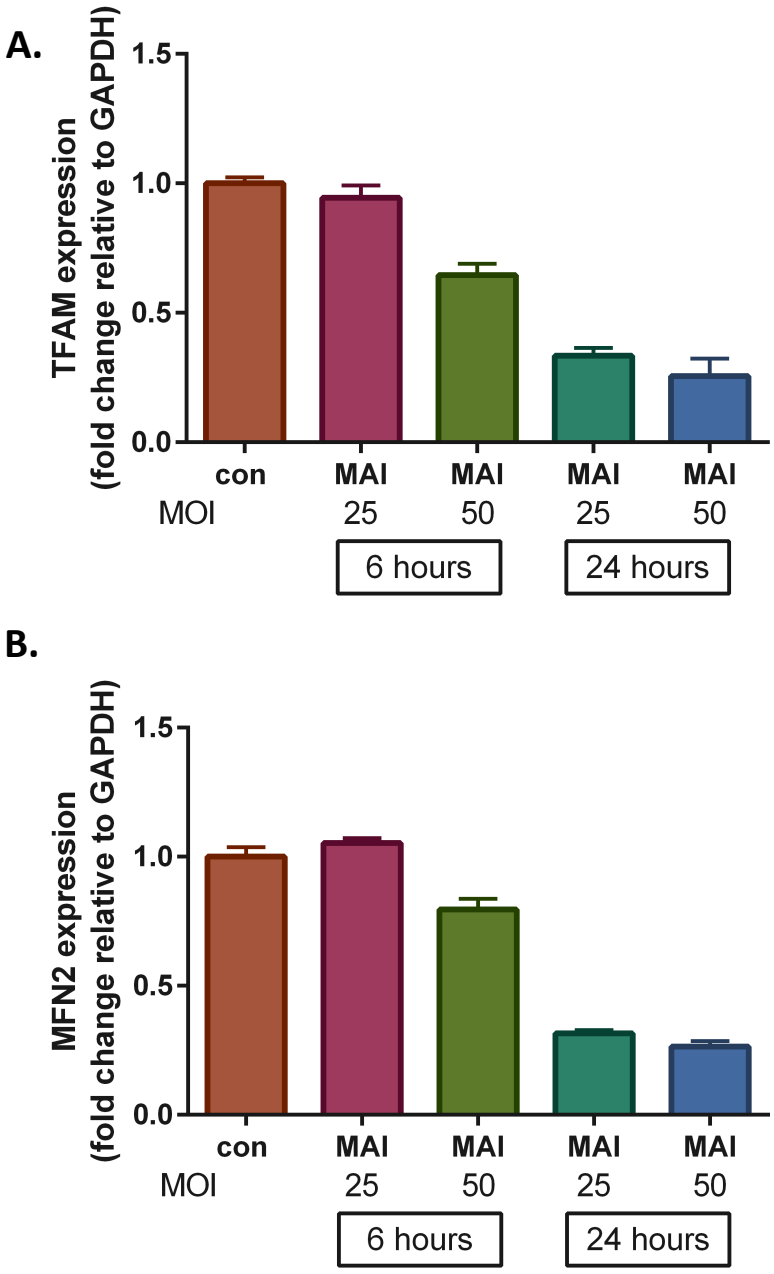
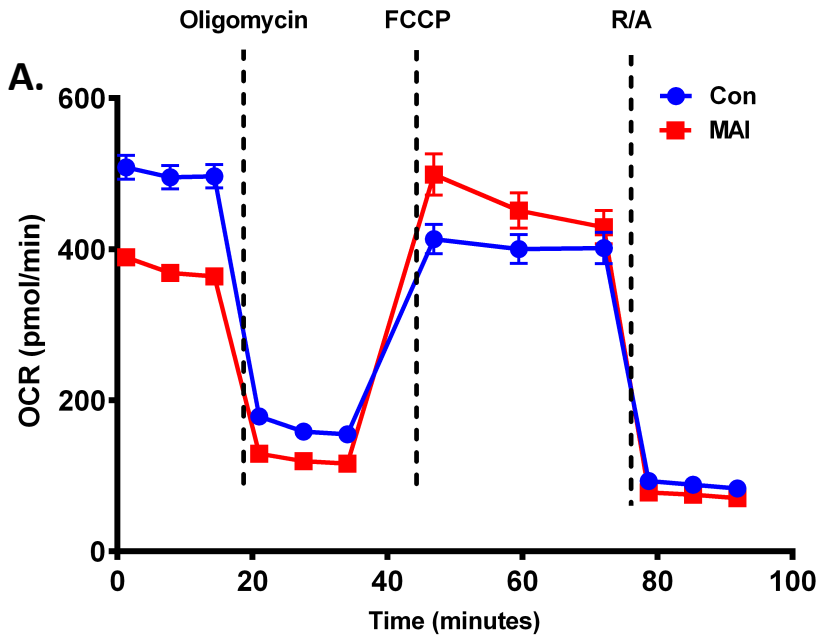
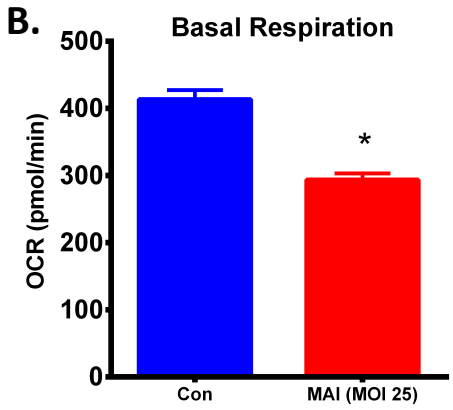


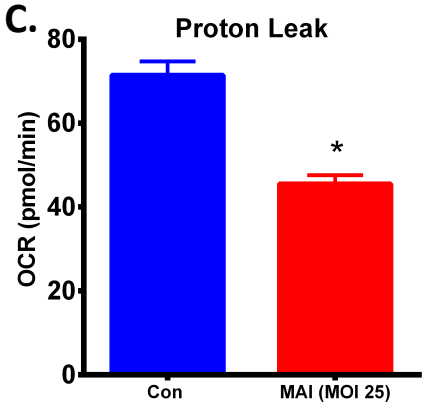
Figure 3.5. Effects of MAI infection on TFAM and MFN2 mRNA expression. **A)** TFAM expression measured in BMDMs by RT-qRT-PCR following 6 and 24 hours of treatment with *M. avium-intracellulare* at an MOI of 25 and 50 (n=1, 2 replicates). **B)** MFN2 expression measured in BMDMs by RT-qRT-PCR following 6 and 24 hours of treatment with *M. avium-intracellulare* at an MOI of 25 and 50 (n=1, 2 replicates). Error bars represent mean ± SEM.

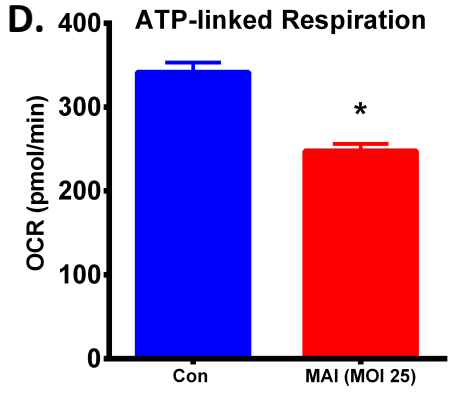
Effects of NTM exposure on Mitochondrial Bioenergetics

To better understand how MAI can affect macrophage mitochondrial bioenergetics, we examined detailed bioenergetic profiles of RAW 264.7 mitochondria using metabolic extracellular flux measurement in the absence or presence of MAI supernatant treatment for 24 hours. The analysis of mitochondrial bioenergetics data (Figure 3.6A) determined that treatment with NTM supernatant results in impaired basal respiration (Figure 3.6B), decreased proton leak (Figure 3.6C), and decreased ATP-linked respiration (Figure 3.6D) in RAW 264.7cells treated with MAI supernatants. Total ATP also decreased after 24 hours of MAI supernatant treatment (Figure 3.6E).


A.
Oligomycin
FCCP
R/A
Con
MAI
OCR (pmol/min)
600
400
200
0
0
20
40
60
80
100
Time (minutes)


B.
Basal Respiration
OCR (pmol/min)
500
400
300
200
100
0
*
Con
MAI (MOI 25)


C.
Proton Leak
OCR (pmol/min)
80
60
40
20
0
*
Con
MAI (MOI 25)


D.
ATP-linked Respiration
OCR (pmol/min)
400
300
200
100
0
*
Con
MAI (MOI 25)

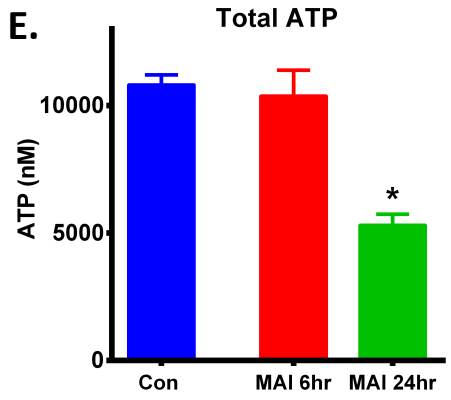

E.
Total ATP
ATP (nM)
10000
5000
0
*
Con
MAI 6hr
MAI 24hr

Figure 3.6 (on previous page). MAI exposure impacts mitochondrial bioenergetics. **A)** Oxygen consumption rate of RAW 264.7 cells assessed with extracellular flux measurement following MAI supernatant treatment. Measurements include **(B)** basal respiration, **(C)** proton leak, and **(D)** ATP-linked respiration (n=5). **E)** Total ATP in RAW 264.7 cells determined with fluorometric test following 6- and 24-hour MAI supernatant treatments (n=3). *$p<0.05$; multiple comparison by one-way ANOVA. Error bars represent mean ± SEM.

Effects of NTM on Mitochondria-derived ROS

Macrophage's mitochondrial function was shown to be impaired by MAI, and impaired mitochondria function can lead to higher mitochondrial ROS. Therefore, studies were completed to determine whether MAI exposure affects mitochondrial ROS levels and whether mitoTEMPO can reduce ROS levels in macrophages. In the presence or absence of mtTEMPO, RAW 264.7 cells were treated with MAI supernatants for 16 before ROS levels were evaluated. MAI-infected cells had higher fluorescence intensity (Figure 3.7A) and more cells positive for ROS detection (Figure 3.7B) compared to control and MAI-infected cells treated with mitoTEMPO.

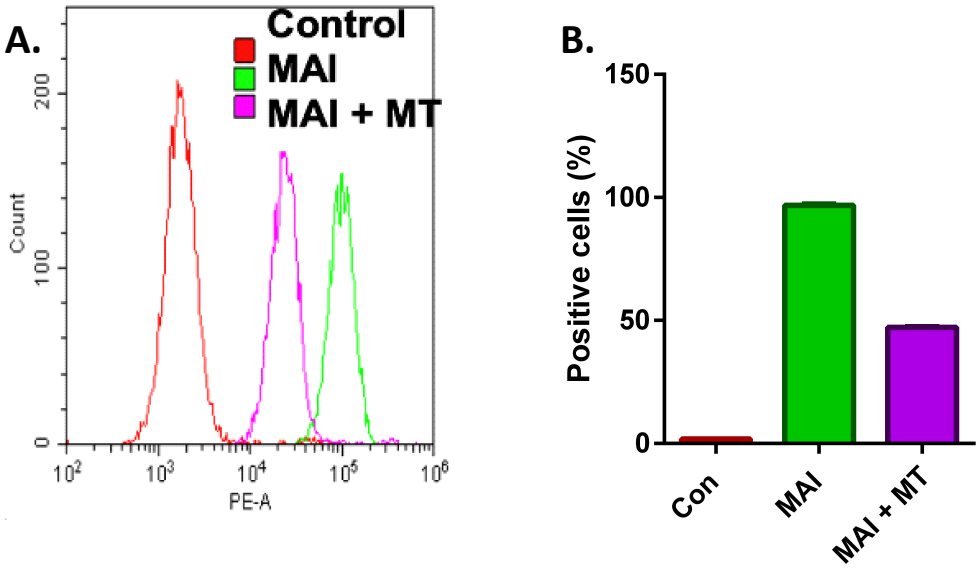
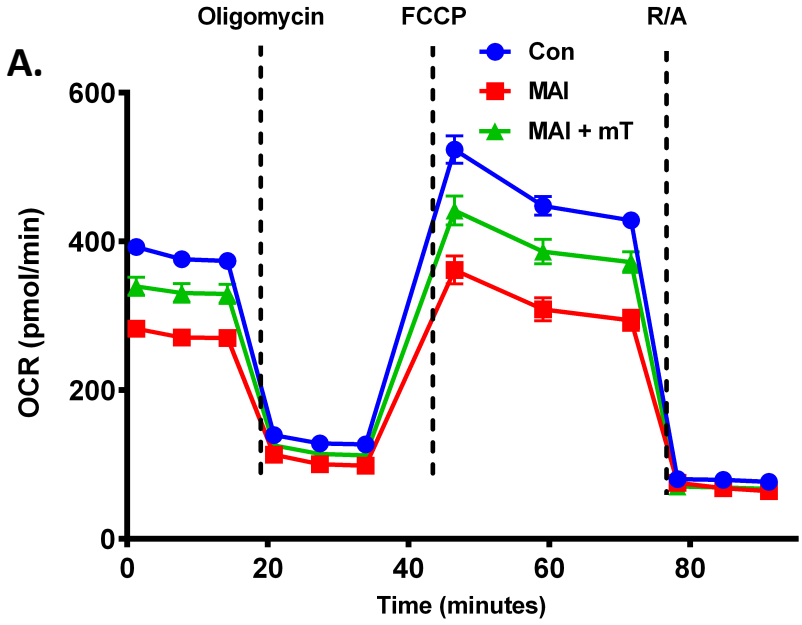


Figure 3.7 Effects of MAI on mitochondrial ROS levels. **A)** Fluorescence intensity (x-axis) of RAW 264.7 cells following MAI and MAI + mitoTEMPO treatments. **B)** Percentage of cells positive for presence of intracellular ROS obtained through CytExpert software (n=1, 2 replicates). Error bars represent mean ± SEM.

Treatment with mitoTEMPO Restores NTM Impairments in Mitochondrial Bioenergetics

To examine whether eliminating mitochondrial ROS with mitoTEMPO can improve mitochondrial bioenergetics, we performed metabolic extracellular flux measurement on RAW 264.7 cells treated with MAI supernatants and 1mM mitoTEMPO for 24 hours. The analysis of mitochondrial bioenergetics data (Figure 3.8A) revealed that infected cells treated with mitoTEMPO had higher proton leak (Figure 3.8C) and improved basal respiration (Figure 3.8B) and ATP-linked respiration (Figure 3.8D) compared to cells treated with MAI-only treatment.



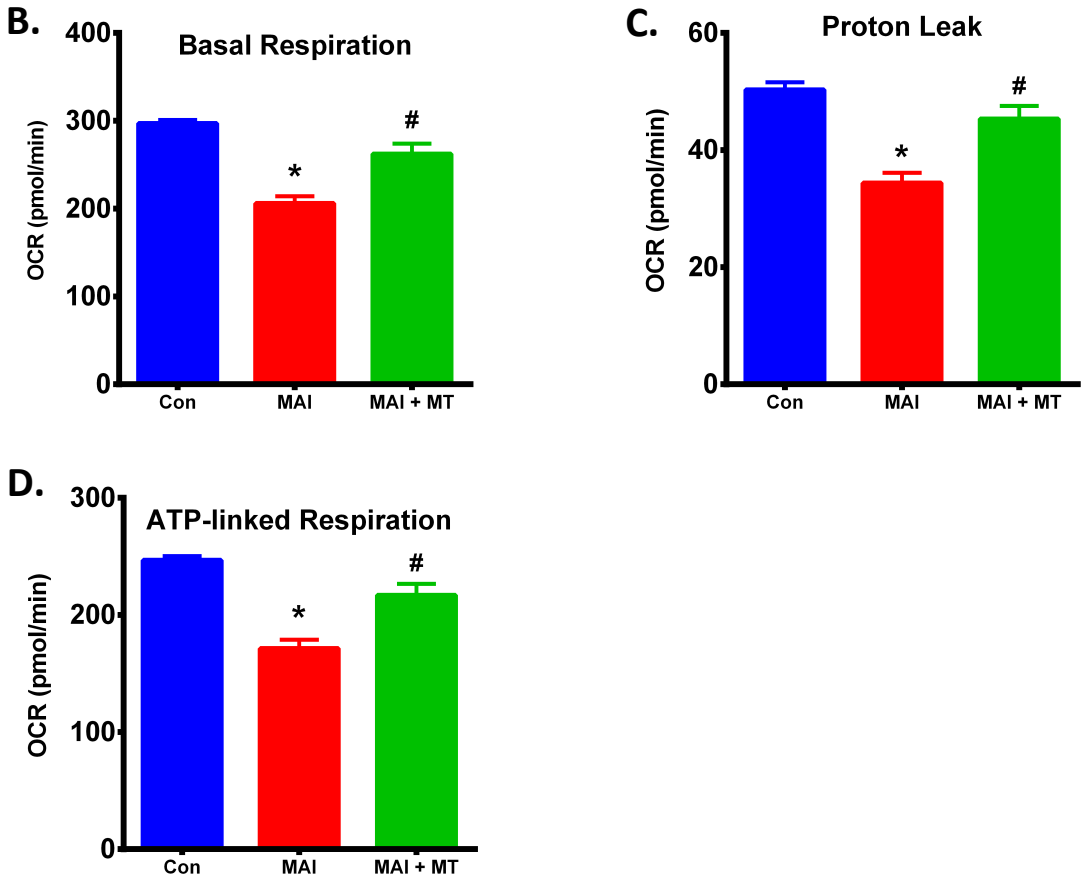


Figure 3.8. Effects of mtTEMPO pretreatment on mitochondrial bioenergetics in MAI infection. **A)** Oxygen consumption rate of RAW 264.7 cells assessed with extracellular flux measurement following exposure to MAI supernatant ± mtTEMPO. Measurements include **(B)** basal respiration, **(C)** proton leak, and **(D)** ATP-linked respiration (n=5). *$p<0.05$ compared to control, #$p<0.05$ compared to MAI; multiple comparison by one-way ANOVA. Error bars represent mean ± SEM.

Targeting Mitochondrial Superoxide in MAI Infection

To determine if reducing mitochondrial ROS with mitoTEMPO impacts NO levels in NTM-infected cells, MAI-infected cells (MOI = 25) were treated with mitoTEMPO at various concentration (10, 100, and 1000 μM), and NO present in the cell media was quantified. Cells treated with mitoTEMPO had significantly higher NO levels in their media compared to uninfected control and MAI-infected cells without mitoTEMPO treatment (Figure 3.9).

To assess whether eliminating mitochondrial ROS with mitoTEMPO can improve intracellular bacterial killing, MH-S cells were treated with MAI at an MOI of 50 in the presence or absence of mtTEMPO, and the bacterial killing assay was conducted. Following 4, 24, and 48 hours of incubation, cells were lysed and plated on agar plates. Across all incubation times, mitoTEMPO-treated cells had lower intracellular CFU per milliliter than control (Figure. 3.10).

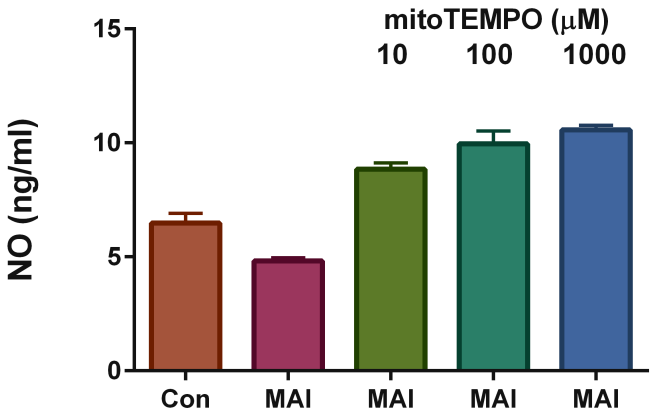


Figure 3.9. Effects of mitoTEMPO on NO levels in MAI infection. NO concentration in cell media measured in RAW 264.7 cells by NO quantification assay following treatment with various mitoTEMPO concentrations – 10, 100, 1000 μM – in addition to MAI infection at an MOI of 25 (n=1, 2 replicates). Error bars represent mean ± SEM.

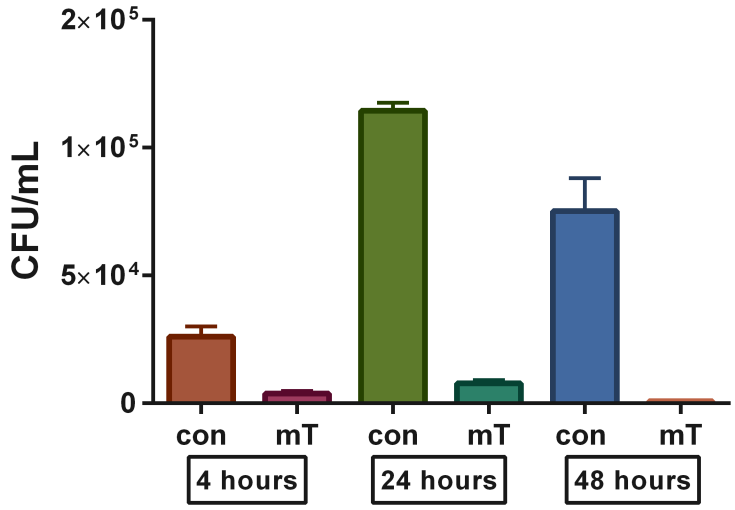


Figure 3.10. Effects of mitoTEMPO on intracellular CFU in MAI infection. Intracellular CFU determined in mitoTEMPO-treated MH-S cells by bacterial killing assay following 2 hours of infection with *M. avium-intracellulare* (MOI of 50) and 4, 24, and 48 hours of incubation (n=1, 2 replicates). Error bars represent mean ± SEM.

CHAPTER 4

Discussion

The current study provides supporting evidence that iNOS is involved in the immune response to NTM infection as shown by the significant increase in its mRNA expression in MH-S cells after 48 hours of infection by MAI and MAB, while, on the other hand, nNOS and eNOS play little or no role in macrophages' immune response (Figure 3.1). This is consistent with previous investigations that observed the induction of iNOS mRNA and protein in macrophages after mycobacterial infection[30]. During the time before the 48-hour mark, however, no change in iNOS expression was observed, which could be a result of blockage of phagosome maturation by mycobacteria observed previously [22-27]. After 48 hours, macrophages may have overcome the blockage and increased iNOS expression to kill pathogens. More experiments need to be conducted in order to determine the delayed response of the macrophages to infection.

Decreased NO levels in RAW 264.7 cells were observed in MAB infections for up to 48 hours and in MAI infection for up to 11 hours (Figure 3.2), which could be explained by no change in iNOS expression at 6 and 24 hours of infection (Figure 3.1). However, despite the increase in iNOS expression at 48 hours, no change in NO levels was observed. A possible explanation for this contradictory finding is that NO produced during an infection is scavenged by ROS, whose concentration was observed to increase in mycobacterial infections[69, 70], thereby decreasing NO levels.

Higher intracellular NTM is observed in iNOS-inhibited MH-S cells treated with iNOS siRNA or L-NNA. Intracellular NTM levels were elevated 6 and 24 hours post-incubation in siRNA-treated cells and 24 hours post-incubation in L-NNA-treated cells (Figure3.3). Consistent

with our hypothesis regarding the importance of iNOS, this finding suggests that, in the absence of iNOS, macrophages may not effectively eliminate the phagocytosed NTM and allow NTM to survive intracellularly. Furthermore, the observation that CFU increased in siNOS-transfected cells in a time-dependent manner may indicate intracellular bacterial replication occurs more in cells that lack iNOS.

As mentioned previously, MTb infection can lead to higher ROS levels[69, 70], which has been shown to cause mtDNA damage[43]. In our study, mitochondrial DNA damage also was observed in BMDMs infected by MAI, as shown by higher mtDNA 79:230 ratio after 24 hours of infection (Figure 3.4). Furthermore, a two-fold decrease in the mRNA expression of TFAM and MFN2 was observed in BMDM during MAI infection (Figure3.5). TFAM is a key mitochondrial transcription factor necessary for mitochondrial respiration and mitochondrial DNA replication, repair, and packaging, and decreased TFAM expression is associated with reduced proliferation and maintenance in regulatory T cells[73]. The mitochondrial structure is dynamic and constantly undergoes fission and fusion, and such changes in morphology help mitochondrial function properly. MFN2 is a pivotal regulator of mitochondrial fusion, and silencing MFN2 prevents the increase in mitochondrial length and oxygen consumption rate[74]. Together with mtDNA damage, the observed decrease in two of the most important genes for mitochondrial function provides an insight into how mitochondria can be affected by NTM infection. To further understand the effect of MAI infection on mitochondria of macrophages, mitochondrial bioenergetic profiles during an infection was obtained. Decreased basal respiration, ATP-linked respiration, and total ATP were observed, which suggest slowing down of the electron transport chain (Figure 3.6 B, D, & E). Decreased proton leak was also observed, suggesting mitochondrial

structural damage (Figure 3.6 C). The bioenergetic findings are consistent with a previous observation that demonstrated MTb infection led to decreased rate of oxidative phosphorylation[68].

Many pathways that we identified so far are related to mitochondrial ROS. In order to provide support for our hypotheses, the effect of MAI infection on mitochondrial ROS levels was investigated. In our fluorometric study using mitoSOX, a higher fluorescence intensity was observed in MAI-infected RAW 264.7 cells, indicating an increase in ROS levels in MAI infection (Figure 3.7). Moreover, to confirm that the observed signal was mitochondria-dependent, the cells were treated with a mitochondrially targeted antioxidant, mitoTEMPO, and a decrease in fluorescence intensity was found in the mitoTEMPO treated cells (Figure 3.7).

To investigate whether eliminating mitochondrial ROS can improve mitochondrial bioenergetics and be the basis of future treatments developed to combat NTM, mitochondrial bioenergetic profiles of MAI-infected macrophages treated with mitoTEMPO were obtained. The NTM-induced decrease in basal respiration, ATP-linked respiration, and proton leak was attenuated in MAI + mitoTEMPO treatment compared to MAI-only treatment suggests that mitoTEMPO can restore mitochondrial bioenergetics and limit mitochondrial damage in MAI infection (Figure 3.8). Furthermore, mitoTEMPO treatment during MAI infection increases NO levels in cell media, indicating better NO availability for immune response (Figure3.9). However, the reduction in ROS in macrophages may have an undesirable effect on host defense since NO and ROS can react to generate highly reactive and antimicrobial molecules in the phagolysosome to disable pathogens[38-40]. To test the effects of mitoTEMPO treatment on

macrophages' ability to eliminate intracellular bacteria, a bacterial killing assay was performed. Lower intracellular CFU was observed in mitoTEMPO-treated cells, suggesting mitoTEMPO treatment do not negatively affect bacterial killing but, instead, enhances bacterial killing. Similar to our findings, the therapeutic properties of an antioxidant were reported previously. Treatment with another mitochondrial targeted antioxidant, 10(6'-plastoquinonyl) decylrhodamine 19 (SkQR1), was found to be beneficial in acute pyelonephritis, a life-threatening upper urinary infection. SkQR1 treatment in this urinary tract infection led to lower cell death and animal mortality and alleviated renal dysfunction associated with the infection[75].

Nevertheless, there may be some weaknesses in the presented research. Our results are based on immunocompetent models, while the development of NTM-PD is most likely in the immunocompromised[11, 12]. Predominately mouse cell lines are used in our experiments. Although mouse is a good model organism having similar physiology to humans, NTM pathogenesis in human may not be the same as seen in mouse models. Furthermore, some of our studies are not appropriately powered, and their significance cannot be determined.

For our future direction, we will investigate how NO regulates mitochondrial function and how this may play a role in NTM infection. Since NO was reported to have an anti-inflammatory characteristic previously[49], we will characterize the inflammatory response during NTM infection and the pathways that NO may exert its anti-inflammatory effects. Moreover, we will conduct *in vivo* studies in immunocompromised models to have a better understanding of NTM pathogenesis in hosts with immune deficiency.

In summary, while many of these studies need to be replicated to fully support the hypotheses, these findings indicate that NTM infection leads to expression of iNOS, which produces microbicidal NO, and that NTM infection is associated with mitochondrial dysfunction and higher production of ROS, which can scavenge NO and reduce its availability. Mitochondrially targeted antioxidants such as mitoTEMPO can eliminate excessive ROS and improve NO levels and intracellular bacterial killing. Therefore, treatments targeting mitochondrial ROS may potentially alleviate NTM infection, and may be a therapeutic target, especially in those patients that develop antibiotic resistance[16, 17]. To our knowledge, this is the first study characterizing changes in NO levels, mitochondrial bioenergetics of macrophages, and mitoTEMPO's therapeutic properties in NTM infection.

Tables and Figures

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