ABSTRACT

Title of Document: MOLECULAR MECHANISMS OF THE INHIBITION OF HOST CELL APOPTOSIS BY *MYCOBACTERIUM TUBERCULOSIS*.

Jessica Lynn Miller, PhD, and 2009

Directed By: Assistant Professor, Volker Briken PhD, Cell Biology and Molecular Genetics

The capacity of infected cells to undergo apoptosis upon insult with a pathogen is an ancient innate immune defense mechanism. Consequently, the ability of persistent intracellular pathogens, such as the human pathogen *Mycobacterium tuberculosis* (*Mtb*), to inhibit infection-induced apoptosis of macrophages is important for virulence and to achieve persistence in the host. The *nuoG* gene of *Mtb*, which encodes the NuoG subunit of the type I NADH dehydrogenase NDH-1, is important in *Mtb*-mediated inhibition of host macrophage apoptosis. Here I determine the molecular mechanisms of this host-pathogen interaction. Apoptosis induced by the *nuoG* deletion mutant (Δ*nuoG*) is caspase-8 and TNF-α dependent. This cell death was also reduced in the presence of neutralizers and inhibitors of reactive oxygen species (ROS) and in macrophages derived from NOX2 deficient mice, suggesting that Δ*nuoG* induced death is dependent upon NOX2 derived ROS. Correlatively, Δ*nuoG* infected macrophages also produced more phagosomal ROS

than those infected with *Mtb*, or cells derived from NOX2 deficient mice. NuoG also inhibited apoptosis in human alveolar macrophages in a NOX2 dependent manner. These data suggest that reduction of phagosomal ROS is important for inhibition of apoptosis. Consistent with this hypothesis, *Mtb* deficient in the ROS neutralizing catalase, KatG, also accumulated ROS in the phagosome and was pro-apoptotic in macrophages. The specific mechanism by which NuoG reduces phagosomal ROS is still unknown. We could not detect secretion of NuoG, so direct neutralization of ROS is unlikely. Interestingly, preliminary data suggests that Δ*nuoG* may be defective in secretion of SodA and KatG, enzymes known to be important for neutralizing ROS. In conclusion, these studies revealed that *Mtb* inhibits host cell apoptosis by neutralizing phagosomal ROS. Furthermore, this research suggests a novel function for NOX2 activity in innate immunity, which is the sensing of persisting intracellular pathogens and subsequent induction of host cell apoptosis as a second line of defense for pathogens resistant to the respiratory burst.

MOLECULAR MECHANISMS OF THE INHIBITION OF HOST CELL APOPTOSIS BY *MYCOBACTERIUM TUBERCULOSIS*.

By

Jessica Lynn Miller

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Advisory Committee:
Professor Volker Briken, PhD, Chair
Dr. Karen Elkins, PhD
Professor Kenneth Frauwirth, PhD
Professor David O'Brochta, PhD
Professor Steven Salzburg, PhD

Dedication

I dedicate this dissertation to:

My sister Kristen who kept me balanced.

And to:

My parents Matthew and Mary Jo Miller who always reminded me to

“Ride the Waves”.

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List of Abbreviations

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5-LO – 5-lipoxygenase
AP1 – Activator protein 1
B6 – C57/B6
BCG - Bacillus Calmette-Guérin
BCIP – 5-Bromo-4-chloro-3-indolyl phosphate
BH – Bcl-2 homology
BMDM – Bone marrow derived macrophage
C3I, C8I, C9I -A – Caspase 3, 8, or 9 inhibitor –analog
cAMP – Cyclic adenosine monophosphate
CARD – Caspase recruitment domains
CD4+ – Helper T cells
CD8+ – Cytotoxic T cells
c-FLIP – Cellular FLICE like inhibitory protein
CFU – Colony forming units
CGD – Chronic granulomatous disease
CIA – Caspase independent apoptosis
DC – Dendritic cell
DCFDA – 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
DED – Death effector domain
DHE – Dihydroethidium
DISC – Death inducing signaling complex
DMEM – Dulbecco's modified eagle medium
DPI – Diphenylene iodonium
ELISA - Enzyme linked immunosorbent assay
ESAT – Early secreted T cell antigen
FADD – Fas-associated death domain
FCS – Fetal calf serum
gp91-/- – *gp91* knockout
HBSS – Hank's Buffered salt solution
HIV - Human immunodeficiency virus
IAP – Inhibitors of apoptosis
IKK – IκB kinase
IL – Interleukin
IFNγ – Interferon gamma
iNOS – Inducible nitric oxide synthase
IR – Immune response
JNK – June N-terminal kinase
K – Kinase
LAM – Lipoarabinomannan

LXA4 – Lipoxin A4
MAP – Mitogen activated protein
MAPK – MAP kinase
MDR – Multi-drug resistant
MHC – Major histocompatibility complex
MKP – MAP kinase phosphotase
MOI – Multiplicity of infection
MOMP – Mitocondrial outer membrane permeabilization
Mtb – Mycobacterium tuberculosis
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NF-κB – Nuclear factor-κB
NK – Natural killer
NOX – NADPH oxidase
NRAMP – natural resistance-associated macrophage protein
O2- – Superoxide
PAI2 – Plasminogen activator inhibitor type 2
PAMP – Pathogen associated molecular patterns
PBS – Phosphate buffered saline
PBST – PBS-tween
PCD – Programmed cell death
PFA – Paraformaldehyde
PGE2 – Prostaglandin E2
PknE – Protein kinase E
PMA – Phorbol myristate acetate
RNI – Reactive nitrogen intermediates
PRR – Pattern recognition receptor
ROS – Reactive oxygen species
SOD – Superoxide dismutase
TB – Tuberculosis
Th1 – CD4+ T helper cell type 1
Th2 – CD4+ T helper cell type 2
tBid – Truncated Bid
TDM – Trehalose dimycolate
TLR – Toll like receptors
TNF-α – Tumor necrosis factor alpha
TNF-α-/- – *TNF*-α knockout
TNFR – TNF-α receptor
TRADD – TNFR-associated death domain
Trx – Thioredoxin
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
WHO – World Health Organization
WT – Wild type
XDR – Extensively-drug resistant
XO – Xanthine oxidase

CHAPTER 1. INTRODUCTION

1.1 Tuberculosis (TB)

1.1.1 The disease and its progression

Tuberculosis is a problem of global importance as it is one of the most prevalent bacterial diseases in the world and is responsible for the deaths of nearly 2 million people each year. Its etiologic agent, *Mycobacterium tuberculosis* (*Mtb*), infects approximately 2 billion people, a fact that is not surprising as TB is endemic in almost all of Africa, India, and in parts of Eastern Europe [3]. Tuberculosis typically manifests itself as a pulmonary disease and is transmitted via the aerosol route. Once in the lungs, *Mtb* is taken up by alveolar macrophages, and/or phagocytic white blood cells, in which the bacteria can survive and multiply. Infected macrophages may undergo cell death via necrosis, releasing infectious bacteria that will then infect new macrophages. New macrophages and other white blood cells are recruited to the site inducing a small localized inflammatory response at the site of infection. These macrophages become new hosts for the bacteria and form the basis for a granuloma, a collection of immune cells that "wall off" particles that are sensed but cannot be cleared. Here the bacteria can persist for long periods of time without causing any new pathology. This stage of the disease is called latency. Individuals with latent TB often never develop any symptoms and are generally not contagious. However, 10% of subjects infected with TB will develop active tuberculosis. This often happens when the patient becomes immunocompromised, either from old age, malnutrition, infection with human immunodeficiency virus (HIV), or treatment with immunocompromising drugs. In these cases the infecting bacteria will induce

necrosis and caseation in the granuloma, resulting in release of bacteria into the tissue and the airway. This can often result in dissemination of the bacteria leading to pathology in other tissues including the spleen, liver, bone, and brain[4,5]. Patients with active TB are contagious and experience symptoms associated with the disease including incessant coughing, coughing up blood or sputum, weakness or fatigue, loss of appetite, chills, and fever. People with late stage tuberculosis often appear pale and lifeless, a trait that awarded the disease the label of "White plague".

1.1.2 History

Tuberculosis is an ancient pathogen that has caused disease in humans for thousands of years. Mummies from ancient Egypt and pre-Columbian South America show evidence of tuberculosis [6,7], and *Mtb* DNA has been isolated from mummified human lesions from all over the world, the oldest being a Mediterranean man who lived approximately 9,000 years ago [8]. Tuberculosis caused massive amounts of mortality in Europe where it was known as consumption or the "white plague". In England alone one out of every four deaths was attributed to TB in the 1800's [9], and TB was responsible for taking the lives of many famous historical figures including the Brontë sisters, Frédéric Chopin, Robert Louis Stevenson, and Eleanor Roosevelt.

1.1.3 Treatment, chemotherapy, and prevention

Drug remedies

Prior to the development of preventative and curative medical measures, the best "treatment" for Tuberculosis was thought to be rest, proper nutrition, and fresh air. Such therapy was usually administered at specialized sanatoriums, which ranged from mountain resorts for the wealthy, to forced quarantine prisons for the poor. The tubercle bacterium, *Mycobacterium tuberculosis*, was identified and isolated by Robert Koch in 1882, work for which he received a Nobel Prize in 1905. Koch claimed to have found the cure for tuberculosis in the *Mtb* extract tuberculin. Tuberculin was immediately found to be ineffective as a cure, although it is still used today for TB diagnosis and in adjuvant formulations [10]. The first effective drug for tuberculosis treatment was Streptomycin, discovered by Selman Waksman and Albert Schatz in 1943. Over the years new anti-tuberculosis drugs were discovered including isoniazid (1951), rifampicin (1959), pyrazinamide (1952), and ethambutol (1961)[11]. While treatment with these first line antibiotics resulted in a substantial drop in tuberculosis associated mortality, there remained several problems. Many of these drugs are fairly toxic, especially isoniazid which has high toxicity in the liver. Also, the course of therapy is long, about 6 to 9 months for "short course" treatment, which is often extended to for up to two years. Finally, drug susceptible bacteria often persist in the patient even after chemotherapy. The reasons for this persistence are unknown, but may be due to the confinement of bacteria in drug-impermeable granuloma and/or general insusceptibility of bacteria *in vivo* [12].

BCG Vaccine

Twenty-two years before the discovery of successful TB drugs, the first, and to date only, effective tuberculosis vaccine was created by the French bacteriologist Albert Calmette and veterinarian Camille Guérin. They created the vaccine, Bacillus Calmette-Guérin (BCG), by passaging *M. bovis* in culture for 11 years until it was no longer virulent in humans. Although the vaccine was available to the public as early as 1921, it was not widely used until just after World War II. Now it is administered to children in most countries world wide, with the exception of the United States and the Netherlands, although use of the vaccine has been declining in recent years. Unfortunately, the effectiveness of BCG in preventing pulmonary tuberculosis is quite variable, ranging from providing no protective effect in some regions to 60-80% efficacy in others. These differences appear to be regional, with effectiveness declining the closer one gets to the equator [13]. Such disparities may be due to genetic differences in the BCG strains used for vaccination [14], or in immunological effects in the population due to differing environmental exposure. Even in regions of high vaccine efficacy, the protective effect of BCG only lasts about 15 years, although one study found that a group of American Indians remained protected 60 years after immunization with almost no loss in efficacy [15].

Resurgence of TB

Improvements in public health combined with the creation of the BCG vaccine and the discovery of tuberculocidal drugs resulted in significantly decreased morbidity and mortality associated with TB. However, TB was never eradicated and in the 1980's incidence of tuberculosis began to rise. This increase was due to the emergence of multidrug resistant strains of *Mtb* and the concurrent rise in the

incidence of AIDS. Resistance of *Mtb* to drugs has been observed since the first days of chemotherapy [16], and currently *Mtb* strains resistant to at least one drug have been found in every country surveyed by the World Health Organization (WHO)[3]. Multi-drug resistant *Mtb* (MDR), which is resistant to at least isoniazid and rifampicin, was acknowledged by the WHO in 1993 after a TB outbreak in New York city was found to be a due to a MDR-*Mtb* strain [17]. Ineffective treatment of MDR with second line TB drugs led to the development of extensively drug resistant tuberculosis (XDR) in the early 2000s. This form of MDR is resistant to not only isoniazid and rifampin, but also to second-line medications: fluoroquinolones and at least one of three injectable drugs (i.e., amikacin, kanamycin, or capreomycin) [3]).

HIV-TB co-infection has also led to a sharp increase of TB incidence. HIV-positive patients are much more likely to reactivate or have active TB, which can then be spread to others. Also, TB is one of the major causes of death in patients co-infected with HIV [16]. In some cases, infection of HIV patients with XDR-TB has been observed to be almost 100% lethal as made known by the Tulega ferry disaster, where 52 out of 53 HIV patients infected with XDR-TB died, with a median survival of 16 days post diagnosis [18]. The need for new TB control measures is obvious in light of the emergence of drug resistant TB, lack of efficacy of the BCG vaccine, and the increasing incidence of HIV co-infection.

1.2 Mycobacteria and the immune response

The immune response (IR) can be simply separated into two categories, the innate response and adaptive response. The innate IR is a localized, nonspecific

reaction that occurs early in the course of infection. It is the first line of defense against pathogens and does not result in any lasting protection. In contrast, the adaptive IR is a highly specific, cellular, and humoral response that results in immunological memory. As a successful intracellular pathogen, *Mtb* has the capacity to manipulate the host immune response. Here I review the basics of the immune response and its exploitation by *Mtb*.

1.2.1 The immune system

Cytokines and TLRs:

Cytokines are protein signaling molecules secreted by immune cells to shape an immune response. The bouquet of cytokines expressed by immune cells can direct whether the resulting immune response is cell-mediated or humoral. They are also involved in the recruitment of new immune cells to a site of infection. These particular cytokines are called chemokines. Some of the cytokines that are most important for control of tuberculosis are interleukin 12 (IL-12), Tumor necrosis factor (TNF-α), and interferon gamma (IFNγ), which are all pro-inflammatory. IL-12 is produced by macrophages and can stimulate T cells to produce IFNγ, which can then activate macrophages to become more efficient killers. TNF-α is produced by macrophages and T cells and also plays a role in macrophage activation and induction of IL-12. All of these cytokines are necessary for controlling TB as deletion of any of the three results in reduced control of *Mtb* by the IR [19,20,21]. TNF-α in particular is important for controlling tuberculosis as patients on anti-TNF-α therapy are much

more likely to reactivate latent TB [22,23]. In addition, TNF-α is also important for the induction of apoptosis of *Mtb* infected cells (sections 1.4 and 1.5)

Toll-like receptors (TLRs) are pathogen recognition receptors (PRR) present on leukocytes that recognize microbial products or pathogen associated molecular patterns (PAMP). Signaling downstream of TLR ligation can result in the activation of immune transcription factors such as nuclear factor –κB (NF- κB) or activator protein 1 (AP1). These transcription factors then induce expression of multiple immune response genes including those for cytokines. Cell wall extracts and several proteins from *Mtb* have been shown to bind to TLR-2 [24,25].

Cell types involved in innate immunity:

The main cells types that compose the innate immune response are macrophages, dendritic cells (DC), and neutrophils. Several other cell types also play a role in innate immunity, but are not relevant to this discussion. All of these cell types are phagocytes, meaning they can readily ingest large particles or microorganisms.

Neutrophils are often the initiators of an immune response. They are the most abundant white blood cells type and can secrete cytokines to activate and recruit other immune cells. Neutrophils are also able to kill phagocytosed pathogens by inducing a strong respiratory burst, which is an influx of ROS into the phagosome. This burst is dependant upon the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or NOX2 complex (section 1.3). While neutrophils are one of the first cell types recruited during tuberculosis [26], their role in the immune response to TB is still unclear [27]

Macrophages are known as professional phagocytes. They are particularly important in the removal of dead tissue, apoptotic cells, and foreign substances, including pathogens. Similar to the neutrophils, macrophages can induce a respiratory burst upon phagocytosis, however it is shorter than and not as robust as the one induced by the neutrophil. After phagocytosis, the resulting vacuole, or phagosome, matures, acidifies, and eventually fuses with lysosomal vesicles to create the phago-lysosome. These lysosomal vesicles contain degradative enzymes that can break down the contents of the phagosome and kill invading microbes. Macrophages can also secrete a multitude of cytokines such as pro-inflammatory TNF-α and IL-12, but also anti-inflammatory cytokines such as IL-10. The naïve macrophage can become activated in the presence of TLR agonists, TNF-α, and IFNγ. Upon activation, the macrophage upregulates its antimicrobial capacity by expressing the nitric oxide producing inducible nitric oxide synthase (iNOS) and the ROS producing NOX2 complex. Activation also upregulates the expression of major histocompatibility complex (MHC) II molecules, transforming the macrophage into a potent antigen presenting cell. Macrophages are the main host cell for *Mtb*, which can inhibit the formation of phago-lysosomes to create a hospitable environment for the bacterium. However, activated macrophages can override these inhibitory effects and are better able to kill phagocytised *Mtb*[28].

Dendritic cells are known as professional antigen presenting cells. Antigen specific T cells recognize and are activated by antigens presented on the cell surface of antigen presenting cells. Macrophages are also able to present antigens, but the process is much more efficient in dendritic cells. Antigens contained in phagosomes

or endosomes are processed in the endosome, bound to MHC II, and trafficked to the cell surface for presentation. Cytoplasmic antigens, produced by intracellular bacteria or viruses, are processed in the cytoplasm by the proteosome and transported to the endoplasmic reticulum where they bind MHC I before being exocytosed to the membrane. Phagosomal antigens can also be presented on MHC I molecules in a process known as cross presentation. During tuberculosis, dendritic cells are necessary for activation of tuberculosis specific T cells [29], and are also thought to be bacterial hosts [30].

Cell types involved in adaptive immunity:

Cells involved in adaptive immunity are called lymphocytes. Lymphocytes recognize and respond to specific antigens. B cells are lymphocytes that make antibodies in response to antigen and mediate the body-wide humoral immune response. T cells recognize antigens presented by antigen presenting cells and generate a cell-mediated immune response. This response is pro-inflammatory and localized to the site of infection. T cells are separated into two functionally distinct cell types, helper T cells (CD4+) and cytotoxic T cells (CD8+). CD4+ T cells secrete cytokines which recruit and activate leukocytes to kill invading pathogens. Depending on the cytokine environment, CD4+ cells can dictate a pro-inflammatory response (Th1) or an anti-inflammatory, humoral response (Th2). During tuberculosis, macrophages exposed to bacterial products secrete TNF-α and IL-12, which stimulate T cells to induce an inflammatory Th1 response. One of the most important cytokines that these CD4+ T cells produce in tuberculosis is IFNγ, which activates macrophages allowing them to become more efficient killers. The necessity

of CD4+ T cells in controlling TB is shown by the increased susceptibility HIV patients to TB. CD8+ T cells recognize and are activated by antigens presented on MHC I. Upon activation these cells secrete IFNγ to activate macrophages, but they can also directly kill cells infected by intracellular pathogens by inducing apoptosis. T cells are also the driving force in the formation of the TB granuloma. Furthermore, both CD4+ and CD8+ T cells are necessary for control of TB as mice that are deficient in one or both lineages are more susceptible to TB [28].

1.2.2 Manipulation of the immune response by *Mtb*

Mycobacterium tuberculosis is a persistent intracellular pathogen. Thus, in order to be successful *Mtb* has evolved mechanisms to influence the immune response of the host. Both the innate and the adaptive host immune responses can be manipulated by *Mtb* infection.

Mtb survival strategies in the phagosome:

In 1969 D'Arcy Hart's group discovered that the vacuoles containing Mycobacteria did not fuse with lysosomes[31]. This ability of *Mtb* to stall phagosomal maturation and inhibit lysosomal fusion is a necessary step for bacterial survival. It not only allows for bacterial replication within macrophages, but also circumvents the bactericidal capabilities of the lysosome. *Mtb* cell wall lipids such as lipoarabinomannan (LAM) [32,33]and trehalose dimycolate (TDM) [34], and multiple proteins including SapM [35] and PknG [36] have all been implicated in blocking phagosome-lysosome fusion. In addition to being unable to deliver *Mtb* to the lysosomal compartment, the impediment of phagosomal maturation also prevents

the fusion of the phagosome to MHC II containing vesicles. Thus, *Mtb* can diminish the ability of its host macrophages to present antigens to CD4+ T cells [37]. Arrest of phagosomal maturation also allows for reduced cross presentation as mycobacterial antigens remain sequestered in the phagosome away from the processing machinery necessary for presentation on MHC I molecules. However, it has been suggested that the host cell compensates for this by undergoing apoptosis (section 1.5).

Mtb can modify its phagosomal vacuole and resist its degradative mechanisms in several ways. First, the vacuolar-ATPase is excluded from the *Mtb* phagosome, resulting in only a slight acidification of the phagosome to a pH of 6.4[38]. Inhibition of vacuolar-ATPase activity also impairs the normal routing of membranes and cargo along the endocytic pathway, although the molecular mechanisms in this process are still unclear [39]. Secondly, *Mtb* is also able to neutralize ROS produced in the phagosome by NOX2 complex via the secretion of superoxide dismutastes (SOD) A and C, and the bacterial catalase G (KatG) [40,41,42]. All of these enzymes appear to be necessary for effective virulence as silencing or deletion of these ROS neutralizing enzymes renders the bacterium less virulent [43,44,45]. Through these mechanisms *Mtb* is able to create a livable niche in the macrophage phagosome in which it can divide and persist.

Activation of the *Mtb* host macrophage with IFNγ results in the upregulation of multiple anti-bacterial mechanisms in the cell. These include the induction of iNOS and phagosome-lysosome fusion. While macrophage activation often results in increased bacterial death, the effect is not sterilizing and many bacteria are able to resist the effects of iNOS and/or persist in the acidic environment of the phago-

lysosome [38]. Multiple groups have shown that *Mtb* can resist the effects of iNOS. For instance, *Mtb* contains a four protein NADH-peroxynitrite reductase, which is involved in detoxification of both ROS and iNOS dependent reactive nitrogen intermediates (RNI). Deletion of one of these proteins, dlaT, from *Mtb* resulted in a mutant hypersuceptible to RNI [46]. Furthermore, Darwin and colleagues discovered that the mycobacterial proteosome was necessary for bacterial resistance to iNOS, although the mechanisms involved in this resistance have yet to be determined [47,48]. Macrophage activation also results in the maturation and acidification of the *Mtb* containing vacuole [49]. However, *Mtb* possesses innate resistance to acid that allows the bacteria to persist even in the acidic environment of the phago-lysosome. *Mtb* can maintain its internal pH and survive both *in vitro* and *in* vivo at a pH of 4.5 [50]. This is likely due to modification to the bacterial cell envelopee amongst other mechanisms [51]. Thus, even though macrophage activation is necessary for control of tuberculosis, *Mtb* is able to resist clearance by the macrophage and persist in the immune cell.

Formation of the granuloma

In addition to manipulating the host cell immune response, *Mtb* is also able to drive formation of the granuloma, the structure in which *Mtb* survives and persists over time. The granuloma is often thought of as being advantageous to the host as it allows for containment and sometimes sterilization of the pathogen. However, in recent years the benefit of this protective structure to the bacillus has become more appreciated [52]. The granuloma in humans has a relatively defined structure, consisting of a core of infected, sometimes necrotic macrophages, surrounded by both

foamy and activated apoptotic macrophages, which are in turn surrounded by activated T cells. These lymphocytes secret IFNγ and TNF-α, which activate the enclosed macrophages, increasing their ability to kill phagocytised bacteria [53]. A relative equilibrium exists between pathogen and host within the granuloma as the bacterial population appears to remain constant, even over several years. In some cases the balance can shift, either toward the host, resulting in bacterial elimination, or in favor of the bacillus, promoting bacterial replication and active disease progression. Several groups suggest that *Mtb* actively induces an immune response in order to establish granuloma formation [52,54]. Mycobacterial products, such as the early secreted T cell antigen 6 (ESAT-6) [54]and mycobacterial cell wall lipids [55], can induce granuloma formation. Interestingly, beads coated with cell wall lipids, in particular TDM, induced formation of a rudimentary granuloma in the absence of any live bacteria [55]. Thus, *Mycobacterium tuberculosis* is able to manipulate not only the environment of the host cell, but also drive the immune response of the tissue in order to survive and persist.

1.3 NADPH Oxidases

1.3.1 The NOX family

The NADPH oxidases (NOX) are trans-membrane enzymes that transfer electrons across biological membranes to produce ROS in a regulated manner. They have been found in many cells types and across kingdoms [56]. NOX is regulated by several other proteins that must bind to the cytoplasmic side of the subunit in order to activate it. This fully functional enzyme is referred to as NOX-complex. To date,

seven members of the NOX family of enzymes have been identified in humans; NOX1-5 and the dual oxidases DUOX1 and DUOX2. All of the NOX family members preserve the ability to accept and transfer electrons, and thus are structurally similar. These NOX family proteins conserve structures such as NADPH- and FAD-binding sites, six trans-membrane domains, and four highly conserved heme-binding histidines [57]. The NOX proteins differ mostly in localization, with different NOXs being more highly expressed in different tissues, although most are also expressed at low levels in numerous tissues. For instance, NOX1 is mostly highly expressed in the colon [58], while NOX3 is almost exclusively expressed in the inner ear[59], and the DUOX proteins are concentrated in the thyroid [60].

The Phagocytic oxidase- NOX2

The phagocytic NADPH-oxidase (NOX2 or $gp91^{phox}$) is the best studied member of the NOX family of ROS-generating NADPH oxidases. It is expressed most highly in phagocytes, but has low level expression in a multitude of other tissues [57]. As ROS are detrimental to the cell, the activation of NOX2 is very tightly regulated. NOX2 alone is inactive and in order to be fully functional it must associate with several other components in order to form the NOX2 complex. The $gp22^{phox}$ subunit is constitutively associated with NOX2 and both are expressed in the plasma membrane and on intracellular vesicles. Upon macrophage activation or stimulation the cytosolic components $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ are recruited to the cytoplasmic side of the complex. In addition, the cytosolic GTPase Rac has to be recruited in order to form a fully active NOX2 complex [57,61,62]. Once fully

formed, the complex can then initiate the formation of large amounts of superoxide (O_2^-) either in the phagosomal compartment or extra-cellularly.

1.3.2 NOX2 in the immune response

Direct microcidal effects

NOX2 is most famously known for initiating the respiratory burst, or the flood of phagosomal ROS associated with phagocytosis [63,64]. This increase in superoxide and its derivatives is thought to be an important component of the bactericidal activity of the macrophage and especially the neutrophil phagosome [65]. Many ROS, especially H_2O_2 and HOCL, have been shown to have direct microcidal effects [66,67]. These effects of NOX2 may also be due to the change in ion content of the phagosome. The influx of electrons into the phagosome by NOX2 results in a charge imbalance that is compensated by the influx of cations, either H^+ or K^+. In neutrophils, the influx of K^+ results in bacterial killing through changes in phagosomal osmolarity and unbinding of cationic proteases from their proteoglycan matrix [68,69]. Whether the microcidal affects of NOX2 activity are due to direct oxidation or change in osmolarity, it is evident that NOX2 plays an important role in the control of pathogens.

NOX2 in signaling

While NOX2 generated ROS may be important for direct microbial killing, more recent studies have shown that these ROS may also play a role in immune signaling. ROS have been shown to inactivate protein tyrosine phosphatases by oxidizing catalytic cysteine residues [57,70]. This decrease in phosphatase activity

can result in enhanced tyrosine phosphorylation and thus amplify multiple signaling pathways including those involved in immunity. Additionally, NOX2 has been shown to play important roles in TLR signaling, as NOX2 deficient mice are unable to activate NF-κB via TLR 4 [71] and TLR2 [72].

The most well characterized NOX2 mediated signaling pathways involve its role in activation of mitogen activated protein kinase (MAPK) signaling pathways, specifically in the activation of Ask-1. Ask-1 is a MAPKKK that can lead to activation of either the june N-terminal kinase (JNK) or p38, both of which can induce apoptosis [73]. Ask-1 is maintained in the cytoplasm complexed to thioredoxin (Trx), an inhibitor of Ask-1 activation. However, only the reduced form of Trx is able to bind to Ask-1. NOX2 generated H_2O_2 can oxidize Trx to free up Ask-1 for phosphorylation and activation. Furthermore, the fully active Ask-1 signaling complex consists of a protein complex including TRAF2 and TRAF6, the recruitment of which is also dependent upon ROS [74,75]. Recent studies have also implicated NOX2 derived ROS as important in the induction of TNF-α induced NF-κB activation [76,77]. These studies suggest that NOX2 derived ROS are necessary for efficient recruitment of TRAF2 to the TNF-α signaling complex (complex1). The complex then activates IkB kinase (IKK), and promotes NF-κB activation. Thus, NOX2 derived ROS seem to be heavily involved in TNF-α signaling, leading either to survival and inflammation or apoptosis [77] (section 1.4.1 and Figure 2).

Chronic Granulomatous Disease (CGD)

The significance of the NOX2-complex for innate immune response is illustrated by the pathology of human subjects that have genetic defects in

components of the complex and develop CGD. This is usually due to genetic mutations in either the $gp91^{phox}$ or $p22^{phox}$ subunits. CGD is named for the chronic inflammatory granulomas that are characteristic of those with the disease. However, the most salient phenotype of CGD is the increased susceptibility of patients to fungal and bacterial infections, especially *Staphylococcus aureus* [78,79]. Mice deficient in the NOX2 subunits are consistently more susceptible to infections with bacterial pathogens such as *Salmonella typhimurium* for example [65,79].

Cross presentation

In addition to playing a role in direct microbial killing and cell signaling events, NOX2 activity also plays an important role in antigen presentation. Upon phagocytosis, the NOX2 complex is recruited to the phagosomes of dendritic cells. In a subset of these DCs, NOX2 steadily produces low levels of ROS, resulting in maintained alkalinization of the phagosome. This alkaline environment is adapted for efficiently processing antigens for cross-presentation rather than pathogen killing. These DCs can then effectively present antigens to CD8+T cells to induce an adaptive immune response [80,81]. Thus, NOX2 also plays an important role in the adaptive IR.

Pathogen response to NOX2

Considering its role in the immune response, it is not surprising that pathogens have developed strategies to counter the NOX2-complex. Several pathogens can inhibit NOX2-complex assembly on the phagosome, as is the case for *S. typhimurium*[82] and *Helicobacter pylori* [83]. Others can reduce steady-state levels of NOX2 components as illustrated by *Anaplasma phagocytophila* [84,85] or

Ehrlichia chaffeensis [86] (for review [87]). Finally, several pathogens, including *Anaplasma phagocytophila* and *Mycobacterium tuberculosis* [43,44], possess the ability to directly neutralize NOX2 products as previously described (Section 1.2.2).

1.4 Programmed cell death pathways

Programmed cell death (PCD) is any form of cell death that is mediated by an intracellular signaling pathway. These include forms of death like apoptosis, programmed necrosis, and autophagy and are usually beneficial to the host in some way. PCD is an important part of development, homeostasis, and the immune response.

1.4.1 Apoptosis

Apoptosis is a controlled method for clearance of unwanted cells in many multicellular organisms. It is necessary for general homeostasis, but also for tissue development, selection of lymphocytes in the immune system, and host defense. During apoptosis the cytoskeleton is broken down causing the cell to shrink and become rounded. The nucleus becomes more compact due to the condensation and cleavage of chromatin, an impermeable cellular or apoptotic envelopee forms [88], and the cell eventually breaks apart into vesicles called apoptotic bodies. These vesicles are ingested by neighboring phagocytic cells. The phagocytes recognize apoptotic "eat me" markers on the surface of the vesicle, such as phosphatidylserine, which allow them to phagocytize the apoptotic bodies in an immunologically silent fashion. Apoptosis is mediated by effector enzymes called caspases and can be induced via either intrinsic or extrinsic means (reviewed in [89]).

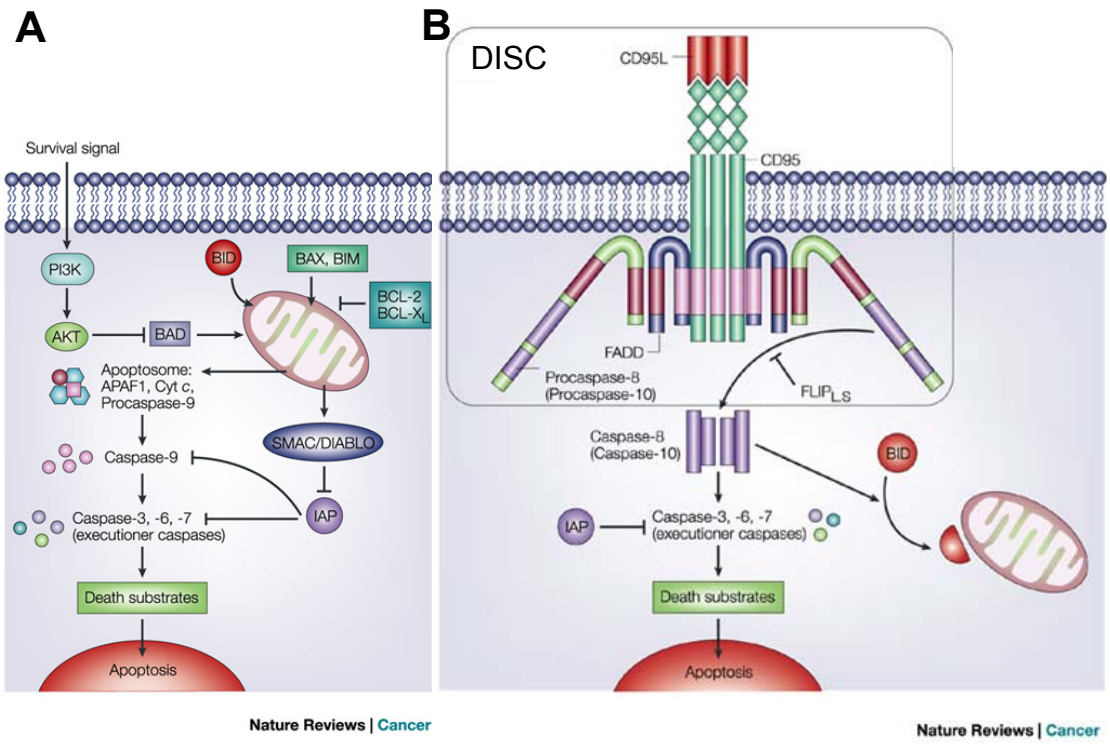
Caspases

Caspases, or cysteine-aspartic acid proteases, are cysteine proteases which cause the systematic break down of cellular contents during apoptosis. Caspases are maintained in an inactive form or, pro-caspase, which needs to be cleaved in order to become active. The caspase family can generally be divided into three groups, initiator, effector, and inflammatory. Initiator caspases are the first to be activated in response to apoptotic stimuli. These include caspase 8 and 10, the initiator caspases for the extrinsic pathway induced by receptor ligation, and caspase 9, the initiator caspase for the intrinsic pathway induced by the apoptosome. Caspase 2 is also an initiator caspase that is activated by the PIDDosome. Many of these enzymes contain a caspase recruitment domain (CARD), which is necessary to interact with other caspases, and a death effector domain (DED) through which it can interact with its activation platform (DISC, apoptosome, or PIDDosome). The initiator caspases can self-cleave in order to become active, and continue to activate the effector caspases (caspase 3, 6, and 7) in a caspase cascade that eventually results in apoptosis [90]. Caspases 1, 4, and 5 are termed inflammatory caspases as they are thought to be involved in inflammation. Of these three, only caspase 1 has been extensively studied. Caspase 1 complexes with several other proteins to form the inflammasome, which initiates the cleavage and activation of IL-1β and IL-18 [91]. The inflammasome has also been shown to be able to induce a form of cell death called pyroptosis, which is a form of PCD that is distinct from apoptosis [92]. In short, caspases are proteolytic enzymes that are often involved in apoptosis and other PCD mechanisms.

Intrinsic pathway

The intrinsic apoptosis pathway is usually activated in response to internal stress conditions such as extensive DNA damage, radiation exposure, or loss of survival factors. The determining event in the intrinsic pathway is the mitochondrial outer membrane permeabilization (MOMP) or depolarization of the mitochondria. The result of MOMP is the release of cytochrome C from the mitochondria amongst other proteins. Cytochrome C can bind to and activate Apaf-1, which contains a caspase binding CARD domain, and can form the oligomeric apoptosome. This complex then binds and activates procaspase 9 via a DED, releasing active caspase 9. Caspase 9 in turn cleaves and activates caspase 3, which turns on other effector caspases and proteases to result in apoptosis [90](Figure 1A).

MOMP is generally the result of an imbalance between pro- and anti-apoptotic Bcl-2 family proteins. Bcl-2 family proteins share one or more of the four characteristic Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4) that are necessary for function. The anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL, Bcl-w, A1, and Mcl-1) contain all four conserved BH domains. The remaining pro-apoptotic proteins conserve several domains (Bax and Bak) or only the BH3 domain (Bid, Bim and Bad). Normally the anti-apoptotic Bcl-2 family proteins would bind to and sequester the pro-apoptotic proteins, making them inaccessible for the induction of cell death. When the ratio of pro-apoptotic proteins to anti-apoptotic proteins is high, the pro-apoptotic proteins can promote MOMP and induce apoptosis.



(A,B) Modified from [94]

Figure 1. Intrinsic and extrinsic mechanisms of apoptosis. (A) The intrinsic apoptosis pathway. In the presence of a death signal or the absence of pro-life signals, levels of the pro-apoptotic Bcl-2 family proteins (Bax, Bad, Bim) can overcome the number of anti-apoptotic proteins (Bcl-2, Bcl-xL) and induce MOMP. This mitochondrial permeabilization allows for the release of the pro-apoptotic proteins cytochrome C and SMAC/Diablo. Cytochrome C binds to APAF1 to create the apoptosome, which can bind to and activate the initiator caspase 9, which in turn activates the executioner caspases 3,6, and 7 to induce apoptosis. SMAC/Diablo can bind to inhibitor of apoptosis proteins that can inhibit caspase activation. (B) The extrinsic apoptosis pathway. Upon Fas ligation (CD95 in this figure) FADD and pro-caspase 8/10 are recruited to the cytoplasmic side of the receptor forming the DISC complex. Caspase 8/10 becomes activated and goes on to cleave and activate the effector caspases. Caspase 8 can also cleave the BH3 only protein, Bid, into tBid, which can induce MOMP and the activation of caspase 9. Although these two pathways utilize different initiator caspases, they both converge at the level of caspase 3 [94].

For example, Bcl-2 can bind to and sequester Bax and Bak. However, in the absence of Bcl-2, Bax and Bak can form a channel in the mitochondrial membrane resulting in the release of cytochrome C and the subsequent induction of apoptosis. In addition to the release of cytochrome C, Bcl-2 family proteins can also promote the release of other pro-apoptotic proteins such as SMAC/Diablo. These proteins competitively inhibit the anti-apoptotic IAPs (inhibitors of apoptosis proteins), which normally bind to caspases, inhibiting their activation and targeting them for proteosomal degradation. Thus, the intrinsic apoptosis pathway is mediated by an equilibrium of pro- and anti-apoptotic Bcl-2 family proteins, which when out of balance can induce MOMP, and subsequent activation of caspase 9 to induce apoptosis (Figure 1A)[93].

Extrinsic pathway

The extrinsic pathway is initiated by external signals such as the binding of Fas ligand to Fas, or TNF-α to TNF-α Receptor 1 (TNFR-1). The cytoplasmic ends of these receptors contain a death domain motif, which bind to the Fas-associated death domain (FADD). This interaction can either be direct (Fas:FasL) or, as in the case if TNFR signaling, through an adaptor molecule such as the TNF-α receptor-associated death domain (TRADD). FADD binds to pro-caspases 8 and 10 through a DED. During Fas signaling the FADD/Caspase-8 -10 complex is referred to as DISC (death inducing signaling complex), which is sufficient to activate caspase 8. The analogous complex in TNFR signaling, complex II, requires more adaptor molecules and subsequent signaling events in order for caspase 8 activation. Once bound to FADD, caspase 8 can self activate via cleavage. Activated caspase 8 is then released from the DISC and activates apoptosis effecter molecules including caspase 3, at

which point the external and internal pathways converge (Figure 1B). It is important to note that in addition to activating caspase 3, caspase 8 will also cleave the Bcl-2 family member protein Bid into tBid (truncated Bid), which causes destabilization of the mitochondrial membrane and induces release of cytochrome C [94] (Figure 1B). Thus, the external and internal pathways are not mutually exclusive, but intersect and influence the other.

Dual nature of TNF-α signaling: involvement of JNK and ROS

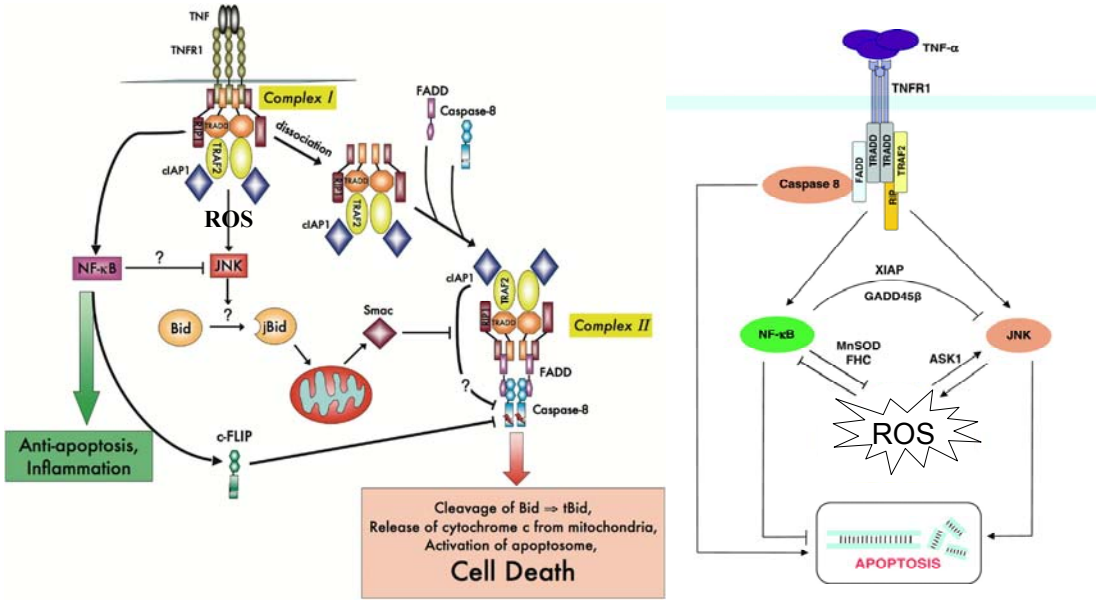
Although TNF-α is considered an inducer of apoptosis, it is also an important inflammatory signaling molecule, and can actually promote cell survival. Typically, treatment of cells with TNF-α results in the activation of NF-κB and subsequent increase in expression of inflammatory and anti-apoptotic molecules. Correspondingly, protein synthesis usually needs to be inhibited in order to induce TNF-α mediated apoptosis *in vitro*. The dual nature of TNF-α signaling is partially controlled via the spatial and temporal separation of NF-κB inducing signaling complex, complex I, and the apoptosis inducing signaling complex, complex II. Immediately upon ligation of TNF-α to TNFR-1, the proteins RIP1, TRADD, TRAF2, and cIAP1 are recruited to form complex I, which transduces signals leading to NF-κB translocation. Later, most likely after internalization of the receptor complex, RIP1, TRADD, and TRAF2 dissociate from TNFR-1 and recruit FADD and caspase-8 to form complex II. Subsequently, caspase 8 can become activated leading to apoptosis (Figure 2A). The apoptotic activity of complex II is inhibited in the presence of sufficient amounts of NF-κB regulated anti-apoptotic proteins. These include the cellular FLICE like inhibitory protein (c-FLIP$_L$), which directly inhibits

caspase 8 activation, Bcl-2-family member proteins, and cellular inhibitor of apoptosis proteins (c-IAP 1 & 2) amongst others [1](Figure 2A,B).

NF-κB mediated factors can also inhibit TNF-α induced apoptosis by dampening signaling by the June N-terminal kinase (JNK). A short, but powerful induction of the JNK signaling cascade is induced upon formation of complex I. However, this cascade is usually suppressed by NF-κB mediated factors. In the absence of the factors, complex I induces sustained JNK signaling, which results in a pro-apoptotic response. Interestingly, increased JNK signaling alone appears to be sufficient for promoting TNF-α induced apoptosis as constitutive JNK activity can over-ride NF-κB mediated apoptosis suppression [95]. JNK signaling induces apoptosis is via the cleavage of the pro-apoptotic protein Bid into jBid (distinct from the previously mentioned tBid), although the exact mechanism of truncation is unknown. jBid induces the preferential release of SMAC from the mitochondria, which can free complex II from the inhibitory effects of c-$FLIP_L$ on caspase 8 (Figure 2A)[1].

It is known that intracellular levels of reactive oxygen species can activate the MAP kinase cascade necessary for JNK activation (Figure 2B), and although the specific mechanism by which complex I activates JNK is still unknown, it is thought to be via a ROS mediated mechanism. Intracellular ROS are typically a byproduct of respiration and thus are generated by the mitochondria. As JNK signaling can induce release of SMAC from the mitochondria, it may also result in increased levels of ROS via depolarization of the mitochondrial membrane, subsequently generating a positive

A **B**



(A) Modifyed from [1]. (B) Modifyed from [2]

Figure 2. TNF-α signaling leads to both pro- and anti-apoptotic outcomes. (A) Ligation of TNF-α to TNFR-1 results in the recruitment of RIP1, TRADD, TRAF2, and cIAP1 to form complex I. Complex I signaling results in the activation of NF- κB and subsequent induction of multiple pro-inflammatory and anti-apoptotic proteins. At later time points, RIP1, TRADD, TRAF2, and cIAP1 disassociate from TNFR-1 and bind to FADD and pro-caspase 8 to form the apoptosis inducing complex II. Activation of caspase 8 is inhibited by NF- κB regulated apoptosis inhibitors such as c-FLIP. NF- κB signaling can also inhibit sustained JNK signaling induced by complex I. In the absence of NF- κB mediated factors, JNK signaling can result in the cleavage of Bid into jBid, which can cause the release of Smac from the mitochondria, a pro-apoptotic protein that can counter-act c-FLIP. (B) Increased levels of ROS can shift TNF-α signaling from pro-survival (NF- κB activation) to apoptotic by inducing sustained activation of JNK.

feedback loop and increasing the levels of active JNK. However, the mitochondria are not the only ROS generators in the cell. The NADPH oxidases can also induce ROS production in cells (section 1.3). Several reports have shown activation of NADPH oxidases to be upstream of JNK activation during apoptosis [96,97,98]. JNK activation by ROS may be due to the activation of Ask-1 [99] or the inhibition of MAP kinase phosphatases [100].

1.4.2 Other pathways and forms of cell death

Necrosis:

Necrosis is an uncontrolled form of cell death that is typically induced by damage to the cell. Such damage can be in response to infection and inflammation, or trauma from wounding, oxygen deprivation, or toxins. It is characterized by cell and organelle swelling, chromatin degradation, membrane disruption, and eventual cell lysis. Since necrosis is an uncontrolled form of cell death, it is immunologically stimulating and can induce recruitment of several types of inflammatory cells, including activated macrophages, neutrophils, and other leukocytes. Interestingly, cells can be programmed to die via necrosis, such as in response to TNF-α, but the biochemical pathways involved in this form of PCD are not fully understood [101]. Infections with certain pathogens, such as many viruses, Group A streptococcus (*Streptococcus pyrogenes*), or *Mycobacterium tuberculosis*, can induce necrosis in cells. In the case of *Mtb*, necrosis is generally believed to be pro-bacterial and *Mtb* is thought to preferentially induce necrosis in host cells [102]. Also, necrosis in the granuloma (termed caseous necrosis) is the seminal event in re-activation and transmission of *Mtb* [53].

Caspase independent apoptosis (CIA)

Apoptosis has technically been defined based on its physical characteristics, particularly DNA cleavage. Apoptosis is usually defined as caspase independent if the addition of caspase inhibitors has no effect on the apoptotic phenotype. CIA is often mediated via the release of proteolytic enzymes from the mitochondria that are not dependant upon caspases for activation. For example, the nuclease activator apoptosis inducing factor, or AIF, can induce chromatin condensation and cleavage [103,104]. Also, endonuclease G is an apoptogenic enzyme that can induce DNA cleavage and apoptosis after being released from the mitochondria [105]. Lysosomal enzymes have also been shown to play a role in CIA [106,107]. "Leaky lysosomes" can release small amounts of cathepsins into the cytoplasm, which can induce MOMP and apoptosis [108]. This lysosomal pathway has been shown to be induced in *Mtb* infected cells at high MOIs and is thought to aid in the spread of bacteria to new host cells [109].

Autophagy

Autophagy is the process of self-digestion by which cells recycle proteins and organelles. It is characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles, which are delivered to and digested in lysosomal compartments. Autophagy has an important function in cellular remodeling due to differentiation, stress, or damage induced by cytokines. Excessive autophagy can result in cell death in a caspase independent fashion. Autophagy also plays a role in host defense as autophagic cells infected with *Mtb* are able to reduce bacterial viability [110,111], probably by overcoming the phagosome maturation block imposed by *Mtb*.

1.5 Manipulation of host cell death by *Mtb*, and its consequence on the immune response

Mtb can modulate the host IR in order for survival and persistence (section 1.2). Apoptosis is an important component of the immune response as it plays roles in both the innate and adaptive immunity to TB. *Mtb* poorly induces apoptosis in host cells, but is also able to inhibit both the intrinsic and extrinsic apoptosis pathways in response to apoptotic stimuli. Thus, the role of apoptosis in the immune response to TB is controversial. However, apoptosis inducing mutants of *Mtb* are less virulent *in vivo* suggesting that the ability of *Mtb* to inhibit apoptosis is a virulence mechanism.

1.5.1 Role of apoptosis in the immune response to *Mtb*

Innate IR

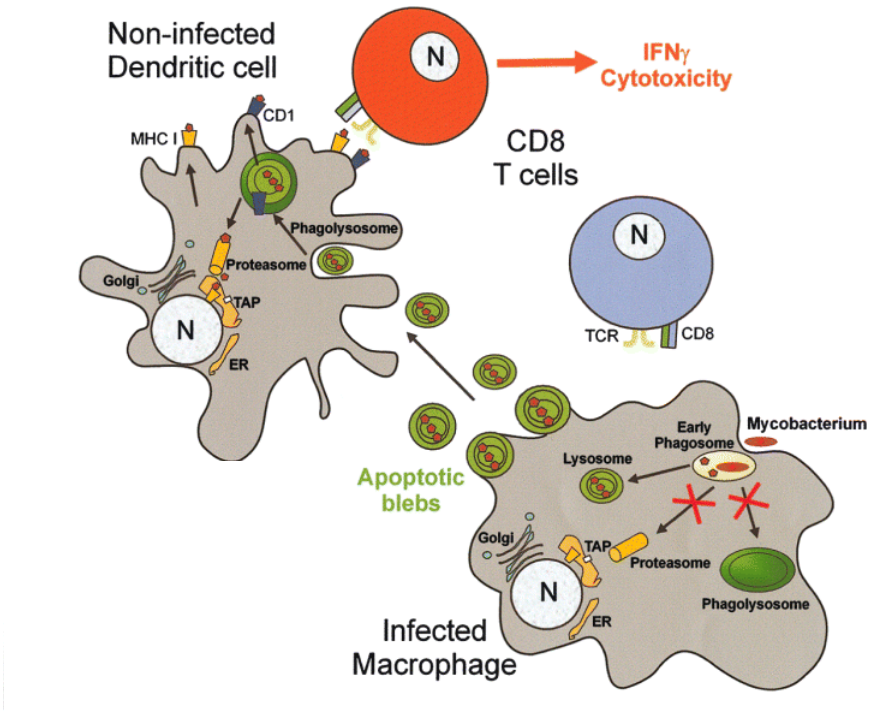
It has long been known that apoptosis plays an important role in the innate IR. In order to achieve persistence, intracellular pathogens have evolved mechanisms to inhibit apoptosis, a trait that has been utilized by multiple bacterial pathogens including *M. tuberculosis*, *Legionella pneumophila*, and *Chlamydia* species amongst others (for detailed review [112,113]). Infection of cells with *Mycobacteria spp* results in differing levels of apoptosis in host cells [114]. Non-virulent or facultative species, such as *Mtb*-H37Ra, BCG, *M. smegmatis*, and *M. kansasii* induce significantly higher levels of apoptosis in human alveolar macrophages than virulent species of Mycobacteria (*Mtb*-H37Rv, *Mtb*- Erdman, *M. bovis*)[114]. This trend was also observed in THP-1 [115,116] and J774 [117] cell lines and by our lab in murine

bone marrow derived macrophages (BMDM)(A. Bohsali, unpublished). While avirulent mycobacterial species induce apoptosis in host cells, virulent species induce much higher levels of necrosis, a host cell death fate that is advantageous for infection and promotes bacterial survival [118,119,120]. Conversely, apoptosis of mycobacteria infected cells resulted in bactericidal effects [102,118,119,121,134,122]. Early studies showed that induction of TNF-α or Fas mediated apoptosis in infected cells resulted in enhanced bacterial killing [118]. Also, Fratazzi *et al*. showed that fresh macrophages added to apoptotic cells infected with *M. avium* were able to reduce the amount of viable bacteria. Furthermore, infection of BMDMs from 5-lipoxygenase deficient mice, which induce high levels of apoptosis upon infection with *Mtb*, resulted in increased bacterial killing when compared to wild-type (WT) mice [102]. The *Mtb-nuoG* deletion mutant, which induces elevated levels of apoptosis in host cells, is less virulent in immnocompromised SCID mice [123]. In conclusion, the ability of pathogenic *Mycobacterium spp* to inhibit host cell apoptosis is one important aspect of the pathogen's capability to counteract the innate immune response.

Adaptive IR

Induction of host cell apoptosis during mycobacterial infection has a direct bactericidal effect, and is thus part of the innate IR. However, apoptosis also helps initiate an adaptive IR by priming cytotoxic T cells against TB antigens. During apoptosis the cell membrane begins to bleb, resulting in the formation of apoptotic bodies, which contain the contents of the cytoplasm. These apoptotic blebs are quickly cleared by nearby macrophages and/or dendritic cells, a process that typically

does not induce an IR. However, if these apoptotic blebs contain foreign antigens capable of stimulating PRRs, then the exogenous peptides within the apoptotic bodies can be presented on MHC I molecules in a process called cross presentation [124,125]. These APCs are capable of cross priming, which is the process of activating naïve CD8+- T cells specific for foreign peptides that would generally be presented on MHC II molecules (Figure 3). The role of cross-priming in tuberculosis was demonstrated by Schaible *et al*. who showed that DCs co-cultured with apoptotic *Mtb* infected macrophages were able to activate CD8+ T cells *in vitro*. Furthermore, the addition of caspase inhibitors prevented cross-priming [126]. This same group later established the importance of apoptosis *in vivo* by showing that inoculation of mice with apoptotic vesicles from *Mtb* infected macrophages resulted in activation of CD8+ T cells [125]. In addition, these inoculated mice were protected against mycobacterial challenge[125]. Thus, induction of apoptosis is a key component in the induction of an adaptive IR, and in particular for the activation of CD8+ T cells during tuberculosis.



Adapted from [133]

Figure 3. Model of the detour pathway; the role of apoptosis in cross-priming. The machinery necessary to process antigens for cross presentation cannot gain access to *Mtb* antigens as the bacterium persists in a stalled phagosomal compartment. Apoptosis of the host macrophage releases mycobacterial antigens within apoptotic blebs, which can be taken up by neighboring antigen presenting cells like dendritic cells. These cells can then process the bacterial antigens for cross presentation of MHC I molecules, which can be recognized by cytotoxic CD8+ T cells and initiate an adaptive immune response.

Host susceptibility and apoptosis

Just as the ability to inhibit apoptosis is a bacterial virulence trait [114], the capacity of the host to induce apoptosis confers carrier resistance to mycobacterial infection. This role of apoptosis in host defense against TB was shown after the identification of the mouse susceptibility-to-tuberculosis locus 1 (sst1), which seems to be involved in switching the cell death pathway of infected macrophages from necrosis to apoptosis [127]. This phenotype was later mapped to the *Ipr1* gene. Correlatively, mice with defective *Ipr1* genes induce more necrosis and less apoptosis than wild-type and are also more susceptible to *Mtb* infection [128]. A link between host susceptibility and polymorphisms in the human Ipr1 homologue SP110 has also been observed [129], clearly indicating that apoptosis plays an important role in the immune response against *Mtb*.

Induction of apoptosis by Mtb

It is generally understood that apoptosis induction in host macrophages by *Mycobacteria* is relative. While *M. tuberculosis* stimulates significantly less apoptosis than non-virulent species, it still consistently induces low levels of PCD in host cells. *Mtb* contains several factors that have been shown to induce apoptosis such as ESAT-6 [130] and the 19kDa lipoprotein [131]. In the battle between host and pathogen, it is possible that this background level of apoptosis could be the result of the host cell apoptotic response over-riding the bacterial anti-apoptotic mechanisms. An alternative hypothesis is that *Mtb* actively induces low levels of host cell apoptosis in order to induce granuloma formation. Studies in *M. marinum* suggest that early granuloma formation is contingent upon induction of ESAT-6

dependent host cell apoptosis by the bacillus [54]. These data suggest that primary infected cells must undergo apoptosis in order for bacteria to passively infect new infiltrating macrophages. However, the same group also showed that in the absence of TNF-α, *M. marinum* infected cells underwent necrosis, not apoptosis, which accelerated granuloma formation [132]. Together these data indicate that it is cell death in general that is necessary for granuloma expansion and not necessarily apoptosis.

It has also been observed that at high MOIs *Mtb* can induce a caspase independent form of cell death. This form of cell death was observed to be more rapid than previously described *Mtb* induced apoptosis and showed characteristics of necrosis at later stages. It was also found to be dependant upon cathepsins, and thus is thought to be initiated by lysosomes. Importantly, death induced at high MOIs was not bactericidal and was in fact permissive of bacterial growth and dissemination. Thus, this distinct form of PCD is thought to be a bacterial driven mechanism to promote extracellular spread of the pathogen, and perhaps may also be involved in the caseation of late stage granuloma [109].

In conclusion, *Mtb* induces low levels of apoptosis in the host cell that may be important in pathogenesis. However, higher levels of apoptosis appear to impair mycobacterial infection and promote host containment of the infection. This is supported by the observations that apoptosis is bactericidal and is important in the induction of an adaptive immune response. Furthermore, virulent strains induce less host cell apoptosis than non-virulent strains, and host populations defective in induction of apoptosis are more susceptible to TB.

1.5.2 *Mtb* inhibits both the extrinsic and intrinsic apoptosis pathways

The field of apoptosis signaling is constantly changing and evolving. The simplest delineation between pathways is to group the signaling into the intrinsic pathway, or mitochondrial pathway, and the extrinsic, or death receptor, mediated pathway. The intrinsic pathway is characterized by permeablisation of the mitochondrial membrane resulting in the release of cytochrome C and the subsequent activation of caspase 9. The extrinsic pathway is initiated by ligation of a death receptor, which subsequently results in the activation of caspase 8 (section 1.4). *Mtb* has the ability to inhibit both the intrinsic and the extrinsic apoptosis pathways.

Inhibition of the intrinsic pathway

The intrinsic apoptosis pathway is characterized by the depolarization of the mitochondrial membrane, resulting in the release of cytochrome C, and the subsequent activation of caspase 9. *Mtb* has been shown to manipulate several of the pro- and anti-apoptosis proteins which modulate the intrinsic pathway. For instance, macrophages upregulate the anti-apoptotic protein Mcl-1 post infection with virulent H37Rv, but not avirulent H37Ra [134]. These results were corroborated by functional data using anti-sense oligonucleotides to knock-down Mcl-1. Upon infection with H37Rv, knockdown macrophages treated underwent higher levels of apoptosis than WT. Importantly, the increase in apoptosis corresponded with increased bactericidal effects, suggesting a role for apoptosis in bacterial killing [134]. Mcl-1 is a Bcl-2-like protein which, like other Bcl-2 like proteins, resides on the outer mitochondrial membrane and inhibits mitochondrial permeabilization by stabilizing the mitochondrial membrane. Infection with *Mtb* also induced the mRNA

expression of another Bcl-2 like protein, bfl-1/A1. Notably, upregulation of A1 was not observed upon infection with *Mtb*-H37Ra [116]. Other studies have also shown that the anti-apoptotic protein Bcl-w is upregulated in *Mtb*-H37Rv, but not *Mtb*-H37Ra, infected cells [135], while the pro-apoptotic protein Bad is inactivated following *Mtb* infection [136].

Inhibition of the extrinsic apoptosis pathway

Several lines of data have shown that *Mtb* can inhibit the extrinsic apoptotic pathway. This pathway is characterized by the cleavage and activation of caspase 8, which is usually initiated by the ligation of a death receptor, such as TNFR-1 or Fas. One way by which *Mtb* manipulates the extrinsic pathway is by mediating the expression of death receptors. Cell surface levels of Fas were shown to be lower in host cells upon infection by *Mtb* [117,118], which may help to protect infected cells from Fas mediated apoptosis. Additionally, *Mtb*-infected macrophages have been reported to exhibit increased secretion of soluble TNFR2 (sTNFR2). This receptor sequesters TNF-α in the extracellular milieu, competitively inhibiting its binding to TNFR-1, the activation of which leads to an apoptotic cell fate [137,138]. Furthermore, infection with *Mtb*-H37Rv can actively protect host cells from TNF-α mediated cell death, while imfection with *Mtb*-H37Ra cannot. When macrophages infected with non-virulent *Mtb*-H37Ra were exposed to TNF-α, they exhibited significantly increased levels of apoptosis induction (from 28.6% to 47.9%). However, exposure of cells infected with *Mtb*-H37Rv to TNF-α led to only a slight increase in apoptosis (12.6% to 17.4%)[139]. These data suggest that virulent *Mtb* is able to inhibit the extrinsic apoptosis pathway induced by Fas and TNF-α.

Lipid mediators and their roles in Mtb induced host cell apoptosis

Pro- and anti-inflammatory lipids have been shown to be important mediators of the immune response. Recently, two of these lipids, Lipoxin A_4 (LXA_4) and prostaglandin E2 (PGE_2) have been implicated in the apoptotic response of host cells to mycobacterial infections. LXA_4 is an anti-inflammatory eicosanoid that supports and augments *Mtb* infections [140]. Presence of LXA_4, which is created in *Mtb* infected macrophages, downregulates both iNOS and inflammatory cytokine production [140,141]. Accordingly, 5-lipoxygenase (5-LO) deficient mice, which can not make LXA_4, control mycobacterial infections better than wild type [140]. PGE_2 is an inflammatory lipid that has been shown to be important for controlling infections with Mycobacteria. The effects of LXA_4 and PGE_2 on the host cell are opposing, with higher cellular levels of PGE_2 resulting in mycobacterial control and higher levels of LXA_4 resulting in a pro-mycobacterial response.

Increased production of LXA_4 in macrophages infected with H37Rv has recently been linked to the inhibition of host cell apoptosis by blocking cell membrane repair [102] and inhibiting the formation of the apoptotic envelope [142]. Infection with *Mtb* induces plasma membrane disruptions in macrophages. Cells infected with H37Ra, but not those infected with H37Rv, are able to repair these perforations via delivery of Golgi and lysosmal membranes to the injured site, a process which is necessary for preventing apoptosis and inducing necrosis. Lysosomal membrane repair is dependant upon the production of cyclic AMP (cAMP), which is generated as a result of PGE_2 signaling. LXA_4 can inhibit PGE_2 mediated lysosomal repair by reducing transcription of COX2, the inducible PGH

synthase, which is required for PGE_2 synthesis [102,143](Figure 4A). Accordingly, infection of LXA_4 deficient *alox5*$^{-/-}$ macrophages with H37Rv resulted in higher levels of apoptosis and lower bacterial viability compared to infection in WT macrophages. LXA_4 has also been shown to inhibit the formation of the apoptotic envelope, an insoluble cellular casing composed of cross-linked proteins that forms prior to apoptosis [88,142]. Formation of the apoptotic envelope is dependent upon the deposition of the phospholipid-binding protein annexin-1 to the cell surface and subsequent transglutaminase-mediated crosslinking of annexin-1 [142]. Full length annexin-1 is necessary in order for efficient cross-linking to occur. Infection with H37Ra, or treatment with the apoptosis inducing drug epoptoside, induces the production of plasminogen activator inhibitor type 2 (PAI2), a protease inhibitor also found in the apoptotic envelope which protects annexin-1 from proteolysis. However, the production LXA_4 associated with H37Rv infection inhibits transcription of *PIA2*, which results in the subsequent cleavage of annexin-1, and the inability to form an apoptotic envelope leading to cell death via necrosis [142](Figure 4B).

While both of these anti-apoptotic mechanisms involve increased production of LXA_4, the two pathways do not seem to intersect at any later points. PGE_2 is necessary for lysosomal fusion with the plasma membrane, but does not seem to effect fusion of Golgi derived vesicles [102]. Interestingly, delivery of Golgi derived vesicles to the plasma membrane is necessary for annexin-1 accumulation on the cell surface, a PGE_2 independent process. It is unknown as to whether or not lysosomal fusion may be necessary for localization of PAI2 to the cell membrane. Natural killer cells (NK cells) have been shown to re-orient their Golgi apparatus during target cell

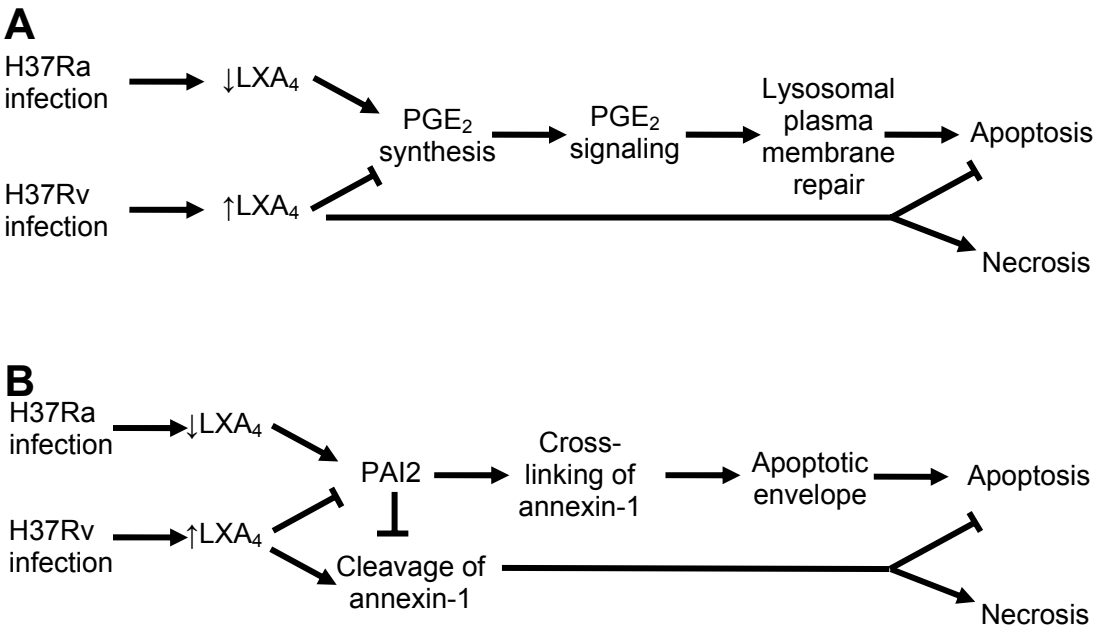


Figure 4. The role of LXA_4 in manipulation of host cell death pathways by virulent *Mtb*. (A) Increases in LXA_4 H37Rv results in the loss of the lysosomal repair pathway. Infection with mycobacteria results in cell membrane damage which can be repaired via the lysosomal repair pathway. Cells infected with avirulent strains of mycobacteria, such as H37Ra, produce high levels of PGE_2, but not LXA_4. PGE_2 signaling modulates the lysosomal repair pathway, allowing the cell to die via apoptosis. However, infection with virulent H37Rv induces production of high levels of LXA_4 which can downregulate PGE_2 transcription. In the absence of PGE_2, the host cell cannot repair its damaged membrane and dies via necrosis. (B) Increases in LXA_4 results in the inability to form an apoptotic envelope necessary for induction of apoptosis. Formation of the apoptotic envelope is dependant upon the ability of full length annexin-1 to cross-link. Upon induction of apoptosis by H37Ra the protease inhibitor PAI2 is produced, which protects annexin-1 from cleavage. However, LXA_4 production via H37Rv infection inhibits transcription of PAI2. Thus, annexin-1 is no longer protected from proteolytic cleavage and cannot cross-link, resulting in the inability to form the apoptotic envelope and a necrotic cell death fate.

cytolysis, directing it towards the cell membrane for delivery of cytotoxic effectors[144]. However, addition of LXA_4 causes disruption of this polarization and the Golgi apparatus becomes randomly oriented inside the cell [145]. It would be interesting to determine if LXA_4 also impedes Golgi orientation in macrophages and thus if it is involved in the delivery of annexin-1 to the plasma membrane.

1.5.3 Apoptosis inducing *M. tuberculosis* mutants

Perhaps the most convincing evidence that *Mtb* inhibits apoptosis is the existence of several pro-apoptotic *Mtb* deletion mutants. Creation of these mutants demonstrates that *Mtb* contains genetic traits that actively inhibit cell death. The first anti-apoptotic protein identified in *Mtb* was NuoG (Section 1.6)[123]. However, in addition to NuoG, two other *Mtb* proteins, protein kinase E (PknE) and SecA2, have been implicated in inhibition of apoptosis. *PknE* encodes a serine threonine kinase whose promoter is induced during nitric oxide stress. The deletion of *pknE* resulted in a mutant that was more susceptible to nitric oxide exposure and also capable of inducing a higher level of apoptosis in human macrophages compared to wild type [146]. *SecA2* codes for the mycobacterial secretion system that secretes SodA, an enzyme that catalyzes conversion of superoxide anions to hydrogen peroxide [147]. SecA2 deficient bacteria induce apoptosis upon infection. However this phenotype is lost when knock out bacteria are complemented with SodA, indicating that it is the secretion of SodA that is involved in inhibiting apoptosis [148].

1.6 NuoG inhibits macrophage apoptosis

A gain-of-function screen was used by Velemurugan *et al* to show that *Mtb* genes can actively inhibit host cell apoptosis induced by non-virulent mycobacterial species [123]. Cosmids containing large fragments of *Mtb* genomic DNA were transformed into the apoptosis-inducing bacterial species *Mycobacterium smegmatis* and *Mycobacterium kansasii*. *M. smegmatis* clones were screened for a reduction in infection-induced apoptosis, resulting in the identification of 3 independent regions of the *Mtb* genome involved in the inhibition of host cell apoptosis. Characterization of one of these loci led to the identification of the first mycobacterial gene identified to be involved in the inhibition of host cell apoptosis. This anti-apoptosis activity was attributed to the type I NADH-dehydrogenase of *M. tuberculosis*, and was mainly due to the subunit of this multicomponent complex encoded by the *nuoG* gene. Expression of *Mtb-nuoG* in the nonpathogenic, apoptosis inducing species *Mycobacteria kansasii* conferred upon it the ability to inhibit apoptosis of infected human and murine macrophages. Conversely, the deletion of *nuoG* from *Mtb* ablated its ability to inhibit macrophage apoptosis in both primary macrophages and in cell lines. Importantly, the *nuoG* mutant was also less virulent than WT *Mtb*. Immunodeficient SCID mice infected with Δ*nuoG* survived longer and induced more apoptosis in the lungs than those infected with wild type. This correlation with virulence was also observed in immunocompetent Balb/C mice infected with Δ*nuoG* [123].

1.7 Summary and significance

Mtb has already infected approximately 2 billion people in the world, making it the most prevalent disease-causing bacteria worldwide [149]. To date, there is no reliable vaccine against this microbe, and the antibiotics available are inconvenient and fast becoming obsolete. It is obvious that new vaccines or drugs must be developed before strains *Mtb* become resistant to all available antibiotics. The ability for *Mtb* to inhibit apoptosis in host cells is a virulence mechanism that can be a target for new potential drugs, or be exploited to make a better vaccine. It is therefore important to identify the molecular pathways by which *Mtb* inhibits cell death. For instance, drugs that prevent the bacterium from inhibiting apoptosis would make the bacillus more susceptible to the host innate immune response. Furthermore, a vaccine strain that induces more apoptosis than the current vaccine, BCG, may more effectively stimulate an adaptive IR. Previous work in our lab has shown that *Mycobacterium tuberculosis* can inhibit host cell apoptosis and that this inhibition is necessary for virulence. We have also shown that one of the several proteins involved in this inhibition of apoptosis is NuoG, the largest subunit of the NADH dehydrogenase. The focus of my research studies was to determine the NuoG dependent molecular pathways by which *Mtb* inhibits macrophage cell death.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials.

C57/B6 (B6) and *gp91* knockout (*gp91*$^{-/-}$) mice were obtained from Jackson laboratories (www. jaxmice.jax.org) and *TNF-α* knockout (*TNF-α*$^{-/-}$) mice were kindly provided by Dr. Karen Elkins (FDA, Bethesda, MD). Caspase specific inhibitors and analogs were purchased from Calbiochem (www.emdbiosciences.com). Neutralizing anti human-TNF-α antibody (#500-M26), the biotinylated detection antibody (500-P31Abt) were purchased from Peprotech Inc (www.peprotech.com). Recombinant human and murine TNF-α, and anti murine-TNF-α antibodies were purchased from BD Pharmingen (www.bdbiosciences.com). CM-DCFDA, DHE, and Vybrant® DiI cell-labeling solution were purchased from Invitrogen (www.invitrogen.com). All other reagents unless otherwise noted were purchased from Sigma (www.sigma.com).TNFα

2.2 Bacteria and culture conditions.

M. tuberculosis H37RV (ATCC 25618) was obtained from the American Type Culture Collection (www.atcc.org), Mtb*ΔkatG* was obtained from TARGET, and Δ*nuoG* has been previously described [123]. All mycobacteria, excluding Δ*katG*, were grown in Middlebrook 7H9 media supplemented with 0.5%glycerol, 0.5% Tween-80, and 10% ADS. Δ*KatG* was grown in the same media supplemented with ADC in place of ADS. Mycobacterial strains expressing plasmid constructs, including NuoG-myc and NuoG-PhoA, were introduced into mycobacterial species via electroporation. Late log phase culture was washed 3 times with 10% glycerol-

0.5% tween and he final pellet was resuspended in 1/10 the original volume of glycerol-tween. Approximately 250-500µl of bacteria were combined with 2-5µl of plasmid in a 0.2cm cuvette (Biorad). Bacteria were electroporated at 2.5kV; 25uFD; 1000Ω in a BioRad GenePulser Xcell™ electroporator. 7H9 media was added to the bacteria, which were allowed to recover for 3hrs for *M. smegmatis* or 24hrs for slow growers (*Mtb*, BCG, *M. kansasii*). The cells were then plated on 7H10 agar plates containing the appropriate selective media and incubated for 3 weeks at 37^{o}C. Resulting colonies were screened by western blot or fluorescence microscopy to ensure take-up of the plasmid. For selective media, 50µg/ml Hygromycin or 25µg/ml Kanamycin were added.

2.3 Cell culture conditions and infection.

Human myelomonocytic cell line THP1 (ATCC® TIB-202™) was cultured in RPMI (ATCC) supplemented with 10% heat inactivated fetal calf serum (FCS) (Hyclone) and differentiated using 20ng/ml phorbol myristate acetate (PMA) as described [123].Bacteria were grown to an OD_{600} ranging from 0.5 to 0.8 and the culture was allowed to settle for 10 minutes. Infections were carried out at a multiplicity of infection (MOI) of 5:1 (5 bacilli to 1 cell) for 4 hours in infection media containing 10% human serum and 10% non heat inactivated FCS. After 4 hours, extracellular bacteria were removed by 2 washes with phosphate buffered saline (PBS) and the cells were incubated in chase media containing 100 µg/ml of gentamicin (Invitrogen) for 1, 3, or 5 days post infection as detailed in the figure legends. Human alveolar lavage was obtained from Mark Cohan at the University of

Maryland-Baltimore. Approximately 10-15ml of obtained lavage was filtered through sterile gauze and cells were washed 3 times in PBS. Cells were resuspended in warm RPMI with 10% heat inactivated FCS, seeded on 8 well slides, and allowed to rest for 1-3 days. Infection was carried out as described above. Bone marrow derived macrophages were flushed from the femur and tibia of B6 and knockout mice and differentiated in Dulbecco's modified eagle medium (DMEM) containing 20% L-929 cell supernatant as previously detailed [123]. Murine cells were infected as described above using 10% FCS and 5-10% L-929 cell supernatant in the infection and chase media. L-929 supernatant was included in order to protect against cytokine withdrawal induced apoptosis. For experiments using caspase inhibitors or analog (20μM), antioxidants (15mM glutathione), and oxidase inhibitor (10μM diphenylene iodonium, DPI), the cells were incubated with the reagents during infection and chase period. In experiments using TNF-α neutralizing antibody (#500-M26, Peprotech) the antibody was included only in the chase medium at a concentration of 5μg/ml.

2.4 Apoptosis assays

The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was preformed to reveal apoptosis-induced DNA fragmentation in tissue culture, primary human, or murine cells using the "In Situ Cell Death Detection Kit-Fluorescein or –TMR Red" (Roche Applied Sciences at roche.com). Post infection, cells were fixed with 4% paraformaldehyde (PFA) overnight to ensure non-viability of bacteria. Cells were then washed 1X in PBS and incubated for 3 minutes at 4^{o}C in permeabilisation buffer (0.1M sodium citrate, 0.1% triton-X). After washing, the

cells were resuspended in 50µl of TUNEL solution and stained for 1hr at 37°C. The percentage of stained cells was analyzed using flow cytometry (Becton Dickinson FACS-Calibur or FACSCanto II) or viewed via fluorescent microscopy (Zeiss AxioCam Mrm) and quantified by counting.

2.5 ROS detection assays

Reactive oxygen species in primary BMDMs and alveolar macrophages were detected at 24hrs or 3 days post infection respectively using the ROS sensitive dyes 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFDA) and dihydroethidium (DHE) (Invitrogen). Bone marrow cells were deprived of L-929 supernatant 16 hrs prior to infection and maintained in L-929 free media without phenol red for the length of the experiment as addition of L-929 abrogated the ROS phenotype (data not shown). Human alveolar macrophages were maintained in normal growth, infection, and chase media. In some cases, bacteria were labeled with lipophilic red dye Vybrant® DiI (Invitrogen). Bacteria were incubated in 7H9 media containing 5ul/ml of DiI for 30 minutes, washed twice with PBS-0.5% Tween-20 (PBST), and then used for infection as normal. Post infection, murine or alveolar macrophages were washed once in Hank's buffered salt solution (HBSS) and then incubated in 10µM DCFDA for 30 minutes or 2µM DHE for 15 minutes at room temperature. Cells were washed 3 times with HBSS, fixed with 4% paraformaldehyde, and analyzed by either flow cytometry or fluorescence microscopy. For staining with Alexa Fluor 555, 1 ml of late log phase wild type and Δ*nuoG* bacteria were washed 1 time in PBST and resuspended in 500ul of 0.1M

sodium bicarbonate solution. Bacteria were incubated with 0.25 mg of Alexa Fluor® 555 carboxylic acid, succinimidyl ester (Invitrogen) for 1 hr at 37°C. Un-bound dye was removed by 2 washes with PBST, and the bacterial pellet was resuspended in 500ul of PBST. Cells were infected, stained, and visualized as described above.

2.6 Enzyme linked immunosorbent assay (ELISA)

ELISA was performed with the supernatants of bone marrow derived macrophages or THP-1 cells infected for 3 or 5 days and treated with or without glutathione or DPI as described above. For detection of human TNF-α, the ELISA-plates were coated with 2μg/ml capture antibody for 2 hours at 37°C. 100μl of cell supernatant was used for the reaction and recombinant human TNF-α (554618, BD Pharmingen) diluted in infection medium was used as a standard. TNF-α was detected using the secondary biotinylated anti human-TNF-α detection antibody, Streptavidin-alkaline phosphatase at 1μg/ml (Zymed), and phosphatase substrate at 1mg/ml (Sigma). The plate was read at an absorbance of 405nm. Murine TNF-α ELISAs were preformed as above using recombinant mouse TNF-α standard (#554589 BD Pharmingen), the capture antibody rat anti murine-TNF-α, and the biotinylated detection antibody rat anti mouse-TNF-α. Specific antibodies and their working concentrations can be found in Table 1.

2.7 *In vivo* aerosol infection

WT B6 and *gp91* knockout mice were purchased from Jackson laboratories and were housed in the BSL3 mouse facility for 10 days prior to infection. Mice

Use	Antibody	Dilution	Clone	Source
WB	Mouse α myc	1/500	9E10	DSHB
WB	Mouse α SodA	1/40	CS-18	CSU
WB	Rabbit α KatG	1/5,000	α-KatG	Donated by Brigitte Saint-Joanis
WB	Rabbit α Rv3881c	1/6,000	TbH4	Donated by Young Gao
WB	Rabbit α Ant85	1/5,000	α-Ag85	CSU
WB	HRP-Goat α mouse IgG	1/40,000	81-6520	Zymed
WB	HRP-Goat α rabbit IgG	1/20,000	81-6120	Zymed
FM	Mouse α myc	1/20	9E10	DSHB
FM	DyLight™ 488-Donkey α mouse IgG	1/100	715-486-150	Jackson Immuno-Research
Elisa-capture	Mouse α human TNF-α	2 μg/ml	500-M26	Peprotech
Elisa-trace	Biotin- α human TNF-α	200ng/ml	500-P31	Peprotech
Elisa-capture	Rat α murine TNF-α	8 μg/ml	551225	BD Pharmingen
Elisa-trace	Biotin-Rat α murine TNF-α	1 μg/ml	554415	BD Pharmingen

Table 1. Concentrations and names of utilized antibodies. Primary antibodies were used either for western blotting (WB), fluorescence microscopy (FM), or ELISA at the depicted dilutions and concentrations. Antibodies came from Colorado State University (CSU) TB vaccine testing and research materials contract and the Developmental Studies Hybridoma Bank (DSHB) unless otherwise noted.

were infected in a Glas-Col's® inhalation exposure system aerosol machine. Late log phase H37Rv and Δ*nuoG* bacteria were washed once with PBST and the concentration was adjusted to $1x10^6$ bacteria/ml. Mice were added into the nebulizer basket and 5ml of *Mtb* was slowly added to nebulizer using a 10ml syringe. The aerosol infection machine was run according to the following default infection program:

Nebulizing time: 1800 seconds
Cloud decay time: 1800 seconds
Decontamination time: 900 seconds

The process was repeated for infection with Δ*nuoG* bacteria using a new, clean nebulizer. Mice were observed daily and disease progression was scored as agreed by the University of Maryland Institutional Animal Care and Use Committee (IACUC). Mouse lungs were harvested for bacterial colony forming units (CFUs) at 1, 7, 22, 42, 70 days post infection. Briefly, mice were anesthetized with isoflurane and killed via cervical dislocation. The thoracic cavity was opened and the lungs were removed. Approximately one third of the lower lobe on the left lung was removed and fixed in formulin for histopathology. Remaining lung tissue was cut into small sections and placed in a stomacher bag containing 4.5ml of sterile PBST. Bacteria were flushed from the lung tissue by homogenation, first by hand for 5 minutes, and then with the stomacher for 5min on the high setting. Homogenate was then strained through a 70μm filter and cells and bacteria were pelleted by centrifugation at 3000RPM for 20 minutes. The pellet was serially diluted in PBST, plated on 7H10 agar plates, and the CFUs were calculated after approximately 3 weeks of growth at 37°C. Lung sections

were sent to AML labs for paraffin embedding, sectioning, and Hemolysin and Eosin staining.

2.8 *In vitro* superoxide susceptibility assays

In vitro superoxide killing assays were performed using a hypoxanthine/xanthine oxidase system to generate superoxide. Mid-log-phase cultures of bacteria grown in 7H9 medium were washed and adjusted to a density of approximately 10^6 CFU/ml. Bacteria were exposed to superoxide generated by combining 250 μM hypoxanthine with 0.1 U of xanthine oxidase per ml in PBS. Catalase (1 U/ml) was added to prevent killing by H_2O_2 during the assay. Percent survival was determined at 0, 1, and 3 hours post exposure by plating serial dilutions of the bacteria on 7H10 plates. The means from triplicate tubes were calculated, and the data were expressed as a percentage of the values at time zero [45].

2.9 Preperation of cell lysates, culture filtrates, and western blotting

Cell lysate preperation

Pellets from 10-30ml of late-log phase Mycobacterial cultures grown in 7H9 were washed once in PBST and pellets were resuspended in 500μl of protein extraction solution (TRIS, NaCl, 1.0% Triton-X, protease inhibitor cocktail) with 100μl of 106μm beads. The bacterial suspension was bead beated for 3 minutes using a BioSpec™ Mini bead beater, and the beads and unbroken cells were spun down by centrifugation and the CL was collected.

Culture filtrate preperation

Mycobacteria strains were grown in 7H9 media until late-log phase, pelleted and then diluted in BSA-free Sauton's media. The cultures were passaged at least once before being transferred into 30ml of Sauton's media. Bacteria were cultured for about 4-5 days, or until they reached mid-log phase (OD_{600} 0.4-0.7), before being harvested by centrifugation. The culture filtrate was prepared by filtering the resulting supernatant once through a 0.22μm filter and concentrated approximately 50-fold in an Amicon Ultra® centrifugal filter concentration device.

Electrophoresis and Western blotting

The protein concentration of the cell filtrates and cell lysates were determined using the BCA assay according to the manufacturer's protocol (Pierce). Samples were diluted with 4x sample buffer and boiled for 5min at 95°C+. Equal amounts of protein from each sample were loaded into 4-20% protein gels, and the proteins were separated via electrophoresis (100V for 1hr). Proteins were transferred onto a PVDF membrane using the Genie Blotter (Idea Scientific Co.)(12V for 1hr) and the membrane was blocked with 5% milk in PBS for 1hr before blotting with specific primary antibodies (Table 1). The secondary antibody HRP-Goat α Mouse, or α Rabbit IgG, and ECL (Pierce) were used for detection.

2.10 Immunofluorescence microscopy

Approximately 10ml of late log phase Δ*nuoG*:nuoG-myc bacteria were pelleted and fixed overnight in 4% PFA at 4°C. Droplets (around 20μl) of bacteria in 4% PFA were fixed to microscope slides by drying. Specimens were blocked with 2% BSA in PBS for 20 minutes before adding anti-myc primary antibody (Table 1).

After 1 hour of incubation at room temperature, the wells were washed 3 times with PBS and the bacteria were incubated with AlexaFluor 488-α murine IgG for 2 hours. Bacteria were also stained with PI for 10 minutes before washing. Slides were mounted using ProLong® Gold anti-fade reagent and visualized via fluorescence microscopy.

2.11 Creation of Mycobacterial knockouts

Mycobacteria tuberculosis gene knockouts were created using a specialized phage transduction strategy (Figure 5) [150]. The regions of the *Mtb* genome flanking the genes of interest were amplified by PCR and cloned into a pjsc347 based vector containing a Hygromycin resistance gene (P004). Once the orientation and sequences of the flanking regions were confirmed by sequencing, the construct was digested with the PacI restriction enzyme and ligated into the mycobacterial phage phae159 to create a phasmid. This phage is lysogenic when grown at 30^{o}C, but not at 37^{o}C. The phasmid DNA was then packaged using *E. coli* phage packaging protein (Gigapack® Stratagene Inc), and used to infect *E. coli* for phasmid amplification. Phasmids were extracted from *E. coli* expressing Hygromycin resistance, and were electroporated into *M. smegmatis*. Transformed and non transformed bacteria were mixed together with noble agar, plated onto 7H10 agar plates, and grown at 30^{o}C for 3-4 days. At this temperature the phages lysed infected bacteria, releasing more phage into the media, and infecting neighboring cells. After 3-4 days, plaques consisting of lysed bacteria were visible and phages were harvested by adding MP buffer (50mM Tris-Hcl, 150mM NaCl, 10mM $MgSO_4$, 2mM $CaCl_2$) and shaking

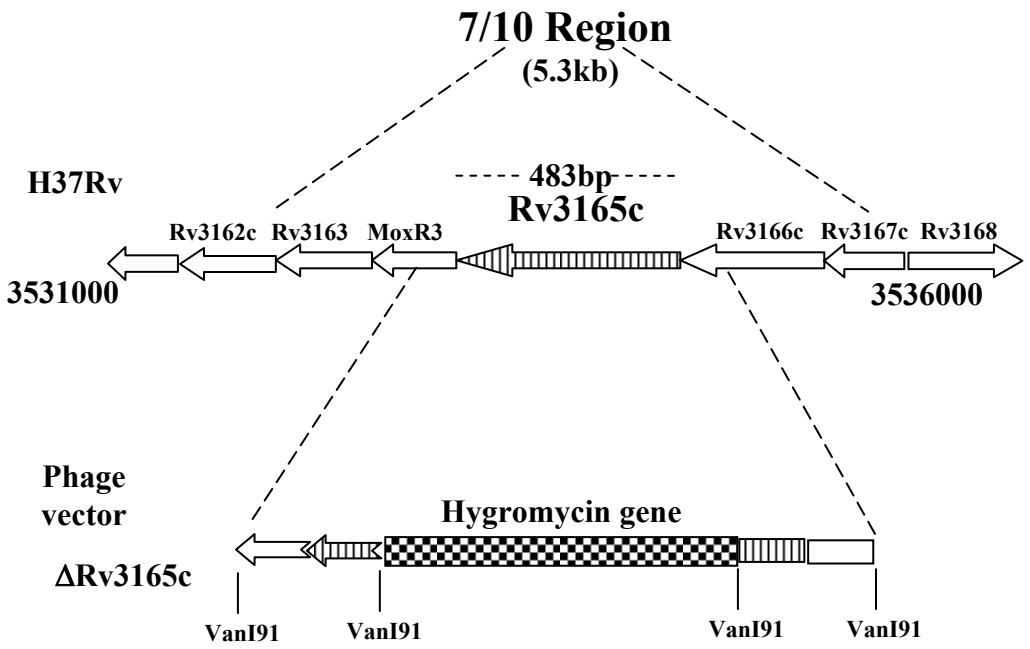


Figure 5. Strategy of gene knockout in *Mycobacterium tuberculosis* by specialized phage transduction. **N**ull mutants in *Mtb* were created by introducing a hygromycin cassette into the genome by specialized phage transduction (creation of Δ*3165c* shown). In brief, PCR fragments of DNA regions flanking Rv3165c, including portions of surrounding genes, were ligated into VanI91 sites of the P004 vector, which contains a Hygromycin resistance gene. This vector was then incorporated into a phage and introduced into *Mtb* via transduction. Knockouts were created via double recombination, during which the Hygromycin cassette replaced the gene in the genome.

slowly for 2 hours. The phage solution was filtered through a 0.22μm filter before being used for transduction [150].

Transduction

H37Rv was grown to an OD_{600} of 0.5-1.0 and approximately 10 ml of culture was washed once with MP buffer (50mM Tris, 150mM NaCl, 10mM $MgSO_4$, 2mM $CaCl_2$) before being resuspended in 1.0ml of the same buffer. The bacteria were then incubated with 1ml of phage (~ 10^{7-8} plaque forming units /μl) at 37°C for 24 hours. Bacteria were then allowed to recover in 7H9 for 24hrs before being plated on 7H10 agar plates with 50μg/ml of Hygromycin [150].

2.12 Statistical analysis

Statistical analyses were performed on three independent experiments each with two experimental replicates (ANOVA with Tukey post-test) unless otherwise noted in the figure legends. Significance indications are as follows: *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$. Graph-bars represent the mean ± the standard error of the sample and in-text values are depected as (mean ± standard deviation). Percentages of DCFDA or DHE positive cells found in the sample and not the control (Figure 9 and Figure 11) were calculated by subtracting the histogram of uninfected cells from experimental histograms using Overton cumulative histogram subtraction (FlowJo version 8.8.6 DMV). Differences were compared via ANOVA.

CHAPTER 3. RESULTS AND DISCUSSION

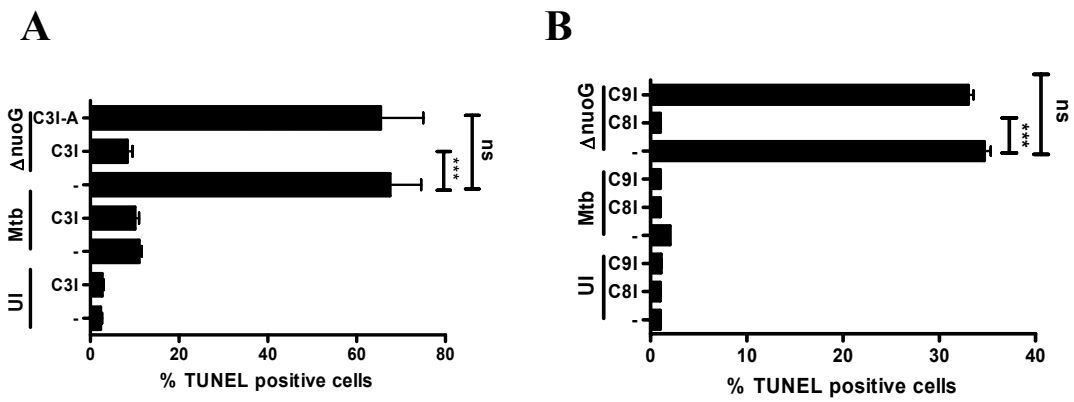
3.1 Molecular mechanisms of Δ*nuoG* induced apoptosis in macrophages.

NuoG of *Mtb* was previously shown to be involved in the inhibition of apoptosis by *Mycobacterium tuberculosis* [123]. The *nuoG* deletion mutant was shown to both less virulent in mice and induce more apoptosis in macrophages than WT *Mtb* [123]. The following studies address the molecular pathways that NuoG manipulates in effort to inhibit cell death in macrophages. Several of these experiments were joint efforts between Dr. K.K. Velmurugan and myself.

3.1.1 The Δ*nuoG* mutant induces apoptosis via an extrinsic, caspase-dependent pathway

Velmurugan *et al*. previously demonstrated that a Δ*nuoG* mutant of *Mtb* induced more apoptosis in host cells than wild type bacteria [123]. In order to analyze the mechanism of the NuoG/NDH-1 mediated host cell apoptosis inhibition, we first determined the involvement of caspases in the pro-apoptotic phenotype of Δ*nuoG* using specific caspase inhibitors. PMA-differentiated THP-1 cells were pre-treated with Caspase-3 inhibitor (C3I) or a chemical analog with no inhibitor activity (C3I-A) at 20 μM for 3h before infection, during infection, and after infection. Cells were either left uninfected, or were infected with *Mtb* or Δ*nuoG*. After five days cells were harvested and stained for genomic DNA degradation using TUNEL assay. The percentage of TUNEL positive cells was determined by flow cytometry analysis. This analysis revealed that the uninfected population contained low percentage of apoptotic cells (2.3±0.6%), *Mtb* infection slightly increased this amount to 11±1.0%.

As expected from previously published results [123], cells infected with the *nuoG* mutant showed a very significant increase in apoptosis (67.7±18.9%). Treatment of THP-1 cells with the C3I reduced the percentage of Δ*nuoG* induced apoptosis to 8.3±2.1%, whereas the C3I-A had no significant effect on apoptosis induction (65.3±16.8%)(Figure 6A). The C3I did not have an effect on the low level of *Mtb*-induced apoptosis, as 10.0±1.7% of C3I-treated *Mtb*-infected cells were TUNEL positive, suggesting that *Mtb* may be inducing low levels of apoptosis via a caspase independent mechanism. In order to determine if the *nuoG* mutant induces apoptosis via the extrinsic (i.e., death receptor mediated), or the intrinsic (i.e., mitochondrial) pathway [151], cells were treated with Caspase-8 and Caspase-9 inhibitors, respectively. The experimental conditions and analysis were identical to the previous experiment with the exception that cells were harvested 3 days post infection. Analysis of TUNEL staining after this shorter time period resulted in similar rates of apoptosis for *Mtb* infected and uninfected cells (2.1±0.1% and 1.1±0.1%, respectively). Treatment of these populations with either C8I or C9I had no effect. The *nuoG* mutant induced apoptosis in 34.7±1.1% of cells, which was not significantly affected by the addition of C9I (33.2±0.8), but was significantly reduced by the addition of C8I to levels similar to uninfected cells (1.2±0.2%)(Figure 6B). These results indicated that the *nuoG* mutant induced host macrophage apoptosis via an extrinsic, caspase-8 dependent signaling pathway.

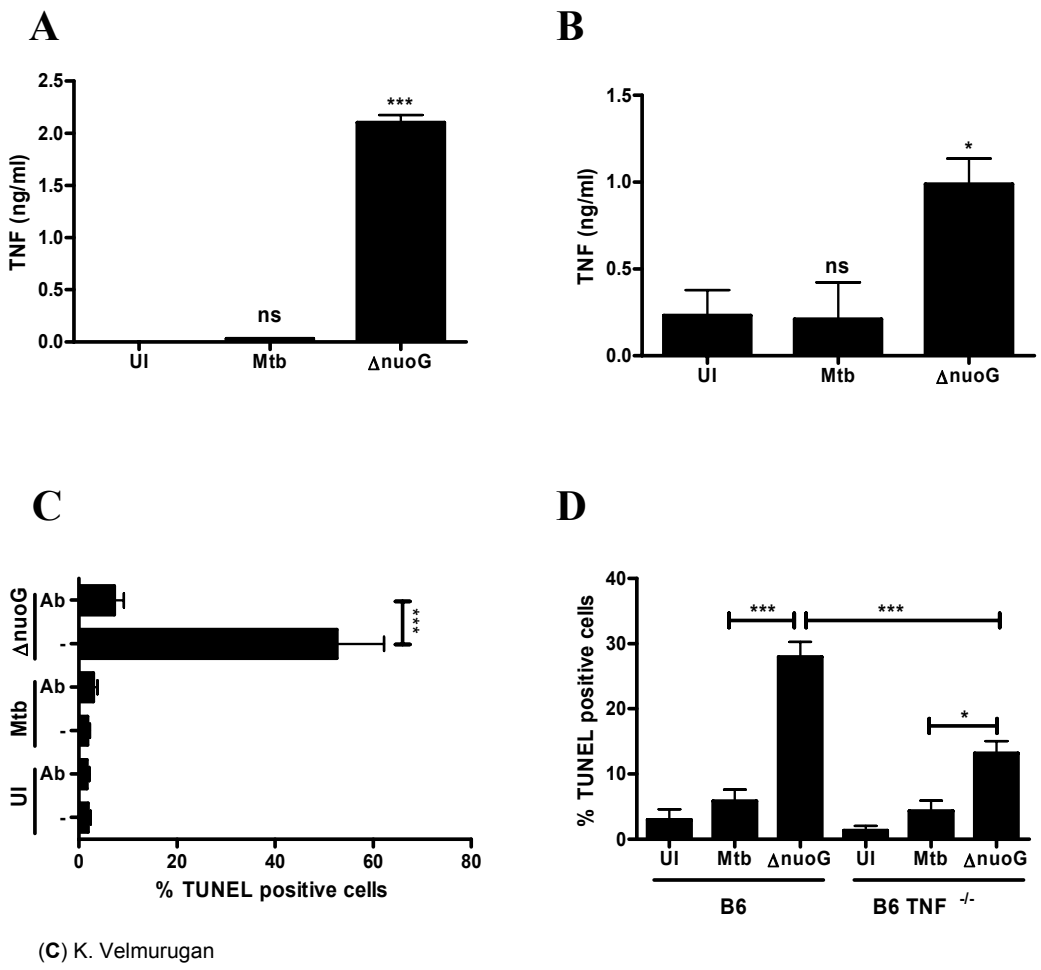


(**A & B**) K. Velmurugan.

Figure 6. *Mtb* NuoG mediates inhibition of extrinsic but not intrinsic apoptosis pathways. (A and B) THP-1 cells were either infected with *Mtb* or the *nuoG* mutant (Δ*nuoG*) for 4h at an MOI of 5, or left uninfected (UI). (A) Cultures were either treated with a 20 μM of Caspase-3 inhibitor (C3I), an inactive analog of the inhibitor (C3I-A) or medium only (-), and analysis of TUNEL+ cells by FACS was performed after 5 days. (B) As in (A), THP-1 cells were infected or left untreated (UT), and cultured with specific inhibitors of Caspase-9 (C9I), Caspase-8 (C8I) or in medium alone (-) for 3 days, followed by analysis of $TUNEL^{+}$ cells. Statistical analysis was performed on three independent experiments (ANOVA with Tukey post-test) and significance is indicated as follows: *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$.

3.1.2 Host macrophage TNF-α is important for the apoptogenic phenotype of the *nuoG* mutant

TNF-α is of major importance for a successful host defense against mycobacterial infections, and has also been implicated in the apoptosis response to mycobacterial infection by the macrophage [21,137]. Since TNF-α receptor signaling can result in cellular apoptosis, we tested whether autocrine TNF-α production and signaling were involved in apoptosis of Δ*nuoG* infected THP-1 cells. We first determined if infection with Δ*nuoG* resulted in an increase of secreted TNF-α. Supernatants from infected THP-1 (Figure 7A) and BMDM cells (Figure 7B) were collected 3 days post infection and levels of TNF-α were measured by ELISA. In both systems, Δ*nuoG* infected cells secreted significantly more TNF-α than those infected with wild type (30 pg/ml to 2.1 ng/ml for *Mtb* and Δ*nuoG* in THP-1 cells; 0.2 ng/ml to 1 ng/ml for *Mtb* and Δ*nuoG* in murine cells). Having established the presence of higher levels of TNF-α, we next evaluated the effect of TNF-α signaling on the pro-apoptotic phenotype of the *nuoG* deletion mutant. This was first addressed by addition of human TNF-α-specific, neutralizing antibodies (5 μg/ml) to the culture media of THP-1 cells during and after infection. The addition of anti-TNF-α-antibody significantly inhibited macrophage apoptosis induced by Δ*nuoG* infection, as the percentage of apoptotic cells was reduced from 62.3±8.6% to 8.7±4.0% after addition of antibody (Figure 7C). PCD in uninfected or *Mtb* infected cells was not significantly affected by the addition of antibodies (Figure 7C). The involvement of TNF-α in the pro-apoptotic phenotype of the *nuoG* mutant was further analyzed by utilizing BMDM from *TNF*-$\alpha^{-/-}$ mice. The *nuoG* mutant induced apoptosis in



(**C**) K. Velmurugan

Figure 7. *Mtb* NuoG mediates inhibition of TNF-α-induced apoptosis and TNF-α secretion. (A and B) TNF-α secretion was measured in human THP-1 (A) or primary murine (B) macrophages 3 days post infection with *Mtb* and *nuoG* knockout bacteria by ELISA. Results in (A) are a representative example. (C) THP-1 cells were infected with wild type (*Mtb*) and *nuoG* knockout (Δ*nuoG*) bacteria in the presence of 5 μg of TNF-α-neutralizing antibodies. Apoptotic cells were quantified by TUNEL staining 5 days post infection. (D) BMDM derived from C57B/6 (B6) and *TNF*-α knockout mice (B6 *TNF* $^{-/-}$) were infected with wild type and mutant bacteria and were assayed for apoptosis 5 days post infection.

28±5.7% of wild type C57B/6 (B6) cells, as compared to 5.8±4.4% of *Mtb* infected cells (Figure 7D). In contrast, the pro-apoptotic phenotype of the *nuoG* mutant was partially reduced in *TNF*-$\alpha^{-/-}$ BMDM, resulting in levels of apoptosis of 13.3±4.3%, whereas *Mtb* infected cells were not significantly different at 4.3±4.0%. Overall, these experiments confirmed the involvement of TNF-α in the pro-apoptosis phenotype of the *nuoG* mutant.

3.1.3 Host macrophage NOX2-derived reactive oxygen species are necessary for Δ*nuoG*-induced apoptosis and increase in TNF-α secretion

Reactive oxygen species are involved in shifting the balance of TNFR-1 mediated signaling from anti-apoptotic to pro-apoptotic [100,152] (Section 1.4). We investigated the role of ROS in Δ*nuoG* induced apoptosis by utilizing a general ROS scavenger (the antioxidant glutathione) and an oxidase inhibitor (diphenylene iodonium or DPI) during infections of THP-1 cells [57]. THP-1 cells were incubated with 15 mM glutathione or 10 μM DPI 3 hours prior to and throughout infection with *Mtb* and Δ*nuoG*. Untreated cells infected with the *nuoG* mutant induced apoptosis in about 44.0±2.0% in the population, as compared to 1.0±0.1% in uninfected, and 3.7±0.6% in *Mtb* infected cells (Figure 8A). The presence of DPI and glutathione reduced apoptosis induced by the mutant to 6.3±0.6% and 5.3±1.5% of cells, respectively (Figure 8A). Thus, both of these agents greatly suppressed apoptosis induced by Δ*nuoG* in THP-1 cells, a finding consistent with a strong dependence of the apoptotic death response on ROS accumulation (Figure 8A) [100]. Increased ROS levels in the cytosol can also lead to increased gene transcription of an array of

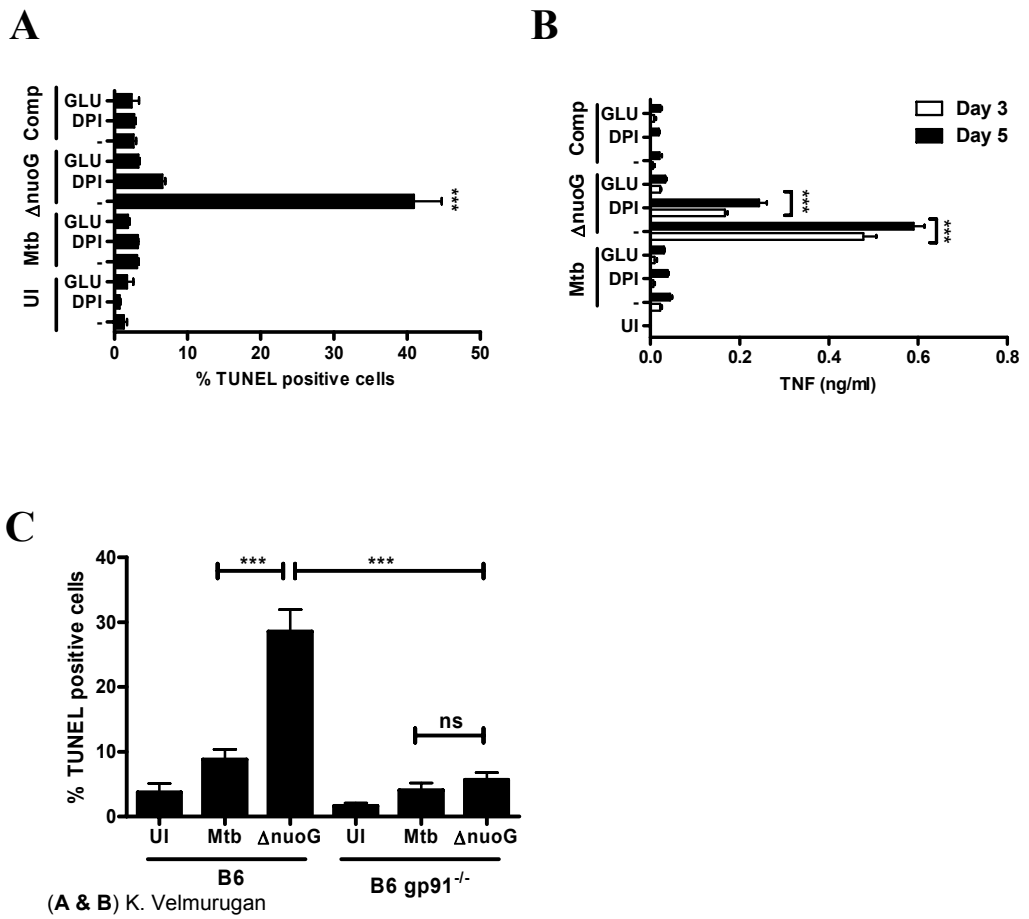


Figure 8. *Mtb* NuoG mediates inhibition of ROS-dependent induction of apoptosis and TNF-α secretion. (A) THP-1 cells were infected with wild-type (*Mtb*), Δ*nuoG*, or complemented mutant (Comp) strains of *Mtb* or left uninfected (UI). Cultures were incubated in medium alone (-), or in medium containing 15 mM glutathione (GLU) or 10 μM DPI. Apoptotic cells were quantified via TUNEL staining 5 days post infection. (B) Supernatants of the cultures from (A) were harvested on indicated days, and levels of TNF-α were determined by ELISA. (C) Macrophages derived from wild type C57B/6 (B6) or NOX2 deficient *gp91* knockout mice (B6 *gp91*$^{-/-}$) were infected with *Mtb* or Δ*nuoG*. Apoptosis was assayed after 5 days by TUNEL staining.

genes involved in oxidative stress and immunity, including TNF-α [152]. For that reason, the TNF-α levels in the supernatant of infected THP-1 cells were analyzed after 3 and 5 days by ELISA. Insignificant amounts of TNF-α were detected in supernatants of uninfected cells, and only low concentrations of TNF-α (below 50 pg/ml) were detected in supernatants of cells infected with *Mtb* or the complemented *nuoG* mutants strains (Figure 8B). In contrast, the *nuoG* mutant increased secretion of TNF-α by a factor of 10 to 0.5-0.6 ng/ml. This increase was partially reduced to about 0.1-0.2 ng/ml by treatment of the cells with DPI, and almost completely reduced by the addition of glutathione (0.02-0.03 pg/ml)(Figure 8B). Thus, the increase of intracellular ROS induced by infection of cells with the *nuoG* mutant is required for the increase in TNF-α secretion by infected cells.

Next, we addressed the question of the subcellular origin of ROS during Δ*nuoG* infection. The mitochondrial respiratory chain complex I is an important generator of cellular ROS that is shared by all cells types and might be at the origin of mitochondrial-induced host cell apoptosis. However, the NADPH oxidases are also potent inducers of cellular and extracellular ROS. In macrophages, the phagocyte NADPH oxidase, NOX2, is recruited to phagosomes and generates the production of superoxide in the lumen of the phagosome. These superoxides and their derivates are thought to be important for the killing of ingested bacteria, although their role in pathogenesis is not completely understood. In order to address the importance of NOX2 in the pro-apoptotic phenotype of the *nuoG* mutant, we utilized BMDM derived from mice deficient in NOX2 activity due to the deletion of the major transmembrane subunit of the NOX2 complex, $gp91^{phox}$ ($gp91^{-/-}$). The *nuoG* mutant

induced significantly more apoptosis than *Mtb* in macrophages of wild type C57Bl/6 mice, 31.3±7.6% versus 11.1±5.9%, respectively (Figure 8C). Importantly, this increase was abolished when *gp91*$^{-/-}$ BMDM were used as host cells, since only 7.3±3.2% of Δ*nuoG* infected cells were apoptotic compared to 4.8±3.5% of *Mtb*-infected cells. Therefore, the presence of functional NOX2 is required for the pro-apoptotic phenotype of the *nuoG* mutant of *Mtb*.

3.1.4 Macrophage infection with Δ*nuoG* induces phagosomal ROS accumulation

If the ROS responsible for the pro-apoptotic phenotype of the *nuoG* mutant originate from NOX2, then macrophages infected with Δ*nuoG* should have higher intracellular levels of ROS than those infected with *Mtb*. In order to address this hypothesis, two dyes for detection of ROS were utilized: DCFDA, which is more sensitive to H_2O_2, and DHE, which is more sensitive to O_2^-. Macrophages were infected and after 24hr the amount of ROS was detected in uninfected, *Mtb* and *Mtb*Δ*nuoG* infected cells using flow cytometry analysis. *Mtb* infection induced only slightly elevated levels ROS as detected either by DCFDA or DHE since the histogram overlays closely with that of uninfected cells (Figure 9A, B). Conversely, both dyes detected a significant increase in ROS levels after infection of wild type cells with the *nuoG* mutant as depicted by the positive shift in fluorescence (Figure 9A, B). Importantly, this increase in ROS staining was abolished in *gp91*$^{-/-}$ BMDM, thus clearly indicating that ROS are being generated by the NOX2 complex (Figure 9A, B). In order to directly observe ROS localization on a subcellular level, macrophages were infected with DiI-labeled mycobacteria (Figure 9C), stained with

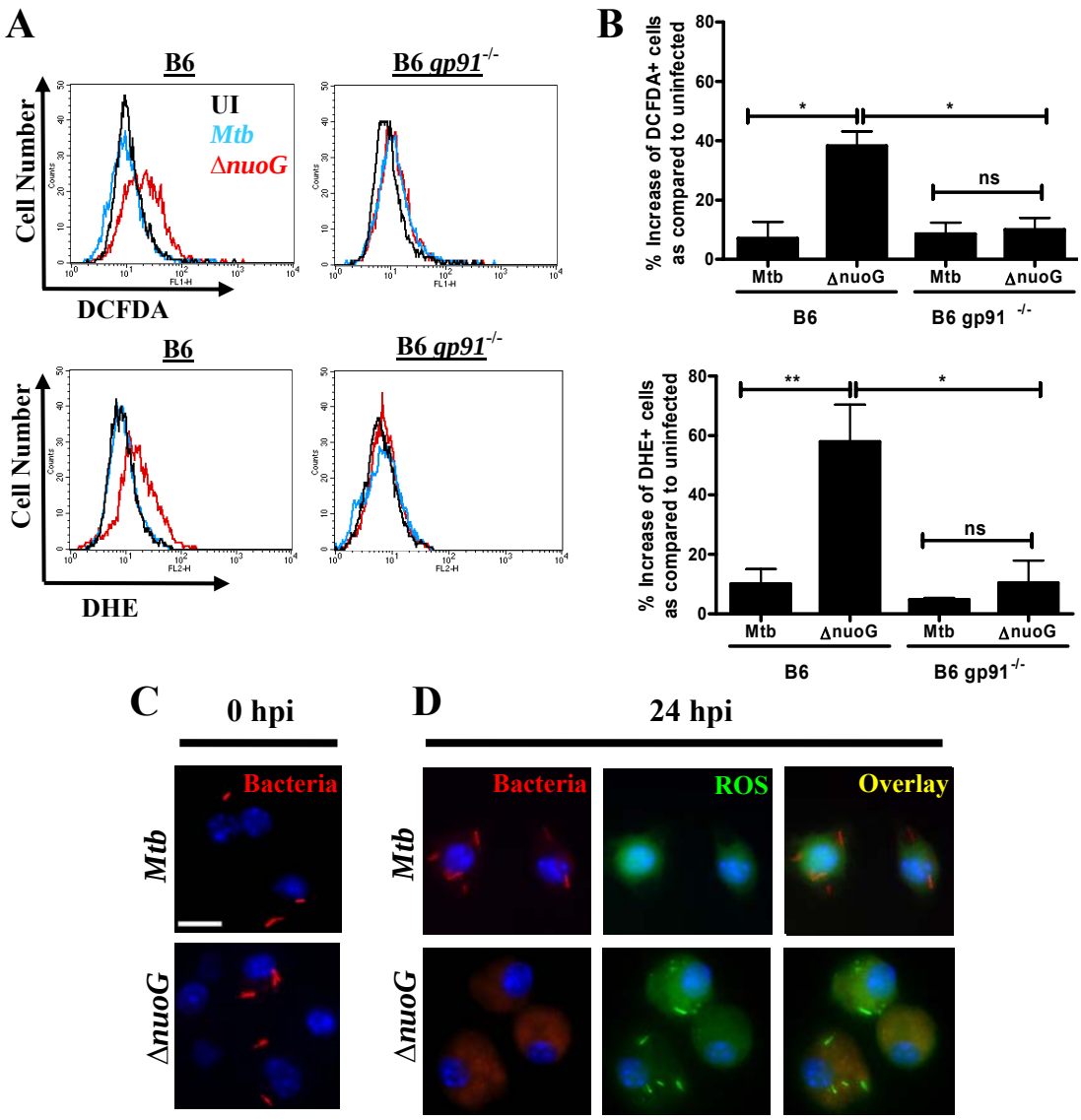


Figure 9. *Mtb* NuoG mediates inhibition of infection-induced phagosomal ROS production. (A) Macrophages from C57B/6 (B6) or gp91 deficient mice (B6 *gp91*$^{-/-}$) were infected with *Mtb* or Δ*nuoG* and stained 24 hours post infection (hpi) with the ROS sensitive dyes DCFDA and DHE, which are more sensitive for H_2O_2 and O_2^- respectively. ROS production was measured by flow cytometry. (B) Quantification of the increase in ROS levels as detected by DCFDA or DHE fluorescence intensities. Net increases in individual fluorescence intensities were obtained by subtracting the fluorescence intensity distributions of untreated cells from the corresponding *Mtb* or Δ*nuoG* infected distribution. (C and D) C57/B6 macrophages were infected with wild type (*Mtb*) and NuoG deficient (Δ*nuoG*) bacteria labeled with the lipidphilic dye DiI. Cells were visualized via fluorescence microscopy (scale bar = 10 μm) at (C) 0 hpi, or stained with DCFDA and visualized at (D) 24 hpi. Note that the DiI fluorescence is lost in the presence of ROS (D, overlay), but not in the absence of ROS (C and D). (A, C, D) Representative samples shown.

DCFDA, fixed, and analyzed by fluorescence microscopy. Only in the Δ*nuoG* infected macrophages were phagosomes stained with the ROS sensor DCFDA, whereas phagosomes of *Mtb*-infected macrophages remained DCFDA negative (Figure 9D). This data also revealed that the DiI fluorescence is quenched in the presence of ROS and thus the bacterial staining is lost during infection with Δ*nuoG*, but not during infection with *Mtb* (Figure 9D). Other dyes were used for external labeling of bacteria with similar results (data not shown). These results not only confirmed the flow cytometry analysis in which an increase of ROS signal was detected only after infection of macrophages with the Δ*nuoG* mutant (Figure 9A), but furthermore localized this increase of ROS to the host cell phagosome (Figure 9D).

3.1.5 Primary human alveolar macrophages undergo apoptosis upon Δ*nuoG* infection in a ROS-dependent fashion

In order to analyze if the ROS-dependent mechanism of apoptosis induction upon infection with the *nuoG* mutant is conserved in human cells, primary alveolar macrophages were used as host cells. Due to the source of the cells, only a limited number of cells were available, and therefore the apoptosis assay was adapted to be performed on slides, which were analyzed by fluorescence microscopy. For each donor triplicate wells were infected with *Mtb*, Δ*nuoG*, or were left uninfected. Cells were stained for the TUNEL assay 3 days post infection (Figure 10A). Approximately 500 cells were counted on each slide in blinded fashion and the number of TUNEL positive cells was recorded (Figure 10B). In the uninfected cell

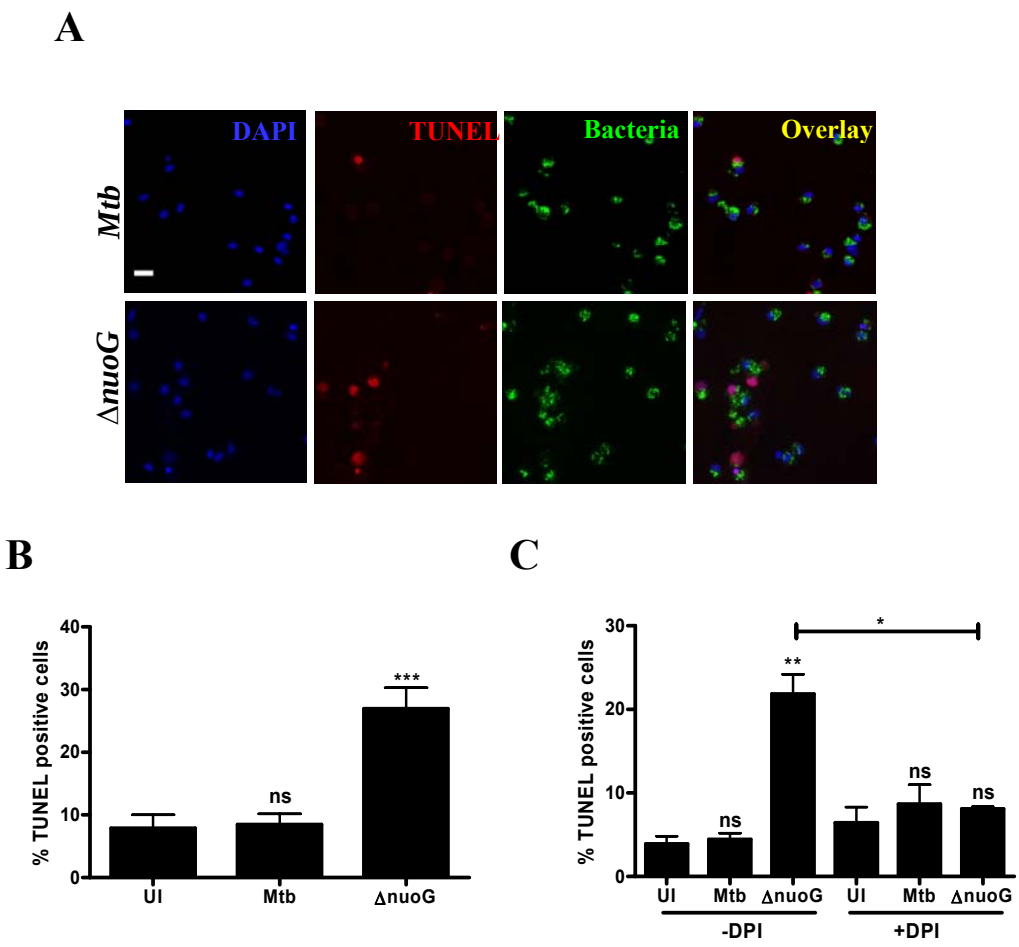


Figure 10. The pro-apoptotic phenotype of the *nuoG* mutant is conserved in primary human alveolar macrophages and is dependant on ROS. (A) Fluorescence microscopy of human alveolar macrophages infected with wild type (*Mtb*) or NuoG deficient bacteria (Δ*nuoG*) and stained with TUNEL 3 days post infection (scale bar = 20 μm)(representative sample). (B) Quantification of TUNEL positive macrophages (500 cells counted per condition; average of 5 donors). (C) Human macrophages were infected in the presence of the NOX2 inhibitor DPI (10 μM) and assayed for apoptosis 3 days post infection (500 cells counted per condition; average of two donors). Error bars are the standard error of the mean.

population, 7.9± SEM of 2.2% of macrophages were apoptotic, a percentage which was not significantly different from that of *Mtb* infected cells (8.5± SEM of 1.7%). In contrast, there was roughly a 3fold increase in the percentage of apoptotic macrophages infected with Δ*nuoG* (26.9± SEM of 3.3%). These results were pooled from five different donors, indicating that the phenotype of NuoG-mediated apoptosis inhibition is consistently conserved among different human subjects. The dependence of this pro-apoptotic phenotype on the generation of intracellular ROS was analyzed in two different donors using the inhibitor DPI as described above. Approximately 5 times as many human cells infected with the *nuoG* mutant underwent apoptosis as compared to those infected with *Mtb* (21.9± SEM of 2.4% and 4.5± SEM of 0.75% respectively). However, this difference between the two strains was abolished by the treatment of cells with the inhibitor DPI, as about 8.7± SEM of 2.3% of *Mtb* and 8.1±0.4% of Δ*nuoG* infected cells were apoptotic under these conditions (Figure 10C). These data strongly suggests that in primary human alveolar macrophages, as in murine BMDM, the NOX2 complex is critical for the pro-apoptotic phenotype of the *nuoG* mutant. Lastly, the intracellular ROS levels in *Mtb* or *Mtb*Δ*nuoG* infected cells were analyzed using DCFDA staining. The percentage of infected cells containing one or more ROS-positive phagosomes was quantified from two donors. The amount of cells containing ROS-positive *Mtb* phagosomes was similar from both donors (19.1±4.1% and 21.8±1.5%). However, these percentages were increased at least 3 fold in *Mtb*Δ*nuoG* infected cells to be 69.3±2.7% and 69.5±10.4 for the two donors (Figure 11A). Also of note, cells

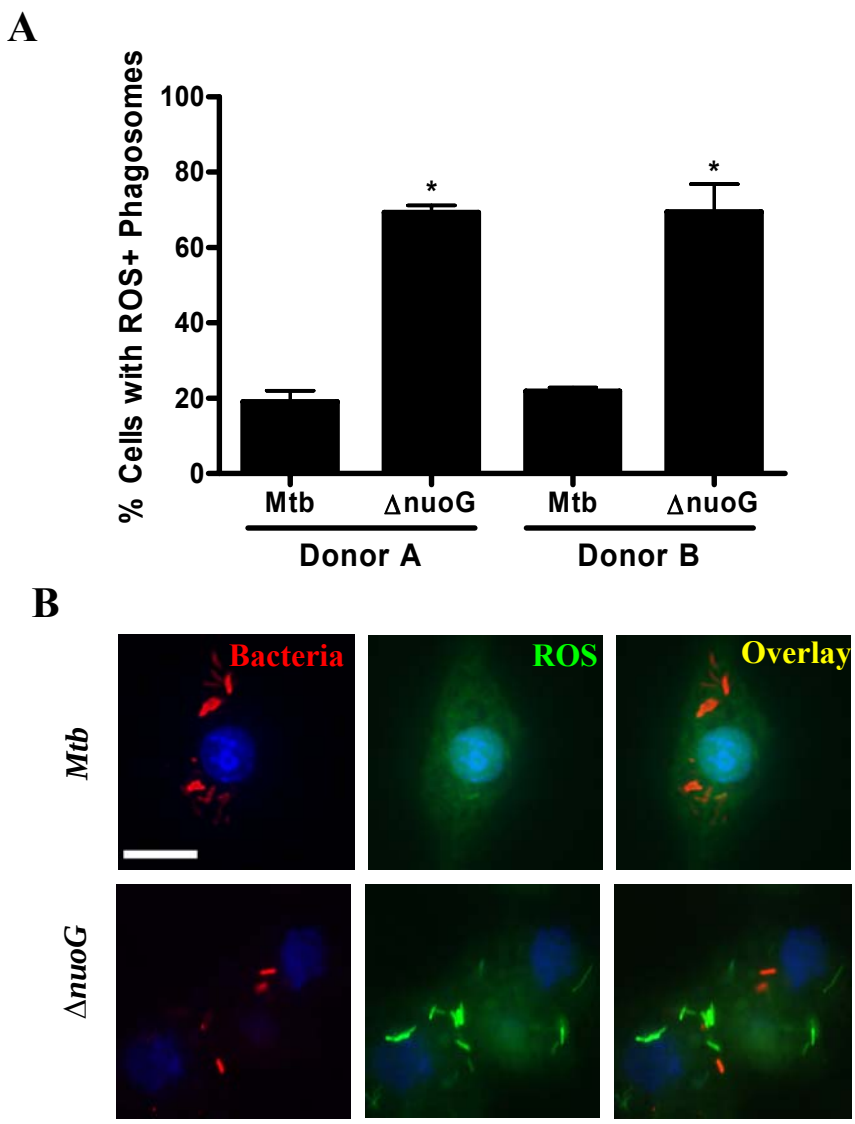


Figure 11. Δ*NuoG* induces phagosomal ROS production in infected primary human alveolar macrophages. (A and B) Alveolar macrophages were infected with *Mtb* and *nuoG* knockout bacteria (Δ*nuoG*) and stained with the ROS sensitive dye DCFDA after 3 days. (A) Quantification is of cells containing one or more ROS positive phagosomes (two donors shown). (B) Fluorescence microscopy of DCFDA stained alveolar macrophages infected with DiI labeled bacteria (scale bar = 10 μm)(representative sample).

infected with Δ*nuoG* contained many more ROS-positive phagosomes than those infected with *Mtb* (Figure 11B and data not shown).

3.1.6 Δ*KatG* infection increases ROS production and induces apoptosis in macrophages

Since the pro-apoptotic phenotype of Δ*nuoG* is dependent upon the accumulation of ROS in the phagosome, we hypothesized that neutralization or countering of phagosomal ROS may be a general mechanism of inhibition of apoptosis. If this hypothesis were true, other known ROS neutralizing proteins could potentially play a role in inhibition of PCD in host cells. *M. tuberculosis* contains several enzymes involved in the neutralization of ROS including a secreted Mg,Fe superoxide dismutase (SodA), an outer membrane bound Cu,Zn superoxide dismutase (SodC), and a secreted catalase (KatG). Interestingly, a previous report established the involvement of SodA in the inhibition of apoptosis[148]. To determine if SodC or KatG could likewise affect cell death pathways THP-1 cells were infected with *Mtb sodC* and *katG* deletion mutants and stained with TUNEL after 3 days. Δ*SodC* did not induce more apoptosis than the wild type *Mtb* (strain Erdman) (Figure 12B), possibly due to the redundant presence of secreted SodA. However, Δ*katG* induced significantly more apoptosis than *Mtb* (63±5.1% and 23±3.2%, respectively)(Figure 12A). Similar to cells infected with Δ*nuoG* bacteria, Δ*katG* infected cells secreted 30-fold more TNF-α (0.5 ng/ml) than those infected wild type bacteria (16 pg/ml)(Figure 12C). Infection of murine macrophages with the *katG* knockout also resulted in the increase of NOX2-dependent phagosomal ROS (Figure 13A-C) as B6 macrophages infected with Δ*katG* exhibited increased DHE fluorescence as compared to those infected with *Mtb*.

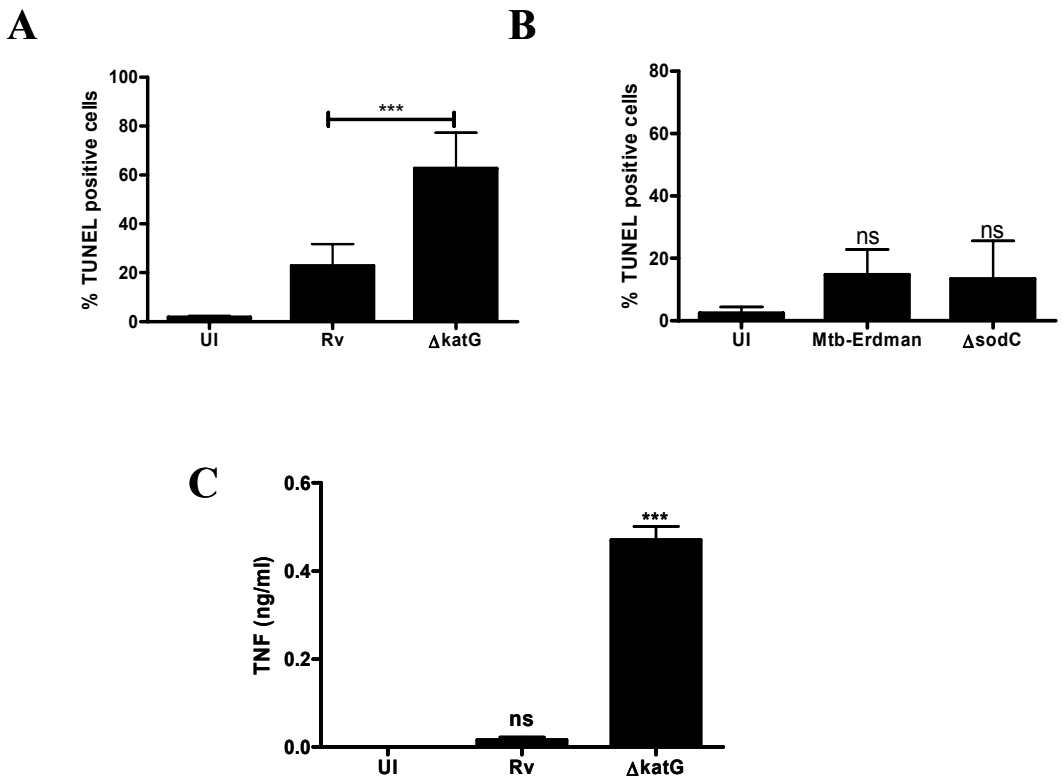


Figure 12. *Mtb* KatG, but not SodC inhibits host cell apoptosis and TNF-α secretion. (A) THP-1 cells were infected with wild type (*Mtb*) and KatG deficient *Mtb* (Δ*katG*) and assayed for apoptosis via TUNEL staining 3 days post infection. (B) The wild type *M. tuberculosis* strain Erdman (*Mtb*-Erdman) and the SodC deletion mutant (Δ*sodC*) were used to infect THP-1 cells. The percentage of apoptotic cells were assessed 3 days post infection via TUNEL staining. (C) TNF-α concentrations in culture supernatants from (A) were assayed by ELISA.

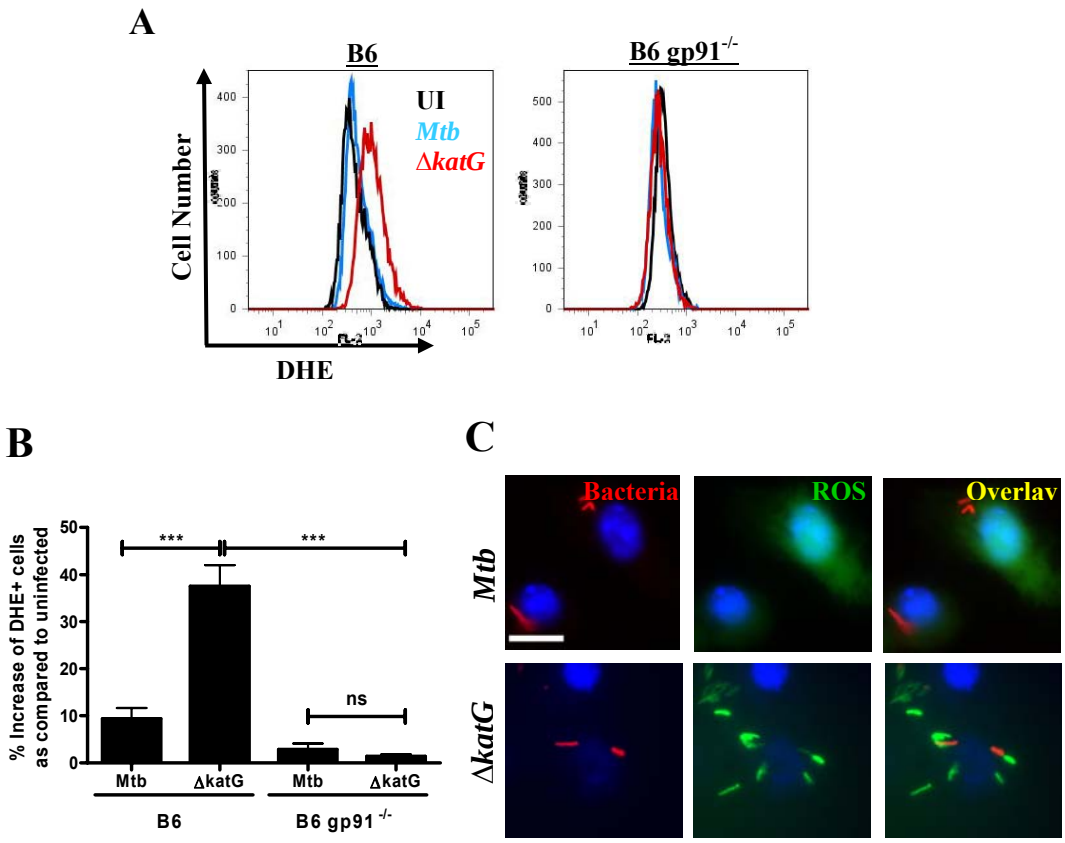


Figure 13. Δ*KatG* induces increased phagosomal ROS in host cells. (A,B) Bone marrow derived macrophages from C57B/6 (B6) or gp91 deficient mice (B6 *gp91*$^{-/-}$) were infected with *Mtb* and Δ*katG*, stained with DHE at 24 hrs post infection, and quantified via flow cytometry. (B) Quantification of the increase in ROS levels as detected by DHE fluorescence intensities. (C) Fluorescence microscopy of macrophages infected with DiI labeled bacteria and stained with DCFDA (scale bar = 10 μm). (A and C) representative samples shown.

These results are consistent with the data obtained from the Δ*nuoG* analysis and reinforce the hypothesis that the NOX2-mediated accumulation of phagosomal ROS can lead to induction of host cell apoptosis.

3.1.7 Discussion

The search for anti-apoptosis genes in the genome of *M. tuberculosis* led to the identification of NuoG as being important in host cell apoptosis inhibition and virulence [123]. Here I show that macrophages infected with the *nuoG* mutant responded with a NOX2-mediated increase in phagosomal ROS and TNF-α secretion, both of which were essential to its pro-apoptotic phenotype when compared to *Mtb*-infected cells. It is to our knowledge the first time that a direct connection between phagocytosis of a pathogen, NOX2-generated phagosomal ROS levels, and TNF-α-mediated apoptosis signaling has been demonstrated in infected macrophages.

TNF-receptor 1 (TNFR-1) mediated signaling has either pro-survival or pro-apoptotic consequences [152]. The ligation of TNFR-1 results in either activation of NF-κB, leading to survival of the cell, or activation of the c-Jun N-terminal kinase (JNK), which entails an apoptotic response [152,153](Figure 2, section 1.4.1). A major determinant in the outcome of TNF-α-mediated cell signaling is the concentration of cytosolic ROS [154]. High ROS levels lead to oxidation and inactivation of the MAP kinase phosphatase (MKP), which in its active form inhibits JNK activity. Without active MKP, TNF-α-signaling leads to prolonged activation of JNK and subsequent cell death [100]. Increased ROS can also effect activation of the MapKKK Ask-1 via oxidation of its inhibitor thirodoxin (section 1.3)[99]. Activation

of Ask-1 initiates a MAP kinase cascade that results in the eventual phosphorylation of JNK. Interestingly, the ROS involved in this Ask-1 activation is thought to be NOX2 generated [75]. It will be of interest to determine if the increased ROS during TNF-α-signaling target the oxidation of MKP, and/or the activation of Ask-1, or if other components are involved. Furthermore, TNFR activation has been shown to rapidly induce a MAP kinase cascade and NF-κB activation in a NOX2-dependent manner during very early time points (<1hr post infection)[72]. However, our data suggests that ROS signaling may also play a role at later stages of infection as NOX2-derived ROS are necessary for induction of apoptosis several days post infection (Figure 8).

Fluorescence microscopy revealed elevated ROS in the phagosomes of infected mutant infected macrophages (Figure 9 and 11). Interestingly, Δ*nuoG* does not appear to be more susceptible to external superoxide than WT bacteria (Figure 17). So how does increased phagosomal ROS lead to increased cytoplamic ROS to affect TNF-α signaling? The most simple possibility is that NOX2 generated O_2^- is dismutated into membrane permeable H_2O_2, which can leak out of the phagosome to elevate cytoplasmic ROS levels. Differences in cytoplasmic ROS levels may not be large enough to be detected by our ROS sensitive dyes and thus a more sensitive read-out may have to be implemented to address this hypothesis. Another theory is that *Mtb* utilizes NuoG-mediated ROS reduction to affect signaling cascades localized to the phagosome. In this case ROS levels would be raised only within the phagosome. This theory is supported by the finding that NOX2 activity has been shown to be necessary for TLR2 signaling and JNK activation in mycobacterial

infections [72]. Interestingly, TLR2 and NOX2 both localize to *Mtb* phagosomes, and appear to physically interact as they can be co-immunoprecipitated with one another [72]. Since both the TNFR and active NOX2 are localized to endosomal and plama membranes [155], it is possible that these molecules may also interact and that NOX2 derived ROS can affect TNFR signaling in a spatially regulated fashion. Studies using *Salmonella* suggest that TNFR-1 is important for localization of NOX2 to the bacterial phagosome, as $TNFR^{-/-}$ (p55 knockout) mice do not recruit NOX2 to phagosomes [82]. In light of this, it would be interesting to determine if TNFR accumulates on *Mtb* phagosomes, or if TLR2 signaling is necessary for the pro-apoptotic phenotype of the *nuoG* mutant.

Considering our results it is tempting to hypothesize that the NDH-1-encoding *nuo*-operon in *M. tuberculosis* might have acquired a different function when compared to other prokaryotes. Accordingly, regulation of the *Mtb* nuo-operon is opposite to that in *E. coli*. In *Mtb*, gene expression of the *nuo*-operon is down-regulated under hypoxic conditions *in vitro* and at late stage infections in the lungs of mice, whereas it is upregulated under these conditions in *E.coli* [156]. Interestingly, it is the type II dehydrogenase complex, NDH-2 (ndh, ndhA), of *Mtb* that is upregulated under hypoxic, nonreplicating conditions [156]. Under these conditions NDH-2 is crucial for maintaining a minimal PMF which is essential for survival[157], suggesting a possible alternative role for the *Mtb* NDH-1 system. The *nuo*-operon is under positive control by the two-component system PhoPR [158], which is important for virulence of *Mtb* and is one of the targets for attenuating mutations in *Mtb* H37Ra[159,160]. The *phoP* mutant fails to replicate in macrophages and infected

mouse organs; however bacteria are able to survive in a state of nonreplicating persistence, suggesting that the dormancy regulon is not affected by the *phoP* mutation and that the PhoPR system is important for early steps of *Mtb* infection[161], or later stages when bacteria are escaping from the phagosome. This is consistent with a role of the NDH-1 complex during the replicative phase of *Mtb* infections when the host cell NOX2 system is the most active.

The NOX2 complex has been demonstrated to be of great importance for innate immune defense against a variety of pathogens[79]. In order for bacterial or protozoal pathogens to survive inside the macrophage they must have developed strategies to overcome NOX2 activity. One approach is to directly inhibit NOX2 activity by either perturbing the recruitment of the subunits to the phagosome[65,162] or by decreasing the steady state levels of NOX2 complex subunits[85,86]. A novel mechanism employed by *Helicobacter pylori* is to misdirect the assembly of functional NOX2 complex away from the membrane of phagosome to the plasma membrane, so that superoxides are being released into the extracellular space instead of the phagosomal lumen[83]. Furthermore, a common strategy used by several pathogenic bacteria, including *M. tuberculosis*, is the enzymatic detoxification of NOX2 generated superoxides via the secretion of enzymes such as superoxide dismutases and catalases[147]. In the case of *Mtb*, the secretion of large amounts of SodA and KatG may account for the relative insensitivity of the bacteria to bactericidal effects of NOX2 produced superoxides[163]. If our discovery that the NuoG-mediated neutralization of NOX2 activity is important for inhibition of host cell apoptosis is of general importance, one would predict that any mutant deficient in

inhibition NOX2 activity should have a pro-apoptotic phenotype. There are few defined mutants for any pathogen described that are deficient in neutralizing NOX2 activity, and could thus be used to confirm or disprove this hypothesis. In the present study, we utilized a *Mtb* deletion mutant of the only catalase in the *Mtb* genome (KatG) and demonstrated that it had a similar phenotype to the *nuoG* mutant of *Mtb* in regard to an increase in phagosomal ROS and host cell apoptosis induction, both of which were dependent upon functional NOX2 (Figure 12 and 13). Interestingly, the *katG* mutant has been described as being attenuated and the attenuation was dependent on the presence of functional NOX2 complex in the host [44]. Furthermore, inhibition of SodA secretion by *Mtb*, achieved either via deletion of *secA2* or via inhibiting *sodA* transcription, also leads to a pro-apoptotic phenotype of the bacteria[148]. This increase in apoptosis is likely to be due to increases in phagosomal ROS levels and dependent upon host cell NOX2 activity, although that has not been investigated to date. We also tested a *sodC* deletion mutant, but found that the deletion of this second SOD did not affect apoptosis (Figure 12). This may be due to the probable presence of secreted SodA in the phagosome, although this has not yet been tested. The identification of both SodA and KatG as anti-apoptotic proteins indicate that for *Mtb*, mutants deficient in countering host cell NOX2 activity are generally pro-apoptotic. It will be interesting to know if this mechanism can be extended to other pathogens such as *Leishmania donovani*, which is able to inhibit host cell NOX2 recruitment to the phagosome [162]. In conclusion, *Mtb* inhibits infection induced apoptosis via reduction of NOX2 derived phagosomal ROS. These

data suggest that the inhibition of ROS accumulation may be an effective method to prevent cell death.

3.2 *In vivo* effect of Δ*nuoG* in *gp91* deficient mice

3.2.1 The *nuoG* mutant is not more virulent in *gp91*$^{-/-}$ mice

The correlation between induction of apoptosis and decreased virulence was made by our group and others [123]. As previously shown, the pro-apoptotic phenotype of Δ*nuoG* was reduced in cells derived from the NOX2 deficient *gp91* knock out mice (Figure 8). We hypothesized that since the *nuoG* mutant induced less apoptosis in *gp91*$^{-/-}$ cells, it would also be more virulent in these mice as compared to wild-type. To test this hypothesis, wild-type B6 and *gp91* knockout mice were infected with 10^2 *Mtb* and Δ*nuoG* bacteria via the aerosol route. Viable bacteria present in the lungs were determined by CFU counts at 1, 7, 22, 56, and 70 days post infection in two replicate experiments (Figure 14). In all four conditions, bacterial burdens increased exponentially until day 22, at which time they leveled out due to the onset of an adaptive IR. B6 mice infected with *Mtb* and Δ*nuoG*, and *gp91*$^{-/-}$ mice infected with the mutant, were able to maintain bacterial loads of approximately 10^6 bacteria throughout the length of the experiment. However, bacterial burdens of *Mtb* in the *gp91*$^{-/-}$ mice were approximately 0.5-1 log higher than those of Δ*nuoG*, or either bacteria in the B6 background (Figure 14). This increased load was especially apparent in the second experiment during which multiple *Mtb* infected *gp91*$^{-/-}$ mice died during the course of the study (*, Figure 14B). Visible lesions were evident on the lungs from all the mice, but there was no apparent difference in the gross-lung

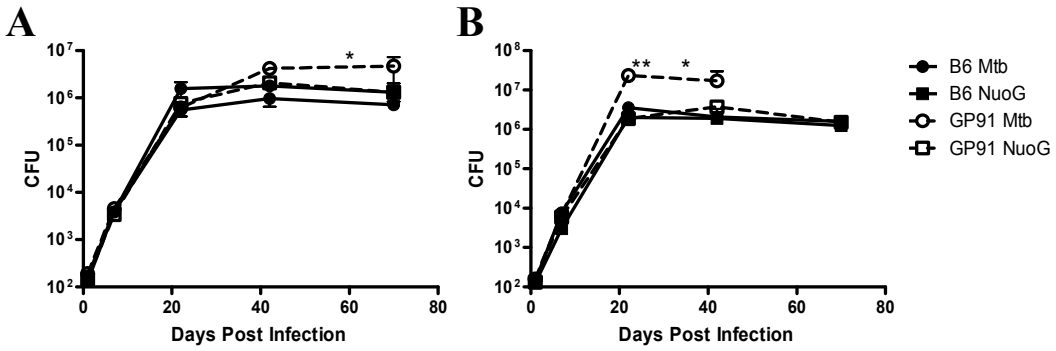


Figure 14. CFU of of B6 and *gp91*$^{-/-}$ mice infected with *Mtb* and *Mtb*Δ*nuoG*. (A,B) B6 and *gp91*$^{-/-}$ mice were infected via the aerosol route with approximately 10^2 CFU of *Mtb* (-●- and --○--) or Δ*nuoG* (-■- and --□--). The lungs from infected mice were harvested 1, 7, 22, 42, and 70 days post infection and bacterial burden was determined by calculating CFUs. During the course of the experiments, several *gp91*$^{-/-}$ mice infected with *Mtb* died as depicted by (*). (B) Results of repeat infection.

pathology (Figure 15) or histopathology (Figure 16) between *Mtb* and Δ*nuoG* infected WT mice and Δ*nuoG* infected *gp91*$^{-/-}$ mice. Conversely, lungs from *Mtb* infected *gp91*$^{-/-}$ mice were larger, with fewer obvious granuloma like structures than those from the other conditions (Figure 15). Also, histopathology of these lungs showed more cellular infiltration, as well as more extensive and less organized pathology than those infected with the *nuoG* knockout, or B6 mice infected with either bacterial strain (Figure 16). The increased size of lungs from *Mtb* infected *gp91*$^{-/-}$ mice could be due to swelling induced by cellular infiltration.

3.2.2 Discussion

We hypothesized that since Δ*nuoG* induces NOX2 dependent apoptosis in B6 macrophages (Figure 8), that this strain would be less virulent in B6 mice, and that this virulence would be diminished in NOX2 deficient *gp91*$^{-/-}$ mice. However, we were unable to show either a decrease in virulence of the *nuoG* deletion in WT mice, or complementation in *gp91*$^{-/-}$ mice (Figure 14-16). It was surprising that the *nuoG* knockout did not have a significant phenotype in B6 mice as we previously reported the growth of this mutant to be reduced in the lungs of Balb/C mice [123]. Several differences in the experimental design may account for this disparity. First, the time points at which bacterial burdens were calculated were different between the two experiments. In the current study bacterial burderns were determined up to 10 weeks post infection. However, the original Balb/C infection was a survival study and the CFU timecourse was carried out to 20 weeks post infection [123]. As the most sizable differences between bacterial loads of *Mtb* and Δ*nuoG* in the lungs of Balb/C

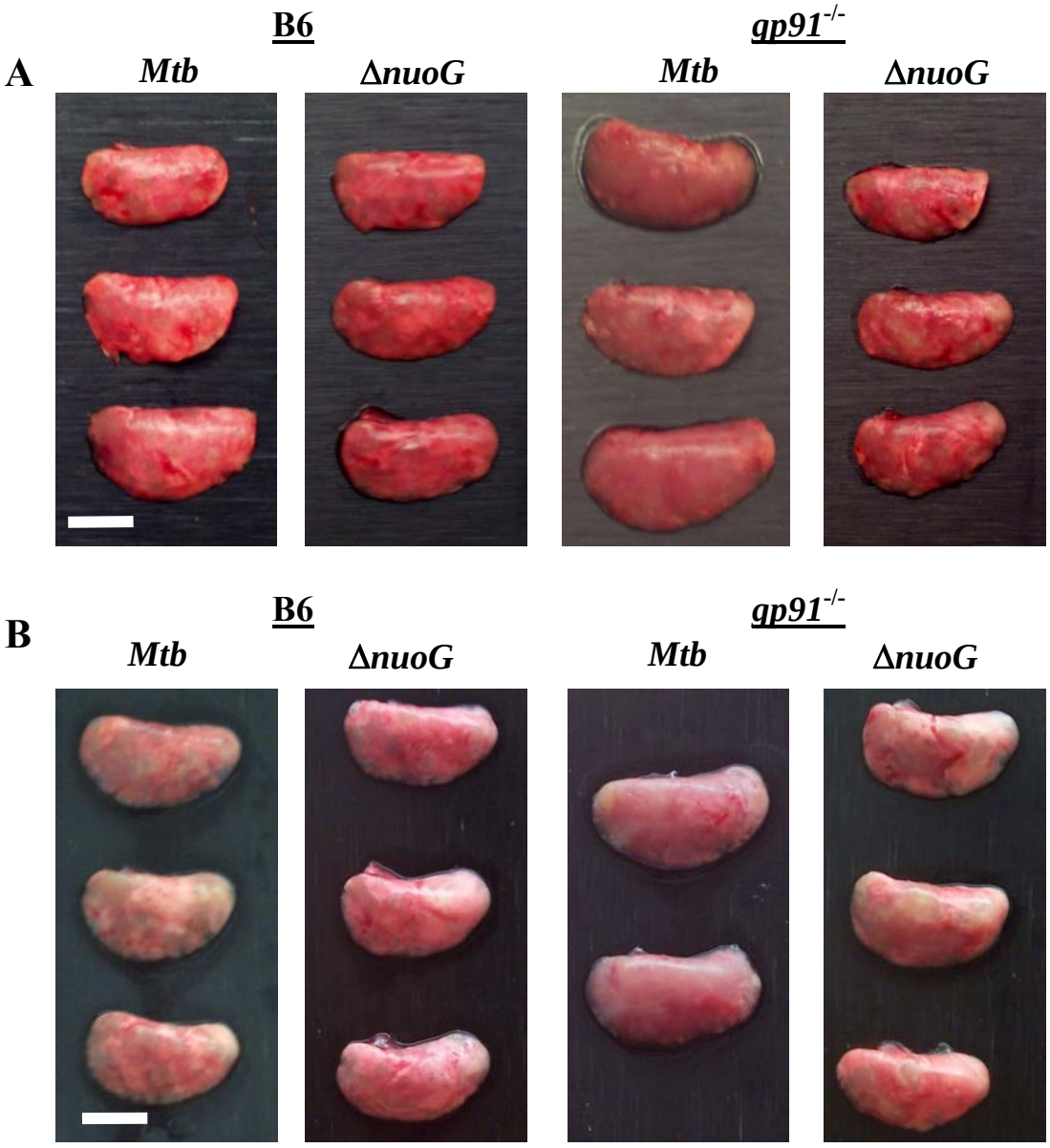


Figure 15. Gross lung pathology of B6 and *gp91*$^{-/-}$ mice infected with *Mtb* and the *nuoG* knockout. (A,B) B6 and *gp91*$^{-/-}$ mice were infected via the aerosol route with approximately 10^2 CFU of parental *Mtb* or the *nuoG* deletion mutant (Δ*nuoG*). The left lung was harvested after (A) 6 weeks or (B) 10 weeks post infection. Note granuloma appear as grey splotches. Note the increase in size of *gp91*$^{-/-}$ mice infected with *Mtb*. (A,B) Results are from the first experiment. Scale bar = 2cm.

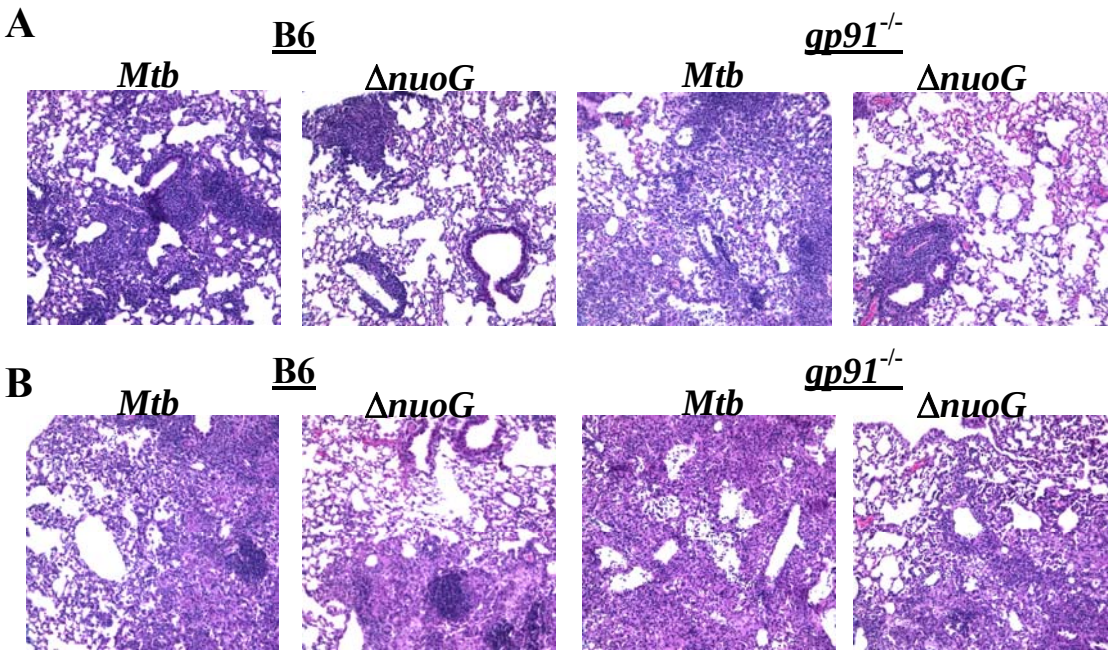


Figure 16. Lung histopathology of of B6 and *gp91*$^{-/-}$ mice infected with *Mtb* and the *nuoG* knockout. (A,B) Representative lung sections from B6 or NOX2 deficient mice (*gp91*$^{-/-}$) infected with WT *Mtb* or the *nuoG* deletion mutant (Δ*nuoG*) after 3 (A) or 10 (B) weeks post infection. Small portions of the lower right lung were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin.

mice were observed at later timepoints during the infection, the B6 timecourse may simply have been too short to observe significant growth differences between bacterial strains. Secondly, the strains of mice used in these experiments were different and have differing immune responses to *Mtb*. Balb/C mice are known to preferentially induce a Th2 type immune response, where as B6 mice induce a more robust Th1 responses [164]. This is noteworthy as the Th1 response is necessary for the control of *Mtb*, whereas the Th2 response is permissive of *Mtb* growth [165]. Correlatively, Balb/C mice succumb to *Mtb* infection more rapidly than B6 mice. Phenotypic variation, such as deviating expression of NRAMP1 (natural resistance-associated macrophage protein) and NOS2, may account for their varying immune responses [164]. Interestingly, B6 macrophages may also express less NOX2 than Balb/C macrophages (personal communication S. Grinstein), however this has not been well studied. Induction of a Th1 response is necessary for effective granuloma formation and maintenance, thus part of the reason Balb/C mice are more susceptible to *Mtb* may be because they cannot effectively contain the infection. This is supported by the lack of an apparent stationary growth phase of *Mtb* in Balb/C mice in our previous experiments [123]. These data also suggest a possible role for the inhibition of apoptosis at later stages of infection, such as during bacterial escape from the granuloma. Induction of necrosis, and/or caspase independent apoptosis, are thought to be necessary for bacterial growth and expansion in the granuloma [53]. If cells are undergoing apoptosis instead of necrosis, as in macrophages infected with the *nuoG* mutant, re-activation may be delayed. This effect would be more prominent in the Balb/C system than the B6 as the latter are better able to contain *Mtb* infection.

This hypothesis would also suggest that differences in bacterial burden would not be apparent until later stages of the infection, and thus might explain why significant differences between the growth of *Mtb* and Δ*nuoG* were not observed during a 10 week period in B6 mice. To address this hypothesis, future *in vivo* studies should investigate correlation of apoptosis in the murine granuloma and virulence at late time points of infection. Finally, in our previous *in vivo* study, mice were intravenously injected with a high dose of bacteria (approximately 10^6 bacteria), whereas in the current experiment mice were infected with only 10^2 bacteria via the aerosol route. Higher bacterial load early on may result in more rapid disease progression. Thus, it would be interesting to look the role of NuoG and/or apoptosis during chronic and late stages of infections model, development of which may involve higher infection doses.

We hypothesized that bacterial growth of the *nuoG* mutant in *gp91*$^{-/-}$ mice would be similar to that of WT bacteria as the mutant was not pro-apoptotic in NOX2 deficient macrophages. However, our results show that WT *Mtb* grew better and is more virulent in *gp91*$^{-/-}$ mice than the mutant (Figure 14). This suggests that NuoG may play other roles in virulence besides counteracting NOX2 derived ROS. One apparent concern would be how the lack of NuoG might affect intermediary metabolism. *Mtb* may need the extra energy generation capabilities of NDH-1 during the period of active bacterial growth associated with escape from the granuloma. This general fragility of the bacteria might only be apparent at late stages *in vivo*, as the *nuoG* deletion mutant does not appear to have any growth defects *in vitro* or at early time points during infection [123] (Figure 14).

Another possibility as to why the phenotype of the *nuoG* mutant was not lessened in *gp91*$^{-/-}$ mice could be due to a functional adaptation of the mice. NOX2 deficient macrophages secrete significantly more TNF-α than WT mice (Personal communication K. Heinz-Krauss and data not shown). These mice also develop hyper-inflammatory responses similar to those observed in patients with Chronic granulomatous disease. Interestingly, these increased TNF-α levels and hyper-inflammatory responses do not lead to better control of *Mtb* and other infectious agents [78](Figure 14). This enigma could be explained in that high levels of TNF-α have been shown to induce necrosis in addition to apoptosis [166]. Differences in signaling pathways between these two fates have yet to be determined, however it is likely that mitocondrially generated ROS may be involved [166]. Increased necrosis would account for the increased inflammation, as well as the ineffectiveness of this hyper-inflammatory response to control infection. Without NOX2, activated macrophages and neutrophils would be unable to kill *Mtb*, or contain the infection, and might die via necrosis. This would lead to increased bacterial growth and induce recruitment of more inflammatory cells to the site without effective clearance of the pathogen. Thus, increased necrosis would also lead to faster disease progression and bacterial expansion. This occurrence is clearly observed during *M. marinum* infections in Zebrafish. Clay *et al* showed that elevated levels necrosis led to more rapid disease progression and the loss of properly formed granulomas [132]. Thus, an increase of necrosis could explain the loss of structured pathology observed in *gp91*$^{-/-}$ mice infected with *Mtb*. Differences in the amounts of apoptotic and necrotic cells between infections in B6 and *gp91*$^{-/-}$ mice would help to address this hypothesis.

3.3 Bacterial mechanism of NuoG mediated inhibition of apoptosis

3.3.1 Susceptibility of the *nuoG* mutant to ROS

As shown in chapter 3.1, the *nuoG* deletion mutant induces apoptosis in host cells via a NOX2 and TNF-α dependant mechanism (Figures 6-13). However, the specific mechanism by which NuoG inhibits phagosomal ROS accumulation, and subsequently apoptosis, has yet to be determined. The inability to effectively neutralize extra-bacterial ROS may result in reduced fitness of the mutant. If so, the pro-apoptotic phenotype could be due to bacterial death in the phagosome. We tested the hypothesis that Δ*nuoG* might be intrinsicly more susceptible to O_2^- by exposing WT and Δ*nuoG* bacteria to superoxide generated by oxidation of hypoxanthine by xanthine oxidase (XO) [45]. Bacterial samples were taken at 1 and 3 hours post addition of XO and plated on 7H10 agar plates to determine viability. There was no significant difference between survival of the mutant and the parental *Mtb* strain as both populations were diminished about 20% over 3 hours (20.4% and 17.5% decrease for *Mtb* and Δ*nuoG* respectively)(Figure 17). Expectedly, there was no significant change in viability of bacteria not exposed to XO over the 3 hour time period (Figure 17). Thus, our data demonstrates that the *nuoG* deletion mutant is not more susceptible *in vitro* to superoxide than WT bacteria.

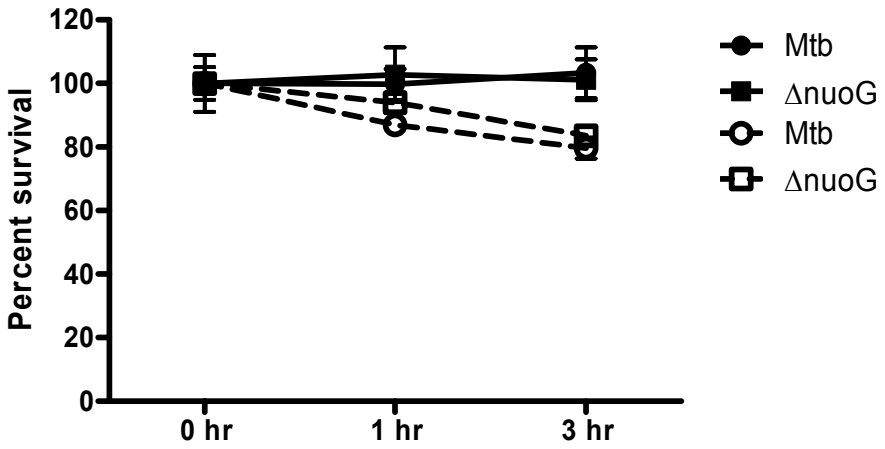


Figure 17. The *M. tuberculosis nuoG* mutant is not sensitive to superoxide-dependent killing. Survival of the *nuoG* mutant (--□--) was compared to survival of WT *Mtb* (--○--) using hypoxanthine/xanthine oxidase to generate 0_2^-. The number of surviving bacteria was determined at 0, 1, and 3 h after exposure to superoxide by plating dilutions of the bacteria on 7H10 plates. Viability of *Mtb* (-●-) and Δ *nuoG* (-■-) not exposed to XO were also determined. The means from triplicate tubes were calculated, and the data are expressed as percentages of the time zero value.

3.3.2 Localization of NuoG

One potential mechanism by which NuoG might be involved in the inhibition of ROS could be via the direct neutralization of NOX2 generated superoxides since they are able to oxidize iron-sulphur ([Fe-S]) clusters with extremely high efficiency [167]. The *Mtb* NuoG protein contains four [Fe-S] clusters which could directly compete for NOX2 generated superoxides. This hypothesis would require NuoG to either be exported to the bacterial cell wall or outer membrane, or be secreted into the phagosomal lumen as O_2^- cannot permeate the bacterial cell wall. In order to determine if NuoG is actively secreted from *M. tuberculosis* we took advantage of the phoA reporter system [168]. PhoA is a bacterial alkaline phosphotase that needs to be exported in order to be active. The phosphotase substrate 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) can be dephosphorylated by PhoA, resulting in a colorametric change of the substrate from clear to blue. Thus, protein secretion can be traced by growing bacteria with phoA fusion proteins on agar containing BCIP. In order to test the hypothesis that NuoG is being secreted, a NuoG-phoA fusion protein was generated by cloning the *nuoG* gene into the reporter plasmid pMB111 containing *phoA*. This construct was then transfected into *M. smegmatis* and transfectents were grown on media containing BCIP. Like *M. smegmatis* transfected with the negative control plamid (empty pmB111), *M. smegmatis*-NuoG-PhoA did not induce a blue color chage such as the one observed with bacteria transfected with the positive control plamid pmB124 (Figure 18). These same results were also obtained with *Mtb* transfected with NuoG-phoA (data not shown), suggesting that NuoG-phoA is not being secreted or exported. However, PhoA is a 466 amino acid

Figure. 18: A NuoG-PhoA-fusion protein is not secreted. *Mtb-nuoG* was cloned into the vector pMB111 to create a NuoG-phoA fusion protein (kindly provided by Dr. M. Braunstein). This plasmid (C) together with the positive control vector pMB124 (A) and empty pMB111 (B) were transfected into *M. smegmatis* and colonies were grown on agar plates containing the phosphatase A substrate (5-bromo-4-chloro-3-indolylphosphate) as described in [170].
long protein.

The addition of such a large tag may interfere with exportation or secretion of NuoG. To address this issue *nuoG* was cloned into a *myc* containing construct inorder to create a NuoG-myc fusion construct (S. Azogue). This construct was then transfected into *Mtb* as well as BCG, *M. kansasii*, and *M. smegmatis* (Figure 19 and data not shown). Culture filtrates and cell lysates of *Mtb* clones expressing NuoG-myc were assayed by immuno blot for the presence of NuoG-myc. NuoG-myc is clearly present in the bacterial cell lysate, but absent from the cell filtrate. This suggests that NuoG-myc is not actively secreted into the cell filtrate. However, the absence of NuoG-myc from the culture filtrate does not negate the possibility that NuoG is exported to the bacterial cell wall or cell membrane. Furthermore, the orientation of NuoG in the cell membrane is unknown. Previous structural studies of NuoG done in *E.coli* show that the NuoG containing arm of NDH-1 is on the cytoplamic side of the bacterial membrane [169], however no structural studies have been done in *Mycobacterium spp.* To determine if *Mtb*-NuoG localized to the interior perimeter of the bacteria, Δ*nuoG* complemented with NuoG-myc were fixed to slides, treated with lysozyme, and the location of NuoG-myc was determined by immuno-fluorescence microscopy using the anti-myc antibody. Lysozyme was added in effort to determine if NuoG resided intra- or extra-cellularly. For example, if intact, unlysed, bacteria stain positive for NuoG-myc, then NuoG-myc likely resides outside the bacterium. Staining was consistently observed around the perimeter of the bacteria independent of lysozyme treatment (Figure 19A,B) implying that NuoG-myc does indeed localize to the bacterial cell membrane or cell wall. Attempts at determining the structural integrity of the stained bacteria were inconsistent as many, but not all, of the bacteria

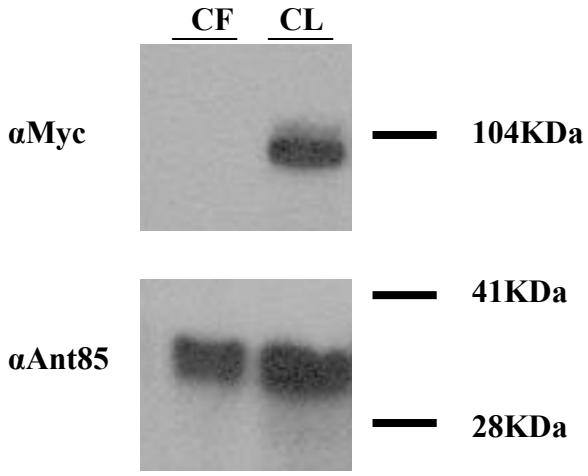


Figure 19. NuoG is not secreted in the culture filtrate. *NuoG* knockout bacteria were complemented with a NuoG-myc construct and grown in Sauton's media. Shown is a western blot of NuoG-Myc (92KDa) on culture filtrate (CF) and the cell lysate (CL). Equal ratios of protein were loaded for CF and CL. Antibodies against Antigen 85 (Ant85)(32KDa) were used as a loading control and to show that the CF contained protein (representative sample shown).

stained P.I. positive, including many of those not treated with lysozyme (Figure 19). However, due to the lack of necessary reagents, specifically functional antibodies for cytoplasmic proteins, the specific localization of NuoG in the bacterial perimeter is still uncertain.

3.3.3 Discussion

The specific mechanism by which NuoG inhibits ROS accumulation in the phagosome remains to be determined. We showed that the *nuoG* mutant was not more susceptible to superoxide *in vitro* than WT *Mtb* (Figure 17). Also, previous results demonstrated that the addition of NuoG alone to the apoptogenic *M. kansasii* was sufficient to inhibit bacterial induced apoptosis of host cells [123]. Combined, these data suggested that NuoG, potentially through the scavenging of ROS through its [Fe-S] groups, may be directly involved in the neutralization of ROS to inhibit apoptosis. Using a phoA reporter system we tested the hypothesis that NuoG was being exported or secreted in order to bind superoxide, but we failed to detect secretion of the NuoG-phoA construct (Figure 18). Furthermore, we were also unable to detect secretion of a NuoG-myc construct in culture filtrate, suggesting that NuoG is most likely not being secreted (Figure 19D). NuoG-myc does localize to the perimeter of the cell as detected by immuno-fluorescence microscopy (Figure 20), but specific localization was inconclusive as the necessary controls are currently unavailable. However, the inability to detect secretion of NuoG with both the myc and the phoA tag suggest that it is not likely being exported.

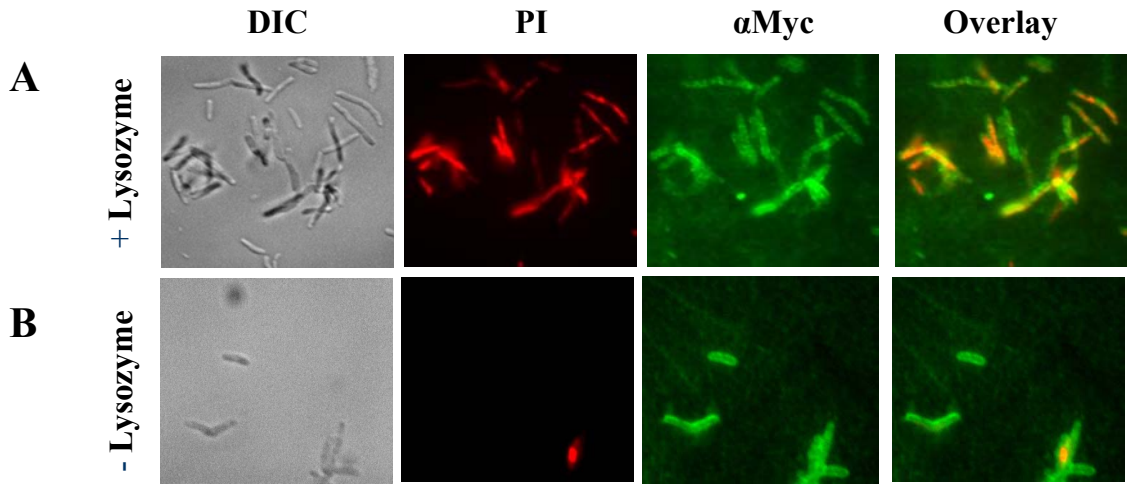


Figure 20. NuoG-myc localizes to the bacterial perimeter. Δ*NuoG* bacteria complemented with a NuoG-myc construct were incubated with (A) or without (B) lysozyme. The bacteria were then fixed on lysine coated slides and the presence of NuoG-myc was determined via immuno-fluorescence microscopy. Bacteria were also stained with PI in order to determine the permeability of the bacterial membranes.

The inability to detect NuoG secretion is not surprising as NuoG does not contain a known signal peptide, and structural analyses of other bacterial NDH-1 systems predict NuoG to be on the cytosolic side of the bacteria [169]. This is further supported by the identification of NuoG in the membrane fraction of *M. tuberculosis* H37Rv [171]. However, another group found NuoG in both cell membrane and the cell wall fractions of *Mtb* [172]. Thus, it is still possible that NuoG may reside in the cell wall. This is not unprecedented as several lower molecular weight, but functionally and structurally related dehydrogenases are secreted into the periplasmic space of gram negative bacteria such as *E.coli* [173]. However, exportation of these subunits depends on the TAT secretion system and no TAT signal sequences can be found anywhere in the NuoG protein sequence (data not shown). Nonetheless, the lack of a signal peptide does not prove that NuoG is not being exported, as proteins secreted via the SecA2 secretion system of *Mtb* do not contain signal peptides. While the hypothesis of secretion and direct neutralization of ROS by NuoG is intriguing, there is presently no evidence in favor of it.

The inability to observe NuoG secretion in the culture filtrate suggests that it is not NuoG by itself that is important for the superoxide neutralization. Another possibility is that the enzymatic activity of the NDH-1 complex is required for ROS neutralization. NDH-1 is H^+ translocating NADH dehydrogenase that is important in energy generation. The translocation of H^+ results in the formation of a proton gradient that provides the proton motive force necessary to drive ATP generation and protein secretion [174]. Previous experiments determined that NDH-1 is inactive in

BCGΔ*nuoG*, suggesting that deletion of *nuoG* is sufficient for the disruption NDH-1 function (C. Vilcheze, unpublished). However, NuoG is the largest subunit of NDH-1 and deletion of it is likely to disrupt NDH-1 oligomerization. Thus, this data alone does not prove that NDH-1 function is necessary for inhibition of cell death. This hypothesis could be addressed experimentally by characterizing deletion mutants of the NuoL and NuoM subunits of NDH-1 (B. Hurley, unpublished). In homology with other prokaryotic NDH-1 complexes the L and M subunits are proposed to be important in translocation of protons across the membrane during the dehydrogenase activity of NDH-1, thus their deletion should abolish the enzymatic activity of the NDH-1 complex[169]. If the hypothesis that the enzymatic activity of the NDH-1 complex is important for NOX2 neutralization is valid, then these deletion mutants should have a similar phenotype to the *nuoG* mutant in regard to ROS and apoptosis increases in host macrophages.

One potential mechanism by which the enzymatic activity of NDH-1 could mediate host cell apoptosis inhibition is by maintaining a proton gradiant. The resulting NDH-1 dependent proton motive force (PMF) could potentially be important in driving protein secretion, specifically secretion of ROS neutralizing enzymes such as SodA and KatG. We tested this hypothesis by determining levels of SodA and KatG in the culture filtrate and cell lysate of WT and knockout bacteria and found that Δ*nuoG* was defective in the secretion of these ROS neutralizing enzymes (Figure 21A). Interestingly, both SodA and KatG secretion were previously reported to be SecA2 dependent, however the presence of KatG in culture filtrate of Δ*secA2* bacteria in this experiment indicates that KatG can also be secreted by other

mechanisms (Figure 21B). This occurrence cannot be explained by lack of specificity of the KatG antibody as no bands were detected in the cell lysate of Δ*katG* bacteria (Figure 21C). The *nuoG* deletion does not affect secretion of Rv3881c, which is secreted via a mycobacterial specific ESX-Type VII secretion system (Figure 21A), suggesting that this secretion defect is not ubiquitous. While these initial results appear promising, they are still preliminary findings that need to be confirmed. Also, several controls, most importantly a bacterial lysis control, need to be established and implemented. Future studies should also include determining whether expression of the J21 region or *Mtb*-nuoG in *M. kansasii* would induce secretion of SodA and/or KatG, which could potentially explain the anti- apoptotic phenotype observed in the original gain-of-function screen [123]. It would also be interesting to determine if the pro-apoptotic phenotype of Δ*nuoG* can be diminished by restoring KatG/SodA secretion via a different secretory pathway (SecA1/TAT/ESX-Type VII). However, the effects of NDH-1 deletion of each of these pathways would first have to be evaluated. In conclusion, the preliminary finding that *nuoG* knockout bacteria do not secrete SodA and KatG suggest an interesting mechanism by which NuoG could be involved in neutralization of ROS.

3.4 **Identification of other pro-apoptotic mutants in *Mtb***

3.4.1 Creation and screening of 7/10 region *Mtb* mutants

NuoG was originally identified during a gain-of function screen for genes involved in the inhibition of host cell apoptosis. In brief, large regions of *Mtb* DNA were ligated into cosmid backbones, and transfected into pro-apoptotic *M. smegmatis*.

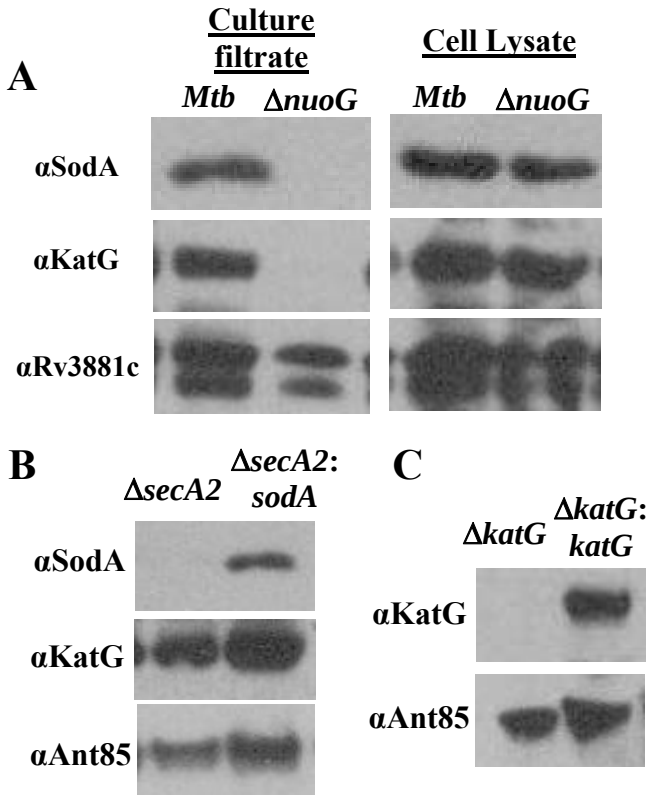


Figure 21. *Mtb*Δ*nuoG* does not secrete SodA and KatG. (A) Culture filtrates and cell lysates of WT *Mtb* and Δ*nuoG* bacteria were assayed via western blot for the presence of SodA and KatG. Both SodA and KatG are present in culture filtrates from *Mtb* and in cell lysates from *Mtb* and Δ*nuoG* but are absent from the culture filtrate from Δ*nuoG*. The *nuoG* deletion does not appear to affect Esx-Type VII secretion as Δ*nuoG* can secrete the ESX dependant protein Rv3881c. (B, C) The antibodies for SodA and KatG are specific as bands are not seen in the culture filtrate of Δ*secA2*, which cannot secrete SodA (B), or in the cell lysate of Δ*katG* (C). KatG was present in the culture filtrate of Δ*secA2* (B).

These clones were then screened in THP-1 cells for the conferred ability to inhibit cell death. Several clones containing cosmids with separate non-overlapping *Mtb* genomic DNA inserts gave significantly reduced levels of apoptosis, one of which was designated J21 (Appendix I). In effort to narrow down the region(s) specifically responsible for the inhibition of apoptosis phenotype, four sections of *Mtb* DNA corresponding to regions contained within J21 were knocked out via phage mediated transduction in the chromosome of *Mtb* (Figure 5). These knockouts were then used in THP-1 cell infections to assay for consequent loss of the ability to inhibit apoptosis. In addition to Δ*nuoG*, another knockout, designated as Δ7/10, also showed an increase in apoptosis of infected macrophages *in vitro* (Figure 22A, originally shown by K. Velmurugan, unpublished). Furthermore, Δ7/10 bacteria were shown to be less virulent in SCID mice than *Mtb* (K. Velmurugan, unpublished).

The 7/10 region spans from position 3531000 to 3536300 in the genome and contains 5 genes in their entirety: *MoxR3* and *Rv3167c*, both probable transcriptional regulatory genes, *Rv3165c* and *Rv3166c*, both conserved hypothetical proteins, and *Rv3163c* a secreted protein that previous data suggests is not involved in apoptosis (data not shown). Individual knock outs were made of *MoxR3-Rv3167c* via phage transduction, and were used in THP-1 macrophage infections. Two of these knockouts, Δ*3165c* and Δ*3167c*, showed an increase in apoptosis of infected macrophages *in vitro*, while Δ*3166c* and Δ*MoxR3* did not induce significantly more apoptosis than WT bacteria (Figure 22B). Complementation and characterization of Δ*3165c* and Δ*3167c* are currently being pursued by Serdar Gurses.

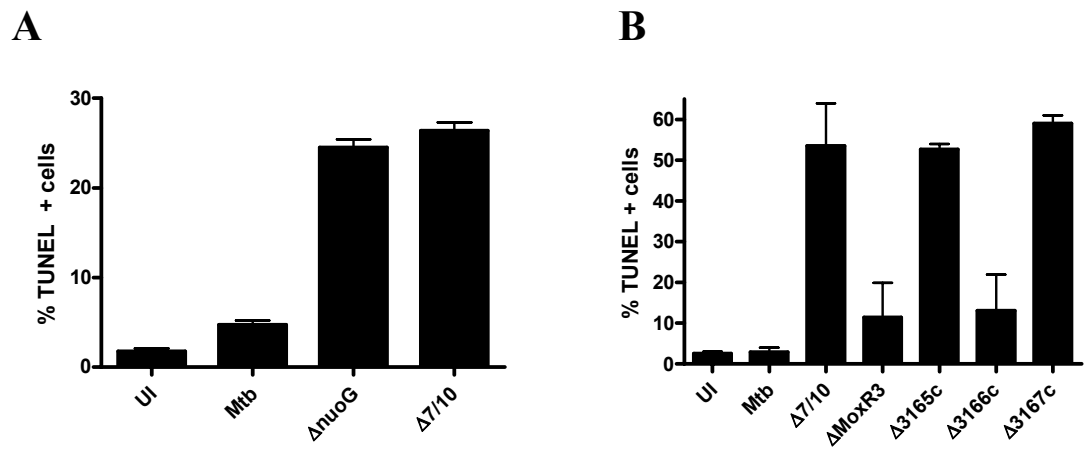


Figure 22. Induction of apoptosis by Δ7/10 region and screening of individual deletion knockouts. (A) Both Δ*nuoG* and Δ7/10 from the J21 cosmid region induce apoptosis in THP-1 cells. Apoptosis was determined by TUNEL staining after 3 days. (B) The four created knockouts from the 7/10 region were screened for the ability to induce apoptosis in THP-1 cells after 5 days. Of the mutants screened, Δ*3165c* and Δ*3167c* showed pro-apoptotic phenotypes.

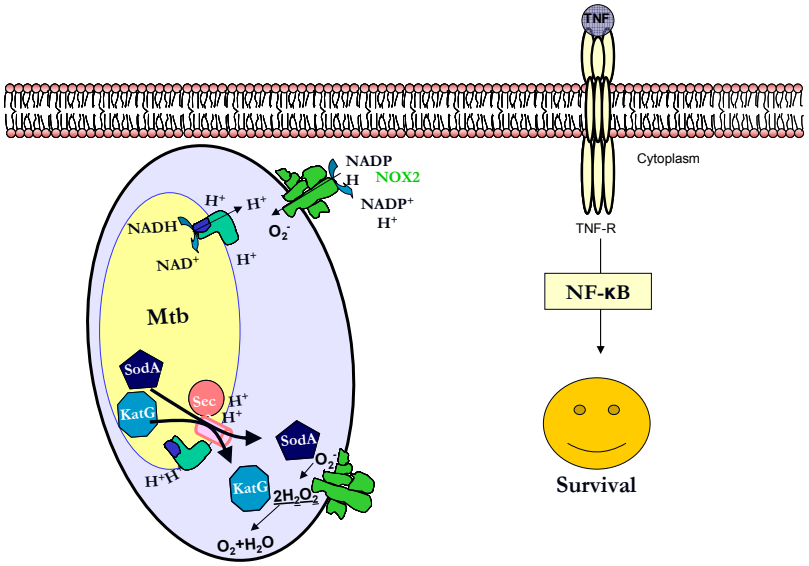
3.4.2 Discussion

The J21 cosmid contains at least three genes involved in the inhibition of apoptosis, *nuoG*, *Rv3165c*, and *Rv3167c*. Rv3165 is a hypothetical protein of unknown function and Rv3167 is predicted to be a transcriptional regulatory protein in the TETR-family (Tuberculist). Initial protein localization studies indicate that Rv3165 is present in the bacterial membrane and that Rv3167 is located in the cytosol of the bacterium (S. Gurses, unpublished). These data suggest that Rv3165 could potentially be a sensor that can activate the transcriptional regulatory protein Rv3167. It light of the relative proximity of the 7/10 genes to NDH-1 operon, it is tempting to speculate that the 7/10 genes might be regulating *nuoG*. Intriguingly, preliminary data revealed that NuoG is down regulated in both Δ*3165c* and Δ*3165c* bacteria, suggesting that *nuoG* transcription might be regulated by these 7/10 region proteins. These preliminary data suggests the following possible model: Rv3165 is a membrane stress sensor, possibly sensing ROS, which signals to activate the transcriptional regulator Rv3167. Rv3167 can then induce transcription of stress response genes including *nuoG* and/or the entire NDH-1 operon, which can neutralize phagosomal ROS to inhibit apoptosis. Current and future studies to test this hypothesis will include RT-PCR to identify genes regulated by Rv3167, particularly those which encode ROS neutralizing enzymes, as well as to determine changes in the transcriptional response to the presence of ROS. TNF-α secretion and ROS production by Δ*3165c* and Δ*3167c* infected macrophages should also be ascertained in order to determine if these mutants induce apoptosis in the same manner as Δ*nuoG*.

3.5 Summery and General Discussion

The inhibition of host cell apoptosis is an important mechanism for *Mtb* survival as it is necessary for maintaining a habitable niche and inhibiting induction of an adaptive immune response from the host [126]. We previously showed that NuoG from *M. tuberculosis* is involved in the inhibition of host cell apoptosis and that this ability was important for virulence [123]. Here I have described some aspects of the molecular mechanism by which NuoG can prevent cell death as elucidated during my research project. These data indicate that NuoG inhibits TNF-α and NOX2 mediated apoptosis. My results further imply that the countering of NOX2 derived ROS is an important mechanism for the inhibition of cell death as *Mtb* deficient in the catalase KatG also induce apoptosis in a similar manner as Δ*nuoG*. Preliminary data indicates that NuoG mediated NDH-1 activity may be necessary for the secretion of ROS neutralizing enzymes SodA and KatG. In view of the data presented here, I propose that *Mtb* can inhibit apoptosis of host cells by secreting ROS neutralizing enzymes in a NuoG dependant fashion in order to prevent induction of the ROS dependent pro-death TNF-α signaling pathway (Figure 23). In the presence of increased ROS concentrations TNF-α signaling can shift from an inflammatory, pro-survival pathway to a pro-apoptotic pathway [152]. During macrophage infection with *Mtb*, NDH-1 activity creates and maintains a H^+ gradient and PMF that are necessary to drive SodA and KatG secretion. These ROS neutralizing enzymes can counteract the effects of NOX2 by neutralizing NOX2 generated ROS. Consequently, less ROS is available to influence TNF-α signaling, which by default results in NF-κB activation and cell survival (Figure 23A).

A



B

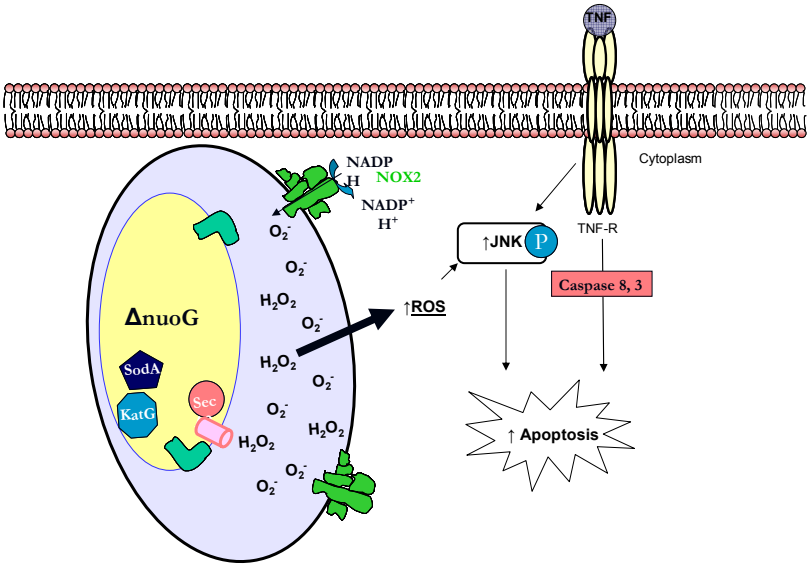
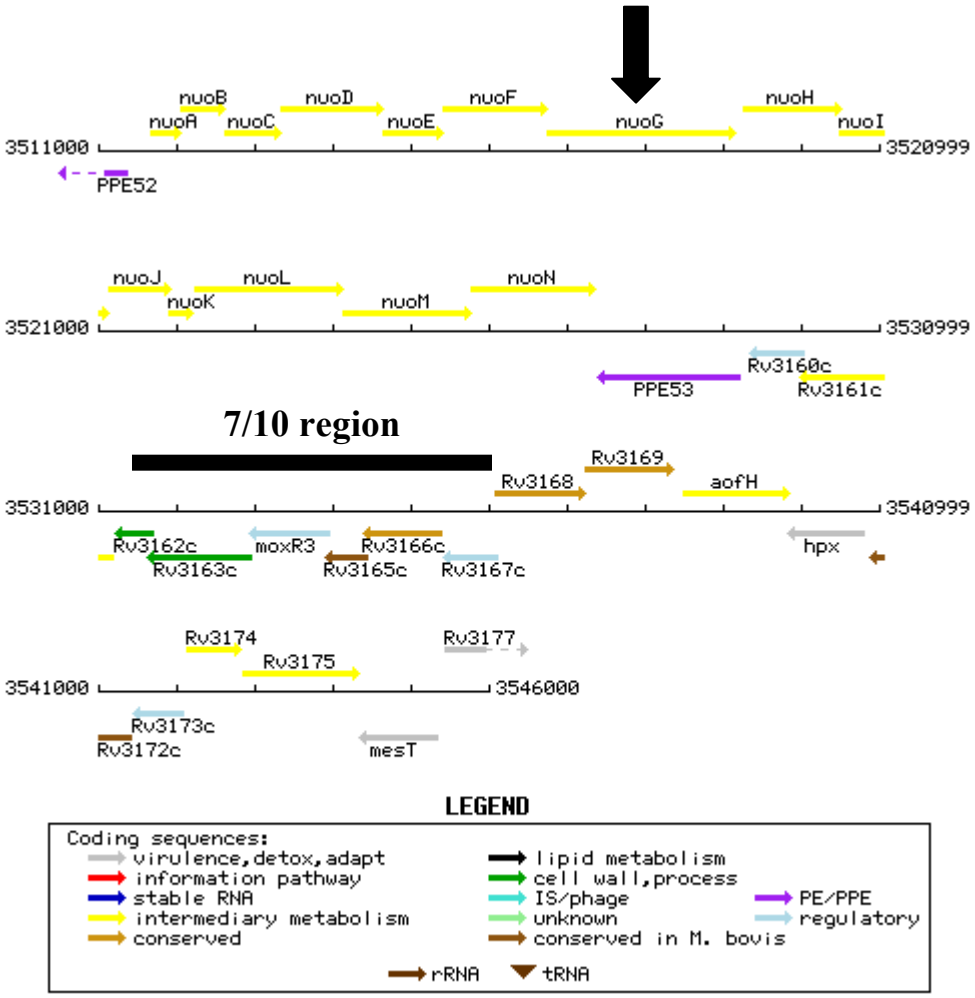


Figure 23. Model for NuoG dependent inhibition of apoptosis by *Mtb*. (A) Upon phagocytosis, NOX2-complex is localized the phagosomal membrane and pumps superoxide into the phagosomal compartment. As part of NDH-1, NuoG is involved in the creation of a proton gradient and accordingly the PMF necessary to drive secretion of ROS neutralizing enxzymes SodA and KatG. Thus, *Mtb* is able to neutralize the NOX2 generated phagosomal ROS. In the absence of increased ROS TNF-α signaling will results in activation of NF-κB and cell survival. (B) In the absence of NuoG, NDH-1 is non-functional and cannot maintain the necessary PMF to power SecA2 mediated secretion of SodA and KatG. Consequently, *Mtb* cannot neutralize NOX2 derived ROS, which will build up in the system. This increase in ROS can lead to sustained JNK activation and shift the TNF-α signaling pathway toward apoptosis.

However the loss of NDH-1 function by deletion of NuoG results in the inability to maintain a H^+ gradient and the subsequent loss of the PMF necessary to secrete SodA and KatG. NOX2 derived ROS may then build up in the phagosome, and potentially be leaked to the cytoplasm in sufficient quantities to initiate the pro-apoptotic signaling pathway (Figure 23B). This model explains a possible mechanism by which NuoG can inhibit apoptosis. However, WT *Mtb* is hypervirulent in *gp91*$^{-/-}$ mice (Figure 14), suggesting that NuoG/NDH-1 most likely plays an additional role in virulence other than inhibition of apoptosis. This could be due to metabolic deficiencies as previously discussed (Section 3.2.2). Alternatively, since preliminary results suggest that NuoG may be necessary for effective protein secretion, it is possible that *Mtb* may secrete other virulence factors in a NuoG dependant fashion. Indeed, other preliminary results indicate that the mutant may also be impaired in the secretion of other proteins (data not shown). Perhaps it is the effects of these unknown factors that might account for the lack of complementation in the *gp91*$^{-/-}$ mice. NuoG may also be important in secretion of non-protein effectors such as lipids. Specific mechanisms of lipid transport in *Mtb* are still being investigated, though it is thought that the mmpL family of proteins are involved, as deletion of *mmpL7* and *mmpL8* from *Mtb* has resulted in mutants deficient in lipid transport [175,176]. Intrestingly, the *mmpL7* mutant also demonstrated decreased bacterial burdens in the lungs during the chronic phase of infection [175] in a similar manner to the *nuoG* mutant in Balb/C mice. Comparisons between the secretion-proteome and lipidome of *Mtb* and the *nuoG* mutant might illuminate possible effectors involved in virulence.

The investigation of the pro-apoptotic phenotype of a mutant of *Mtb* deficient in functional NDH-1 complex serendipitously revealed a novel important function of host cell NOX2 complex in macrophages. Our results demonstrate that continuous NOX2 activity will ultimately lead to host macrophage apoptosis induction. The classical respiratory burst is transient, since this generates sufficient amounts ROS to kill susceptible bacteria and thus reduce NOX2 activity. Persistent pathogens which have adapted to the macrophage as a survival niche are able to survive this initial ROS burst. Thus, infection with these pathogens could potentially lead to continuous NOX2 activity on the phagosome. The results presented here enable us to formulate the following hypothesis: successful intracellular pathogens need strategies to inhibit prolonged activation of NOX2 and/or neutralize the generated superoxides since this will otherwise be sensed by the host cell and will lead to host cell apoptosis. This hypothesis expands the function of NOX2 from the previously described ROS generation for bactericidal activity, to postulate that the host cell macrophages use the NOX2 complex as a mechanism to detect persisting intracellular pathogens.

Appendices



Appendix 1. The J21 cosmid region. The J21 cosmid region was identified in a gain-of-function screen to identify anti-apoptotic loci from *Mtb* [123]. Deletion of the *nuoG* gene (arrow) and the 7/10 region from *Mtb* resulted in pro-apoptotic mutants (Velmurugan unpublished, Figure 22).

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