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M.tb Killing by Macrophage Innate Immune Mechanisms: A Dissertation

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M.TB KILLING BY MACROPHAGE INNATE IMMUNE MECHANISMS

A Dissertation Presented

By

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DEDICATION

This work is dedicated to everyone who has taken the time to invest in adopted kids everywhere. We were not born into the best circumstances, but you changed that. You offered us loving homes to grow up in, taught us to make better choices than our predecessors, and gave us a life full of opportunities we would have never had otherwise. You make the world a better place.

For Bruce & Birdy Hartman who adopted me, and the Riel and Hartman families who gave me lots of love throughout my life.

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ABSTRACT

Macrophages infected with a heavy burden of *M.tb* Erdman undergo a cell death that initially resembles apoptosis but quickly transitions to necrosis. Unlike the previously reported TNF dependent apoptosis induced by avirulent Mycobacterium [1], this form of macrophage cell death is not microbicidal [2]. Microbicidal effects are observed however, when the heavily infected macrophage encounters an uninfected naïve macrophage. My studies describe in part, the crosstalk between the uninfected and infected macrophage that results in the killing of the intracellular *M.tb*. Cell contact between the two cell populations is not necessary for this killing of bacilli to occur and the soluble "signal" of communication between the two cell populations is transferrable, without naïve macrophages present, to newly infected cells also resulting in the reduced viability of the bacilli. We have found that when the IL-1 receptor is absent in the naïve macrophage population that the co-culture antimycobacterial effect is abrogated, suggesting that IL-1 released by the infected dying macrophage is critical for naïve macrophages to respond in a way that results in the decrease in mycobacterial viability. The signaling between the two cell population ultimately converges on activation of iNOS in the infected cell however ROS appears not to be involved.

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List of Abbreviations

1,25D3	1α,25-dihydroxycholecalciferol
AA	Arachidonic Acid
AB	Add-Back
AIDS	Acquired Immunodeficiency Syndrome
AOBS	Acousto-Optical Beam Splitter
Autophagy	used in place of “Macroautophagy”
ATG	Autophagy Related Protein
ATPase	Adenosine Triphosphatase
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
CLR	C-type Lectin Receptor
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CTX	Cholera Toxin Subunit B
DC	Dendritic Cell
DMEM	Dulbecco’s Modified Eagle Medium
EBP50	Ezrin/Radixin/Moeisin (ERM) binding phosphoprotein 50
EDTA	Ethylenediaminetetraacetic Acid
(E)GFP	(Enhanced) Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESAT-6	Early Secreted Antigenic Target Protein-6
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
Gag	Group Antigens
GFP-LC3	EGFP-MAPLC3
HEK	Human Embryonic Kidney 293 Cells
HIV	Human Immunodeficiency Virus
Hoechst	bisBenzamide H 33258
HRP	Horseradish Peroxidase
IFNϒ	Gamma Interferon
IL-1	Interleukin 1
iNOS	Inducible Nitric Oxide Synthase
IRAK	IL-1 Receptor Associated Kinase
Irgm1	Murine Interferon-Inducible Immunity Related GTPase Family M Member 1
IRGM	Human Interferon-Inducible Immunity Related GTPase Family M Member 1

LB	Luria-Bertani
LC3	(MAP-LC3) Microtubule Associated Protein 1 Light Chain 3
LO	Lipoxygenase
LPS	Lipopolysaccharide
LRG-47	47 kilodalton Guanosine Phosphatase family protein 47
Mϕ	Macrophage
ManLAM	Mannose Capped Lipidarabinomannan
MCS	Multiple Cloning Site
MDP	Muranyl Dipeptide
MDR-TB	Multi-Drug Resistant Tuberculosis
MOI	Multiplicity of Infection
M.tb	*Mycobacterium tuberculosis*
mTOR	Mammalian Target of Rapamycin
MyD88	Myeloid Differentiation Response Gene 88
NECA	N-ethylcarboxamidoadenosine
NFκB	Nuclear Transcription Factor κB
NK	Natural Killer Cell
NO	Nitric Oxide
No AB	No Add-Back
NOD	Nucleotide-Binding Oligomerization Domain
NLR	NOD-Like Receptor
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCD	Programmed Cell Death
PE	Phosphatidylethanoloamine
PI3K	Phosphatidylinositol-3-Kinase
Pol	Reverse Transcriptase
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene Fluoride
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering Ribonucleic Acid (RNA)
TAK1	TGFβ-activated Protein Kinase 1
TB	Pulmonary Tuberculosis Disease
TBST	Tris-Buffered Saline and Tween 20
TCR	T-cell Receptor
TDM	Trehalose 6,6'-Dimycolate (aka: “Chord Factor”
TGF-β	Transforming Growth Factor β
THP-1	Human Acute Monocytic Leukemia Cell Line
TLR	Toll-Like Receptor

TRIF	Toll-Interleukin Receptor Domain-Containing Adaptor-Inducing Interferon β
VSV-G	Vesicular Stomatitis Virus Protein G
WT	Wild Type
XDR-TB	Extensively Drug Resistant Tuberculosis

CHAPTER I: Introduction

The TB Pandemic

Tuberculosis (TB) is one of the highest infectious killer of adults worldwide, only second to human immunodeficiency virus (HIV) killing 1.3 million people in 2009 (WHO, 2010). Approximately one third of the world's population is estimated to have latent infection and around 9 million new cases are reported annually. Though many people with TB will never suffer from clinical disease, 5-10% of patients will suffer from active TB and around 13 million people suffer from active disease globally at any one time. TB has an economic impact of between $1-3 trillion dollars annually and is most prevalent in some of the world's poorest countries (Fig.1.1). TB has the highest incidence rates in countries that also have widespread HIV infections and continues to remain the leading cause of death among people with HIV/AIDS. [3]

Two of the major recent events that hindered containment and treating of TB is the increased spreading of multi-drug resistant (MDR)-TB, *M.tb* resistant to first-line anti-TB drugs rifampicin and isoniazid, and the emergence of extensively drug resistant (XDR)-TB, *M.tb* resistant to first-line anti-TB drugs as well as to drugs in the quinolone family and additionally to at least one of the second-line anti TB drugs kanamycin, capreomycin and amikacin [4]. In 2009, there were 5.8 million new cases of TB of which 12% were MDR-TB. In July 2010 there were 58 countries with at least one case of XDR-TB (WHO, Report on Global

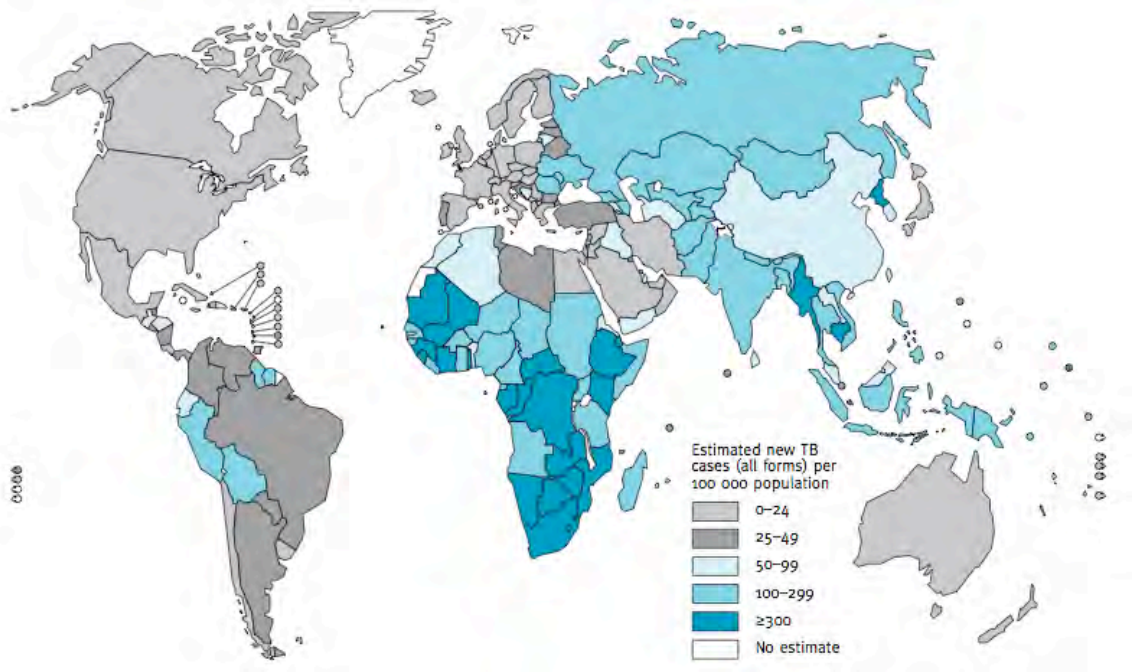

Figure 1.1: Estimated TB incidence rates by country, 2009. From the 2010 World Health Organization report on Global Tuberculosis Control.

Tuberculosis Control 2010). Due to its high cost and global incidence, TB should be a concern for everyone to consider. Advancement is necessary in the basic sciences to help establish not only better drugs and treatment strategies, but better diagnostics as well.

TB Infection and the Granulomatous Response

TB is spread from person to person via inhalation of aerosolized mycobacteria. In humans TB is most commonly caused by *Mycobacterium tuberculosis* (*M.tb*), but can also be caused by *M. bovis*. After *M.tb* is inhaled, it is phagocytosed by an alveolar macrophage. The infected alveolar macrophage then expresses chemokines and cytokines resulting in the influx of mononuclear cells to the site of infection. The *M.tb*-infected macrophage becomes the center of the granuloma which becomes surrounded by infected and uninfected macrophages in addition to foamy macrophages and other monocytic cells [5, 6]. The granuloma can be solid (non-necrotic), caseous (necrotic) or end-stage cavitary (consolidation) [7-9]. Infected macrophages in the granuloma often undergo necrosis upon activation of the T-cell response [10]. Within the granuloma, *M.tb* is subjected to nutrient starvation and hypoxia which limits its growth significantly [11]. *M.tb* containment and restriction of growth is maintained until the host immune system is weakened to the point at which it can no longer maintain the infection resulting in the dissolution of the granuloma into caseous necrosis into the lung, resulting in pulmonary TB [6, 12].

M. tuberculosis Pathogenesis

M.tb is an intracellular pathogen that can survive and replicate inside macrophage phagosomes. Upon infection of a host, *M.tb* is phagocytosed primarily by alveolar macrophages and is able to grow logarithmically for approximately two weeks until the adaptive immune response is activated and Interferon (IFN)ϒ contributes to restricting mycobacterial growth [13]. *M.tb* continues to survive as a successful pathogen avoiding complete clearance from the host by interfering with the cell death of the infected macrophage, altering the cytokine and cytokine receptor expression profile and protecting its endosome from exposure to various innate antimicrobial responses and acidification. *M.tb* is able to persist comfortably for months, years or decades within the host.

M.tuberculosis Evasion of Immunity

M.tb is uniquely able to evade host cellular killing mechanisms by interfering with the fusion of the mycobacteria-containing compartment with lysosomes such that it resembles an early endosome/phagosome. One way that it does this is by inhibiting an increase of Ca^{2+} in the cytosol. By doing so, calmodulin and CaMKII-facilitated recruitment of lysosomal hydrolases such as Cathepsin D is abrogated [14, 15], and the *M.tb*-phagosome is incompletely acidified [16-18], maintaining a pH of 6.4, and retaining early endosomal marker Rab5 GTPase [19-22].

Alternatively, *M.tb* interferes with the endocytic pathway and prevents the acidification of its compartment by blocking the insertion of vacuolar ATPase into

the phagosomal membrane [23]. *M.tb* can also interfere with the localization of inducible nitric oxide synthase (iNOS) to its phagosome by limiting the amount of time that it's scaffolding protein EBP50, a Na/H^+ exchange regulatory factor, is associated with the compartment in the cell thus preventing the release of reactive nitrogen intermediates (RNI) into the phagosome [24]. By blocking the progression of its phagosome to harsher climates in early infection, *M.tb* creates an environment amenable to replication and survival within the host.

Recent studies using *Mycobacterium bovis* BCG have demonstrated a zinc metalloprotease 1-mediated arrest of phagosome maturation. Using Zmp1 mutants, the group demonstrated an increase in presentation of MHC class II restricted antigens. This suggests that *M.tb*'s ability to block phagosomal maturation not only aids in the evasion of lysosomal delivery, but also decreases *M.tb*'s immunogenicity [25]. Zmp1 has also been shown to inhibit caspase-1 dependent activation and secretion of IL-1β [26]. The two are possibly linked because IL-1β is known to be sequestered in autophagosomes [27], a compartment known to become acidified, similarly to lysosomes, in macrophages with a high burden of *M.tb* [28].

Immune evasion of *M.tb* is also achieved through components of the cell wall. One glycolipid in particular, mannose capped lipidarabinomannan (ManLAM), inhibits the function of $CD4^+$ T-cells by interfering with early events in T-cell receptor (TCR) signaling. *M.tb* does this by inserting ManLAM into lipid

rafts in the membranes of T cells thus interfering with the T-cell's ability to signal properly [29].

A unique capability of *M.tb* is that it virulent strains are able to influence the mode of cell death of it's infected macrophage. This contributes to it's immune evasion repertoire because a cell destined for necrosis is unable to restrict mycobacterial growth. However, infected macrophages that die via apoptosis are antimicrobial in nature. One major mechanism that *M.tb* uses to influence the mode of cell death in macrophages is based on the balance of eicosanoid production upon infection. In the case of Mycobacteria, virulent strains are known to stimulate LXA_4 which is permissive for mycobacterial growth compared to avirulent strains conversely induce PGE_2 [30-32]. Infection with virulent *M.tb* causes microdisruption of membranes, specifically the disruption of mitochondrial membranes leading to necrosis of the infected cell which is beneficial to the survival of *M.tb* within the host [33, 34]. Membrane repair in infected macrophages is dependent on the production of the eicosanoid prostaglandin E_2 (PGE_2), however infection with virulent *M.tb* stimulates the production of the eicosanoid Lipoxin A_4 (LXA_4) which blocks PGE_2 and thus it's membrane repair effects leading to necrosis of the infected macrophage and avoiding death by apoptotic cell death mechanisms of the host [35].

Innate Immune Response to *M. tuberculosis*

Immune recognition of *M.tb* was recently well reviewed by Kleinnijenhuis *et. al.* describing toll-like receptors (TLRs) 2, 4 and 9 with their adaptor molecule MyD88 as the predominant method of initiating an immune response to *M.tb* infection [36]. In addition to TLR's, other pattern recognition receptors (PRRs) such scavenger receptors, NOD2, Dectin-1, Mannose receptor and DC-SIGN are involved in sensing *M.tb*. In general, upon *M.tb* binding to TLR receptors on a macrophage, the signaling cascade involving IL-1 receptor associated kinase (IRAK), TNF receptor associated factor (TRAF) 6, TGFβ-activated protein kinase 1 (TAK1) and mitogen-activated protein (MAP) kinase leads to the activation and nuclear translocation of nuclear transcription factor (NF)-κB. This leads to the production of critical cytokines TNF, IL-1β and IL-12, and nitric oxide is produced [36]. Though several receptors are involved in the detection of *M.tb* by the immune system, the primary pathogen associated molecular pattern (PAMP) of *M.tb* that is sensed is the predominant cell wall glycolipid trehalose 6,6'-dimycolate (TDM/cord factor). Innate sensing of this molecule is mediated by class A scavenger receptor MARCO and TLR2 in a MyD88-dependent fashion resulting in a pro-inflammatory cytokine response [37]. Though TLR 2 and 4 have been shown to be essential for controlling chronic TB disease in mice [38, 39], they have a minimal impact and/or are dispensable for the acute response to *M.tb* infection [38, 40-44].

In contrast, IL-1 receptor signaling via MyD88 is required for acute control of *M.tb* infection [45, 46]. Mice deficient in MyD88 are extremely susceptible to TB infection despite that they are able to mount an antibody response [47, 48] due to defective IL-1R signaling [46]. Defective IL-1R signaling in mice not only results in diminished production of nitric oxide (NO) [49], but also lower production of IFNY, defective granuloma formation and lower survival [50].

Cytokines in the Immune response to *M. tuberculosis*

Janeway defines cytokines as “proteins released by cells that affect the behavior of other cells that bear receptors for them” [51]. There are many cytokines involved in the immune response to *M.tb* and are reviewed very briefly in Table 1.1 [52]. Cytokines can be pro- or anti-inflammatory and both classes are required to combat infections successfully. Cytokines are produced by a wide array of cells and include cells involved in innate immunity, adaptive immunity and in barrier protection of the host, such as endothelial and epithelial cells.

One cytokine of particular is of critical importance in the innate immune defense against *M.tb* infection and deserves special attention here: IL-1 [46, 53, 54]. IL-1 family cytokines can be produced by macrophages (Mϕ), dendritic cells (DC), epithelial and endothelial cells. IL-1 family cytokines are pro-inflammatory and consist of two types, IL-1α and IL-1β. Both mature cytokines are initially found as pro-cytokines that require further processing for bioactivity, though

ProIL-1α is biologically active. All of the effects of IL-1α are unknown however it is known that ProIL-1α can bind to DNA and is localized in the nucleus [55, 56]. IL-1β is inactive until processed by caspase-1 and until that point, is maintained in the cytoplasm of the cell and is then exported [57, 58]. Signaling for both forms of IL-1 occur through binding to one of nine variations of the IL-1R. Not all of the receptors are very well characterized though it is known that signaling through these receptors results in the release of IL-1β [58].

Important discoveries focusing on the role of IL-1 in *M.tb* infection have been recently made. In a study using human peripheral blood mononuclear cells (PBMC's), it has been shown that caspase 1-dependent IL-1β production is stimulated by *M.tb* by both the TLR2/TLR6 and NOD2 receptors. The same study demonstrated that the the subsequent secretion of IL-1β from infected human PBMC's was dependent on P2X7 receptor activation by endogenous ATP in supernatants [59]. Mayer-Barber *et. al.* have shown that IL-1R-/- mice are extremely susceptible to *M.tb* infection and that both IL-1α and IL-1β are important for host defense against *M.tb* and that these are regulated by interferons [53, 54]. There is evidence with some bacteria that components of the bacteria themselves can bind to ICAM molecules resulting in the activation of the NLRP3 inflammasome components resulting in the production of IL-1 though evidence of this with *M.tb* is not yet defined [60].

Though IL-1β plays a role in protecting the host from devastation by TB, as a successful pathogen, virulent *M.tb* is able to inhibit IL-1β production. One

way *M.tb* modulates IL-1β is by stimulating type 1 interferon (IFN) resulting in decreased production of IL-1β at the mRNA level (as opposed to the level of caspase activation of pro-IL1 to its mature form or by IL-1β autocrine amplification) in addition to decreased expression of IL-1R [61]. Additionally, *M.tb* encodes a Zn^{2+} metalloprotease, Zmp1, that reduces IL-1β production by about 10-fold by inhibiting caspase 1 activity [26]. These studies suggest a prominent role for IL-1β in the host defense against TB.

The role of Eicosanoids in *M.tuberculosis* Infection

Arachidonic acid (AA) released from cell membranes by the enzyme phosphlipase A2 is the originating source of eicosanoids. Following release from the membrane, AA is converted to specific molecules by cyclooxgenase (COX) and lipoxygenase enzymes. The resulting molecules are classified into four families: prostaglandins, prostacyclins, thromboxanes and leukotrines [62]. Eicosanoids range in function and are known to be both pro- and anti-inflammatory in nature. These molecules exert their effects by acting on G-protein coupled receptors and are known to be involved in asthma, cancer, rheumatoid arthritis, tissue remodeling and other autoimmune disorders [63].

Additionally, infection with various species of bacteria can trigger very different eicosanoid production profiles. As described above, virulent strains of mycobacteria are known to stimulate LXA_4 which is permissive for mycobacterial growth compared to avirulent strains conversely induce PGE_2 [30-32] and *M.tb*'s

Cytokine	Producing Cells	Effect
IL-12	Mϕ, DC's	-induced after phagocytosis of *M.tb* -stimulates Th1 response
IFNγ	$CD4^+$, $CD8^+$ T-cells, NK's	-activates macrophages -sufficient to control *M.tb* infection
TNFα	Mϕ, DC's, T lymphocytes	-synergizes with IFNγ to induce NOS2 expression
IL-1	Mϕ, monocytes, DC's, epithelial cells	-important for the acute phase response -facilitates T lymphocyte expression of IL2R and IL-2 release
IL-2	$CD4^+$ T-cells	-lymphocyte expansion
IL-10	Mϕ, T-cells	-Mϕ deactivation and downregulation of IL-12 and IFNγ -reduces $CD4^+$ T-cell response
TGFβ	monocytes	-deactivates Mϕ production of ROI & RNI -inhibits T-cell proliferation
IL-8	Mϕ, monocytes	-recruits neutrophils, T-lymphocytes and basophils -neutrophil activating factor

Table 1.1: Cytokines in the immune response to M.tuberculosis. A brief description of the general cytokines involved in the immune response to M.tb as reviewed by Raja **[52, 54]**.

ability to promote the production of LXA_4 as opposed to PGE_2 allows for *M.tb* to evade clearance from the host via apoptosis-dependent antimicrobial pathways [35]. Generally, Leukotrines and hepoxilins are involved in mounting an inflammatory response and prostaglandins and lipoxins are more involved in reducing an immune response. Cells of the innate immune system, including macrophages, neutrophils and dendritic cells (DC's) are known to produce eicosanoids in order to influence the immune response to a pathogen [63]. For example, macrophages will decrease production of pro-inflammatory TNFα and increase production of anti-inflammatory IL-10 in response to prostaglandin E_2 (PGE_2) [64, 65].

Adaptive Immune Response to *M. tuberculosis*

The current understanding of the adaptive immune response to *M.tb* is predominantly based on cell-mediated immunity by T-lymphocytes. Literature supporting protective effects of humoral immunity of TB mediated by B-lymphocytes is extremely unclear and contradictory in nature [66]. The human T-cell response to *M.tb* involves $CD4^+$,$CD8^+$ and γδ T-cells though it was originally thought that $CD4^+$ T-cells would have a greater role in the control of *M.tb* infection because MHC class II molecules have access to phagosomes and other compartments within cells which increases *M.tb* specific antigen presentation [67]. The primary role of *M.tb* specific $CD4^+$ cells is in producing cytokines, specifically tumor necrosis factor (TNF), IFNγ and IL-2.

The precise role of cytotoxic T-cells ($CD8^+$) is unclear. Winau *et. al.* hypothesize that $CD8^+$ T-cells are activated as a result of infected macrophages undergoing apoptosis thus serving as a cross-priming mechanism [68]. What is clear however is that lungs and granulomas in non-human primates infected with *M.tb* contain equal proportions of $CD8^+$ and $CD4^+$ T-cells [69]. It is well understood that patients with latent *M.tb* infection have increased levels of $CD8^+$ T-cells [70, 71]. Mouse model studies suggest that $CD8^+$ cytotoxic T-cells could be protective in *M.tb* infection in two ways. The first is by a perforin & granulysin mechanism resulting in the lysis of a *M.tb*-infected macrophage followed by direct killing of *M.tb* bacilli, or secondly via Fas-FasL mediated lysis of an infected macrophage that results in the release of *M.tb* allowing nearby activated macrophages to take up and kill the bacilli [72].

Though both adaptive and innate immune mechanism contribute to controlling *M.tb* infection, it is unclear where one system ends and another begins. One cell type in particular, γδ T-cells, play a significant role in bridging the gap between the adaptive and innate immune responses to *M.tb* infection [73-77]. Some insightful studies have demonstrated that upon infection with *M.tb*, macaque's show a significant increase of γδ T-cells in tissues and lymph nodes but not in the blood [78]. These studies also demonstrate that γδ T-cells undergo clonal expansion and display functional memory in response to *M.tb* phosphoantigens, a classically adaptive immune function in addition to producing the cytotoxic granule molecule granzyme B in response to a high *M.tb* burden

within lung granulomas [78]. Furthermore, there is a significant number of studies supporting the role of γδ T-cells in the immune response to *M.tb*. γδ T-cells can recognize *M.tb* antigens via monocytes in a non-MHC restricted manner can be directly activated by mycobacterial phosphoantigens [79-81]. γδ T-cells can also be activated by TLR signals, especially TLR3 and TLR9 ligands [82-84], and additionally by IL-15, a T-cell specific growth cytokine, produced by monocytes in response to *M.tb* infection [85]. Both the memory functions of γδ T-cells and the ability of the subset of cells to respond directly to *M.tb* and other innate immunity signaling molecules suggests a unique role of these cells in the immune response to *M.tb*.

Autophagy

Autophagy is cellular homeostatic process most widely recognized for enabling the cell to clean up the cytoplasmic space and rid itself from potentially harmful cellular constituents such as protein aggregates and damaged mitochondria [86]. It was first identified, however, as a mechanism used to degrade cellular contents under nutrient depletion conditions as a means to maintain cellular anabolic needs [87]. In addition to being a constitutive cellular "housecleaning" mechanism, autophagy is activated by cell stress responses, for example in response to nutrient deprivation, activated in response to intracellular infections and also as a mode of type II cell death [88].

The activation mechanism differs between the different stimuli of autophagy, however, they all result in the formation of a double membrane (often referred to as “isolation membrane”) and converge on LC3 recruitment to the phagophore. This membrane comes from a variety of sources, namely the endoplasmic reticulum (ER), the plasma membrane and mitochondria [89-95] and the formation of the phagophore from cellular membranes is mediated by autophagy related protein (ATG) 16L [93]. ATG16L is critical for coupling membrane acquisition and autophagosome biogenesis and additionally appears to influence whether or not the phagophore will mature into a LC3-positive autophagosome [94] required for the formation of mature autophagosomes.

Autophagy and the immune response to *M. tuberculosis* infection

Autophagy can be induced not only by environmental stimuli, but also by a number of molecules associated with the immune response to pathogens, both from the host and the pathogen. It is a cellular mechanism that responds effectively to numerous innate immune signals yet also regulates cytokine secretion. It is a process that bridges innate and adaptive immunity by increasing antigen presentation to T cells [96]. Because of its antigen presentation abilities, it is not surprising that autophagy can be regulated by Th1 cytokines, which promote autophagy and antimicrobial mechanisms, and Th2 cytokines, which are known to inhibit IFNϒ induced autophagy and yet again,

presents another mode of interference with antimicrobial mechanisms for pathogens to manipulate within its host [97].

Treating *M.tb*-infected macrophages with ATP, IFNϒ and rapamycin induces autophagic killing of mycobacteria *in vitro* at least in part by stimulating maturation of its phagosome [98-100]. This effect can be reversed by siRNA treatment against Beclin 1, Irgm1 or Atg7 [99, 101, 102]. It has been suggested that IFNϒ induced autophagy may depend on TNFα autocrine signaling based on findings that TNF blockers inhibit IFNϒ induced autophagy though the mechanism is not well understood [103, 104]. Evidence of *M.tb*-induced autophagy without addition of exogenous IFNϒ and direct *in vivo* relevance of autophagy in the immune response to TB has yet to be determined.

M. tuberculosis-induced Cell Death

In the last 15 years, it has become widely recognized that the fate of the infected cell also determines the fate of *M.tb* [105]. A unique feature of virulent strains of mycobacteria, such as *M.tb* H37Rv and *M.bovis,* is that at a low multiplicity of infection (MOI) it evades TNFα-mediated apoptosis in primary murine macrophages, a mode of cell death that restricts bacillary growth that attenuated and avirulent strains of mycobacteria such as *M.bovis* BCG and *M.tb* H37Ra are unable to inhibit. [1, 106] The original idea that *M.tb* might manipulate the host cell death mechanism for an exit strategy to not only infect more cells within a single host but to increase host-to-host spreading was

suggested by Lee *et. al.* [2]. This idea stemmed from evidence that cytolytic viruses are able to modulate antiapoptotic signals to support their own replication and ultimately allow for their spread [107].

To mimic the point during infection that *M.tb* would be ready to escape from its initially infected macrophage, the high MOI model was established. This is a model that remained unvalidated until recently due to the long-term latency associated with TB infection in patients. Most studies have focused on the time during infection when *M.tb* is not able to replicate and is minimized to approximately 1-2 bacterium per cell. However, one recent *in vivo* study showed that in *M.tb* lesions in control mouse lungs at 15 days post-infection by aerosol, had numerous occurrences of heavily infected cells (Fig. 1.2) [108].

The findings of the high MOI study established a novel mode of cell death initiated by the live, virulent strain *M.tb* Erdman that initially mimicked apoptosis but rapidly transitioned to a necrotic mode of cell death, a process here on out referred to “apoptonecrosis”. Apoptonecrosis is unique from TNF-dependent apoptosis and favors the survival of the mycobacteria and independent from the typical caspases and Fas signaling usually present in apoptotic cell death [2]. This was the first evidence of a cell-death induced escape mechanism for *M.tb* and suggested that apoptosis of *M.tb* -infected cells is good for the host and necrotic-predominant forms of cell death favors *M.tb* survival.

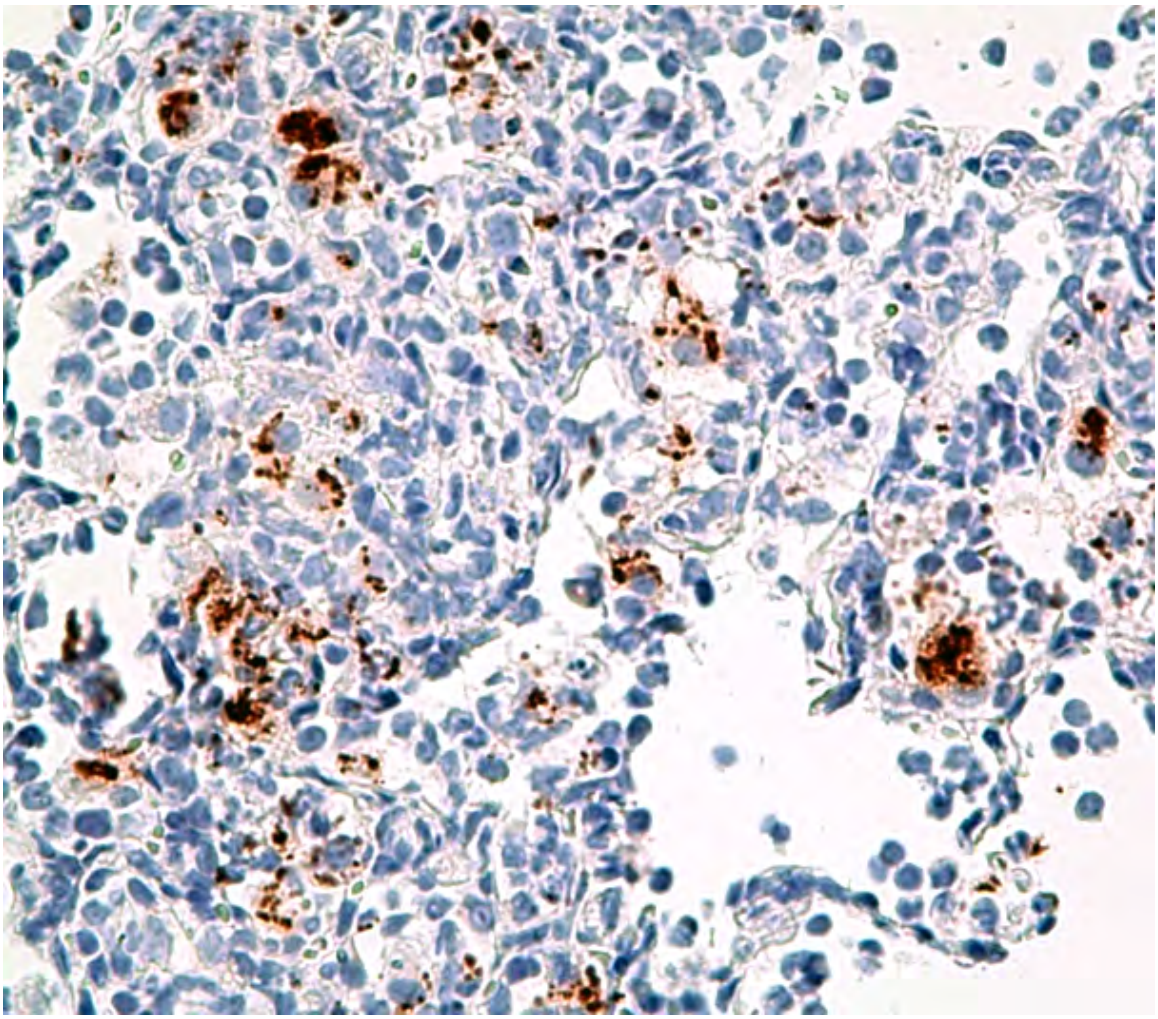

FIGURE 1.2: Anti-PPD immunohistochemistry of lung sections from control mice with TB. Mice were infected with ~100 CFU Mtb Erdman by aerosol and then lungs were isolated 15 d later for immunohistochemistry with H&E counter stain. *M.tb*-infected macrophages are distinguished by brown staining (X 400 magnification). [108]

Though the mode of high MOI cell death induced by *M.tb* is not directly microbicidal, depending on the circumstances surrounding the infected macrophage, the opportunity for antimycobactericidal effects remain. The idea that programmed cell death of Mycobacterium-infected macrophages would serve as a way to sequester the infection and enhance the immune response was originally presented by Fratazzi *et. al.* [105]. Their data showed that the interaction between *Mycobacterium avium* (*M.avium*)-infected, apoptotic human peripheral blood mononuclear cells (PBMCs) and non-apoptotic PBMCs reduced the viability of *M.avium* yet when the infected cells were made necrotic by sonication, there was no reduction in *M.avium* viability. This experiment was repeated by our lab using *M.tb* Erdman in the context of the high MOI model using early time points, 3 hours post infection, of heavily infected macrophages to model the apoptotic cell death state and later time points, 24 hours post-infection, to model necrosis of infected cells [2]. These experiments confirmed the early observations by Fratazzi *et. al.* but additionally identified approximate kinetics of when macrophages with a heavy burden of *M.tb* would be either apoptotic or necrotic [105]. Additionally, experiments by Lee *et. al.* identified a window in time where heavily infected, dying macrophages would be able to exert antimicrobial effects on intracellular *M.tb* with the help of naïve macrophages added to the culture, which we believe models the recruitment of naïve macrophages to the site of infection in the lung [2]. Together, these data suggest that manipulation of the mode of cell death of the infected macrophage

is critical to the survival mechanism of *M.tb*. The goal of the work in presented in this dissertation was to identify the mechanism and factors involved in the cross-talk between macrophages with a high burden of *M.tb* and naïve ones.

PREFACE to Chapter II

A modified version of this chapter has been published:

Hartman, ML, Hardy Kornfeld. Interactions between naive and infected macrophages reduce Mycobacterium tuberculosis viability. PLoS One. 2011; 6 (11): e27972.

This manuscript represents the main thesis project of Michelle Hartman, who contributed to its conception, experimental design, generated all data with subsequent analysis, and assisted in writing the associated manuscript.

Acknowledgements

I’d like to acknowledge Dr. Shruti Sharma of Dr. Kate Fitzgerald’s lab at University of Massachusetts Medical School for the countless discussions we had regarding the possibility of the role of IL-1b and activation of the inflammasome in the co-culture conditions. Without her suggestions, IL-1 would likely not have been pursued and we would have missed out on it’s involvement in our system. I’d also like to thank Dr. James Harris from Trinity College Dublin for his overall insightfulness and contribution of ideas for the direction of the project described in this chapter.

CHAPTER II: Interactions between Naive and infected Macrophages Reduce *Mycobacterium tuberculosis* Viability

ABSTRACT

A high intracellular bacillary load of *Mycobacterium tuberculosis* in macrophages induces an atypical lysosomal cell death with early features of apoptosis that progress to necrosis within hours. Unlike classical apoptosis, this ultimately necrotic macrophage cell death does not directly impair *M. tuberculosis* viability. We previously reported that culturing heavily infected macrophages with naïve macrophages produced an antimicrobial effect, but only if naïve macrophages were added during the pre-necrotic phase of *M. tuberculosis*-induced cell death. In the present study, we investigated the mechanism of antimicrobial activity in co-culture settings, anticipating that efferocytosis of bacilli in apoptotic bodies would be required. Confocal microscopy revealed frustrated phagocytosis of *M. tuberculosis*-infected macrophages with no evidence that significant numbers of bacilli were transferred to the naïve macrophages. The antimicrobial effect of naïve macrophages was retained when they were separated from infected macrophages in Transwell® plates, and conditioned supernatants from co-cultures transferred antimicrobial activity to cultures of infected macrophages. Antimicrobial activity in macrophage co-cultures was abrogated when the naïve population was deficient in IL-1R or when the infected population was deficient in inducible nitric oxide synthase. The participation of nitric oxide suggested a conventional antimicrobial mechanism requiring delivery

of bacilli to a late endosomal compartment. Using macrophages expressing GFP-LC3 we observed the induction of autophagy specifically by a high intracellular load of *M. tuberculosis*. Bacilli were identified in LC3-positive compartments and LC3-positive compartments were confirmed to be acidified. Thus, the antimicrobial effect of naïve macrophages acting on *M. tuberculosis* in heavily-infected macrophages is contact-independent. Interleukin-1 provides an afferent signal that induces an as yet unidentified small molecule which promotes nitric oxide-dependent antimicrobial activity against bacilli in autolysosomes of heavily-infected macrophages. This cooperative, innate antimicrobial interaction may limit the maximal growth rate of *M. tuberculosis* prior to the expression of adaptive immunity in pulmonary tuberculosis.

Introduction

A role for apoptosis in innate defense against *Mycobacterium tuberculosis* (*M.tb*) was suggested by evidence demonstrating that attenuated *M.tb* H37Ra and *M. bovis* BCG induced tumor necrosis factor (TNF) α stimulated apoptosis in infected macrophages, which was associated with a reduction in bacillary viability [1, 109]. In addition to the direct antimicrobial processes occurring in apoptotic macrophages, a previous study by Fratazzi *et al.* [105] demonstrated that the addition of naïve macrophages to macrophages infected with an apoptosis-inducing strain of *M. avium* strongly inhibited bacillary growth. This co-culture effect was observed when the infected cells were apoptotic, but not if they were made necrotic by sonication.

The host-protective effects of apoptosis in tuberculosis (TB) is now an accepted paradigm, but its biological relevance is uncertain since virulent *M.tb* inhibits the apoptotic death of infected macrophages [1] and uses these cells as a replication niche. Lee et al. [2] reported that virulent *M.tb* induces an atypical, ultimately necrotic mode of macrophage cell death at a threshold intracellular burden of 25 bacilli per macrophage. In the first several hours after high multiplicity of infection (MOI) challenge, infected macrophages have apoptotic features of nuclear condensation and phosphatidylserine (PS) externalization without apoptotic vesicle formation. Adding naïve macrophages during this pre-necrotic interval was shown to inhibit *M.tb* replication, whereas adding naïve

macrophages at 24 h post-infection when the infected population was necrotic had no antimicrobial effect.

In the present study we investigated the mechanism responsible for inhibiting *M.tb* replication in co-cultures of naïve and infected macrophages, initially testing the hypothesis that this depended on phagocytosis of apoptotic bodies (efferocytosis) by the naïve population. Results did not support that hypothesis: confocal microscopy demonstrated incomplete engulfment of infected macrophages ("frustrated efferocytosis") and the antimicrobial effect contributed by naïve macrophages was found to be contact-independent. We show that naïve macrophages are stimulated in an interleukin-1 receptor (IL-1R)-dependent manner to produce a soluble factor that acts back on heavily infected macrophages to restrict *M.tb* growth in a nitric oxide-dependent manner. This implies the delivery of bacilli to a late endosomal compartment, and in that regard we showed that a high intracellular *M.tb* load is sufficient to induce autophagy in macrophages. Our data reveal cross talk between infected and uninfected macrophages that inhibit *M.tb* replication, most likely by promoting antimicrobial processes in autolysosomes prior to the completion of *M.tb*-induced necrosis. These interactions may serve to limit the maximal proliferative potential of *M.tb* during the early phase of pulmonary TB before the induction of adaptive immunity.

Results

Cell contact is not required for co-culture antimycobacterial activity

Previous studies of macrophages infected with *M. avium* or *M.tb* demonstrated that co-culture with naïve macrophages restricted mycobacterial growth, but only when the originally infected cells exhibited features of apoptosis [2, 105]. It was speculated in those reports that antimycobacterial activity might depend on uptake of bacilli by uninfected macrophages via efferocytosis of apoptotic host cells, but this was not formally tested. To investigate that question we first assessed whether efferocytosis occurs in co-cultures where naïve immortalized cell line macrophages, with Cholera toxin subunit B (CTX)-488 stained membranes, were added to *M.tb* infected (MOI 50) macrophages, with membranes stained with CTX-647, which had been infected 3 h earlier with. Under these conditions the infected population is committed to death and externalizes phosphatidylserine (PS) but would not have progressed to necrosis, which is not widespread until 24 h post-infection. Samples were fixed at 4, 6, and 8 h after co-culture and examined by confocal microscopy. Interactions between infected and uninfected macrophages consistent with early or attempted engulfment were evident at all time points. Figure 2.1A shows pedestal formation and partial engulfment of whole infected cells at the synapse between these cell populations (white arrows). Complete internalization of infected macrophages was not observed even after 24 hours of co-culture. These interactions were

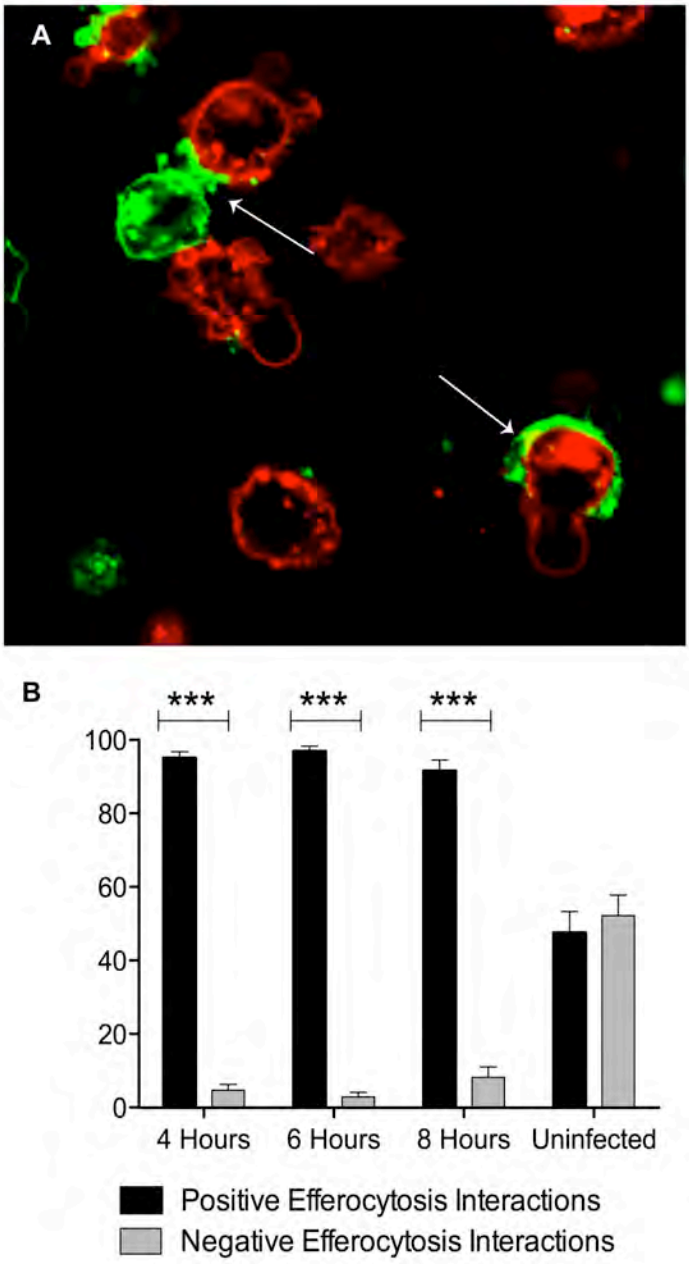

Figure 2.1: Attempted engulfment of *M.tb*-infected macrophages by naïve macrophages is incomplete and unidirectional. (A) Infected macrophages (red) were co-cultured with uninfected macrophages (green) for 3 h. Samples were fixed and observed by laser-scanning confocal microscopy. White arrow identifies early contact points where naïve macrophages form pedestals or partially engulf infected macrophages. **(B)** Directionality of frustrated efferocytosis was quantified by counting the number of "positive" interactions, ones in which naïve macrophages form pedestals or partially engulf infected macrophages, compared to "negative" interactions where cell-cell contact occurred without pedestals or engulfment (all interactions not described as "positive"). Results are expressed as the % positive interactions vs. % negative interactions at each time point ± SD. ***, $p < 0.0001$ for all time points

almost exclusively unidirectional, with uninfected cells forming pedestals on infected macrophages only partially engulfing them (Fig. 2.1B). This indicated that these interactions were not random but mediated through an “eat me” signal such as PS on the surface of the infected cells. The apparent failure to completely internalize all or parts of infected macrophages suggests that these interactions (in the absence of apoptotic vesicle formation) might reflect frustrated efferocytosis by analogy to frustrated phagocytosis [110].

To test whether physical contact between naïve and infected macrophages was required to limit *M.tb* replication, infected and uninfected macrophages were separated with a 0.4µm pore size polycarbonate Transwell® filters for the duration of the co-culture. Colony forming units (CFU) were measured at 3, 48 and 72 h post addition of naïve macrophages. As shown in Figure 2.2a, separation of the macrophage populations by transwell did not change the outcome of the co-culture antimycobacterial effect. This demonstrated that contact between naïve and *M.tb*-infected macrophages is not necessary for this phenomenon to occur, implying the production of a soluble factor by naïve macrophages acting to limit *M.tb* replication in during the early, pre-necrotic phase of cell death in the infected population.

To test whether a soluble factor expressed in co-cultures was sufficient for the observed antimycobacterial effect, we collected supernatants from co-cultures of naïve and *M.tb*-infected macrophages 3 h after co-culture began.

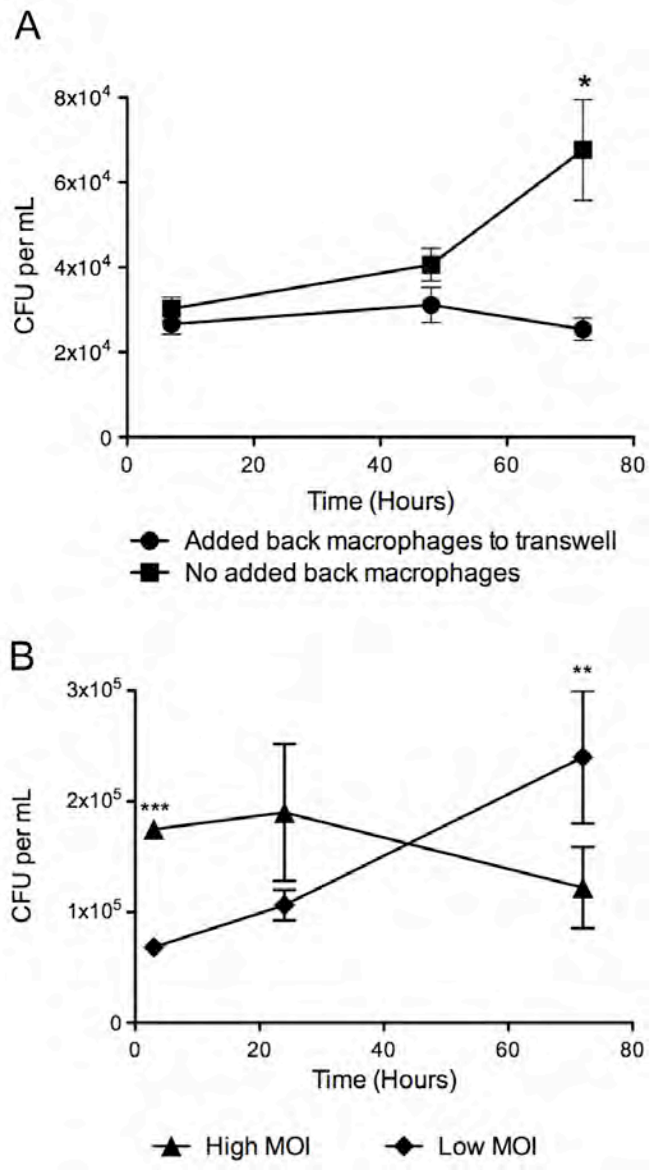

Figure 2.2: Interactions between infected and naïve macrophages results in the production of a soluble factor that reduces M.tb viability. (A) Immortalized cell line macrophages infected with *M.tb* Erdman (MOI 50) were cultured alone or in the presence of uninfected macrophages separated by Transwell® polycarbonate membranes. Results are expressed as mean CFU per mL ± SD at the indicated time points. *, $p = 0.018$ comparing co-cultures of *M.tb*-infected plus uninfected macrophages to infected macrophages alone at 72 h. (**B)** Filter-sterilized supernatant from macrophage co-cultures was added to wells contained isolated, infected macrophages alone. Transfer of co-culture supernatant reduced *M.tb* viability demonstrating that the factor responsible for the effect is soluble. *, $p < 0.0001$; **, $p = 0.002$.

Supernatants were filter-sterilized (0.22 μm) to remove bacilli or debris before being added to new cultures of infected macrophages and then CFU was measured 3, 24 and 72 h later. Antimicrobial activity was transferrable (Fig. 2.2b) and operated only in the context of infected macrophages in the early, apoptotic, phase of *M.tb*-induced death. These results indicated that the co-culture antimycobacterial effect was mediated by a soluble factor produced by naïve macrophages that acted on infected, pre-necrotic macrophages to limit *M.tb* growth. This activity was produced by naïve macrophages within 3 h of co-culture and did not require physical contact with infected macrophages, implying also the existence of a soluble afferent factor produced by the dying cells.

Interleukin-1 is an afferent signal in co-cultures

The involvement of soluble signals in the crosstalk between infected and uninfected macrophages in this high burden condition suggested a possible role for cytokines. Screening co-culture supernatants by ELISA revealed several cytokines whose levels increased following the addition of naive macrophages. (Fig. 2.3); among these were IL-1β. To further investigate the role of IL-1 in the context of high MOI co-culture conditions, we tested whether IL-1R signaling was required as an afferent, death-associated signal to induce production of a secreted efferent factor from naïve macrophages limiting *M.tb* replication in infected macrophages. Co-cultures were established, and IL-1R$^{-/-}$ immortalized cell line macrophages replaced wild type cell line macrophages either as the

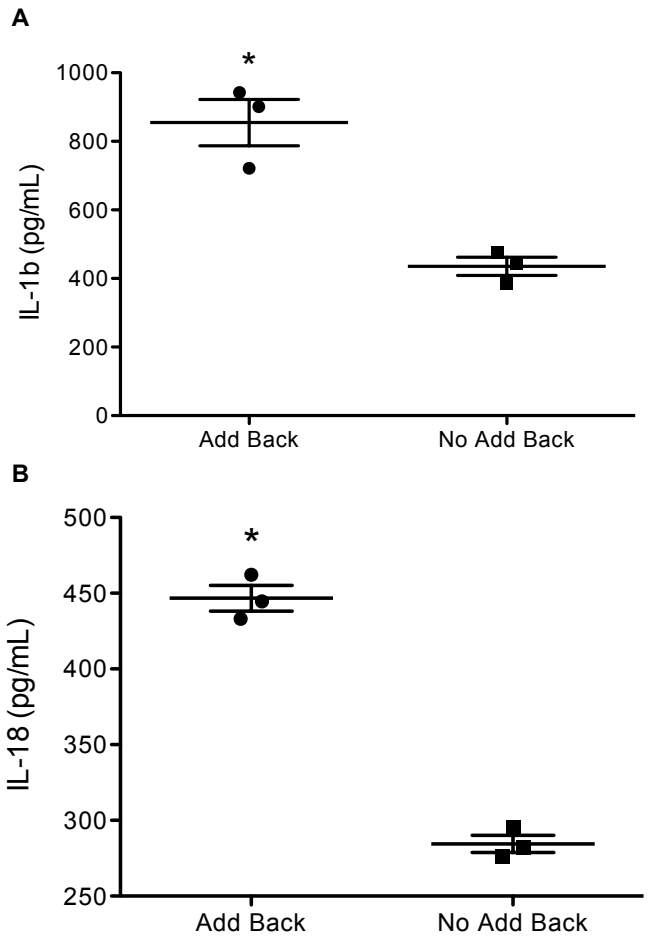

Figure 2.3: Cytokine profile of *M.tb*-infected macrophages in the presence or absence of naïve macrophages. Macrophages were infected with *M.tb* Erdman (MOI 50) for 3 h then cultures were washed and the infected cells were resuspended in medium alone or medium plus an equal number of naïve macrophages. After an additional 3 h in culture, supernatants were collected, and frozen at -80°C until multiplex cytokine analysis. Concentrations of IL-1β and IL-18 are shown for co-cultures of infected and uninfected macrophages (*add back*) or culture of *M.tb*-infected macrophages without naïve macrophages (*no add back*).

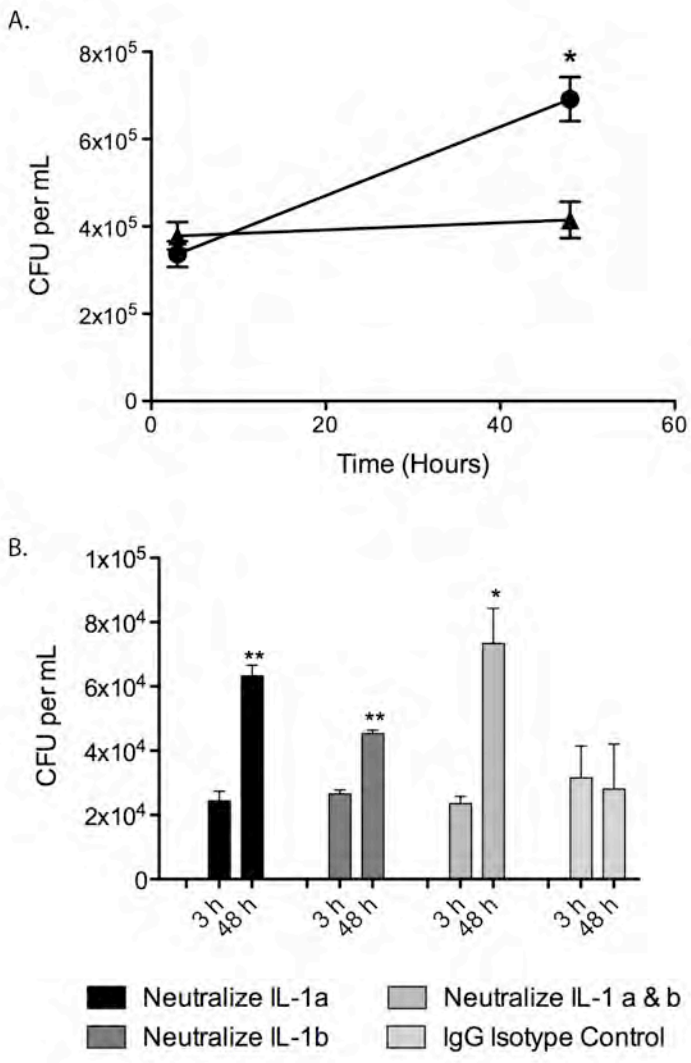

Figure 2.4: (A) Antimycobacterial activity in macrophage co-cultures requires IL-1R expression on the naïve cell population. Co-cultures were established with *M.tb*-infected IL-1R$^{-/-}$ cell line macrophages and uninfected wild type cell line macrophages (*IL-1R$^{-/-}$ AB WT*) or vice versa (*WT AB IL-1R$^{-/-}$*) and co-incubated for up to 48 hours. CFU was measured at 0 and 48 h post-addition of naïve macrophages. Results are expressed as the difference in mean CFU/ml ± SD comparing baseline CFU to $T_{(48)}$. **, $p = 0.009$. **(B) Neutralization of IL-1 with antibodies abrogates antimycobactericidal activity in co-culture supernatants.** Co-cultures were established with *M.tb*-infected and uninfected wild type cell line macrophages. Neutralizing or control antibodies were added to a final concentration of 50µg/mL. CFU was measured at 0 and 48 h post-addition of naïve macrophages. Results are expressed as the difference in mean CFU/ ml ± SD comparing baseline CFU to $T_{(48)}$. **, $p < 0.0055$; *, $p = 0.048$.

infected or naïve population (Fig. 2.4). These experiments demonstrated that the co-culture antimycobacterial activity required IL-1R expression in naïve but not infected macrophages. This suggested that IL-1β served as an afferent signal to directly or indirectly induce the expression of a soluble efferent factor from naïve macrophages.

To further test the involvement of IL-1α and IL-β in the co-culture antimycobacterial effect, we neutralized the cytokines both individually and in unison in a co-culture experiment (Fig. 2.4B). Neutralizing antibodies, were added at the same time as the naïve macrophages and remained in co-culture until CFU/mL was measured. Neutralization of both IL-1α & β was sufficient to abrogate the co-culture antimycobacterial effect. Neutralizing both IL-1α & β in unison was slightly more permissive for *M.tb* growth compared to IL-1α or β separately suggesting the possibility that the two cytokines are working synergistically but separately. This supports the idea that IL-1 is an important signaling molecule in the innate immune response to *M.tb* infection in macrophages. The additive effect of IL-1α & β also suggests that the communication between the infected and uninfected macrophage populations is complicated and likely to involve multiple signals not yet identified.

We then investigated whether activating macrophages with recombinant IL-1α, IL-1β or both was sufficient for the infected macrophages to confer the antimycobacterial activity (Fig. 2.5). Macrophages were pre-treated with IL-1α,

IL-1β or both for one hour prior to infection and 2 hours during infection in the absence of naive macrophages. Pre-treatment with IL-1 was not sufficient for enabling the antimycobacterial effect of infected macrophages, supporting the idea that IL-1 is serving as an afferent signal to the naive macrophages.

The efferent signal in co-cultures is elusive

Having found that IL-1β participates as an afferent factor in the co-culture system, we sought to understand the mechanism of its expression and also to identify the factor present in co-culture supernatant that inhibited *M.tb* replication. It was previously suggested that signaling via ATP receptor, $P2X_{7,}$ ($P2X_7$ R), can activate the NLRP3 inflammasome leading to caspase-1 activation and IL-1β release [111-115] and is modeled to be involved in lung inflammation associated with chronic obstructive pulmonary disease (COPD) [116]. Moreover, stimulation

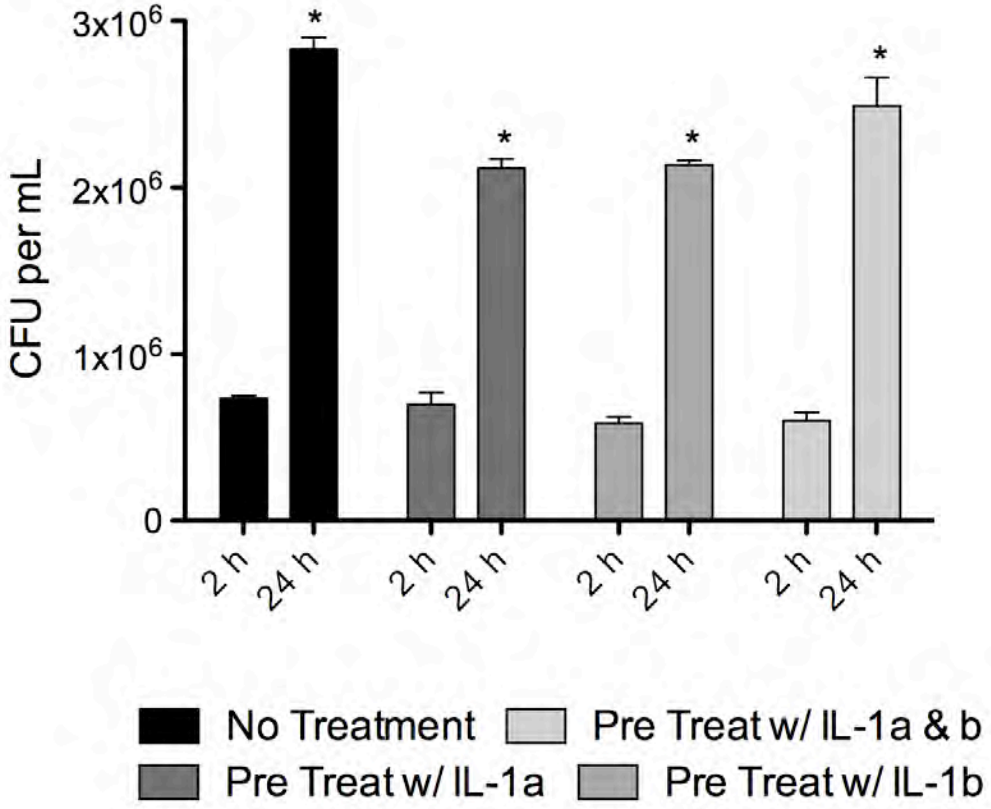

Figure 2.5: Pre-Treating Macrophages with IL-1 before infecting with high MOI M.tb does not reduce bacterial viability. Naïve macrophages were pre-treated for one hour with 100 ng/ mL with IL-1α, IL-1β or both prior to infection. Macrophages were infected with *M.tb* Erdman (MOI 50) for 2 h, with IL-1 remaining in the media, then infected macrophages were washed and kept in DMEM without IL-1 until CFU was measured. CFU was measured at 2, 8 and 24 hours post-infection. Results are expressed as the difference in mean CFU/ml ± SD comparing baseline CFU to $T_{(24)}$. *, $p < 0.0005$

of $P2X_7R$ in *M.tb*-infected macrophages was shown to induce antimycobacterial activity by directing bacilli to autophagosomes [117, 118]. We investigated the involvement of ATP-mediated signaling by establishing co-cultures where either the infected or the uninfected macrophages were derived from $P2X_7R^{-/-}$ mice. Regardless of what population was deficient of $P2X_7R$, there was no reduction of antimycobacterial activity (Fig 2.6a). This result indicated that $P2X_7R$ signaling was not required for either afferent or efferent limbs of antimicrobial activity observed in the co-culture system.

When the nucleotide adenosine is released from stressed and dying cells it can often have immunomodulatory effects. It was reported that IL-1β increases adenosine receptor expression in monocytes and increases responsiveness to adenosine [119]. In co-cultures established with macrophages from adenosine A2a receptor$^{-/-}$ mice we found no reduction of antimycobacterial activity when these cells were used either as the naïve or infected population (Fig. 2.6b). Furthermore, treating infected macrophages with the stable adenosine agonist NECA at 0.1, 1 and 10 μM, did not limit *M.tb* replication in infected macrophages (Fig. 2.6c). Direct antimicrobial activity of NECA was tested at 1 μM and did not affect bacterial replication (Fig. 2.6d). These results effectively excluded a requirement for adenosine for antimicrobial activity.

To define the physical nature of the efferent co-culture factor, supernatants were treated with DNAse, RNAse, proteinase K and at denaturing temperatures (Fig. 2.7). None of these treatments reduced the antimycobacterial effect

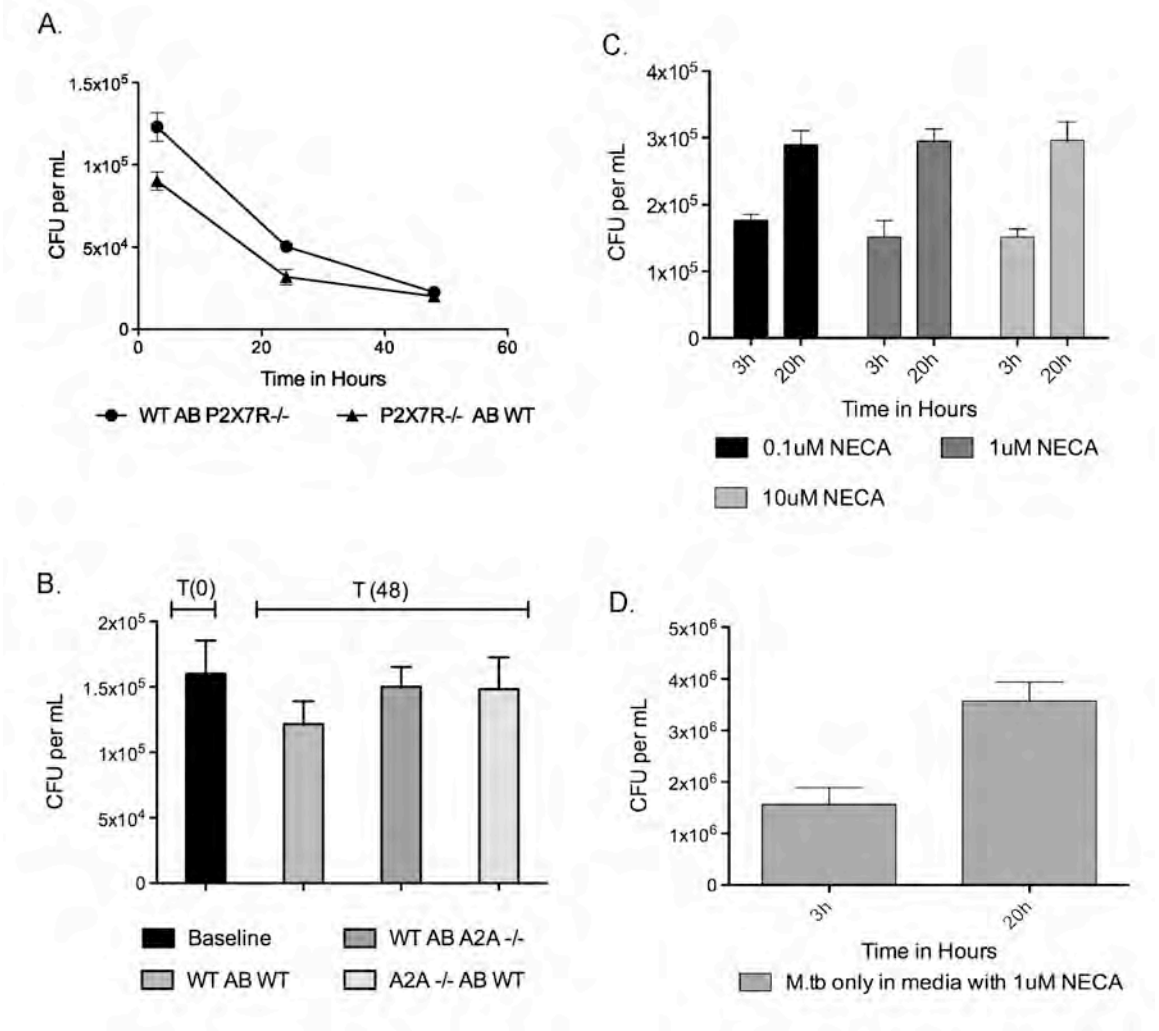

Figure 2.6: Nucleotide signaling does not contribute to the antimycobacterial activity in macrophage co-cultures. (A) Co-cultures were established using wild type immortalized BMDM's infected with *M.tb* and naïve $P2X_7$ receptor-deficient macrophages (*WT AB P2X7R-/-*) or vice versa. CFU per mL was measured at the indicated times. The co-culture antimicrobial effect was observed in both conditions. **(B)** Co-cultures were established with *M.tb* -infected wild type immortalized BMDM's and naïve adenosine A2a receptor-deficient macrophages (*WT AB A2a-/-*) and vice versa. CFU per mL was measured at time zero and 48 hours post-addition of treated supernatants. Adenosine A2a receptor expression on either cell population was dispensable for *M.tb* growth inhibition. **(C)** High MOI co-culture experiment was carried out in the presence of P2X7 receptor antagonist, N-ethylcarboxamidoadenosine (NECA) at 0.1, 1 and 10μM starting at the time of addition of the naïve immortalized BMDM's to the infected ones. CFU was measured at 3 and 20 hours after the addition of NECA and naïve macrophages. To verify that NECA did not have any direct antimicrobial effects, *M.tb* was incubated in media alone containing 1μM NECA **(D)**. CFU was measured at 3 and 20 hours after adding NECA to the bacterial culture.

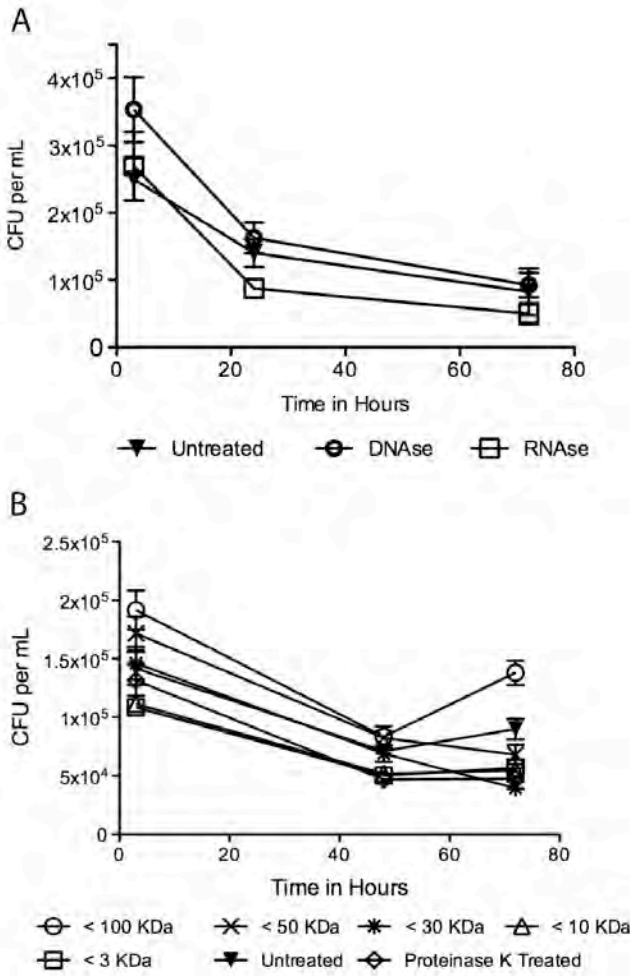

Figure 2.7: Soluble antimicrobial activity in macrophage co-cultures is not mediated by DNA, RNA or protein. Supernatants were harvested from co-cultures of *M.tb*-infected and naïve cell line macrophages, filter-sterilized and then treated as described before being added to isolated cultures of macrophages infected with *M.tb* (MOI 50, 3 h). CFU per mL was measured at time zero and at the indicated times post-addition of treated supernatants. **(A)** Treating co-culture conditioned supernatant with DNase or RNase failed to eliminate antimicrobial activity. **(B)** Co-culture conditioned supernatants were treated with proteinase K and then heated to 95^{o}C for 5 min, or passed through the indicated size-exclusion filters before being added to *M.tb*-infected macrophages. These treatments failed inhibit the ability conditioned supernatant to promote inhibition of *M.tb* replication in the heavily-infected macrophages.

conferred by co-culture supernatants. Passing co-culture supernatant through a series of molecular size exclusion filters to a minimum of 3 kDa also failed to deplete the activity.

Phospohlipase A_2 liberates arachidonic acid from membrane phospholipids which, through activities of cyclooxygenases (COX), lipoxygenases (LO) and cytochrome P450s, can be converted to a multitude of bioactive molecules, including eicosanoids, that affect numerous biological processes [120]. Stimulation of TLRs, especially TLR 4, can up-regulate COX-2 and additionally modulate eicosanoid release [121]. Given the involvement of TLR 4 to *M.tb* recognition, we set out to determine whether inflammatory COX-derived lipids were involved in mediating the co-culture antimycobacterial effect. To do this, naïve macrophages were treated with non-selective COX-1 inhibitor, indomethacin (Fig. 2.8a) and an irreversible COX-1 and COX-2 inhibitor aspirin (Fig. 2.8b). Both failed to diminish the co-culture activity, indicating the efferent factor or its production did not depend on COX enzymes in the naïve cells. These experiments revealed that the efferent factor in this system is a heat-stable small molecule and not likely to be a protein, nucleic acid, prostaglandin or thromoboxane. The identity of the factors involved remains to be determined.

Mechanism of antimycobacterial activity in co-cultures

Having been unable to identify the soluble efferent factor in the co-culture experiments we turned our attention to the cellular requirements for the observed

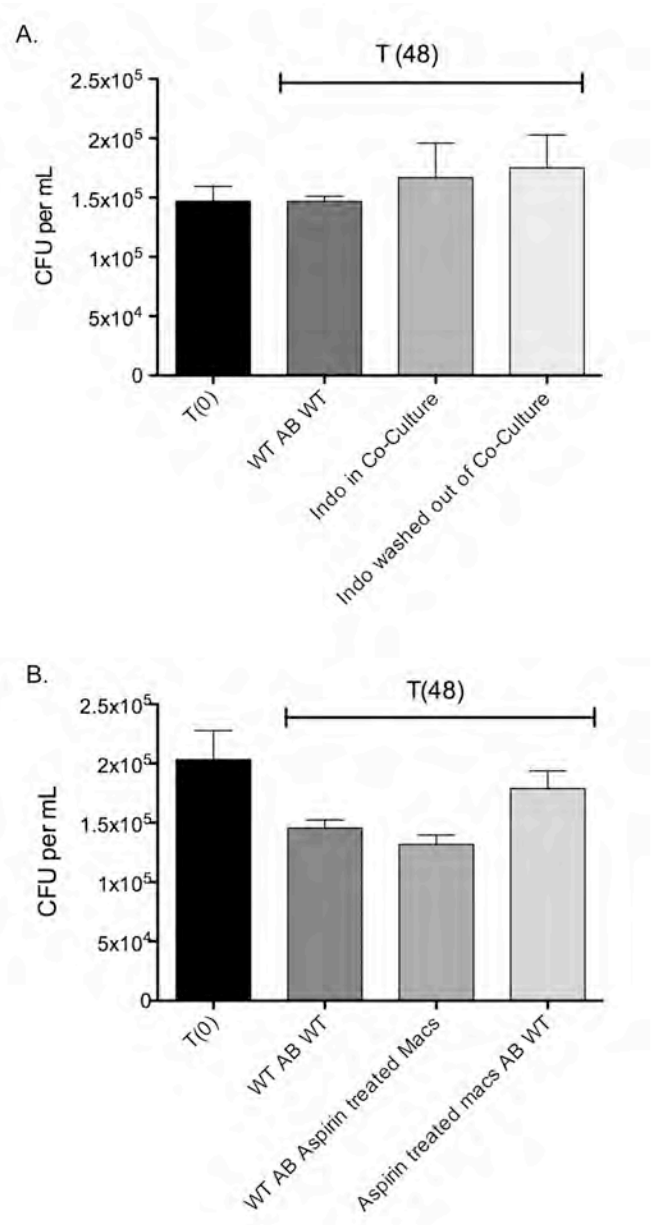

Figure 2.8: Soluble antimicrobial activity in macrophage co-cultures is not dependent on COX-2 generated eicosanoids. (A) Macrophages infected with MOI 50 *M.tb* were co-cultured with either naïve WT macrophages (*WT AB WT*) or macrophages that had been pre-treated with indomethacin for 3 hours prior to being added to the infected cells. For groups with indomethacin pre-treated macrophages, co-culture was carried out both with indomethacin in the co-culture (*Indo in Co-Culture*) or without indomethacin (*Indo washed out of co-culture*). **(B)** Co-cultures were established using wild type macrophages infected with *M.tb* and naïve aspirin pre-treated macrophages (*WT AB Aspirin treated macs*) or vice versa. CFU was measured at 0 and 48 hours after addition of naïve macrophages were added to the infected ones.

antimicrobial effect. Our previous experiments indicated that the factor acted only on dying macrophages before the onset of necrosis, which suggested that the efferent factor induced or facilitated an intracellular antimicrobial process in the infected macrophages. Nitric oxide is a major factor limiting *M.tb* growth in macrophages; a function that has been demonstrated in vitro and in vivo [122-125]. To investigate any involvement of nitric oxide in the antimycobacterial effects observed in our experiments, we established co-cultures using inducible nitric oxide synthase (iNOS)$^{-/-}$ macrophages as the infected or the naïve population(Fig. 2.9). When iNOS$^{-/-}$ macrophages were the infected cell population, co-culture with naïve macrophages failed to limit *M.tb* growth. In contrast, naïve iNOS$^{-/-}$ macrophages were capable of stimulating antimycobacterial activity in wild-type macrophages infected with *M.tb*. This result suggested that the efferent factor released from naïve macrophages facilitated a conventional antimycobacterial function in the infected cells that acts on bacilli in mature phagolysomes. A cardinal feature of *M.tb* virulence is the capacity to arrest phagosome maturation and establish an intracellular compartment resembling an early endosome that is permissive for bacillary replication [19]. In this regard, Davis et al. [24] reported that iNOS is excluded from *M.tb* phagosomes. Results from our experiment with iNOS$^{-/-}$ macrophages imply that in heavily infected macrophages, bacilli may be located in acidified lysosomal compartments.

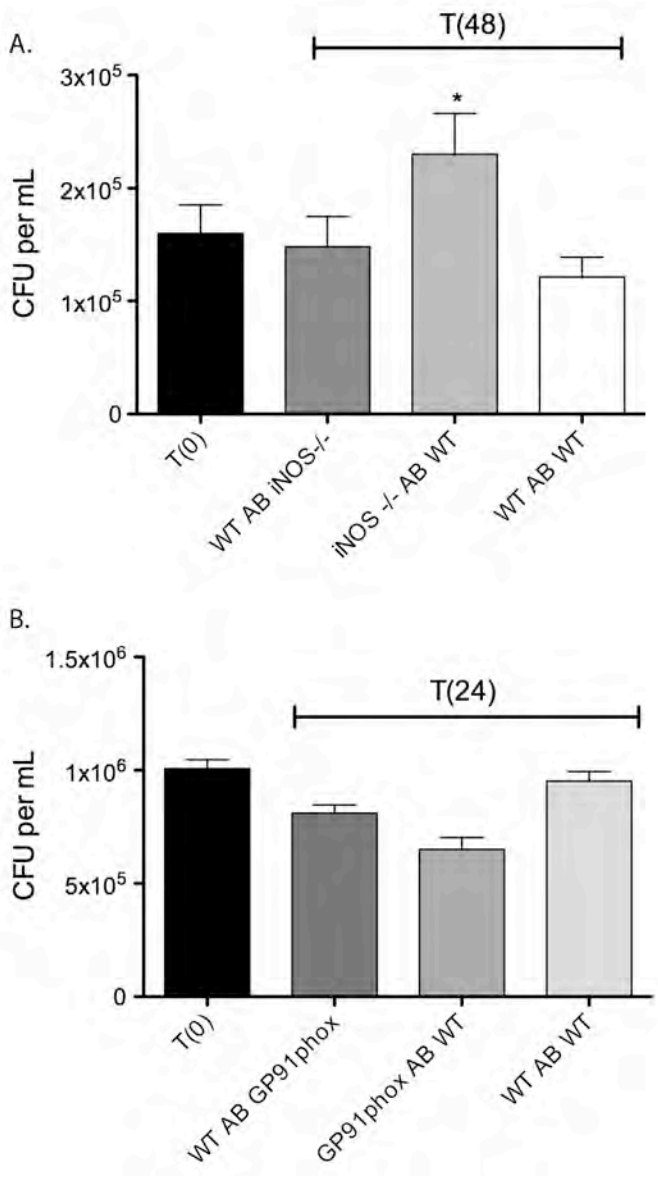

Figure 2.9: Antimycobacterial activity in macrophage co-cultures requires iNOS but not GP91phox expression in the infected cell population. (A) Co-cultures were established with *M.tb*-infected iNOS$^{-/-}$ macrophage cell lines and uninfected wild type macrophage cell lines (*iNOS-/- AB WT*) or vice versa (*WT AB iNOS-/-*) and also wild type macrophage cell lines co-cultured with naive wild type macrophage cell lines (*WT AB WT*). **(B)** Co-cultures were established with *M.tb*-infected wild type macrophages and uninfected wild type macrophages (*WT AB WT*), GP91phox$^{-/-}$ macrophages and uninfected wild type macrophages (*GP91phox AB WT*) or vice versa. CFU was measured at 0 and 24 or 48 hours post-addition of naïve macrophages. Results are expressed as mean CFU/ml ± SD at the indicated times. *, $p < 0.01$ comparing CFU between groups at 48 h.

M.tb infection directly induces autophagy in macrophages

In light of our data showing the involvement of iNOS in co-culture conditions, we postulated that in macrophages proceeding towards death with a high intracellular burden of *M.tb*, bacilli may be delivered to a hostile lysosomal compartment within the cell. Much recent interest has focused on autophagy as a means for macrophages to overcome the arrest of phagosome maturation implemented by *M.tb*. In prior reports, autophagy in *M.tb*-infected macrophages was activated by stimulation with exogenous rapamycin, interferon-γ or ATP [126].

We speculated that bacilli in heavily infected macrophages were being delivered to autophagosomes despite the absence of exogenous autophagy-inducing factors. To test that possibility we generated immortalized mouse macrophages with stable expression of the autophagosome marker LC3 (also referred to as MAP-LC3 for “microtubule associated protein 1 light chain 3”) fused to EGFP (enhanced green fluorescent protein, referred to here on out as “GFP”) [27]. In the absence of bacilli, green fluorescence was homogenously distributed throughout the cytosol of GFP-LC3 macrophages with rare foci of aggregation presumably reflecting an expected basal level of autophagosome formation (Fig. 2.10). Induction of autophagy with rapamycin as a positive control was reflected by a significant increase in GFP-LC3 aggregation. A similarly strong induction of GFP-LC3 aggregation was observed in macrophages infected with viable *M.tb* at an MOI of 50, which is sufficient to initiate cell death

and the antimycobacterial response in co-culture with naïve macrophages. In contrast, GFP-LC3 aggregation was not induced by heat-killed *M.tb* at MOI 50 or by viable *M.tb* at MOI 10; both conditions previously shown not to result in macrophage cell death [2, 127]. The specificity of autophagy induction by viable *M.tb* at high MOI was demonstrated by the failure of viable *E. coli* to stimulate GFP-LC3 aggregation at MOI 50. These results demonstrated that autophagy was specifically induced by a high intracellular burden of *M.tb* in the absence of IFN-γ or other known exogenous factors.

To confirm that autophagy in heavily infected macrophages delivered bacilli to autophagosomes, macrophages were infected with red-fluorescent mCherry-*M.tb* and examined by confocal microscopy. As shown in Figure 2.11a, *M.tb* was located in vacuoles with circumferential green LC3-GFP fluorescence. Co-localization of mCherry-*M.tb* and LC3-GFP was quantified in confocal images of macrophages challenged at low vs. high MOI, confirming a ~60% higher frequency in the high MOI macrophage cultures (Fig. 2.12).

The final stage of phagosome maturation is typically characterized by loss of late endosomal markers and fusion with lysosomes. LAMP1 is a marker commonly associated with lysosomal fusion. To investigate whether LC3 positive compartments arising in macrophages with high intracellular burden of *M.tb* were undergoing such maturation, GFP-LC3 expressing cell line macrophages were infected with *M.tb* and subsequently immunostained with anti-LAMP1 antibodies.

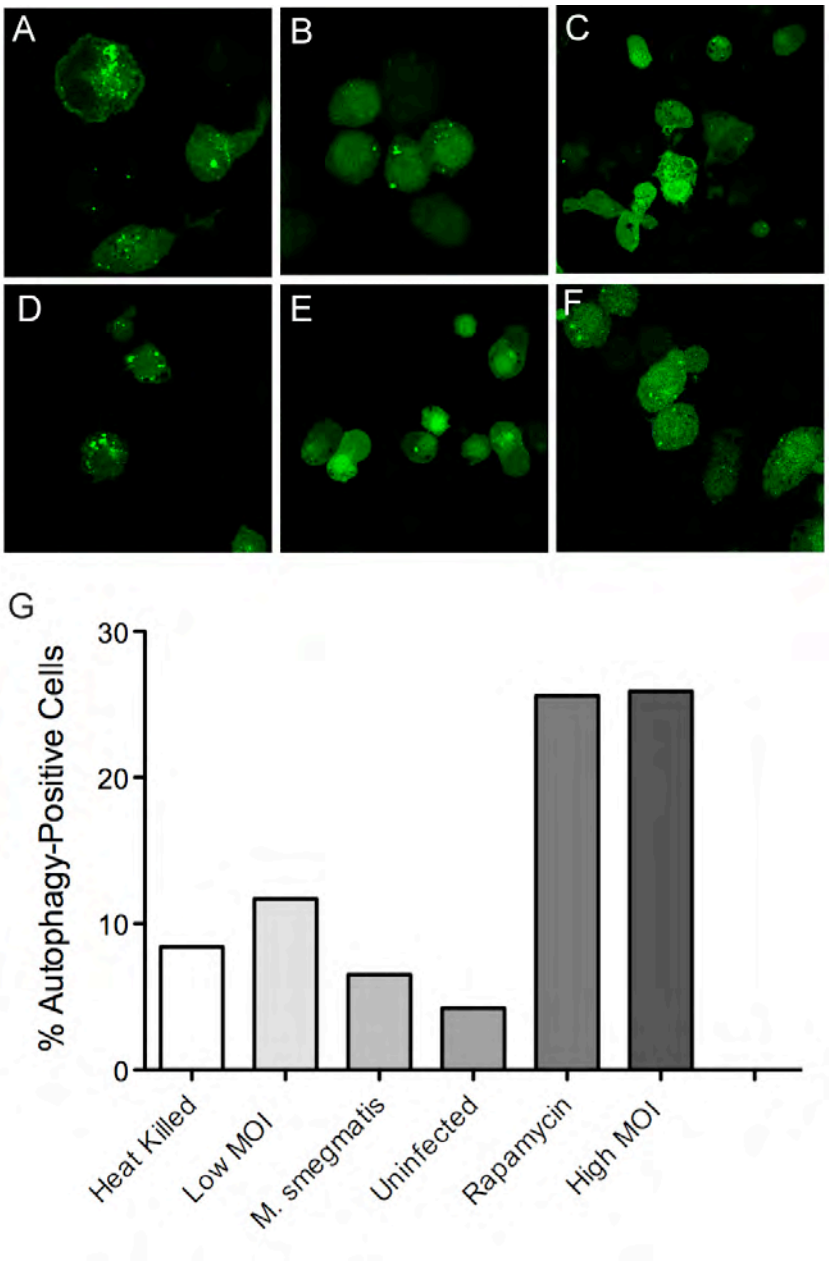

Figure 2.10: High intracellular burden of *M.tb* induces LC3 aggregation. GFP-LC3 expressing macrophages were infected with bacteria for 6 h as indicated below or treated with rapamycin before being fixed and examined by confocal microscopy for aggregation of LC3 indicative of autophagy. Conditions included **(A)** viable *M.tb* Erdman (MOI 50), **(B)** viable *M.tb* Erdman (MOI 10), **(C)** viable *M. smegmatis* (MOI 50), **(D)** rapamycin (25 μg/ml, 3 h), **(E)** heat-killed *M.tb* Erdman (MOI 50), and **(F)** no infection. observed by confocal microscopy. MOI 50 induces LC3 aggregation as strongly as the rapamycin positive control. MOI 10 and *M. smegmatis* infection (B & C) has no significant increase of LC3 aggregation compared to uninfected macrophages **(F)**. **(G)** The induction of autophagy was quantified by counting the % cells in each group with a number of LC3 aggregates above the median basal level of 5, found in untreated, uninfected macrophages.

Using pH-stable alexa-fluor conjugated secondary antibodies, we expect that the lower pH of the compartment will not affect results [128]. Under these conditions, colocalization of LC3 and LAMP1 was evident (Fig. 2.11C). Mature endosomal compartments are also characterized by low pH. In parallel experiments with *M.tb*-infected GFP-LC3 macrophages, we observed co-localization of LC3 with LysoTracker Red DND-99 that partitions to acidified compartments (Fig. 2.11B). Collectively, these results indicate that a high intracellular burden of *M.tb* promotes autophagy in macrophages that delivers bacilli to acidified autolysosomal compartments where nitric oxide-dependent antimycobacterial mechanisms may operate.

To further investigate the role of autophagy in the co-culture antimycobacterial effect, we used siRNA to target specific autophagy related genes. ATG7 is a ubiquitin-like modifying activating (E1-like) enzyme that activates LC3 by PE lipidation thus affecting its localization to the autophagosome [129]. We then investigated whether or not siRNA knockdown of autophagy-related protein 7 (Atg7) would inhibit the antimycobacterial activity in the co-culture experiments (Fig. 2.13). Co-culture conditions consisted of macrophages that had been transfected with siRNA targeted against Atg7 or control siRNA 72 hours prior to infection of wild type macrophages. Neither control or Atg7 siRNA inhibited the antimicrobial co-culture effect suggesting that autophagy is working in conjunction with other cellular mechanisms to decrease

the viability of *M.tb* in the co-culture experiments, but it is not required for clearance.

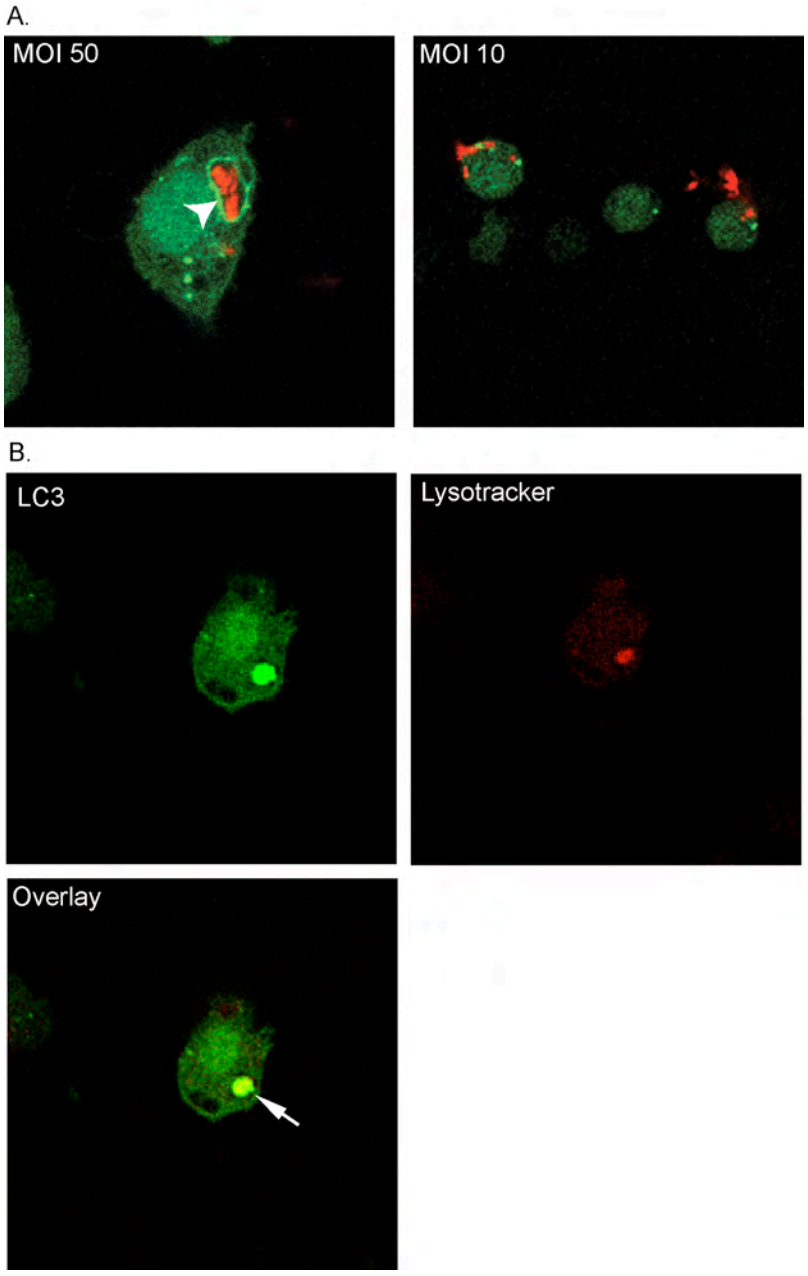

Figure 2.11: *M.tb* localizes to acidified LC3-positive compartments. (A) GFP-LC3 expressing macrophages were infected with mCherry-*M.tb* (MOI 50 or 10, 6 h) and then fixed and observed by confocal microscopy. At high MOI there are GFP-LC3 rings surrounding (*arrowhead*) around mCherry-*M.tb* and co-localization indicated by yellow pixels (*arrow*) that are not seen in macrophages challenged at low MOI. **(B)** Lysotracker Red DND-99 staining of GFP-LC3 macrophages infected with *M.tb* Edrman (MOI 50 or 10, 6 h) demonstrates co-localization of LC3 and LysoTracker (*arrow*). **(C)** LAMP1 immunostaining of GFP-LC3 cell line macrophages infected with *M.tb* Erdman (MOI 50 or 10, 5h) demonstrates co-localization of LC3 and lysosomal markers (*arrowheads*).

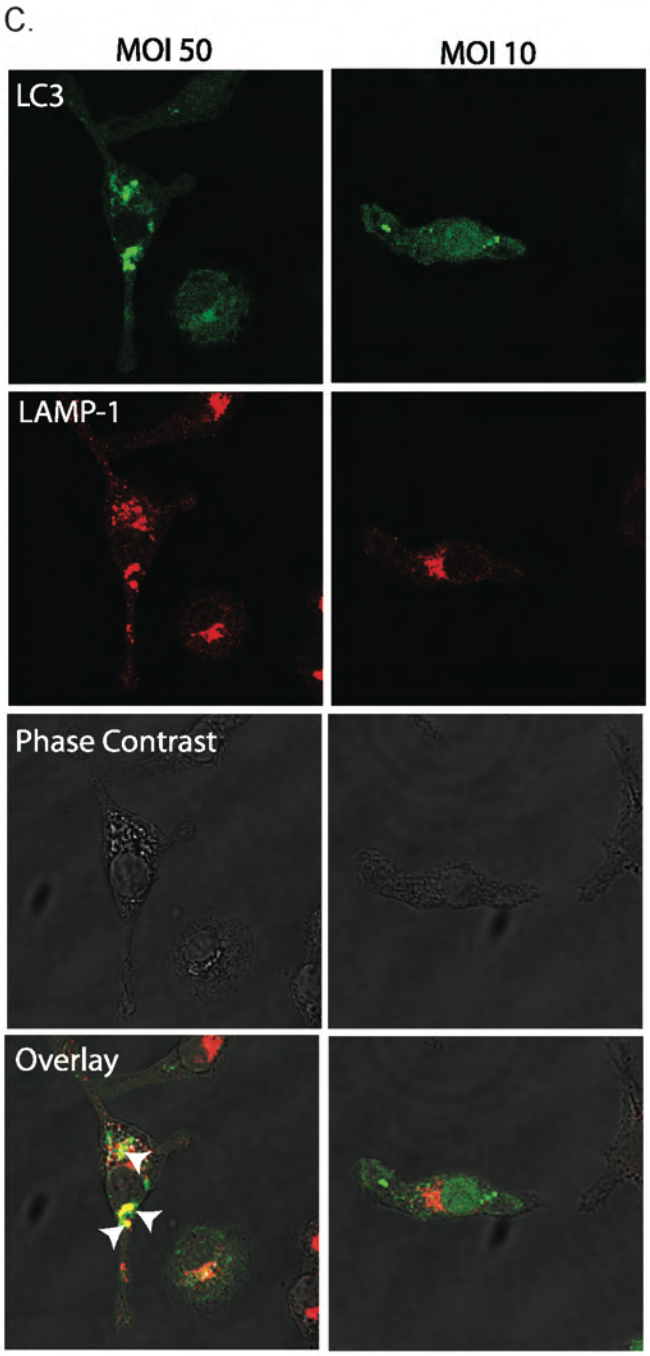

Figure 2.11: *M.tb* localizes to acidified LC3-positive compartments. (C) LAMP1 immunostaining of GFP-LC3 cell line macrophages infected with *M.tb* Erdman (MOI 50 or 10, 5h) demonstrates co-localization of LC3 and lysosomal markers (*arrowheads*).

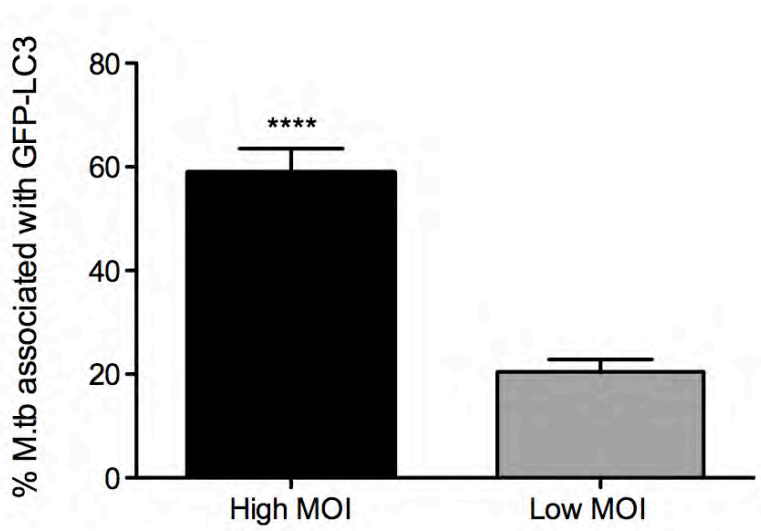

Figure 2.12: Co-localization of *M.tb* with LC3 in infected macrophages. GFP-LC3 expressing macrophages were infected with mCherry-*M.tb* (MOI 50 or 10, 6) and then fixed and observed by confocal microscopy. Cells in the MOI 50 (*high MOI*) and MOI 10 (*low MOI*) groups where *M.tb* was seen to co-localize with LC3 aggregates (see Fig. 2.11A) were counted. Results are expressed at the % cells with co-localization of the total number of cells counted. ****, $p < 0.0001$ comparing co-localization between high and low MOI infected groups.

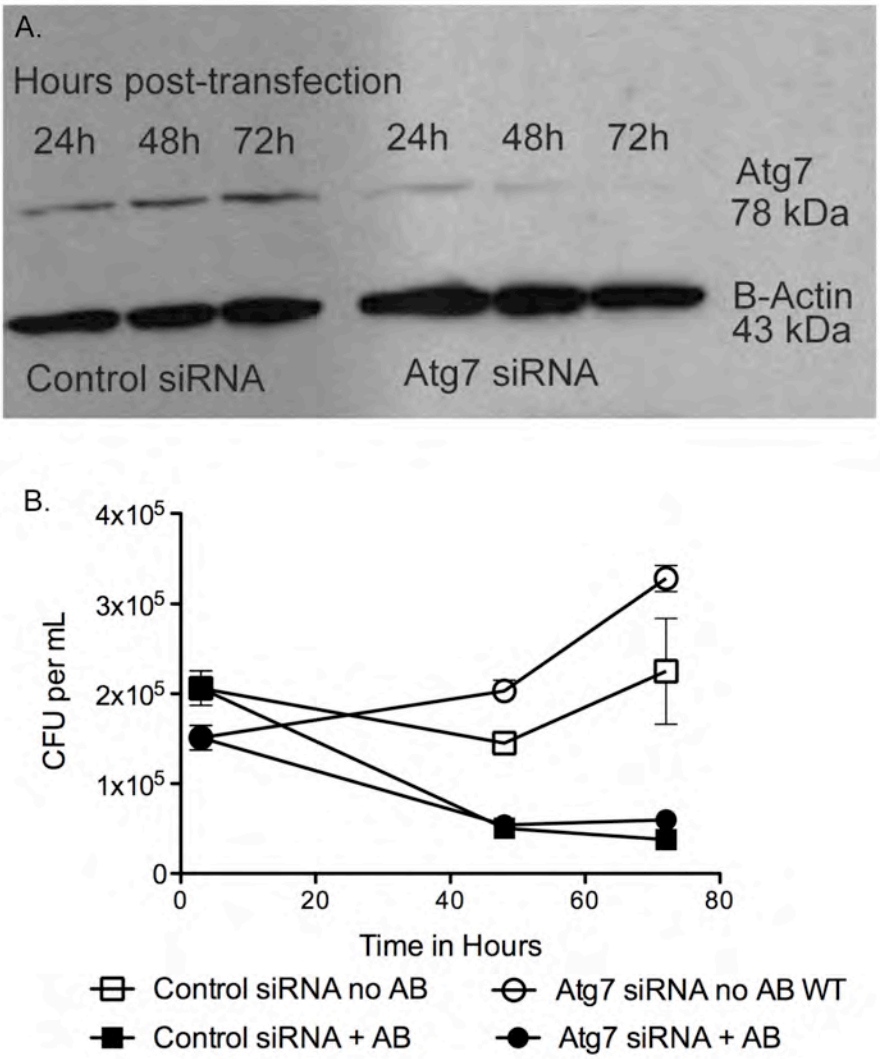

Figure 2.13: ATG7 siRNA does not inhibit the co-culture antimicrobicidal effect. (A) Atg7 was knocked down using Atg7 or control siRNA for 24, 48 and 72 hours. The effectiveness of the knockdown was measured by western blot at each time point. **(B)** Atg7 targeting or control siRNA knocked down cells were plated 72 hours after knockdown with siRNA. siRNA treated macrophages were infected with *M.tb* Erdman at MOI 50 for 3 hours. Infected macrophages then were co-cultured with naïve WT macrophages (+ *AB*) or media alone (*no AB*). CFU was measured at 0, 48 and 72 h post-addition of naïve macrophages. Results are expressed as mean CFU/ml ± SD at the indicated times.

DISCUSSION

Macrophage cell death is thought to play a major role in TB pathogenesis [130]. Previous studies have shown antimicrobial activity when *M.tb*-infected macrophages with features of apoptosis are co-cultured with naïve macrophages [2]. However, when *M.tb*-infected macrophages become necrotic, for example in the context of caseous necrosis in the granuloma, naive macrophages provide no antimicrobial effect. This suggested that efferocytosis of apoptotic bodies containing *M.tb* might enhance antimicrobial activity of naïve macrophages. However, in vivo studies using *M. marinum* infected zebrafish raised doubt by demonstrating a role for efferocytosis to spread infection to naïve macrophages recruited to granulomas and ultimately to seed distal sites [131]. In the present study we demonstrate that the antimicrobial activity against *M.tb* conferred by naïve macrophages in co-culture depends on a lethal intracellular bacillary burden in the infected macrophages, but efferocytosis is not required.

Transwell cultures demonstrated contact-independent crosstalk where an afferent signal from infected macrophages, or possibly from *M.tb*, stimulated an efferent signal from naïve macrophages that restricted *M.tb* replication in the former. The nature of the afferent signal was investigated, with the assumption that cytokines or death-related signals such as nucleotides would be involved. We found an abundance of IL-1β in co-culture supernatants, and additionally, the necessity for IL-1R expression on the naïve macrophage population for antimicrobial activity in co-cultures (Fig. 2.4). *M.tb*-infected macrophages

produced IL-1β and levels were further increased upon exposure to naive macrophages. Giacomini *et.al.* [132] showed that *M.tb* infection increases IL-1β production in macrophages and recent studies demonstrated that efferocytosis of *M.tb*-infected apoptotic neutrophils by naïve macrophages up-regulates IL-1β mRNA expression [133]. The importance of IL-1β in our system is clear, but our experiments did not test whether naïve macrophages also produce IL-1β in co-cultures, nor did they exclude other signals acting synergistically with IL-1β.

Supernatants from co-cultures were capable of stimulating antimicrobial activity in isolated cultures of *M.tb*-infected macrophages. This strongly implied that naïve macrophages produce a soluble factor that augments antimicrobial activity in the infected cell population, but an extensive search failed to reveal the identity of this factor. The activity is heat stable, < 3kDa, and resistant to DNase, RNase and proteinase K (Fig. 2.7a). Previous studies have shown that nucleotide signaling in macrophages stimulated antimycobacterial mechanisms [117, 134] and nucleotides are recognized death-associated molecules. However, our studies failed to identify any involvement of ATP or adenosine signaling. Lipid signaling molecules could fit the physical criteria for the efferent factor in co-culture supernatants, but inhibiting cyclooxygenase with indomethacin had no effect in our system. Our data have so far excluded a wide range of candidates but the identity of the factor that promotes antimycobacterial activity in heavily infected macrophages remains unknown at this time.

Despite being unable to identify the efferent signal in the co-culture system, we were able to gain insight to the antimicrobial mechanisms stimulated by crosstalk between naïve and infected macrophages. Antimicrobial activity is expressed exclusively in the pre-necrotic phase of *M.tb*-induced cell death, when few extracellular bacilli were observed by confocal microscopy. This suggested an antimicrobial mechanism operating within the infected macrophages, a conclusion supported by our finding that the infected cells must express iNOS in order to restrict *M.tb*. This cytoplasmic enzyme normally localizes to phagosomes containing ingested bacteria to efficiently target its product, nitric oxide [23].

A key feature of *M.tb* is the capacity to prevent its endosomal compartment from progressing through the lysosomal pathway [19, 135]. In addition to circumventing exposure to low pH and lysosomal hydrolases, *M.tb* also inhibits iNOS recruitment to phagosomes [24, 136]. Given the requirement for iNOS, we questioned whether autophagy provided an alternate mechanism to overcome the phagosome biogenesis block by *M.tb*. Autophagy has been described as a defense mechanism against intracellular pathogens including *M.tb* [99, 117, 137, 138]. In addition to the conventional antimicrobial properties of acidified phagolysosomes, autophagy results in the generation of neo-antimicrobial peptides from processing of ubiquinated cytosolic proteins that are capable of killing *M.tb* [139]. Published studies have so far relied on starvation or exogenous agents such as IFNγ or rapamycin to induce autophagy in *M.tb*-

infected macrophages. We found that *M.tb* infection can directly induce autophagy in macrophages without any requirement for starvation or exogenous factors. This occurs specifically in the context of a lethal intracellular bacillary burden and presumably reflects a stress response in cells committed to death. The specificity of this response to a high intracellular burden of viable *M.tb* was shown by the lack of autophagy induction by heat-killed *M.tb* at high MOI, viable *M.tb* at low MOI, viable *M. smegmatis* at high MOI or viable *E. coli* at high MOI. In the absence of co-culture with naïve macrophages, this autophagic response does not appear to significantly reduce bacillary viability. That may be due a relatively brief time for exposure of bacilli to hostile conditions before the infected cell progresses to a necrotic state [127]. The soluble factor promoting iNOS-dependent antimicrobial activity in these dying cells could act in any of several ways. It might accelerate delivery of *M.tb* to autolysosomes, it might amplify iNOS enzymatic activity or localization to mycobacterial vacuoles, it might amplify autophagy-specific antimicrobial mechanisms [139], or it might delay macrophage necrosis and thereby prolong the exposure of bacilli to degradative processes within intact autolysosomes.

In conclusion, we studied interactions between *M.tb*-infected and naïve macrophages which model events in pulmonary TB where alveolar macrophages initially infected by inhaled *M.tb* become surrounded by an excess of recruited, naïve macrophages before the expression of adaptive immunity that restricts bacillary replication [108]. This models approximately 7 days post infection, at

which point M.tb has had time to undergo several doublings. We recently showed that in this early, innate phase of TB defense relatively unrestricted replication leads to high intracellular bacillary loads in vivo [127], which results in macrophage necrosis. Since virulent *M.tb* inhibits macrophage apoptosis, but ultimately triggers necrosis at high bacillary loads it is unlikely that antimicrobial mechanisms dependent on efferocytosis play a major role in TB defense. Our data reveal a 2-way interaction where *M.tb*-infected macrophages committed to cell death, produce a signal that causes naïve macrophages to respond by producing a factor which promotes iNOS-dependent inhibition of *M.tb* replication in autolysosomes. While the afferent signal requires IL-1β, additional co-factors may be involved. The identity of the efferent signal is presently unknown but our data permit a focused search by excluding DNA, RNA, proteins and molecules > 3 kDa. The co-culture antimycobacterial effect we describe here may represent a previously unrecognized innate immunological defense mechanism that may limit the maximal replicative potential of *M.tb* prior to the expression of adaptive immunity in the newly infected host or alternatively mimic late stage reactivated TB disease.

MATERIALS AND METHODS

Ethics statement

Experiments with animals were conducted according to the National Institutes of Health guidelines for housing and care of laboratory animals under protocols approved by the Institutional Animal Care and Use Committee (A-1429) and the Institutional Biosafety Committee (I-161) at The University of Massachusetts Medical School.

Reagents

Autophagy inhibitor 3-methyladenine, the non-selective $P2X_7$ receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA), ribonucelase A1, proteinase K (from *Tritirachium album*), Atg7 antibody, LAMP-1 antibody, anti-actin antibody and the cyclooxygenase inhibitor indomethacin were purchased from Sigma Aldrich. Secondary antibody Alexa Fluor 647 goat-anti-rabbit IgG detection for LAMP-1 immunofluorescence was purchased from Molecular Probes. LEAF purified anti-mouse IL-1α and IL-1β neutralizing antibodies were purchased from BioLegend. The mTOR inhibitor rapamycin was purchased from Cell Signaling. TURBO DNase was purchased from Ambion. Centrifugal filter devices used for size fractionation based on Stoke's radius were purchased from Millipore. *E.coli*-derived recombinant murine IL-1α and IL-1β were purchased from R&D systems. HRP-conjugated anti Rabbit antibody, goat-anti-rabbit IgG isotype control for IL-1

neutralization experiments, and West Pico reagent were purchased from Pierce. Non-reversible COX-2 inhibitor Aspirin was purchased from Alexis Biochemicals.

Cell culture and mice

Immortalized cell lines originating from murine bone-marrow macrophages were made from C57/BL6 wild type mice, iNOS$^{-/-}$ mice (Jackson Labs), GP91phox -/- mice (gift from Dr. P. Newburger, University of Massachusetts Medical School), IL-1R$^{-/-}$ mice (gift of Dr. K. Fitzgerald, University of Massachusetts Medical School) using a J2 transforming retrovirus previously described [140, 141]. P2X$_7$R$^{-/-}$ mice were provided by Dr. R. Ingalls (Boston University School of Medicine) and Dr. A. Hise (Case Western Reserve University), and A2a R$^{-/-}$ mice were kindly provided by Dr. J.F. Chen (Boston University School of Medicine). Creation of the GFP-LC3 vector and macrophages are described by Harris et al. [27]. Cell lines were maintained in DMEM medium (Invitrogen Life Technologies) supplemented with 10% FBS (BioWhittaker), 100U/mL penicillin, 100 mg/mL streptomycin and 2mM glutamine and 10% L-929 conditioned media, made in-house, is added for culturing bone marrow derived macrophages.

Bacterial strains and infections

M.tb Erdman, used for all experiments unless otherwise indicated was obtained from Trudeau Institute. *M. smegmatis* mc^{2}155 was purchased from

ATCC. mCherry-*M.tb*, a generous gift from Dr. C. Sassetti (University of Massachusetts Medical School), was synthesized and codon-optimized with the mCherry open reading frame cloned into a pAL5000 based multicopy plasmid under the control of an optimized *E. coli* promoter in the *M.tb* H37Rv background. Bacteria were cultured in roller bottles containing Middlebrook 7H9 broth to OD_{600} of 0.7, washed twice in PBS and resuspended in DMEM medium supplemented with 10% FBS and 2mM glutamine at 1.5 - $2x10^8$ bacteria per mL. Stocks of bacteria were frozen immediately at -80^{o}C. Heat killed *M.tb* was generated by boiling bacteria at 100 ^{o}C for 30 min.

To infect cells, an aliquot of *M.tb* culture was thawed and dispersed with water bath sonicator (Branson Ultrasonics) for 90 sec at room temperature. Cultures were allowed to settle for 20 min to remove clumped bacteria followed by infecting macrophages at MOI 10 or 50 for 3 h at 37 ^{o}C, 5% CO_2. Infected macrophages were washed twice with DMEM and incubated for an additional 1h. CFU was measured at 0, 24, 48 or 72 h after the addition of the naïve macrophages by incubating the cells in 0.05% Tween 20 in water for 5 min at room temperature. Cultures were agitated by pipetting and serial diluted in 0.05% Tween 80 in PBS then plated onto Middlebrook 7H11 agar plates. Plates were incubated at 37 ^{o}C until colonies were counted 16 and 21 days after plating.

MOI was verified by fluorescent microscopy after staining *M.tb* with auramine-O (auramine). Stock solution of auramine is 1% auramine in methanol. Working auramine solution is 0.1% auramine and 33.3% phenol in water.

Samples are stained with working solution of auramine for 20 minutes at room temperature. 1% acid alcohol solution (40% denatured alcohol, 10% HCl in water) is used to counter-stain samples. Counterstain for 3 minutes after auramine staining without rinsing samples in between steps. After counterstain, wash 3x with PBS then slide was mounted with no-fade solution and imaged by fluorescent confocal microscopy. Approximate number of *M.tb* bacilli per were assessed in infection batches for add-back experiments.

Co-culture experiments

2 x10^5 macrophages were incubated for 1 h in 24-well polystyrene tissue culture plates (Nalge Nunc International) and then were infected as described above. At 3 h post-infection, an equal number of naïve macrophages were added either directly to wells containing infected macrophages, or to 0.4 µm pore-size, polycarbonate Transwell® inserts (Corning Inc.). Co-cultures were incubated for various time points before lysis of infected cells and plating for CFU. In supernatant transfer experiments, supernatants were collected from co-cultures of naïve and *M.tb*-infected macrophages 3 h after the naïve cells were added. Supernants were filter-sterilized (0.22 µm) and then added to macrophages that had been infected as described above.

Co-Culture Experiments with Inhibitors

Co-culture experiments were set up as described above. In the NECA experiments, the adenosine receptor agonist was present for the duration of the co-culture. For the aspirin experiments, infected or naive macrophages were pre-treated with 20mM aspirin for 15 minutes prior to being plated before infection or before being added to infected macrophages. For indomethacin experiments, cells were pre-incubated with 10 µM indomethacin for 3 hours prior to infection. In one group, indomethacin was washed out of the co-culture, in the other group, due to the reversible effects of the drug, 10µm indomethacin remained for the duration of the experiment.

Co-Culture Experiments IL-1 Neutralizing Antibodies

Co-culture experiments were set up as described above. At the time of the addition of the naïve cell line macrophages to the infected ones, 50 µg/mL of neutralizing antibodies or IgG isotype control antibody was also added. Antibodies remained in the co-culture until the time at which CFU was measured.

Multiplex

Supernatants from macrophages infected at MOI 50 and supernatants from high MOI co-cultures were collected at 3 h post-addition of naïve macrophages or 7 h post-infection. Supernatants were passed through a 0.22 µm pore size syringe filter then frozen at -80 ^{o}C until shipped for multiplex analysis (Aushon Biosystems).

Macrophage staining and fluorescent laser scanning confocal microscopy

Macrophages were stained with cholera toxin subunit B conjugates 488 or 647 (Molecular Probes) at 4 ^{o}C for 30 min and then washed three times with DMEM. For confocal microscopy, cells were then plated at 10^6 cells per 35mm glass bottom plate with 10 mm glass No. 1.5 thickness (MatTek). After infection and/or co-culturing, contents of plates were fixed with 4% paraformaldehyde in PBS for 30 min then washed three times with PBS. Cells were imaged using a 63x objective at room temperature with a SP2 AOBS confocal laser scanning microscope (Leica Microsystems) running LCS software (Leica Microsystems).

Lysotracker staining

GFP-LC3 expressing macrophages were infected at MOI 50 for 2 h then washed twice with DMEM and incubated 2 h further. 50nM lysotracker red DND-99 (Invitrogen) was added to infected macrophages for 20 min at room temperature. Cultures were then washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 30 min. Before imaging, fixed cells were washed three times with PBS.

LAMP-1 Immunostaining

GFP-LC3 expressing cell line macrophages were infected at MOI 50 for 3 h then washed twice with DMEM and incubated 2 h further. Cultures were then washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 30

min. then permeabilized with 0.1% Triton X-100 in PBS for 10 min. and blocked in blocking buffer (10% goat serum in PBS with 1% BSA and 0.1% Triton X-100) for one hour. Cells were then stained 1:200 with anti-LAMP1 antibodies (Sigma) for one hour in blocking buffer. After washing 5x with PBS, cells were stained with anti-Rabbit secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes) for 1h. Cells were then washed 5x with PBS and mounted as described above for confocal microscopy.

Quantifying cell-cell contact

After confocal imaging, broad fields of cells were assessed for interactions indicative of efferocytosis. While complete engulfment of infected macrophages by uninfected macrophages was rarely observed, the images revealed frequent cell-cell interactions with a distinct directionality of cell contact having the appearance of frustrated efferocytosis. To quantify the direction of these contacts a “positive” efferocytosis interaction was defined as pedestal formation or incomplete engulfment of one cell by another. A “negative” interaction was one where two cells in contact showed no synapse or pedestal formation and no extension of membrane from one cell partially surrounding another. Results are expressed as % positive interactions vs. % negative interactions.

siRNA gene silencing

25mer Stealth siRNA RNA oligonucleotides for silencing mouse ATG-7 were purchased from Invitrogen. The sense strand of the siRNA duplex consisted of a 25 nucleotide target sequence (5'-caa uga ugu ggu ggc ucc agg aga u-3') and the antisense strand was composed of nucleotides complementary to the target sequence. For a negative control, scrambled 25mer stealth siRNA with medium G-C content were used. Two million macrophages were transfected with 1 uM of siRNA using Neon (Invitrogen) according to the manufacturer's instructions. The electromechanical condition for transfection was pulse voltage 1650 V, pulse width 20, and pulse number1. The efficacy of Atg-7 silencing was confirmed by Western blot.

SDS-polyacrylamide Gel Electrophoresis and Western Blotting

Lysates were made from siRNA-knocked down cells by incubating cells in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) by manufacturers instruction shaking gently for 5 minutes. Cell debris was pelletted by centrifugation at 14,000 x g for 15 minutes. Supernatants were frozen at -80℃ until used for gel electrophoresis.

For resolving protein by gel electrophoresis, a 10% SDS-polyacrylamide gel was made with a 6% acrylamide stacking gel. 100µg of protein was boiled at 100℃ for 5 minutes in Laemmili Buffer and loaded onto the gel. The gel ran for

90 minutes at 155 V. Proteins were transferred onto PVDF membrane at 250 mA for one hour.

After transfer, non-specific antibody binding was blocked using 5% non-fat dried milk in TBST for one hour. Membrane was kept rocking at 20℃ overnight in primary antibody (1:2500 in 5% non-fat dried milk in TBST). The next day, the membrane was washed 3x in TBST for 5 minutes each then incubated in secondary HRP-conjugated detection antibody (1:1000) for 4 hours at room temperature. After 3x 5 minute washes with TBST, membrane was developed using West Pico reagent (Pierce). Kodak film was exposed to blotted membrane for 10 seconds and developed.

Quantifying the Number of Colony Forming Units (CFU) per mL

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{\# of colonies on plate}}{(\text{dilution factor})(\text{volume inoculated in mL})}$$

Statistics

Differences between groups at the same time-point were assessed by unpaired t-test using GraphPad Prism. Values of $p \leq 0.05$ were considered to be statistically significant.

PREFACE to Chapter III

The first publication using GFP-LC3 expressing macrophage cell line, listed below, was the debut of the GFP-LC3 expressing immortalized macrophage cell line made by Michelle Hartman with immense technical assistance from Brian Monks and Veit Hornung.

Dr. James Harris at Trinity College Dublin thoroughly tested the GFP-LC3 cell line and generated all data and images for Figures 3.5 and 3.6 of this dissertation.

Citation:

James Harris, Michelle Hartman, Caitrionna Roche, Shijuan G. Zeng, Amy O'Shea, Fiona A. Sharp, Eimear M. Lambe, Emma M. Creagh, Douglas T. Golenbock, Jurg Tschopp, Hardy Kornfeld, Katherine A. Fitzgerald & Ed C. Lavelle. *Autophagy controls IL-1β secretion by targeting pro-IL-1β for degradation.* Journal of Biological Chemistry. 2011. 286: 9587-9597.

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We acknowledge Dr. Tamotsu Yoshimori (Osaka University) and Dr. Noboru Mizushima (Tokyo Medical and Dental University) for the LC3 cDNA as cited in Kabeya *et. al.* [142].

A special acknowledgement to Dr. James Harris and Dr. Ed Lavelle at Trinity College Dublin for exceptional assistance in thorough testing of the cell line to ensure that they functioned properly.

Additionally, we acknowledge Brian Monks, M.S. from the Dr. D. Golenbock lab for the guidance and assistance in making pRP-GFP-LC3 viral vector and Dr. Veit Hornung for assistance with the viral transduction procedure.

CHAPTER III: Construction of a GFP-LC3 Expressing Macrophage Cell Line

Abstract

Autophagy is widely accepted to be a mechanism that macrophages use to rid themselves of *Mycobacterium tuberculosis*. We sought to determine whether autophagy resulted in a high intracellular bacterial burden with *M.tb* and also whether or not it contributed to the antimycobacterial effects observed in the co-culture experiments. To determine the role of autophagy in this context, it was critical to create cells that allowed us to visualize the localization of *M.tb* within the infected macrophage and additionally provide a simple assay to determine whether or not autophagy was stimulated under various conditions. This chapter describes the 8-month process of creating such a cell line.

Introduction

Though macroautophagy, here on out referred to as autophagy, is most widely recognized as a cellular homeostatic process that enables cells to degrade and recycle their contents, it is clear that it also plays a significant role in eliminating some intracellular pathogens, a process alternatively referred to as xenophagy [143-146] Pathogen-induced autophagy appears to be TOR-independent [147].

The role of autophagy in eliminating intracellular *M.tb* was initially demonstrated by Guitierrez *et. al.* [99]. It was shown that upon stimulating autophagy in *M.tb*-infected macrophages by amino acid starvation, there was increased co-localization of the autophagosome marker LC3 to the mycobacterial-containing phagosome, coupled with increased acidification of the compartment. Singh *et. al*. reported that autophagy related to the inhibition of *M.tb* replication is associated with interferon (IFN)-ϒ and its effector Irgm1 (the IFN-inducible immunity related GTPase family M member 1, formerly referred to as LRG-47) [101] They provide data suggesting that Irgm1 increases phosphatidylinositol-3-kinase (PI3K) accumulation in the *M.tb*-containing autophagosome, resulting in enhanced autophagolysosomal maturation. Since the trademark of *M.tb* infection is its phagosome-lysosome fusion block, allowing the pathogen to evade cellular destruction [148-150], the demonstration of autophagy as a mechanism for the macrophage to override this phagosome

maturation block was novel and opened the door to new studies focusing on new innate immune pathways involved in the elimination of *M.tb*.

The precise function and mechanism of every autophagy related protein is unknown. However, what is more widely understood is which proteins are located on the autophagosomal membrane at different times. Yeast autophagy-related (ATG) protein 8, a soluble cytoplasmic protein that associates with the phagophore after being covalently conjugated with phosphatidylethanolamine (PE) is recruited to the autophagosome early on and remains associated with it into maturity. The mammalian orthologs of ATG8 include GABARAP, GATE-16 and MAP-LC3 (or microtubule-associated protein 1 light chain 3- now referred to as “LC3”). All of these orthologs are processed by a cysteine protease ATG4, followed by activation by ATG7 and leading to lipidation by PE. However, only LC3 is observed in pre-autophagosomal structures as well as mature autophagosomes. Though the precise function of LC3 is unknown, it is understood to be regulated by phosphorylation [151] and is cleaved and processed at the induction of autophagy [152]. After its cleavage, LC3 specifically labels the growing phagophore and mature autophagosomes [142, 153]. The major pathways promoting the induction of autophagy converge on the cleavage and lipidation of LC3, which allows for its conjugation to phospholipids and is critical for the formation of autophagosomes and cargo recognition [94, 154, 155] The role of LC3 in the induction of autophagy and its association with mature autophagosomes make it a useful tool to investigate the role of

autophagy in eukaryotic systems. In this chapter, I describe the development of a macrophage cell line that expresses the fusion protein EGFP-LC3, for the purpose of investigating the role of autophagy in the context of *M.tb* infection.

Results

Development of a GFP-LC3 expressing immortalized macrophage cell line

Before control experiments revealed to us that engulfment of infected, apoptotic bodies/macrophages (efferocytosis) was not necessary for the co-culture antimycobacterial effect, we started making a fluorescent cell line to test the hypothesis that autophagy was involved in the destruction of the apoptotic bodies. Though our studies demonstrated that efferocytosis of infected cells was not involved, much recent interest has focused on autophagy as a means for macrophages to overcome phagosome maturation arrest in *M.tb*-infected macrophages. We speculated that bacilli in heavily infected macrophages were being delivered to a hostile lysosomal compartment within the cell via autophagosomes. To test that possibility, we generated immortalized mouse macrophages with stable expression of the autophagosome marker LC3 fused to GFP. [27]

The pEGFP-LC3m plasmid was created by cloning *Rattus norvegicus* microtubule-associated proteins 1A and 1B light chain 3 subunit (LC3) into the multiple cloning sight (MCS) 3‘ of the EGFP coding sequence (Figure 3.1). LC3 was cloned into the pEGFP-C1 plasmid in a manner that both proteins were in the same reading frame [142]. This plasmid was obtained from Dr. Noboru Mizushima at Tokyo Medical and Dental University (Tokyo, Japan) via Dr. Stephen Doxsey at UMass Medical School (Worcester, MA).

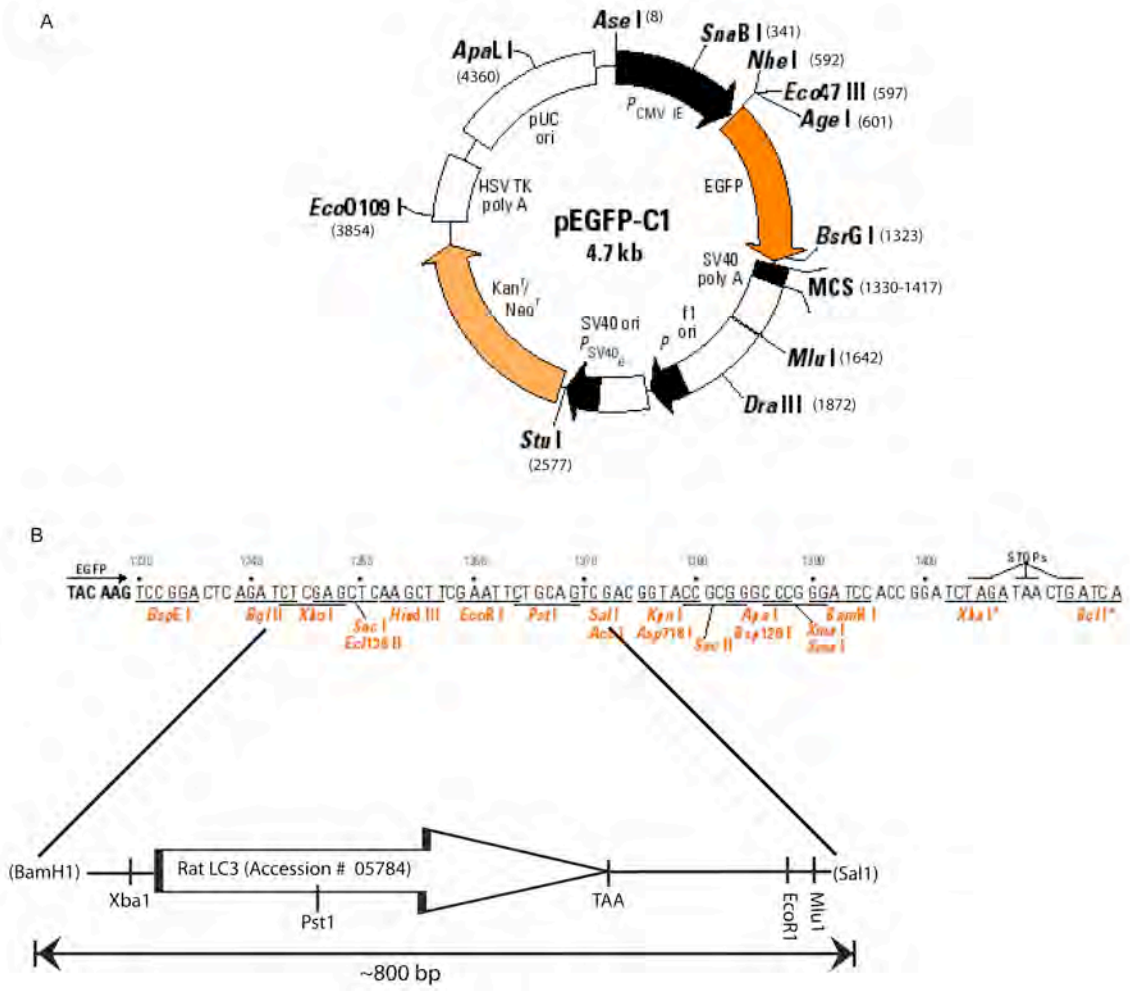

Figure 3.1: Map of pEGFP-LC3m construct. (A) Original pEGFP-C1 plasmid map containing EGFP (dark orange) and a kanamycin resistance cassette (light orange). **(B)** *Rattus norvegicus* LC3 cDNA was cloned into the MCS of pEGFP-C1 plasmid using BamH1 and Sal1.

Plasmid DNA is notoriously difficult to transfect into macrophages [156]. To circumvent this difficulty, a retroviral construct was made to generate virion needed to transduce macrophages to express the vector expressing the EGFP-LC3 construct. pRP vector, a generous gift from Dr. D. Golenbock, was modified from a pcDNA3 backbone (Invitrogen) by removal of the Neomycin resistance cassette and SV40. The SV40 origin was retained however so that the vector could replicate in HEK 293T-cells. pRP::EGFP-LC3 was constructed in the following way: EGFP-LC3 was double digested out of the pEGFP-LC3m plasmid with restriction enzymes AgeI (New England Biolabs) and PspOMI (Fermentas). The viral vector pRP cut with NotI and AgeI. Both the vector and the insert were gel purified (Fig. 3.2a) and directly ligated into into the multiple cloning site (MCS) where expression of the construct is constitutively expressed by the cytomegalovirus (CMV) promoter. Ligated construct was transformed into XL1 Blue *E.coli* and cultured in Luria-Bertani (LB) broth containing ampicillin for generating large amounts of plasmid copies. pRP-GFP-LC3 was then verified by restriction digestion (Fig. 3.2b).

Complete retrovirus expressing the construct was generated in HEK's by transfecting them using Transit® transfection reagent with EGFPLC3::pRP along with two separate plasmids expressing Gag/Pol and VSVG. Three days later retrovirus was isolated from supernatant of the transduced HEK cells. To

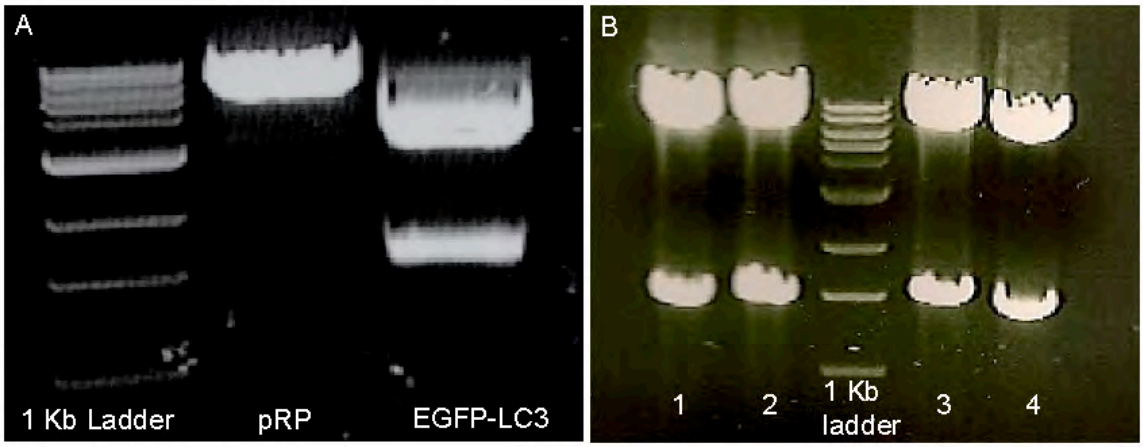

Figure 3.2: Verification of pRP vector and EGFP-LC3 insert before and after ligation. (A) EGFP-LC3 was double digested using out of its original vector pEGFP-LC3m. pRP was also digested. Digestion products were gel purified to optimize ligation reaction. **(B)** EGFP-LC3 was directly ligated into prepared pRP vector using T4 ligase reaction without any PCR modification of the insert. After ligation, plasmid was electroporated into XL1 Blue *E. coli*. Several different XL1 Blue E. coli colonies were tested for expression of the vector, lanes 1-3. Plasmid was digested with EcoR1 to verify insertion of construct into pRP vector. Band at approximately 1Kb signifies proper insertion of EGFP-LC3 into pRP.

transduce the vector into wild type cell line macrophages, the virus-containing supernatant was titrated to 30%, 20%, 10%, 5% and 2.5% of the medium and added directly to the macrophages plated at 70% confluency. 48 hours post-transduction, cells expressing the plasmid were selected for with media containing 5µg/mL puromycin and 30% FBS. One week later, colonies expressing the construct were isolated and expanded under constant selection with 5µg/mL puromycin. The expanded macrophages were sorted (Fig. 3.3) to remove any macrophages not expressing the construct by our FACS (Fluorescence-Activated Cell Sorting) core facility at UMass Medical School FACS CORE facility. FACS-sorted macrophages were grown in bulk and frozen at -80ºC in 1mL aliquots.

Verifying function of GFP-LC3 expressing Macrophages

To verify that the newly-made cell line were performing as expected, cells were treated with 25 µg/mL rapamycin, a pharmacological inducer of autophagy, and an equal volume of its carrier control ethanol, for 3 hours. Treated EGFP-LC3 cell line macrophages were then fixed and imaged by confocal microscopy to assess whether the cells behaved normally. Figure 3.4 shows that carrier control treated macrophages have a diffuse GFP pattern throughout the cytoplasm yet cells treated with rapamycin clearly have an increased amount of GFP puncta which represent the aggregation of LC3, presumably to the phagophore and autphagosome membranes.

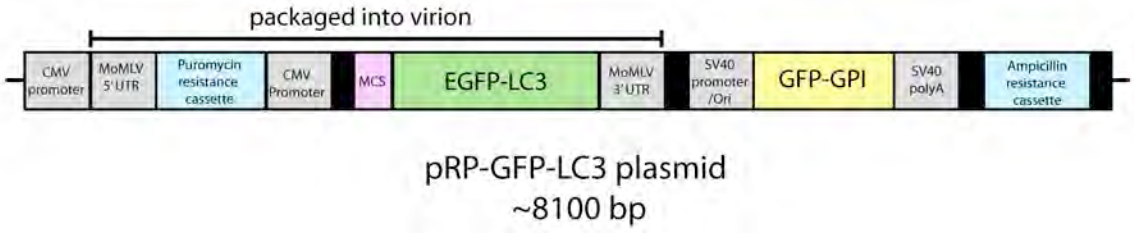

Figure 3.3 Linear map of pRP-EGFP-LC3 plasmid. EGFP-LC3 is inserted into the pRP viral vector so that it is between the 5' and 3' UTR to ensure packaging into virion head and is downstream of the CMV promoter for constitutive expression.

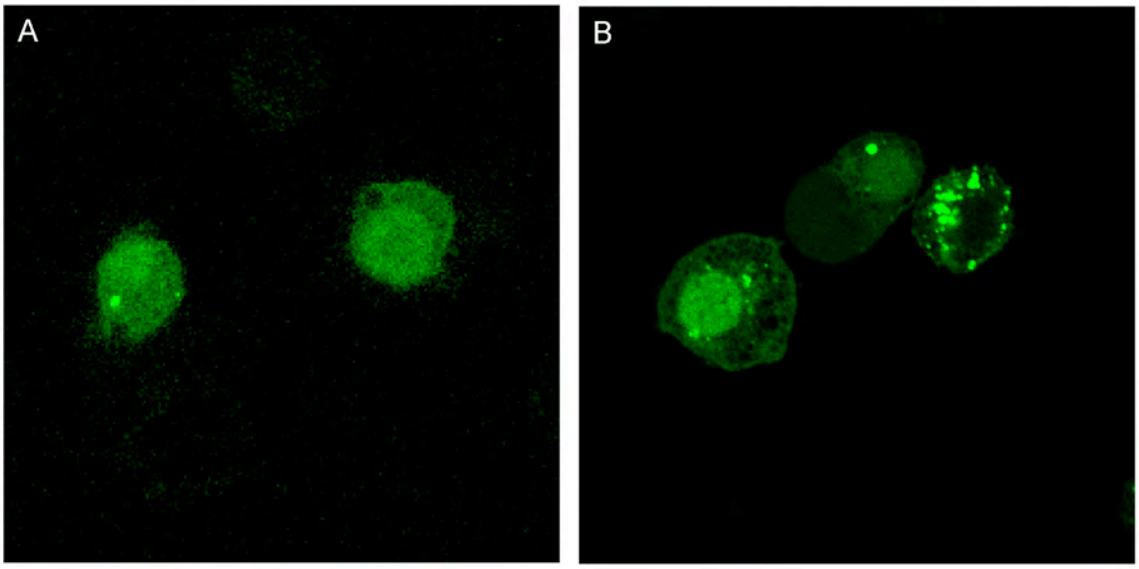

Figure 3.4 Control testing EGFP-LC3 cell line macrophages. Macrophages containing the pRP-GFP-LC3 construct have a diffuse fluorescent green appearance. GFP-LC3 macrophages were treated with **(A)** ethanol only as a carrier control to rapamycin treated macrophages and **(B)** Rapamycin (25 µg/mL). Control macrophages have more GFP in cytoplasm compared to rapamycin-treated group which has increased GFP puncta confirming the validity of the cell line.

Furthermore, to ensure that GFP puncta were not resulting from random clustering of GFP protein in the cell, collaborator James Harris (Trinity College Dublin) stained cells with antibody against LC3B and analyzed co-localization of LC3 with GFP by confocal microscopy After stimulating autophagy with LPS for 18 hours, cells were fixed, permeablized and stained with LC3B primary antibody, followed by secondary antibody staining with Alexa Fluor 568 conjugated goat anti-rabbit antibody. There is clear co-localization of the antibody with the GFP-puncta suggesting that clusters observed are LC3 aggregations (Fig. 3.5).

LPS stimulates autophagy via a Toll-interleukin receptor domain-containing adaptor-inducing interferon β (TRIF)-dependent, MyD88-independent TLR-4 signaling resulting in a p38-mitogen activated protein kinase acting downstream [157]. Induction of autophagy by IFN ϒ is dependent on To verify that the modification of the macrophages by expression of the pRP-GFP-LC3 vector did not alter the cells response to autophagy stimuli, the cells were treated with known stimuli of autophagy and assessed for GFP-LC3 puncta formation by confocal microscopy (Fig 3.6b). This experiment was carried out by Dr. James Harris of Trinity College Dublin. LC3 macrophages were stimulated with LPS or IFN-γ for 18 hours then observed by confocal microscopy and the number of LC3-positive puncta were counted in at least 100 cells per group Figure 3.6a). The significant increase in LC3 puncta in LPS and IFN-γ treated cells suggests that the cells respond normally, in the context of autophagy, to TLR-4 and IFN-γ stimulation.

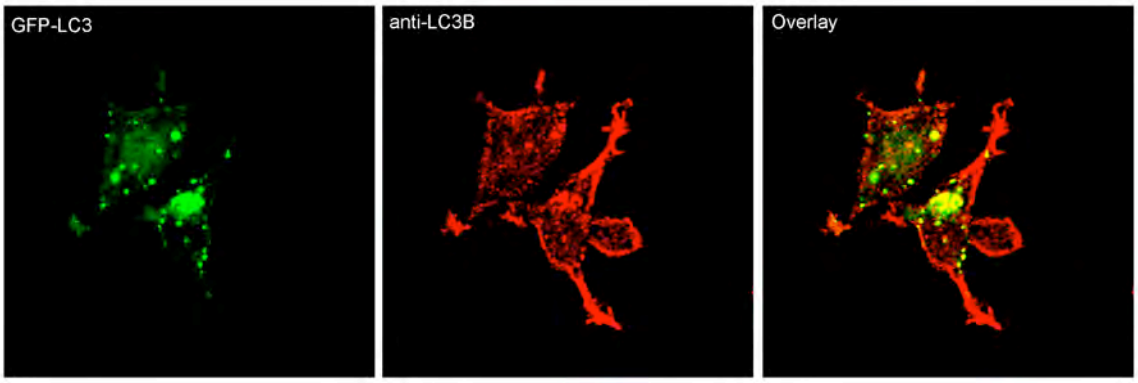

Figure 3.5: Anti-LC3b antibodies co-localize with puncta in GFP-LC3 cell line macrophages. Characterization of GFP-LC3 cell line macrophages by confocal microscopy. Locality of LC3 antibody staining (red) and GFP-LC3 protein expression in macrophages (green) was assessed to ensure that GFP puncta within the macrophages reflected LC3 puncta. Co-localization of antibody labeling with GFP-puncta within the cell is demonstrated by overlay of green and red stains, yielding a yellow appearance.

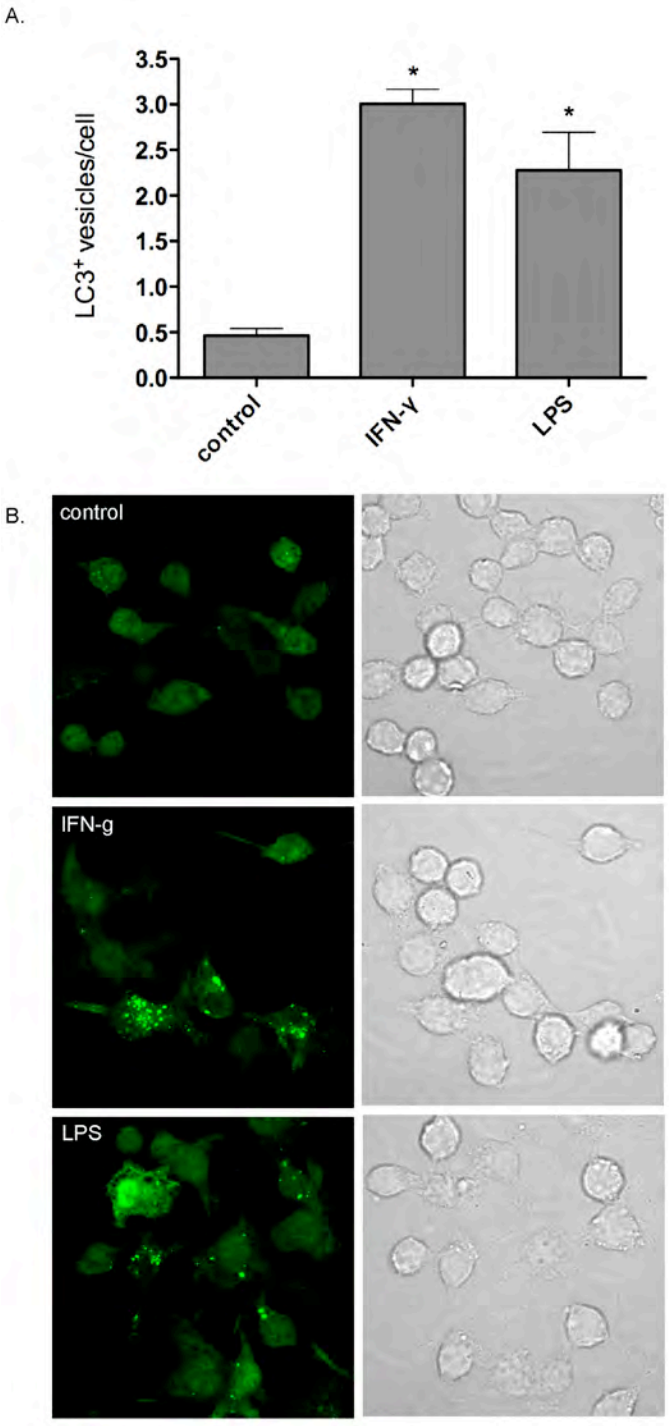

Figure 3.6: Number of LC3-positive vesicles in cells increases with LPS and IFN-γ treatment. LPS and IFN-γ were used to verify that the GFP-LC3 cell line macrophages responded properly to known stimuli of autophagy. Cells were treated with 100 ng/mL LPS or 10 ng/mL IFN-γ for 18 hours then LC3 puncta were counted **(A)** after imaging by confocal microscopy **(B)**. *$p \leq 0.01$ by one-way ANOVA with Tukey's post-test.

Discussion

The appearance of distinct LC3 puncta following stimulation of autophagy suggests that our cell line is useful for detecting upstream events of autophagy since the translocation of LC3 from the cytosol to the autophagosomal membrane is the point at which various autophagic pathways converge **[94]**. However, one limit of the cells may be that the maximum percentage of cells expressing 5 or more puncta with induction by rapamycin is only 30% (Fig. 2.10g). Though there is a 15% difference between untreated and rapamycin-induced groups, the range is limited. However, without immunoblotting for endogenous LC3 it is impossible to exclude the possibility that a greater difference exists between the two groups.

One possible problem with using labeled proteins in transiently transfected cells, specifically GFP-LC3, is that this method commonly causes aggregation of the protein and this event is almost impossible to be differentiated from the induction of autophagy [158]. However, strong co-localization of immunoblotting for LC3B with GFP-LC3 suggests that this effect is minimized in the cell line. Furthermore, in untreated GFP-LC3 expressing macrophages, on average, there were less than five GFP-LC3 puncta per cell. This is no more than one would expect to see due to the ongoing activity of autophagy in cells for housekeeping duties. It is also likely that the GFP-LC3 labeled protein behaves differently from endogenous LC3 in terms of trafficking, size of vacuole and additionally rate of maturation due to any changes in the kinetics of lysosome fusion events.

The major advantage in using an EGFP-LC3 expressing cell line is that you can visualize the activation and maturation of autophagosomes by following LC3. Alternative methods such as immunoblotting are viable alternatives to using the GFP-LC3 cell line, but are more cumbersome and not as convenient. When working with antibodies to label fixed cells, it is more difficult to be certain that the protein that the antibody is binding to is specific. The cell line also enables real-time imaging, which is critical for looking at cellular processes, like autophagy, that are dynamic and often rapidly induced. Other methods for simply detecting the activation of autophagy, such as western blotting, though useful for measuring specific changes to LC3 that are trademark to autophagy, do not allow for visualizing what is inside the autophagosomal compartments themselves. Thus, these GFP-LC3-expressing macrophages represent an extremely powerful tool for dissecting autophagy-dependent processes. (Harris 2010 JBC)

Additionally, LC3 is recycled quickly by removal from PE by the ATG4 protease (Kirisako 2000) and becomes trapped in the autophagosome and degraded after lysosomal fusion with the autophagosome. Therefore, mature autophagosomes lack in-tact LC3, thus indicating that a separate marker is needed to study those structures. However, regardless of the constant recycling of LC3, GFP is quickly denatured in acidified compartments, suggesting an alternative fluorophore might be preferred for following autophagy more closely. Proteins such as RFP and mCherry are more stable and should be considered in

the event of making a construct for a human macrophage cell line, such as human acute monocytic leukemia cell line (THP-1) cells.

Materials and Methods

Reagents

ATG7 antibody, LC3B antibody, anti-actin antibody, and bisBenzamide H (Hoechst) 33258 were purchased from Sigma Aldrich. Alexa Fluor 568 goat anti-rabbit antibody was purchased from Molecular Probes/Invitrogen. The mTOR inhibitor Rapamycin was purchased from Cell Signaling. LPS and IFNϒ for stimulating GFP-LC3 cell line was obtained from Biosera (Ringmer, East Sussex, UK). HRP-conjugated anti Rabbit antibody and West Pico reagent were purchased from Pierce.

Cell culture and mice

Immortalized murine macrophage cell lines derived from C57/BL6 wild type mice was a generous gift from Dr. D. Golenbock (University of Massachusetts Medical School) [20, 21]. Creation of the GFP-LC3 vector and macrophages are described by Harris *et al.* [18]. Cell lines were maintained in DMEM medium (Invitrogen Life Technologies) supplemented with 10% FBS (BioWhittaker), 100U/mL penicillin, 100 mg/mL streptomycin and 2mM glutamine and 10% L-929 conditioned media, made in-house, is added for culturing bone marrow derived macrophages.

Plasmids and constructs

LC3 cDNA came from Dr. T. Yoshimori (Osaka University) and Dr. N. Mizushima (Tokyo Medical and Dental University). The GFP-LC3 plasmid [13] was a generous gift from Dr. S. Doxsey (University of Massachusetts Medical School). pRP viral vector backbone was a gift from Dr. D. Golenbock (University of Massachusetts Medical School).

Immortalized bone marrow-derived macrophages stably expressing EGFP-LC3 (GFP-LC3) were generated by directly cutting EGFP-LC3 out of the pEGFP-C1 vector (22) and inserting it into the multiple cloning site of the retroviral pRP vector (23) using restriction endonucleases NotI and AgeI. Retrovirus carrying the EGFP-LC3 construct was made in HEK cells by transfection with EGFP-LC3::pRP, along with plasmids expressing Gag/Pol and VSVG using *Trans*IT® transfection reagent (Mirus, Madison, WI, USA). Three days later retrovirus was isolated from supernatant of the HEK cells and used to transduce the EGFP-LC3 construct into wild type C57/Bl6 immortalized WT macrophages. 48 h post-transduction, cells expressing the plasmid were selected for using media containing 10 µg/mL Puromycin. One week later, colonies expressing the construct were isolated based on GFP fluorescence by flow cytometry and expanded under constant selection with 10 µg/mL Puromycin. Cells were grown in DMEM medium (Invitrogen Life Technologies) supplemented with 10% FBS (BioWhittaker), 100U/mL penicillin, 100 mg/mL streptomycin and 2mM glutamine.

Verificaiton of GFP-LC3 function by confocal microscopy

For testing induction of autophagy by rapamycin treatment, cells were plated at 10^6 cells per 35mm glass bottom plate with 10 mm glass No. 1.5 thickness (MatTek). After treatment with rapamycin (25 ug/mL) or ethanol (carrier control) for 3 hours, cells were fixed with 4% paraformaldehyde in PBS for 30 min then washed three times with PBS. Cells were imaged using a 63x objective at room temperature with a SP2 AOBS confocal laser scanning microscope (Leica Microsystems) running LCS software (Leica Microsystems).

To verify LC3 antibody staining co-localized with GFP-LC3 puncta in autophagy-induced macrophages, GFP-LC3 macrophages were stimulated with LPS (100 ng/mL) or IFNϒ (10 ng/mL) for 18 h to induce autophagy. Cells were fixed with 2% paraformaldehyde (in PBS) for 30 min at room temperature, then permeabilized with 0.1% Triton X-100 (in PBS) for 10 min. Before antibody staining, permeablized cells were blocked with 3% BSA/5% goat serum (in PBS) for 1 h to minimize non-specific binding of the LC3 antibody to other proteins. Cells were then stained with primary antibody, rabbit anti-LC3B (Sigma) for 1 h at 1/200 dilution (approximately 5 µg/ml) in blocking buffer. After 5 washes with PBS, secondary antibody, Alexa Fluor 568 goat anti-rabbit (Molecular Probes/ Invitrogen), was applied for 1 h at 1/500 in PBS. Cells were washed twice with PBS then incubated with 10 ug/ml bisBenzamide H 33258 (Hoechst 33258, Sigma) for 5 minutes then washed 3 times with PBS and mounted with Dako

Fluorescent mounting medium. Images were taken on an Olympus FV10 confocal microscope with FV10-ASW software.

Agarose Gel Electrophoresis

DNA preparations of constructs were isolated from XL1-Blue *E. coli* by mini-prep procedure (Qiagen). Briefly, *E. coli*, containing various plasmids, was grown in LB medium containing Kanamycin (pEGFP-LC3m plasmid) or Ampicillin (pRP-GFP-LC3 plasmid) to ensure that all replicating bacterium contained the plasmid of interest. Bacterium were re-suspended and lysed under alkaline conditions first re-suspending pelleted bacterium in P1 buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 ug/mL RNAse A, pH 9) to ensure precipitation of all DNA with RNAse present to ensure collection of desired plasmid DNA and not RNA's. Followed by addition of a 5 M potassium acetate solution (P2 buffer: 200 mM NaOH, 1% SDS, pH 8) which neutralizes NaOH allowing small plasmids to remain soluble while genomic DNA, but not plasmid DNA, to precipitate out of the lysis solution. After 5 minutes of incubating, N3 buffer (proprietary buffer containing potassium acetate, pH 4.8) is added, which allows for precipitation of proteins out of the solution. Precipitates are removed from supernatant by centrifugation for 10 minutes at 13,000 rpm. Supernatants are then applied to a spin column containing a silica-gel-membrane, which binds small DNA's. The column is then washed with PE (10 mM Tris-HCl, 80% EtOH, pH 7.5) and PB (5 M Gu-HCl, 10 mM Tris-HCl 30% EtOH, pH 6.6) buffers. After residual wash

buffer is removed, plasmid DNA is eluted in 50-100 µL of elution buffer (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol). Concentration of plasmid DNA is measured by spectrophotometry.

After digesting EGFP-LC3 from pEGFP-LC3m and double digesting pRP backbone with Age1 followed by Not1, products were gel purified using a 1% agarose gel for one hour, 83 V. To test proper insertion of EGFP-LC3 into pRP (pRP::EGFP-LC3), 10µL of miniprepped DNA was test digested with EcoR1 followed by gel electrophoresis of total test digest reaction onto a 1% agarose gel. All agarose gels contained 0.5µg/mL ethidium bromide (EtBr), which fluoresces under UV light when intercalated into the major groove of DNA. Gel images were taken on a BioRad Gel Doc 1000 and image was printed on a Kodak XL 7700 dye sublimation printer.

CHAPTER 4: Discussion

Co-Culture Effectors

The mode of *M.tb*-infected macrophages cell death plays a large role in determining whether *M.tb* survives or is killed by its host [159]. The work described in Chapter 2 examines the how interactions between naïve macrophages added to *M.tb*-infected macrophages in co-culture changes the outcome pre-determined by whether the infected macrophage undergoes apoptosis or necrosis after infection, which was previously identified by Lee *et. al.* [2].

In our pursuit to understand how these two populations of macrophages communicate to reduce viability of *M.tb* in a heavily infected, apoptotic macrophage, we quickly identified inducible host enzyme NOS2, but not GPphox91, as a key player in the co-culture antimycobacterial effect. After identifying the involvement of NOS2 in our system, we moved on to look for molecules and other cell processes that could be acting to stimulate NOS2. We did not consider that the addition of naive macrophages to infected ones could possibly be enhancing NOS2 effects. Furthermore, we did not investigate whether IL-1β was influencing NOS2 expression in heavily infected cells which would be worthwhile investigating as it has been shown to increase iNOS in other systems [160, 161].

We also determined that IL-1 was a key communicator for these events to occur. An interesting point in regards to IL-1 in the co-culture effect is that though

it was critical, it was not functioning in the positive feedback loop typically observed for enhanced immune signaling [162]. However this is consistent with findings that in IL-1β processing in *M.tb*-infected macrophages is independent of inflammasome activation and also that *M.tb* is able to reduce IL-1R expression on macrophages [53, 162]. Together this suggests an alternative role in IL-1β signaling.

The idea that IL-1β could be inducing signaling molecules not previously identified in restricting mycobacterial growth is extremely interesting. We made significant efforts to determine the nature of the molecules involved yet came up with nothing. One possibility that has not been completely eliminated is the involvement of eicosanoids as the efferent signal in the co-culture effect. TLR 4 signaling can induce the production of COX and 5-LO generation of eicosanoids [121]. Since TLR 4 and IL-1R both signal via MyD88 adaptor molecules, as previously discussed, it is not out of the question that IL-1 signaling in macrophages could induce eicosanoid production as well. Additionally, virulent strains of *M.tb* are known to inhibit PGE2 production, as a way to favor necrotic cell death of its host cell as compared to TNFα-dependent, antimicrobial apoptotic cell death [30]. One possible mechanism for the infected macrophages enhanced ability to reduce mycobacterial viability in the context of co-culture is through production of PGE_2 from an uninfected, neighboring cell responding to cytokine signals. In this scenario, the naïve macrophage would be enhancing or stimulating the similar antimicrobial mechanisms involved in the TNF-dependent

apoptotic cell death killing of *M.tb* and could be tested with various caspase inhibitors.

Though indomethacin and aspirin experiments suggest that bioactive eicosanoid molecules resulting from COX modification of arachidonic acid are not involved in the co-culture effect, the effects of indomethacin, but not aspirin, are reversible when not kept in culture media. It is possible that using these drugs to test involvement of eicosanoids is not optimal, and it is not out of the question given the heat stability and small size of the unknown efferent signal that a lipid could be the effecting molecule. Furthermore, using indomethacin and aspirin as drugs to inhibit eicosanoid production in macrophages is limiting because it does not tell us about the involvement of leukotrines such as LTA_4, and the products resulting from 5-LO activity. In fact, the testing of products resulting from 5-LO activity is almost completely absent in our studies and would be worthwhile looking in to especially in light of recent studies by Divangahi *et. al.* showing the important balance between 5-LO and COX products in the immune response to *M.tb* [35].

We also tested the activity of various commercially available eicosanoids on heavily infected cells to assess if they had the potential to be enhancing the co-culture antimicrobial effect. Though we did not observe any reduction in mycobacterial growth after treating macrophages with the molecules, it is possible that they were not as bioactive as the molecules produced by naïve

macrophages. Given these ideas, it is not out of the question to assume the possibility of eicosanoid signaling in the co-culture effect.

The supernatants from co-culturing heavily infected cells with naive macrophages were thoroughly examined. The results demonstrated that the molecule(s) critically involved in the antibacterial effect were heat stable, smaller than 3KDa and resistant to RNase, DNase and Proteinase K treatment. We tested likely candidates that met the qualifications but to no avail. Though the lipid candidates are not totally excluded, other possibilities for the efferent signal include heat-stable small molecules, though HPLC analysis for small molecules didn't reveal anything significantly different between infected macrophages and co-culture supernatants, it is critical to keep in mind that a broad search for small molecules is not the most efficient approach and a more targeted search would likely yield better results.

One possible small molecule that was not examined in the co-culture experiments is Vitamin D. It has been previously reported that *M.tb* derived 19KDa lipopeptide increases vitamin D receptor (VDR) expression on macrophages *in vivo* and *in vitro* [163]. Vitamin D signaling is known to induce expression of antimicrobial peptide cathelicidin which in addition to having antimicrobial properties is an inducer of autophagy and comes into contact with *M.tb* in phagosomes [86, 164]. Recent studies supported this and further shown that vitamin D in its 1,25D3 form, induces cathelicidin-dependent induction of autophagy and phagosomal maturation that is able to inhibit mycobacterial

growth [165]. These new studies are extremely exciting as they demonstrate that the vitamin D3 active metabolite 1α,25-dihydroxycholecalciferol (1,25D3) is sufficient to induce autophagy in *M.tb* infected macrophages. It is possible that in a high MOI model of infection, enough vitamin D is produced to induce autophagy in an autocrine fashion through a similar mechanism. One possible mechanism for enhancing vitamin-D dependent inhibition of mycobacterial growth is through dual action of 1,25D3 and IL-1β. There is evidence that these two molecules are able to synergize in order to activate TLR2/1 antimicrobial pathways for the clearance of *M.tb in vitro* (Lui 2009). It is possible that IL-1β from our heavily infected macrophages is synergizing with bioactive vitamin D from either population of cells to reduce mycobacterial viability in the co-culture conditions described in chapter 2.

***M.tb*-induced autophagy**

One of the most interesting results in this work is that high MOI *M.tb* infection in macrophages induces autophagy in the absence of IFNϒ and that low MOI infection of macrophages does not activate autophagy at all even at later time points. Additionally, in most studies looking at the effects of inducing autophagy in *M.tb*-infected cells have demonstrated that autophagy is antimycobactericidal. Data presented in chapter 2 describes a mode of autophagy that is not directly microbicidal. When heavily infected macrophages are cultured for 24 hours, autophagy is induced at high levels but the residing

bacilli are not growth restricted. Studies have shown that *M.tb* can modulate the infected macrophage to make IL-6 which reduces the macrophage sensitivity to IFNϒ which indicates an indirect mechanism for *M.tb* to evade autophagic-induced clearance from the host [166].

Given that in our experiments there was no exogenous treatment of the infected cells was necessary to induce autophagy, it is unclear what cellular signals induced autophagy in the high MOI conditions. One possible mechanism for high MOI-induced autophagy is *M.tb*-NOD2 stimulation, an idea originally posed by Huynh *et. al.* [162] based on the idea that mycobacteria, contain a N-glycolyl variant of muranyl dipeptide (MDP), a component of peptidoglycan, which activates NOD2 signaling better than the variation found in avirulent strains of mycobacteria [167] and is able stimulate autophagy [168].

What is more interesting to consider is whether or not autophagy plays a role in the co-culture antimicrobial effect. Our data suggests that it is likely that *M.tb* is unable to block progression of its phagosome in the endocytic pathway in heavily infected macrophages. However, this phenomenon is observed without the addition of the naïve macrophage population and is not sufficient for killing *M.tb*. This suggests again, that it would be useful to look for co-culture candidate effector molecules, such as cathelicidin, that have both antimicrobial properties and have been previously associated with autophagic killing mechanisms.

Model & Conclusions

A lucrative model for the possible involvement of IL-1, iNOS, vitamin D and autophagy is extremely plausible and illustrated in figure 4.1. It is possible that IL-1β sensing by the naïve macrophage results in stimulating the conversion of inactive vitamin D (25D3) to active vitamin D (1,25D3) and is released into the supernatant and able to diffuse into the heavily infected macrophages. From there, 1,25D3 can bind to the VDR in the nucleus and affect the transcription of genes affected by the vitamin D response element (VDRE). This activates Beclin 1 and upregulates autophagy in the infected macrophage in addition to generation of anti-microbial peptide cathelicidin. The *M.tb*-containing autophagosome then fuses with lysosomes resulting in exposing *M.tb* bacilli to lower pH and hydrolytic enzymes. Both contributing to the ultimate demise of intracellular *M.tb* in the heavily infected macrophage. However, In addition to these mechanisms, NOS2 expression is induced and NO contributes to the antimicrobial effect. This model takes into account the possibility of vitamin D signaling affecting the induction of autophagy in *M.tb* infected macrophages and the possible contribution of IL-1β.

Overall, this data has broad implications for understanding TB at the early stages of granuloma formation and at the point of reactivation. It is clear from studies in our lab that there is unrestricted bacillary growth resulting in heavily infected cells in the mouse lung which would increase spreading of *M.tb* to numerous macrophages locally and increase chemokine and cytokine production

leading to granuloma formation and as the mechanism a human host uses to confine *M.tb* infection in the lung [108]. In this environment, there are numerous interactions of infected macrophages with immune cells that are recruited to the infection site. Examining the co-culture conditions *in vitro* and figuring out the mechanism of cross-talk between naïve and infected macrophages could shed light on better ways to treat TB in the future.

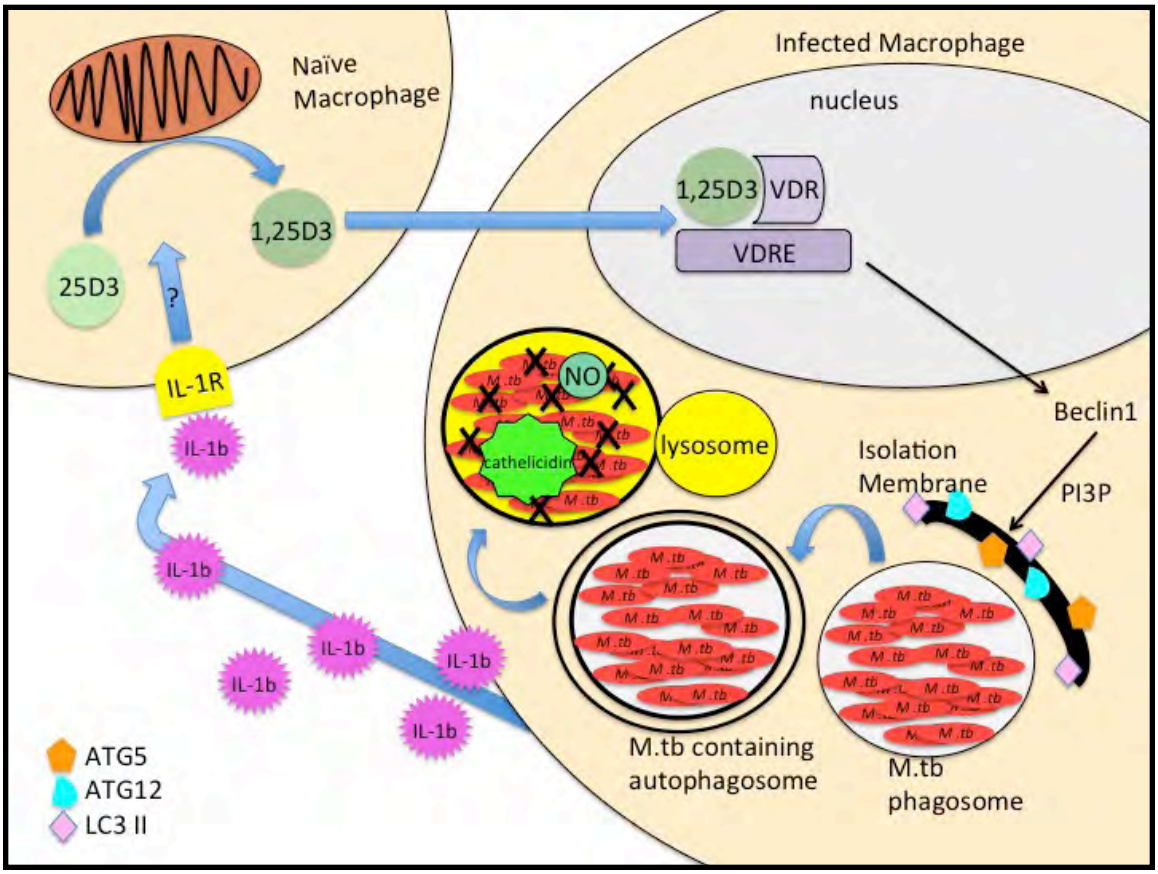

Figure 4.1: Hypothetical model of the high MOI co-culture antimycobactericidal effect. It is possible that IL-1β sensing by the naïve macrophage results in stimulating the conversion of inactive vitamin D (25D3) to active vitamin D (1,25D3) and is released into the supernatant and able to diffuse into the heavily infected macrophages. From there, 1,25D3 can bind to the VDR in the nucleus and affect the transcription of genes affected by the vitamin D response element (VDRE). This activates Beclin 1 and upregulates autophagy in the infected macrophage in addition to generation of anti-microbial peptide cathelicidin. The *M.tb*-containing autophagosome then fuses with lysosomes resulting in exposing *M.tb* bacilli to lower pH and hydrolytic enzymes.

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