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Efferocytosis is an innate antibacterial mechanism of *Mycobacterium tuberculosis* control

Abstract

One third of the world's population is infected with *Mycobacterium tuberculosis,* causing two million deaths annually. The bacteria avoid immune clearance by persisting within macrophages by subverting normal phagosome maturation and acidification. In order to spread, the bacteria induce necrotic death of its host macrophage, broadcasting the infection into neighboring cells. However, it has long been appreciated that the apoptotic, rather than necrotic death of an infected macrophage results in bacterial growth suppression, improved adaptive immune response and survival. The mechanism for apoptosis-mediated bacterial suppression has hitherto remained unknown.

In this dissertation we report that apoptosis itself is not intrinsically bactericidal. We find that following apoptosis, the *M. tuberculosis*-infected macrophage is engulfed by bystander macrophages through the process of efferocytosis. Efferocytosis, or apoptotic cell clearance, is a critical function of macrophages; however, little is known regarding efferocytosis of infected apoptotic cells. We find that *M. tuberculosis*-infected macrophages die by apoptosis more commonly than found previously. By confocal microscopy we observed that apoptotic macrophages are rapidly engulfed by uninfected macrophages. Efferocytosis of *M. tuberculosis*-infected macrophages occurs *in vitro* with all macrophage types tested and *in vivo*- specifically in the lung, indicating that efferocytosis could play an important role during infection. We developed an uninfected macrophage co-culture system in which we observe efferocytosis and

define conditions in which it occurs. Using this co-culture system we observe a suppression of bacterial growth. By blocking efferocytosis, we have found that the engulfment of infected cells is required for *M. tuberculosis* control in the macrophage co-culture system, demonstrating that efferocytosis is a novel antibacterial mechanism. We then demonstrated using transmission electron microscopy that the *M. tuberculosis*-containing efferocytic phagosome is structurally distinct from the traditional *M. tuberculosis* phagosome. Bacteria from within the efferocytic phagosome are unable to halt its maturation, and as such are delivered to lysosomes. Furthermore, we find that following efferocytosis, *M. tuberculosis* are killed. While efferocytosis is recognized as a constitutive housekeeping function of macrophages, our work indicates that is should also be viewed as an antimicrobial effector mechanism.

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List of Abbreviations

+:	having the indicated characteristic (read as ‘positive’)
-/- :	genetically deficient (read as ‘knock-out’)
ANOVA:	analysis of variance
BAL:	bronchoalveolar lavage
BCG:	bacillus Calmette-Guérin
cAMP:	cyclic adenosine mono-phosphate
CD:	cluster of differentiation
CFU:	colony forming unit(s)
CTL:	cytotoxic T lymphocytes (cytotoxic T cells)
DC:	dendritic cell(s)
EEA1:	early endosome antigen 1 protein
ER:	endoplasmic reticulum
FACS:	fluorescence activated cell sorting (flow cytometry)
GFP:	green fluorescent protein
HIV:	human immunodeficiency virus
IFN:	interferon
IL:	interleukin
IP:	intraperitoneal
IT:	intratracheal
iNOS:	inducible nitric oxide synthase
LAMP1:	Lysosomal-associated membrane protein 1
LC3:	Microtubule-associated proteins 1A/1B light chain 3
LPS:	lipopolysaccharide
LXA_4:	leukotriene A4
MHC:	major histocompatibility complex
MOI:	multiplicity of infection
MOMP:	mitochondrial outer membrane permeability
mTOR:	mammalian target of rapamycin
Mtb:	*Mycobacterium tuberculosis*
Mφ:	macrophage
NO:	nitric oxide
PGE_2:	prostaglandin E2
PS:	phosphatidylserine
RNI:	reactive nitrogen intermediates
ROS:	reactive oxygen species
SEM:	standard error of the mean
siRNA:	small interfering RNA
TB:	tuberculosis
TEM:	transmission electron microscopy
Th1:	T helper type one
TLR:	Toll-like receptor
TNF:	tumor necrosis factor
WHO:	World Health Organization
WT:	wildtype

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"Efferocytosis is an innate antibacterial mechanism"

Constance J. Martin, Matthew G. Booty, Tracy R. Rosebrock, Cláudio Nunes-Alves, Danielle M. Desjardins, Iris Keren, Sarah M. Fortune, Heinz G. Remold and Samuel M. Behar

The project was conceived of by Samuel M. Behar and Heinz G. Remold. Experiments were designed by Samuel M. Behar and Constance J. Martin. Experiments were performed and analyzed in large part by Constance J. Martin. Matthew G. Booty aided with apoptosis assays. Tracy R. Rosebrock confirmed the live/dead reporter Mtb. Cláudio Nunes-Alves and Danielle M. Desjardins provided technical support for in vivo work. Iris Keren constructed the live/dead reporter Mtb. Sarah M. Fortune provided reagents and advice.

Chapter 1: Introduction

Tuberculosis: A Global Problem

Despite being an ancient disease, tuberculosis (TB) remains a disease of massive public health importance. It is estimated that a third of the world's population asymptomatically harbors *Mycobacterium tuberculosis* (Mtb), the causative agent, while 12-14 million fall ill each year[1]. An estimated 2 million people die from tuberculosis annually, ranking TB as one of the top infectious disease scourges. TB largely strikes women and working age adults, making the disease a tremendous burden to developing economies and severally impacting children and families[2]. As such, there are an estimated 10 million children in the world orphaned by tuberculosis. The majority of cases and deaths attributed to tuberculosis are found in Southeast Asia and Africa, where the high incidence of HIV has dramatically affected the population and is a major societal and public health problem. And while for 2008, the WHO reported that the global incidence of new TB cases has dropped, TB is still on the rise in many parts of the world[1, 2]. From a clinical and public health standpoint, tuberculosis is a difficult disease to treat, as patients are required to take antibiotics, some with harmful side effects, for 6-9 months or more[3]. Adherence failure has been attributed to the rise in drug resistance, with some clinical isolates showing resistance to multiple antibiotics commonly used to treat TB. Many first line drugs are obsolete in parts of the world, only compounding the difficulty of control of tuberculosis[4].

Tuberculosis, the disease

Tuberculosis most often manifests as a pulmonary infection in adults. Children are more prone to serious systemic infections, including miliary tuberculosis and tuberculosis meningitis. Other common manifestations include infections of the joints, pleural cavity and lymph nodes. Symptoms of tuberculosis include fever and night sweats, loss of appetite and weight, and cough. In fact it is the wasting nature of the disease that gave it its original name- consumption. Patients

suffer from fatigue and reduced lung capacity as normal lung architecture succumbs to inflammation and granuloma formation. It wasn't until 1834 when Johann Lukas Schönlein associated the granulomatous mass that is the hallmark of TB or 'tubercule' in patients suffering from consumption with disease[5]. *Mycobacterium tuberculosis* as the bacterium responsible for disease was identified by Robert Koch in 1882. The development of Koch's Postulates, the foundation of infectious disease microbiology, which outlines how to define disease and its cause by an infectious agent was first demonstrated with Mtb.

Tuberculosis is most often transmitted when an infected individual coughs, aerosolizing the bacteria. It is estimated that fewer than ten bacteria are necessary to establish infection, far less than most other pathogens. The smallest droplets containing bacteria settle into the distal airway, where alveolar macrophages, the sentinels of the lung responsible for clearing inhaled particles and disposing of encountered microorganisms phagocytose Mtb. It is not known how often the initial interaction between host macrophage and Mtb results in productive infection, however Mtb is exquistaly adapted to overtake alveolar macrophages. The infected macrophage sounds the alarm of its infection by releasing pro-inflammatory cytokines, such as IL-12. Most often the infected alveolar macrophage undergoes necrosis, an inflammatory form of cell death that further excites immune cells to the site of infection. Neutrophils are some of the first respondants, as are monocyte-derived macrophages and dendritic cells. Dendritic cells (DCs), after having taken up Mtb or material containing Mtb-derived antigens, traffic to the lung-draining lymph nodes to prime CD4+ and CD8+ T cells. However, this process takes days, during which time the bacteria grow nearly unchecked[6]. When T cells finally arrive, they secrete a variety of Th1-type activating cytokines in an effort to activate antibacterial effector functions within macrophages, induce death and limit the spread of infection. During the immune response to tuberculosis,

the body attempts to 'wall off' the infection behind a granuloma- a mass of fibrous tissue and immune and non-immune cells. The classic structure of the granuloma in humans consists of a ring of B and T cells surrounding an core of myeloid-derived cells and bacteria[7]. The granuloma is a sign of a proper immune response to the bacteria, however it is not without consequences. The granuloma disrupts normal lung function and can spread into and erode into blood vessels and the airway. When the patient coughs, Mtb can then access the airway and be expelled. The granuloma is a structure advantageous to both the bacterium and the host- crucial for both containment and continuation of disease[8].

Most people who encounter the bacteria never display clinical symptoms and are called latently infected. In these individuals an immune response mounted against the bacteria was sufficient to quell the infection. Mtb are still present and replicating within the lungs of these people, however they show no overt clinical symptoms of disease[9, 10]. Bacterial growth is likely countered by bacterial killing in these instances. These people have a 5-10% lifetime risk of Mtb recrudescence, in which bacterial replication is no longer suppressed and granulomas grow and spread. HIV co-infection adds a 10% yearly risk of TB recrudescence[10]. Latent TB infection, while not a drain on the healthcare infrastructure represents a tremendous pool of individuals capable of falling ill. Latently infected individuals can be distinguished from Mtb-naïve individuals based on a positive tuberculin skin test reaction or one of the newly developed IFNγ release assays[11].

The most widely used vaccine in the world is the tuberculosis vaccine, BCG. BCG, or Bacillus Calmette-Guérin was developed by two French doctors at the Pasteur Institute from 1906-1919 by subculturing *Mycobacterium bovis* until it had lost much of its virulence. BCG is thus an attenuated strain of *M. bovis*, harboring many, but not all of Mtb's antigens. Most

notably, BCG lacks the region of difference 1 (RD1) that encompasses ESX-1, a secretion system required for the export of several crucial virulence factors and immunodominant T cell antigens[12-14]. BCG's efficacy is largely debated as studies have shown anywhere from 0-80% protection from pulmonary tuberculosis for vaccinated adults[15]. Boosting strategies have also failed to confer additional immunity[16]. BCG administration to newborns is still recommended, except in HIV^+ neonates, as the vaccine protects young children from disseminated tuberculosis disease[17]. However, as BCG cannot protect adults, especially in high endemic regions, the spread of disease continues. Thus development of novel prophylactic strategies and treatments are paramount. To most effectively counter this pathogen a more complete understanding of the immune response to tuberculosis is required.

Immunity to Tuberculosis

Mycobacterium tuberculosis is one of many bacteria from the genus of mycobacteria capable of infecting humans. Other members of the family include *M. leprae* (leprosy) and *M. ulcerans* (Buruli ulcer) as well as several environmental mycobacteria, some of which are capable of infecting humans, most especially immune-compromised individuals. *M. bovis* especially can infect humans and represents a major pathogen amongst livestock and wildlife. There are several other mycobacteria that infect animals, however tuberculosis exclusively infects humans and is only capable of human-to-human spread. A common characteristic of all mycobacteria, and how they received their name, is their complex waxy outer membrane comprised of mycolic acid. It is this lipidic layer that protects the bacterium from drugs and components of the immune system.

Many animal models have been developed to probe the interactions between Mtb and the immune system. Monkey, guinea pig and zebrafish models have all provided substantial contributions to the field of tuberculosis immunology, but none more so than the mouse. The availability of inbred strains, an array of genetic tools and not least of all, their small size and relatively low cost of maintenance have all contributed to the mouse as the primary model organism to the TB researcher. Knockout mice studies have highlighted many of the important components of the innate and adaptive immune response to TB. $CD4^+$ T cells are most important, but so too are $CD8^+$ T cells. Th1 cytokines such as IL12 and IFNγ are required, as well as downstream effectors, like iNOS[18-21]. MyD88 is uniquely required not just for its role in TLR recognition and signaling, but downstream of the IL1 receptor as well[22, 23]. Genetic differences between inbred mouse strains, with some displaying greater susceptibility or resistance have highlighted additional genes contributing to survival[24-26]. While mouse studies have proven instrumental to the study of tuberculosis, there are significant caveats. Mice do not develop granulomas as human patients do. As tuberculosis is primarily a disease of rampant immunopathology, this is a significant difference. The mouse granuloma generally lacks the caseating necrotic or hypoxic center characteristic of many human granulomas[27]. This is due to genetic differences between mouse and man, loci have been found in immunocometent mice that once deleted result in granulomas bearing greater resemblance to human caseous granulomas[24]. Vitamin D in humans contributes the production of antibacterial peptides, which are capable of killing Mtb[28]. In fact it is speculated that exposure and subsequent Vitamin D production that lead to the successful outcomes of many sanatorium patients in the 1800s. In fact, vitamin D deficiency is linked with disease recrudescence[29]. Mice also lack granulysin, a protein found in cytotoxic granules, which human $CD8^+$ T cells use to directly kill Mtb[30]. There are other

numerous differences, however many findings using mouse studies, both in vivo and in vitro have recapitulated human data.

The study of humans with primary immunodeficiencies, inborn errors of the immune system, with Mendelian susceptibility to mycobacterial diseases (MSMD), including environmental mycobacterial infections and extreme sensitivity to BCG, further confirmed the mouse data. Patients lacking functional components of the IL-12/IFNγ signaling axis struggle with recurrent mycobacterial diseases their entire lives[31]. Other phagocyte-specific genes have been found to play a role in the immune response to Mtb. Mutations in the macrophage/dendritic cell NADPH oxidase and some cathepsins both predispose patients to tuberculosis, highlighting the importance of phagocyte-mediated bacterial killing in control of disease[32, 33]. The anti-rheumatoid arthritis treatments targeting TNF significantly increase patient's risk for TB recrudescence, highlighting the importance of this molecule in defense[34, 35]. As tools develop further, more and more Mtb research will be done using human tissues, which ultimately is required to understand the nuances of the interactions between the bacterium and the host

Mycobacterium tuberculosis infection of the macrophage

Upon inhalation, Mtb most likely first encounters the alveolar macrophage, as alveolar macrophages are required to establish productive Mtb infection[36]. Macrophages use many receptors to mediate recognition and phagocytosis of Mtb[37]. While alveolar macrophages are potent killers, capable of phagocytosing and destroying most pathogens and particles encountered, Mtb is different, and persists within an arrested phagosome. Lysosomes typically rapidly fuse with the nascent phagosome, delivering their caustic cargo responsible for digesting the internalized object. A series of GTPases facilitate the recruitment, tethering and fusion of

lysosomes to the phagosome. Rab5 decorates the early phagosome and is present on Mtb-containing phagosomes[38]. Typically Rab5 gives way to Rab7 in what is called 'Rab conversion', which facilitates fusion to lysosomes via the Rab7 effector RILP[39, 40]. Most studies have found the Mtb excludes Rab7 from its phagosome. This is thought to happen by retaining Rab22a, which required for the Rab5 to Rab7 conversion[41]. Many Rab proteins clearly facilitate phagosomal maturation of non-pathogenic bacteria and particles and many of these are disrupted following Mtb infection, however the exact nature of these interactions are not entirely understood[42]. Over expression of Rab10 rescues the phagolysosome maturation arrest, demonstrating that multiple host GTPases may be targeted by the bacterium to ensure it remains safe within the phagosome[43]. The end result of Rab disruption is the incomplete fusion of the Mtb phagosome with lysosomes and other vesicles responsible for degrading phagosomal contents. One Rab5 effector, EEA1 is required for endosome trafficking and the delivery of proteolytically inactive cathepsins, however Mtb interferes with EEA1 recruitment. The extent to which the Mtb phagosome takes on lysosomal characteristics is also under debate. Some find nearly complete exclusion of lysosomal proteins, such as LAMP1 from the Mtb phagosome, while others find some co-localization of Mtb with lysosomal markers[44].

While there is debate over the exact composition of the Mtb phagosome, there is consensus regarding the block of acidification of the Mtb phagosome. Mtb phagosomes specifically lack vATPase, capable of turning the phagosome into an acidic environment and activating the many lipases and proteases delivered by endosomes and lysosomes[45]. As such, the Mtb phagosome remains nearly neutral, estimated at a pH of 6.4 which is hospitable for the bacteria[46]. While Mtb halts phagolysosome fusion, recycling is not completely inhibited. Vesicles containing transferrin and transferrin receptor readily dock with the Mtb-phagosome,

delivering nutrients such as iron to sustain bacterial growth[47]. Thus Mtb can proliferate within an immature phagosome[48]. Activating cytokines, such as IFNγ can overcome this maturation block and is a major mechanism of Mtb killing both in vitro and in vivo[49]. IFNγ binding to its receptor upregulates an estimated 1000 genes of which only a handful are known to participate in immunity to tuberculosis. Yet, IFNγ represents one of the most crucial tools the immune system possesses to fight Mtb infection. Most notably, IFNγ upregulates the inducible nitric oxide synthase (iNOS), which is responsible for the production of reactive oxygen species capable of damaging the bacterium. Mtb modulates the host macrophages' response to IFNγ, thus further protecting itself from the macrophage's bactericidal activities, including preventing iNOS recruitment to the Mtb-containing phagosome[50, 51].

Mtb infection has other effects on the macrophage. Major Histocompatibility Complex (MHC) class II expression on the surface of Mtb infected macrophages is markedly decreased[52]. As MHC complexed to antigen is required for T cells to recognize Mtb-infected macrophages this is an important strategy Mtb employs to avoid detection. MHC class II antigen processing and surface presentation is upregulated in response to IFNγ. As Mtb limits the macrophages' responsiveness to IFNγ, this is yet another mechanism the bacteria use to evade immune surveillance[53]. Additionally, bacterial lipoproteins bind to Toll-Like Receptors (TLRs) and cause prolonged downregulation of MHC expression[54]. Ultimately this has the effect of hiding the bacteria from the adaptive immune response.

Death of the Mycobacterium tuberculosis-infected macrophage

At some point during intracellular infection, the Mtb-infected macrophage dies. Infection with virulent Mtb most often results in necrosis, while avirulent Mtb mutants and strains cause

more apoptosis[55, 56]. However, it is important to note that the cell death pathways are highly nuanced, and both apoptosis and necrosis are observed following virulent Mtb infection. As a common response to intracellular infection is cell death, it is not clear whether the death program is initiated by the macrophage, or by the bacteria, but it is clear that Mtb influences and alters macrophage cell death to its advantage. Necrosis releases free bacteria, which can infect surrounding macrophages and is a mechanism to spread infection and evade immunity[57]. Apoptosis however confines the bacterium within an apoptotic bleb or cell and is correlated with control of the bacterium and establishment of a robust protective adaptive immune response[58, 59]. Other forms of cell death, including caspase-independent apoptosis and pyroptosis, have been reported following Mtb infection, however is it unclear how great a role they play in vivo, in regards to bacterial control, adaptive immune priming and pathology[60-63]. And while the signaling cascades involved in these forms of cell death are very different from apoptosis and necrosis, morphologically they closely resemble apoptosis or necrosis, and thus might have similar impacts on bacterial growth and immunity.

Apoptosis and necrosis were first described based on morphological changes in cells observed in tissue culture by light microscopy. Apoptosis, or programmed cell death, is a highly coordinated energy-intensive process that packages cellular components into blebs, condenses chromatin and protects the plasma membrane from disintegration[64]. While there are many triggers for apoptosis, classically they all filter into one of two caspase cascades: the extrinsic caspase-8 dominant cascade and the intrinsic caspase-9 dominant cascade. Cell extrinsic stimuli, require the ligation and dimerization of surface receptors comprised of death domains, such as TNF and Fas to stimulate apoptosis via the caspase 8-dependent extrinsic pathway[65]. As TNF and Fas-mediated phagocyte killing occur during Mtb infection, the extrinsic apoptosis pathway

likely occurs in vivo. Stimuli originating from the cell itself, such as microbial danger signals, DNA damage and ER stress trigger caspase 9-dependent intrinsic pathway, relying on mitochondrial outer membrane permeability and release of cytochrome c[66]. Caspase 8 activation in some cases will also lead to BID cleavage and cytochrome c release[67]. Both pathways feed into caspase 3 activation, which helps coordinate and activate intracellular proteases responsible for dismantling the cytoskeleton and partitioning organelles into blebs[68]. Fundamentally, apoptosis maintains the integrity of the plasma membrane[69]. Necrosis however, does not. Previously it was thought that necrosis was an uncoordinated event in which a cell explodes or disintegrates, losing plasma membrane structure and leakage of intracellular contents. Now, some forms and situations of necrosis appear to be as regulated as apoptosis, and may have important immunological function[70]. However it is not known whether this is the case following Mtb infection.

It is not clear what bacterial effector molecules, if any, interact with the host macrophage to induce cell death. Some reports have implicated ESAT-6, a member of the virulence apparatus, ESX-1, in triggering cell death[71]. Alternatively, PE_PGRS33 was found recruited to mitochondria as a prerequisite for cell death[72]. However, these reports remain unsubstantiated. There are bacterial proteins that influence the type of death experienced by the macrophage, but whether or not they specifically start the death cascade or merely influence it while it is started is unknown. Bacterial superoxide dismutase, SodA, inhibits apoptosis[73]. Mutants lacking NuoG, a NADPH dehydrogenase also induce less necrosis and more apoptosis in infected macrophages[56]. Additionally, it is unknown whether or not bacteria are within the phagosome or have escaped into the cytosol at the onset of apoptosis or necrosis. As a loss of organelle membrane integrity is common at the onset of necrosis, determining the subcellular localization of Mtb during or

right before cell death is difficult. An important consideration during apoptosis is the maintenance of the plasma membrane. During apoptosis flipases are activated to facilitate the movement of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexins are simultaneously translocated from the cytosol to the exofacial leaflet of the plasma membrane, although the mechanism is unknown[74]. Annexin I binds PS and then transglutaminase crosslinks the N-terminal tails of annexin I, forming a dense meshwork[75]. This is referred to as the apoptotic envelope and serves to stabilize the dying cell and may facilitate bleb formation[76]. Virulent Mtb infection perturbs apoptotic envelope formation by inhibiting plasminogen activator inhibitor type 2 (PAI2), an enzyme that protects annexin I from proteolysis, dismantling the apoptotic envelope[77]. This promotes necrosis. Another way to stabilize the plasma membrane during apoptosis is via plasma membrane repair. The continual recruitment of lysosome and Golgi-derived membrane to the plasma membrane helps heal and maintain the dying cell. Virulent Mtb infection also interferes with this process. Mtb infection alters the production of many immunologically potent eicosanoids, chief among them lipoxin A4 and prostaglandin E2[78]. PGE_2 signaling via its EP4 receptor aids in synaptotagmin-7 mediated lysosomal plasma membrane repair[79]. LXA_4 antagonizes this. As PGE_2 is inhibited following virulent Mtb infection, but not avirulent, this is yet another mechanism that pushes the dying macrophage towards a necrotic rather than apoptotic death.

Mitochondrial stability is a major regulator of cell death. Mitochondrial outer membrane permeability (MOMP) is a hallmark of intrinsic apoptosis as BID cleavage activates the BAX/BAK pore responsible for the release of cytochrome c and other pro-apoptotic molecules[80]. If the inner mitochondrial membrane is perturbed simultaneously with MOMP the mitochondria loses proton potential, resulting in necrosis[81]. Virulent Mtb infection causes necrosis through

this mechanism, although it is unclear how the bacteria might target the mitochondria[82]. PGE_2 signaling through the EP2 receptor protects the mitochondrial membrane from damage and necrosis[78].

Just as necrosis has been found to benefit the bacteria and further the infection, apoptosis is beneficial for the host and restricts bacterial replication. There are many examples of apoptosis correlating with decreased bacterial load. First, less virulent forms of Mtb and closely related mycobacteria induce apoptosis and grow poorly in macrophage culture[82, 83]. Some of the bacterial mutants discussed above, SodA, NuoG and SecA2 all induce apoptosis and consequently do not proliferate in vitro or in vivo[58, 73, 84]. Even using virulent bacteria, apoptotic death was associated with reduced bacterial burden[85]. Treatment of BCG-infected macrophages with FasL induced apoptosis as well as a decline in bacterial burden[86]. Chemically inducing apoptosis in Mtb-infected human monocyte-derived macrophages similarly lead to decreased bacterial burden[87]. Apoptosis of *S. pneumonia*-infected cells is also associated with reduced bacterial viability, suggesting apoptosis as a general mechanism of antibacterial host defense[88, 89].

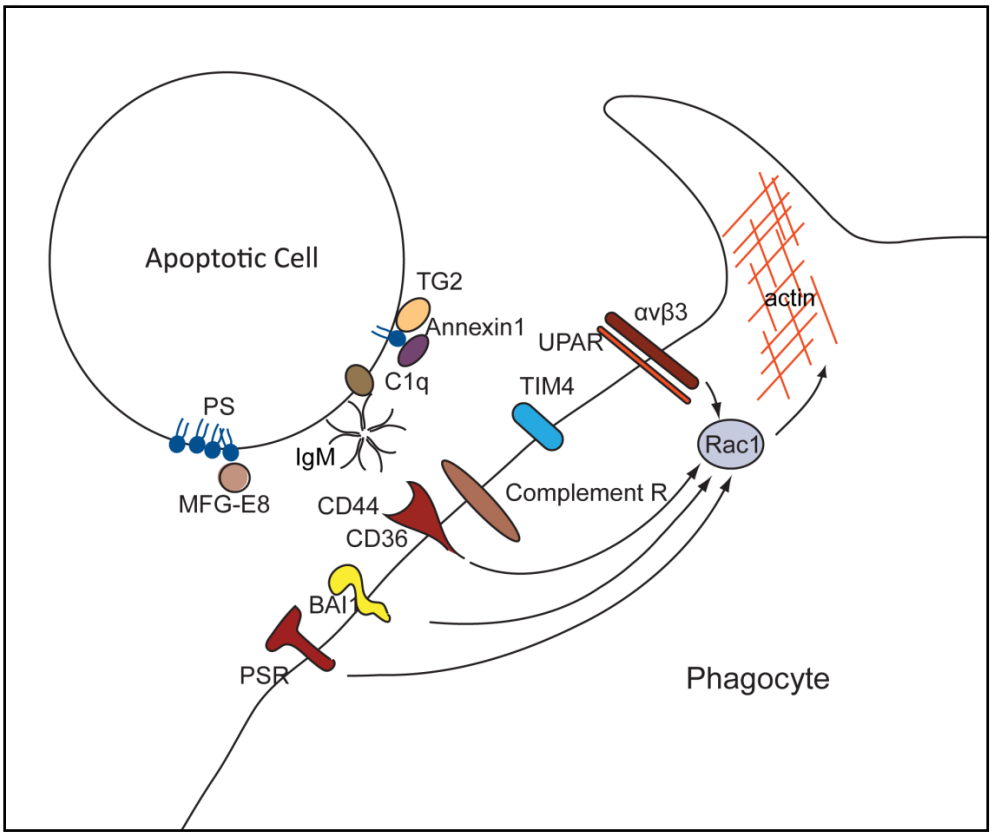

Figure 1-1. Receptors and ligands for apoptotic cell recognition and engulfment.

Many redundant receptors are used by phagocytes to mediated efferocytosis. Some receptors are cell and tissue-specific. Phosphatidylserine (PS) displayed on the surface of the dying cell is the major ligand that directly or indirectly binds to several efferocytosis receptors. This image depicts most of the known receptors and ligands.

Efferocytosis: Apoptotic Cell Clearance

There is constant cell turnover in the body. During development structures form, only to be destroyed, such as the webbing between digits[90]. The end-stage of the immune response to pathogens involves the removal of many of the recruited cells. Clearance of germ cells in the male gonad is required for spermatogenesis[91]. An estimated 10^9 cells apoptose in the human body daily, and they all must be cleared to prevent spillage of their phlogistic cargo[92]. This crucial cell clearance function is the duty of phagocytic cells- mainly macrophages. The engulfment and clearance of apoptotic cells is called efferocytosis, from the Greek 'to take to the grave'. During apoptosis dying cells release 'find me' signals, such as ATP, UTP, and peptides derived from Annexin proteins to attract nearby phagocytes[93-95]. Dying cells also display on their surface a variety of 'eat me' signals, such as PS for which phagocytes have corresponding receptors to mediate the recognition, tethering and engulfment of the apoptotic cell[96]. Most macrophages employ a variety of redundant receptors to facilitate efferocytosis, as inhibition of any one does not dramatically affect the rate or number of internalized apoptotic cells. Many efferocytic receptors have been identified (Figure 1-1). Some, such as TIM4, directly bind PS on the surface of the dying cell[97]. Still others, such as the $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins bind MFG-E8, a soluble bridge protein linking the dying cell to the phagocyte[98]. Both the complement system and Fc receptors can participate in apoptotic cell engulfment. IgM pentamers have been reported to bind apoptotic cells by recognizing unknown epitopes. Furthermore, C1q can bind this structure to mediate complement recognition of the apoptotic cell[99]. However, data so far suggests that both Fc receptors and the complement system appear to play a minimal role in efferocytosis both in vitro and in vivo[100].

While in many ways similar to phagocytosis, efferocytosis employs distinct signaling cascades. For one, efferocytosis most closely mimics macropinocytosis, as the large apoptotic cell is engulfed along with extracellular fluid[101, 102]. This results in a characteristic spacious phagosome[103]. Phagocytosis, however involves tight contact between the particle and plasma membrane during engulfment and the resulting phagosome snuggly fits around the internalized target. The difference in the 'efferocytic gulp' may come from the GTPases that orchestrate efferocytosis. Downstream of several efferocytosis receptors and Fc-receptor, Rac1 and Cdc42 orchestrate the actin cytoskeleton to engulf the apoptotic cell[104, 105]. However, statins which enhance efferocytosis have been found to suppress Fc receptor-mediated phagocytosis, suggesting that there is alternative regulation of these two engulfment pathways beyond the involvement of Rac1 and Cdc42[106]. Perhaps this is due to differential activation of RhoA, a GTPase known to antagonize Rac1-mediated efferocytosis[105, 107]. After internalization, the efferocytic phagosome containing the dead cell matures much like phagosomes, although there is increasing evidence for efferocytosis-specific proteins facilitating Rab conversion on the efferocytic phagosome[108]. Ultimately, the internalized apoptotic cell is destroyed. Another striking difference between phagocytosis and efferocytosis is the effect that dying cells have over macrophages. The ingestion of dying cells promotes an anti-inflammatory program within the macrophage[109-111]. As macrophages engulf apoptotic cells PPARδ is upregulated and acts as a transcriptional sensor for apoptotic cells[112]. PPARδ then orchestrates the dampening of pro-inflammatory cytokine generation as pro-resolving cytokine production takes over[92, 113]. Specifically, TNF, IL-12 and IL-1β production are down-regulated as TGFβ and PGE_2 are produced[114]. These cytokines work in an autocrine manner to inhibit or enforce efferocytosis, respectively. As such, inflammation, specifically driven by TNF inhibits efferocytosis[115]. This

has disastrous consequences during many disease states and contributes to the continuance of inflammation[116]. An apoptotic cell that is not cleared will fall apart in a process known as secondary necrosis[117]. Efferocytosis might be the primary role of the macrophage, especially tissue macrophages who roam the body far from openings to the outside.

Necrotic cells are also engulfed by phagocytes. It is unclear whether the same receptors are involved in the recognition and engulfment of these cells[118]. Early necrotic cells, or cells dying by caspase-independent means, may be engulfed via similar methods as apoptotic cells[119]. Some studies suggest that the uptake of necrotic cells results in an anti-inflammatory program similar to the efferocytosis of apoptotic cells[119, 120]. Others have found that necrotic cell cleanup is not immunologically silent and may form the basis of autoimmunity[121-123]. Furthermore, pyroptosis, caspase –dependent cell death, in many ways is morphologically similar to necrosis and is described as pro-inflammatory cell death as IL1 is released during or immediately preceding death[124]. How efferocytosis functions in response to these types of dying cells is not completely understood Further study into the consequences of necrotic, pyroptotic and atypical cell death clearance is worthy of study.

Efferocytosis initiates immunity

Another aspect of efferocytosis involves the acquisition of antigen. Dendritic cells (DCs) are a large population of highly phagocytic cells, constantly engulfing and sampling the periphery in search of microbes. One way that DC can acquire antigens from intracellular bacteria or viruses to prime protective T cells is to uptake apoptotic blebs from infected cells[125]. Similarly, Yrlid et al. found that virulent Salmonella, an intracellular bacterial pathogen, induces apoptosis of infected macrophages and that DC are able to cross-present the bacterial antigens

onto MHC class I molecules[126]. Cross presentation of antigens derived from efferocytosed apoptotic blebs by DCs can prime and alert protective $CD8^+$ T cells. Apoptosis of Mtb-infected macrophages and the subsequent uptake of these blebs confer protective immunity. Winau et al. used vesicles purified from BCG infected macrophages to immunize mice[127]. The purified apoptotic blebs contained bacterial antigens, but no bacteria. $CD8^+$ T cell priming was observed and required an intact class I MHC pathway. This strategy generated immunity that protected mice from challenge with virulent Mtb. Transfer of Mtb-infected $5LO^{-/-}$ pro-apoptotic macrophages into mice also generated a robust $CD8^+$ as well as $CD4^+$ T cell response much more rapidly than the transfer of wildtype infected macrophages[59]. Thus, the efferocytosis of apoptotic cells containing antigens by DCs aids in the establishment of protective immunity.

Efferocytosis and infection

Cell death is a feature of many intracellular infections. Bacteria and viruses modulate the cell death pathways of their host cells to their advantage. Apoptosis is a general mechanism of host resistance that many pathogens try to avoid[128]. For instance, *Shigella* induces necrosis to aid its escape from the host cytosol[129]. *Streptococcus pneumonia* infected macrophages that die by apoptosis limit bacterial replication[130]. The induction of apoptosis by cytotoxic $CD8^+$ T cells contributes to immunity to influenza. In fact, killing a flu-infected cell eliminates the virus, suggesting that apoptosis itself is virucidal[131]. However, it was later found that viral replication was only inhibited following apoptosis when phagocytic cells were present to engulf the dying infected cells[132, 133]. This was the first evidence that efferocytosis of an infected cell can lead to pathogen destruction. However, there also exists evidence that efferocytosis of an infected cell contributes to pathogen survival and the establishment of infection.

Leishmania enter their host via the bite of an infected sandfly. Neutrophils rapidly respond to the site of injury and phagocytose the parasite. The infected neutrophils subsequently die and are efferocytosed by responding macrophages, allowing the parasite to establish a productive infection[134, 135]. This is known as the 'Trojan Horse' model of Leishmania infection. However intact, infected neutrophils were not found within macrophages in vivo[134]. Thus, it is not certain whether the dying neutrophils are apoptotic, or release the parasite as they die to be subsequently engulfed by macrophages. Chlamydia similarly infects neutrophils with an aim to ultimately enter macrophages through efferocytosis[136]. Phagocytes within the zebrafish embryo were observed taking up material from *M. marinum* dying cells and this contributed to spreading the infection, however it was unclear whether or not dying cells were necrotic or apoptotic[137]. These few instances are the only reports of the efferocytosis of infected cells. In each of these reports efferocytosis was observed and it seemed to correlate with pathogen survival, but efferocytosis was no systematically studied. Some investigators have studied the impact of infection on efferocytosis. Infection with *Fransicella novicidia* impairs macrophage efferocytosis and skews the macrophages towards an 'alternatively activated' M2 phenotype[138]. Conversely, efferocytosis of apoptotic cells was found to inhibit alveolar macrophages' ability to kill *Streptococcus pneumonia*[139]. A DC engulfing an apoptotic cell and receiving TLR stimulus simultaneously initiates a Th17 response, most suitable for clearance of extracellular bacteria[140, 141]. Given the host-protective role of apoptosis during Mtb infection and the lack of data surrounding apoptosis as directly bactericidal, we were interested in investigating the role of efferocytosis during Mtb infection.

Scope of the Thesis

In this thesis I present efferocytosis, the process of apoptotic cell engulfment as a novel innate immune antibacterial mechanism. First, in Chapter 2 I show that there is a significant amount of apoptosis following virulent Mtb infection of macrophages, despite evidence that Mtb favors necrosis (Chapter 2). Following apoptosis, we find Mtb-infected apoptotic cells are taken up by uninfected macrophages by efferocytosis, using both newly developed in vitro and in vivo models (Chapter 2). In Chapter 3 I show that conditions that allow for efferocytosis are associated with decreased bacterial growth in vitro. This growth suppression is in fact mediated by efferocytosis. Finally, Chapter 4 demonstrates that the efferocytic phagosome containing Mtb is a unique structure capable of fusing with lysosomes. This process of efferocytic phagosome maturation is bactericidal. Thus in this work we present efferocytosis as a novel bactrericidal mechanism, capable of killing Mtb.

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Chapter 2: *Mycobacterium tuberculosis* infected apoptotic macrophages are engulfed by uninfected macrophages

Parts of this chapter appears as the submitted manuscript:

"Efferocytosis is an innate antibacterial mechanism"

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Abstract

A variety of intracellular pathogens modulate host cell death pathways as part of their virulence strategies. *Mycobacterium tuberculosis* infection typically ends in the necrotic death of the macrophage it infects to aid its dissemination and evasion of immunity. However the spectrum of cell death is highly nuanced, with infection leading to both apoptosis and necrosis. In vivo, dying cells are rarely observed as they are quickly engulfed through the process of efferocytosis. We report here that *Mycobacterium tuberculosis*-infected macrophages die via caspase-dependent apoptosis and are subsequently engulfed by bystander uninfected macrophages. This process of infected macrophage efferocytosis is observed using a variety of macrophage types both in vitro and in vivo. *Mycobacterium tuberculosis* infection did not specifically inhibit efferocytosis and as such efferocytosis may play an important role in vivo during active disease.

Introduction

Mycobacterium tuberculosis (Mtb) evades host immunity to survive and replicate within macrophages. Despite nearly a century of research, how the bacteria are able to so effectively dismantle the macrophages' innate antibacterial mechanisms remains unknown. One of the bacteria's strategies to persist and cause chronic infection is to modulate the cell death pathways of the macrophages it infects[1]. Apoptosis has long been appreciated for its ability to control bacterial growth; however the mechanism for this remains unknown. Indeed, there are many mutants and attenuated strains of Mtb that induce apoptosis and as such do not proliferate robustly in macrophage culture. H37Ra is a lab-adapted 'attenuated' strain, genetically very similar to the lab-adapted H37Rv 'virulent' strain, save for a few mutations[2]. These mutations have rendered H37Ra less able to grow and proliferate in macrophages, and at the same time,

induce far more apoptosis in macrophage culture than H37Rv[3,4]. Specific genes have been identified in Mtb that influence the infected macrophage's fate as well. NuoG, a NADH dehydrogenase, inhibits apoptosis and contributes to Mtb's virulence[5]. NuoG counters TNF-mediated inducible nitric oxide synthase (iNOS) activity and apoptotic cascade[6]. The SecA2 alternative secretion apparatus is responsible for the translocation of several Mtb effectors into the extracellular space; chief among them is the superoxide dismutase, SodA[7,8]. Mtb mutants in SodA and SecA2 which is responsible for its secretion, cause more apoptosis and have limited virulence[8-10]. There may be many other Mtb genes responsible for apoptosis suppression and induction of necrosis[11]. Closely related non-pathogenic mycobacterial species induce macrophage apoptosis as well, further highlighting the link between apoptosis and suppression of bacterial growth[12]. Host factors also contribute to the death modality of an Mtb-infected macrophage. Prosteglandin E2 (PGE_2) production during Mtb infection can protect a dying cell's plasma membrane, thus preventing necrosis and skews the macrophage to a more apoptotic fate[13]. PGE_2 also stabilizes the mitochondrial membrane to prevent the mitochondrial permeability transition associated with necrosis[14,15]. IFNγ-activated macrophages avoid necrotic cell death potentially through NO-mediated signaling[16]. In all of these examples, apoptosis is related to a decrease in bacterial burden and a favorable outcome for the host.

Apoptosis is often referred to as 'programmed cell death' in that it follows a highly organized cascade of events with the purpose of dismantling the cell while keeping its membrane intact so as to not release inflammatory autoantigens[17,18]. Primarily, apoptosis proceeds down two pathways: intrinsic and extrinsic, that both converge upon the activation of caspase 3. Intrinsic apoptosis is in response to perturbations from within the cell- whether they be pathogen or stress derived. Intrinsic apoptosis relies on the activation of caspase 9. Extrinsic apoptosis,

mediated by caspase 8, requires that the cell receive the signal to die from an external signal, most notably via engagement of the TNF receptor[19,20]. Apoptosis is an innate defense mechanism for bacterial control and is responsible for bacterial growth suppression in vitro, but it also contributes in vivo to the production of a robust T cell response. Dendritic cells can present antigens derived from infected apoptotic macrophages to initiate a protective CD8+ T cell response[21,22].

Necrosis conversely allows the bacteria to escape its macrophage host and infect surrounding macrophages and is a major virulence strategy and source of immunopathology during disease. Virulent Mtb infection induces robust leukotriene A4 (LXA_4), which antagonizes PGE_2, leading to mitochondrial instability, prevention of plasma membrane repair and cell death[3,13]. The end result is necrosis and prolific bacterial growth. Additionally, LXA4 impairs the establishment of a protective $CD8^+$ T cell response and leads to worse outcome in vivo[22]. Necrosis, unlike apoptosis, is a form of cell death that results in the disintegration of the plasma membrane and leakage of intracellular content. It is now appreciated that necrosis is not as 'un programmed' as was first thought and may rely on intracellular signaling events[23]. Apoptosis and necrosis of Mtb infected macrophages has been reported using both human and murine macrophages[13,24-26]. However, the relative contribution of these two forms of cell death during active infection or latency remains unknown. And while necrosis is characteristic of virulent Mtb infection, a spectrum of cell death is observed[27]. In vitro, many apoptotic cells may eventually disintegrate through the process of secondary necrosis, which is phenotypically similar to necrosis. Additionally, many assays used to measure cell death cannot differentiate secondary necrosis from necrosis. Consequently, the majority of macrophage cell death following Mtb infection in vitro is mistakenly attributed to necrosis. However in vivo apoptotic

cells rarely undergo secondary necrosis in this manner. Apoptotic cell clearance is so rapid detection of apoptotic cells in vivo is difficult.

As a cell dies it releases 'find me' and displays on its surface a variety of 'eat me' ligands to attract phagocytes. Phagocytes, mainly macrophages, utilize these 'eat me' signals to mediate the recognition, tethering and engulfment of apoptotic cells[28,29]. The process of apoptotic cell engulfment is called efferocytosis and is a crucial function of macrophages involved in the resolution of infection and organogenesis[30]. The process of apoptotic cell engulfment has been studied extensively and as such many receptors and their downstream signaling components have been identified. Despite a growing body of knowledge surrounding the study of dead cell engulfment, much remains unknown about the consequences of efferocytosis of infected cells.

We sought to understand the consequences of cell death following Mtb infection. First, we sought to better understand virulent Mtb-induced apoptosis in wildtype macrophage. Indeed, we find that a spectrum of death modalities, including apoptosis results after infection. As efferocytosis is an important function of macrophages upon encounter of an apoptotic cell, we asked whether efferocytosis of Mtb-infected apoptotic macrophages occurs during Mtb infection. We found a significant amount of efferocytosis of Mtb-infected macrophages in vitro and in vivo. Additionally, Mtb infection does not impair efferocytosis, indicating that this constitutive macrophage process may occur in vivo in the Mtb-infected lung.

Results

Mtb- infected macrophages undergo apoptosis

While virulent Mtb predominantly induces necrosis of both human and murine macrophages in vitro, particularly after several days or at high multiplicity of infection (MOI), careful analysis revealed that a spectrum of cell death modalities are observed, including apoptosis. Thioglycollate-elicited $CD11b^+$ peritoneal macrophages were infected with mCherry-expressing H37Rv, resulting in the majority of macrophages harboring 1-5 bacteria. This bacterial burden is similar to the burden of infection observed in macrophages in the infected lung. Two days after infection 15% of infected macrophages were apoptotic as determined by TdT-mediated d-UTP nick end labeling (TUNEL) of apoptotic macrophage nuclei (Figure 2-1A). Infected macrophages incubated with 10μM cell-permeable irreversible peptide inhibitors directed against caspase 3 and caspase 9 had markedly less apoptosis compared to untreated and control peptide treated macrophages (Figure 2-1B). Caspase 8 inhibition had no effect, though in other systems 10μM is sufficient to block caspase 8 activity (data not shown). Thus we can conclude that at this time point, apoptosis is executed primarily via the intrinsic apoptosis pathway. There was not significant bystander apoptosis of uninfected macrophages within the infected macrophage conditions (data not shown).

Detection of Mtb-infected apoptotic macrophages engulfed by uninfected macrophages

Phagocytes rapidly clear dying cells in vivo; however, whether Mtb-infected apoptotic cells are engulfed by macrophages has never been examined. We observed $TUNEL^+$ nuclei contained within healthy, intact macrophages, indicating that Mtb-infected apoptotic cells could be engulfed by other macrophages. To formally study efferocytosis of Mtb-infected macrophages we developed a confocal microscopy assay to monitor the fate of infected macrophages (Figure

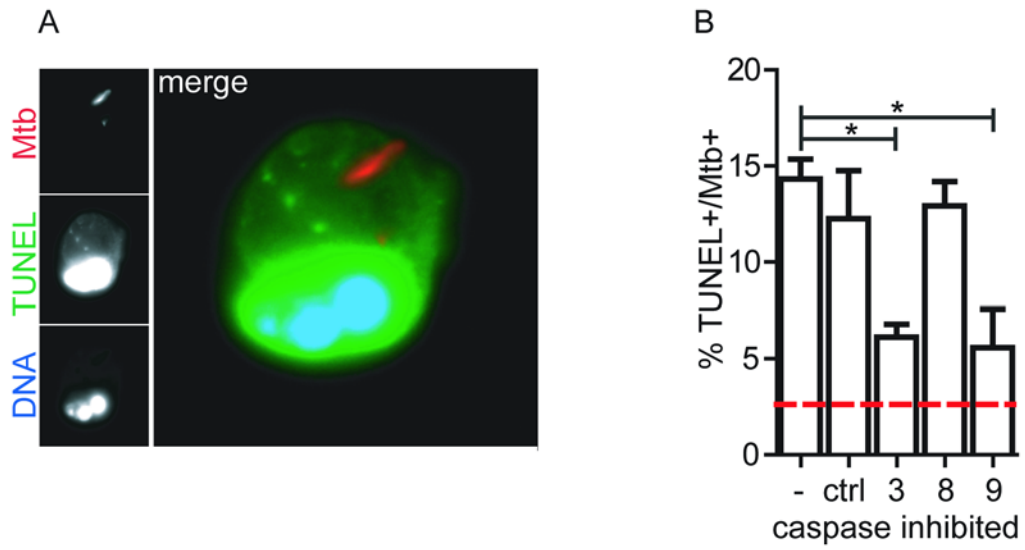

Figure 2-1. Virulent Mtb induces apoptosis of infected macrophages.
A. Apoptotic CD11b^{+} thioglycollate-elicited peritoneal macrophage (pMφ) infected with mCherry-H37Rv two days post infection. Panels show Mtb(red), TUNEL (green), DNA (far red/pseudocolor blue) and merge. Image is representative of 3 experiments. B. Percent TUNEL^{+} infected pMφs of total pMφs. Macrophages were infected with Mtb, washed and 10μM indicated caspase inhibitors or negative control peptide (ctrl) was added before cells were imaged. Red dashed line is mean apoptosis of uninfected macrophages. Data is representative of 3 experiments. Error bars ± SEM; *, $p \leq 0.05$ One-way ANOVA.

2-2A). An important consideration for studying the fate of an Mtb-infected is to distinguish it from uninfected macrophages and to detect the dead cell after it has been engulfed. Many researchers study efferocytosis in non-infectious settings by fluorescently staining thymocytes with an intracellular dye before inducing apoptosis. This allows internalized apoptotic cells to be easily detected by FACS or visualized by microscopy. We based our assay on this standard approach.

Macrophages from C57Bl/6 mice, which express the CD45.2 allele (hereafter referred to as CD45.2 macrophages) were labeled with DiO, a green fluorescent stain that intercalates into membranes and infected with mCherry-H37Rv before adding uninfected congenic CD45.1 macrophages. CD45.1 macrophages were added at a ratio of 2:1. Under these conditions there was no discernible transfer of dye between the macrophage membranes and bacterial membranes (data not shown). There exist three predicted scenarios after overnight co-culture. First, Mtb can remain within the first cell it infects – a primary infected cell (Figure 2-2Ai). Second, necrosis of an infected macrophage allows Mtb to disperse into the culture supernatant and to infect other macrophages (Figure 2-2Aii). These are secondarily infected macrophages. As the uninfected CD45.1 outnumber the DiO-dyed primary infected macrophages, there is a greater chance that the CD45.1 macrophages would take up the extracellular bacteria. However, DiO-dyed macrophages could also become secondarily infected or engulf a DiO^+ Mtb^+ apoptotic macrophage. These instances would be indistinguishable from primary infected macrophages. Thus, we seeded macrophages primary macrophages at low density, and added two times the number of CD45.1 macrophages. Third, an adjacent CD45.1 macrophage could engulf an Mtb-infected apoptotic macrophage (Figure 2-2Aiii). This is efferocytosis. Again, a DiO-dyed

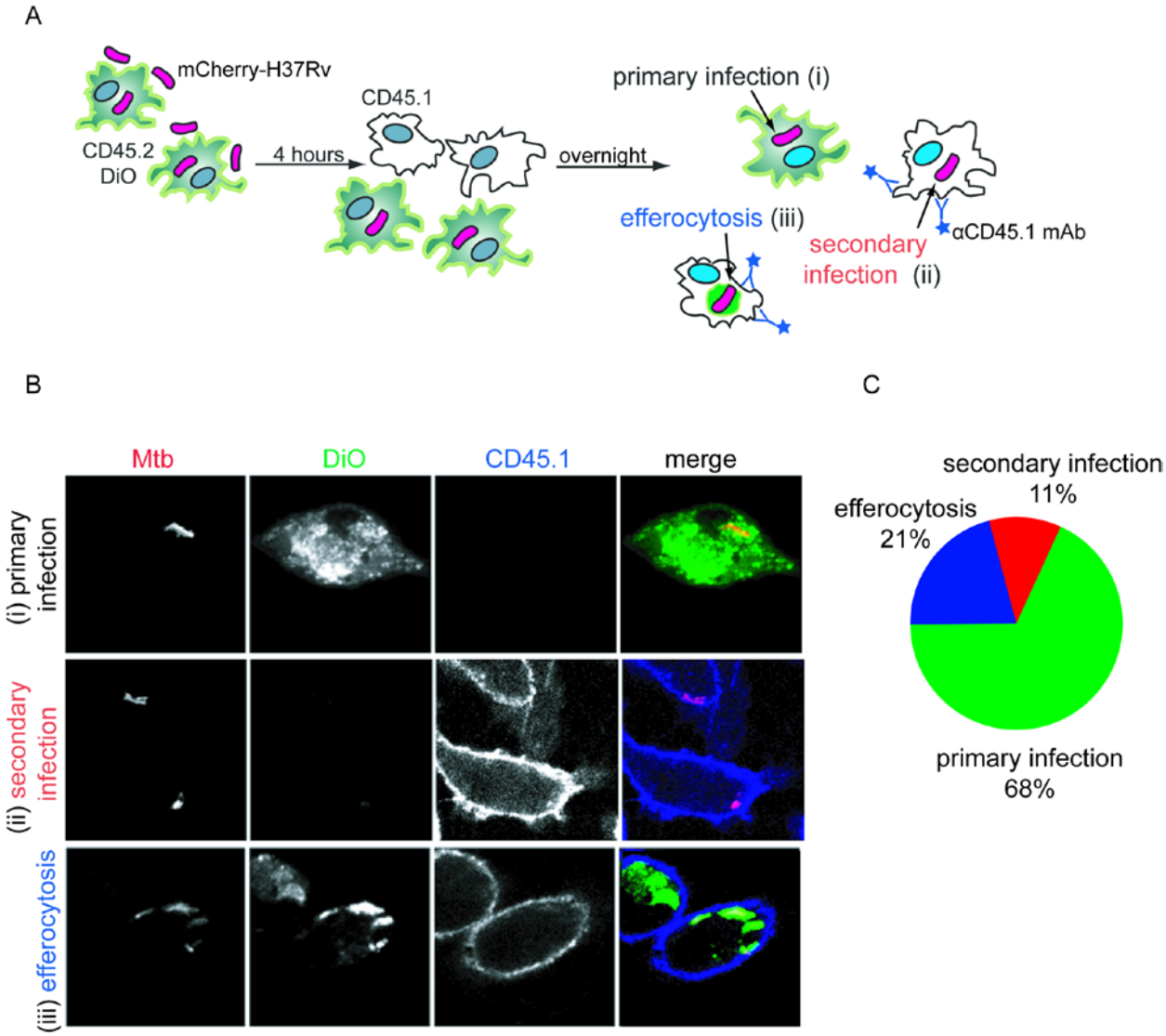

Figure 2-2. Efferocytosis of Mtb-infected macrophages in vitro
A. Assay for detecting efferocytosis of Mtb-infected apoptotic cells in vitro by confocal microscopy and its three predicted outcomes. B. Confocal microscopy images of $CD45.2^{+}$ pMφs dyed with DiO, infected with mCherry-H37Rv and co-cultured with uninfected undyed $CD45.1^{+}$ pMφs for 16 hours before fixation and visualization. Depicted are the three predicted outcomes of the efferocytosis assay; primary infection (i), secondary infection (ii) and efferocytosis (iii). Panels show Mtb (red), DiO (green) and CD45.1 (far-red/pseudocolor blue) and merge. Images are representative of ≥20 experiments. C. Percentage of Mtb within primary infected cells (68%), efferocytic cells (21%) or secondary infection (11%) 1 day post infection. Data is mean of 12 experiments.

CD45.2 macrophage could potentially engulf an infected apoptotic cell, and this would be undetectable.

One day after co-culture of infected and uninfected macrophages, all three scenarios were observed (Figure 2-2B). The majority of Mtb were within primary infected macrophages. However, 21.7±1.7% of Mtb were within efferocytic cells and 9.8±1.2% spread into secondarily infected cells (mean ± SEM; n=12 experiments), which closely corresponds to the amount of cell death observed at this time point (Figure 2-2C). Secondarily infected cells and efferocytic cells are distinguished based on whether or not Mtb colocalizes within discrete DiO^+ vesicles within a CD45.1 macrophage. This analysis may underestimate the frequency of efferocytosis if the DiO label is degraded following efferocytosis. Although we observed loss of the DiO signal in primary infected macrophages after 48 hours, it was not evident at 24 hours, the time point chosen for these experiments. Additionally, secondary infection might be overestimated, as not all extracellular Mtb can be removed, even by extensive washing. Thus a small amount of free bacteria remained when the uninfected macrophages were added.

Efferocytosis was observed using thioglycollate-elicited peritoneal macrophages, resident peritoneal macrophages, and bone-marrow derived macrophages (Figure 2-3C,D). Images are representative of $\geq$ 12 experiments. Thus, this is a general feature of macrophages. We also found that Mtb infection does not significantly impair macrophage engulfment of apoptotic thymocytes, indicating that Mtb infection does not directly inhibit efferocytosis (Figure 2-4A). Furthermore, we found that supernatant taken from Mtb-infected macrophage culture does not inhibit efferocytosis. In this assay, uninfected macrophages were cultured with supernatant from Mtb-infected macrophages for 6 hours before CFSE-labeled apoptotic thymocytes were added. The supernatant from infected macrophages had no effect on uptake of apoptotic thymocytes by

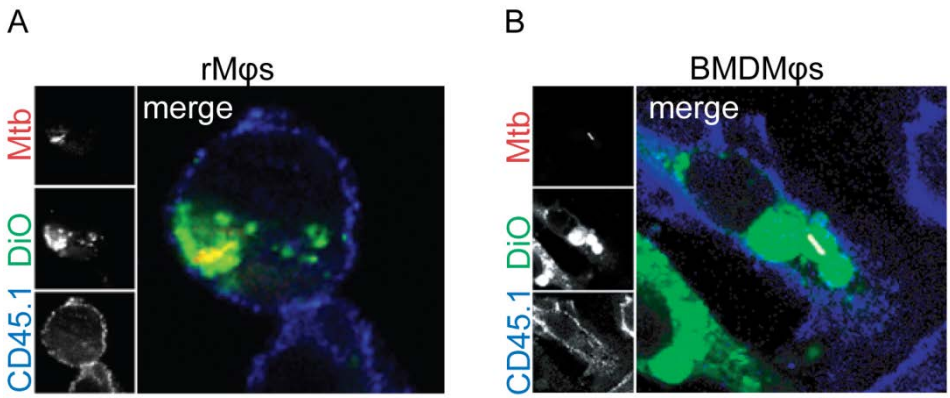

Figure 2-3. Many macrophage types can engulf Mtb-infected apoptotic macrophages
A. Efferocytosis seen using the above assay described in Figure 2-2 performed with resident peritoneal macrophages (rMφs). B. Efferocytosis assay performed with M-CSF matured bone marrow-derived macrophages (BMDMφs). Mtb(red), DiO(green), CD45.1(far red/pseudocolor blue), and merge. Images are representative of ≥ 12 experiments.

uninfected macrophages (Figure 2-4B). As a control, phagocytosis of heat-killed Alexa 488 conjugated *Escherichia coli* was also unaffected. Thus Mtb infection does not specifically inhibit efferocytosis at these timepoints in vitro.

Modulation of apoptosis alters efferocytosis

Next, we sought to confirm that our assay was reliably measuring efferocytosis. As apoptosis naturally precedes efferocytosis, we sought to modulate apoptosis and measure it's effects on efferocytosis. $5LO^{-/-}$ macrophages cannot produce LXA_4 and thus PGE_2 production following Mtb infection is disinhibited following Mtb infection. These macrophages are more likely to undergo apoptosis than wildtype macrophages (Figure 2-5A). $PGES^{-/-}$ macrophages cannot produce PGE_2 following infection, and thus are more prone to a necrotic death after Mtb infection. We infected DiO-dyed $5LO^{-/-}$ macrophages with mCherry-H37Rv before addition of uninfected CD45.1 macrophages. We then measured efferocytosis by microscopy. Significantly more efferocytosis was found following $5LO^{-/-}$ macrophage infection than wildtype and the pro-necrotic $PGES^{-/-}$ macrophage (Figure 2-5B). To further confirm that we have observed efferocytosis of Mtb-infected apoptotic macrophages we sought to inhibit apoptosis in wildtype macrophages. As we have found that caspases 3 and 9 are required for apoptosis of Mtb-infected macrophages early in in vitro infection, we inhibited those caspases and measured efferocytosis by microscopy. Just as caspase inhibition decreased the incidence of apoptosis, they abrogated efferocytosis (Figure 2-5C). Thus, we confirmed that our assay system can be used to monitor the fate of an Mtb-infected macrophage as it dies and is engulfed by a neighboring macrophage.

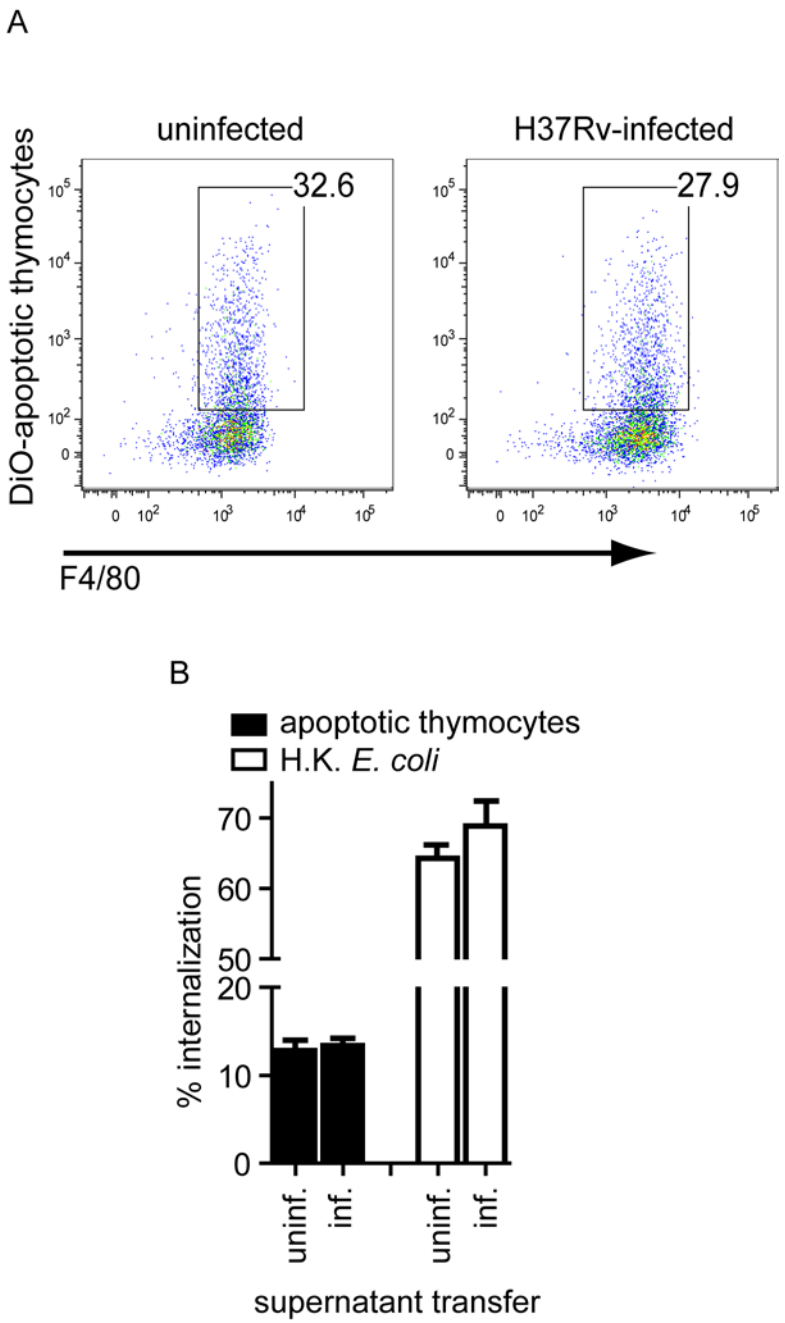

Figure 2-4. Mtb infection does not inhibit efferocytosis
A. Mtb-infected macrophages can efferocytose apoptotic targets. Uninfected macrophages or Mtb-infected macrophages 1 day post infection were cultured with CFSE-labeled apoptotic thymocytes for 1 hour prior to fixation and visualization by FACS. Data is representative of 3 experiments. B. Supernatant from Mtb-infected macrophages does not inhibit efferocytosis. Supernatant from 4 day Mtb-infected culture or 4 day uninfected macrophage culture was added to freshly isolated macrophages. Macrophages were then cultured with CFSE-labeled apoptotic thymocytes or heat-killed Alexa488 *E. coli* for 1 hour prior to fixation and visualization by FACS. Data is representative of 2 experiments.

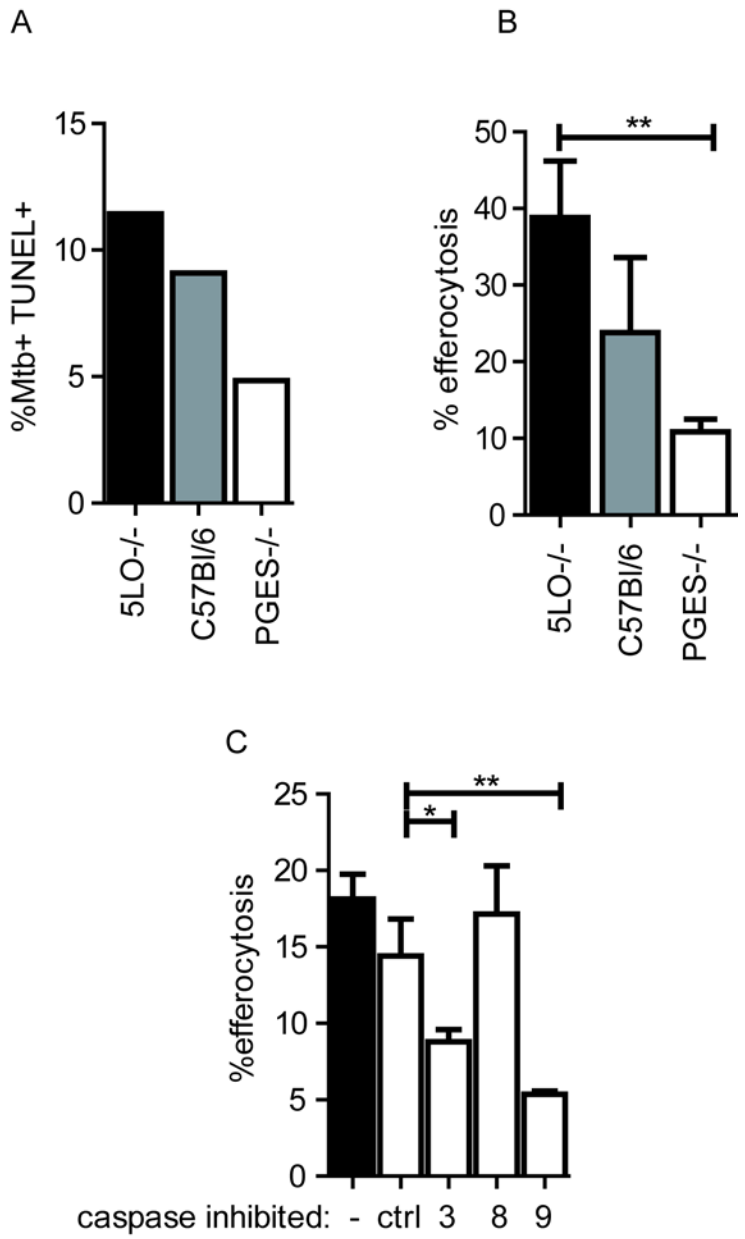

Figure 2-5. Modulation of apoptotic pathways affects efferocytosis

A. % TUNEL^{+} Mtb-infected macrophages of total Mtb-infected macrophages from indicated genotypes. Data is representative of 3 experiments. B. Percent efferocytosed Mtb of total Mtb 1 day post infection of thioglycollate-elicited peritoneal macrophages (pMφs) from indicated genotypes co-cultured with WT uninfected macrophages. Data representative of two experiments. C. Intrinsic pathway apoptosis inhibition restricts efferocytosis. Macrophages infected with Mtb in the presence of 10μM caspase inhibitors for 1 day before fixation and examination by confocal microscopy as described in figure 2. Data is representative of 3 experiments. Error bars ± SEM; *, $p \leq 0.05$; **, $p \leq 0.005$. One-way ANOVA.

Efferocytosis occurs in vivo

As we have found efferocytosis of Mtb-infected apoptotic macrophages in vitro, we sought to confirm that efferocytosis as such could happen in vivo. Efferocytosis has only been studied in a limited way in vivo, due to the multiple redundant mechanisms of dead cell clearance. Thus studying the relative contribution of cell types and specific receptors is limited in vivo. One model for the study of efferocytosis in vivo is through intraperitoneal transfer of apoptotic target cells. In this model thioglycollate was used to recruit macrophages to the peritoneal cavity of CD45.1 mice. CD45.2 DiO-dyed Mtb infected macrophages were transferred intraperitoneally into CD45.1 mice 4 days following thiogylcollate priming. As a control, uninfected macrophages were transferred into separate recipients. The peritoneal cavities were lavaged 16 hours later and the retrieved cells were fixed and stained with antibodies directed against CD45.1 to distinguish the transferred cells from the recipients. Cells were cytospun onto slides and inspected by confocal microscopy (Figure 2-6A). Just as predicted, the three potential outcomes were found: Mtb within primary infected transferred macrophages, secondarily infected recipient macrophages and efferocytosis of transferred macrophage by recipient macrophages (Figure 2-6B). Thus, efferocytosis of Mtb infected macrophages can occur in vivo.

As the lung is the primary site of Mtb infection, we sought to recapitulate the intraperitoneal model of efferocytosis in the lung. DiO-dyed Mtb infected macrophages were transferred intratracheally into the lungs of CD45.1 recipient mice. 16 hours later the lungs were lavaged and the retrieved cells were subject to the same fixation and staining protocols as described in the intraperitoneal model above (Figure 2-7A). Efferocytosis of Mtb infected macrophages was observed by the recipient CD45.1 macrophages as well. As approximately

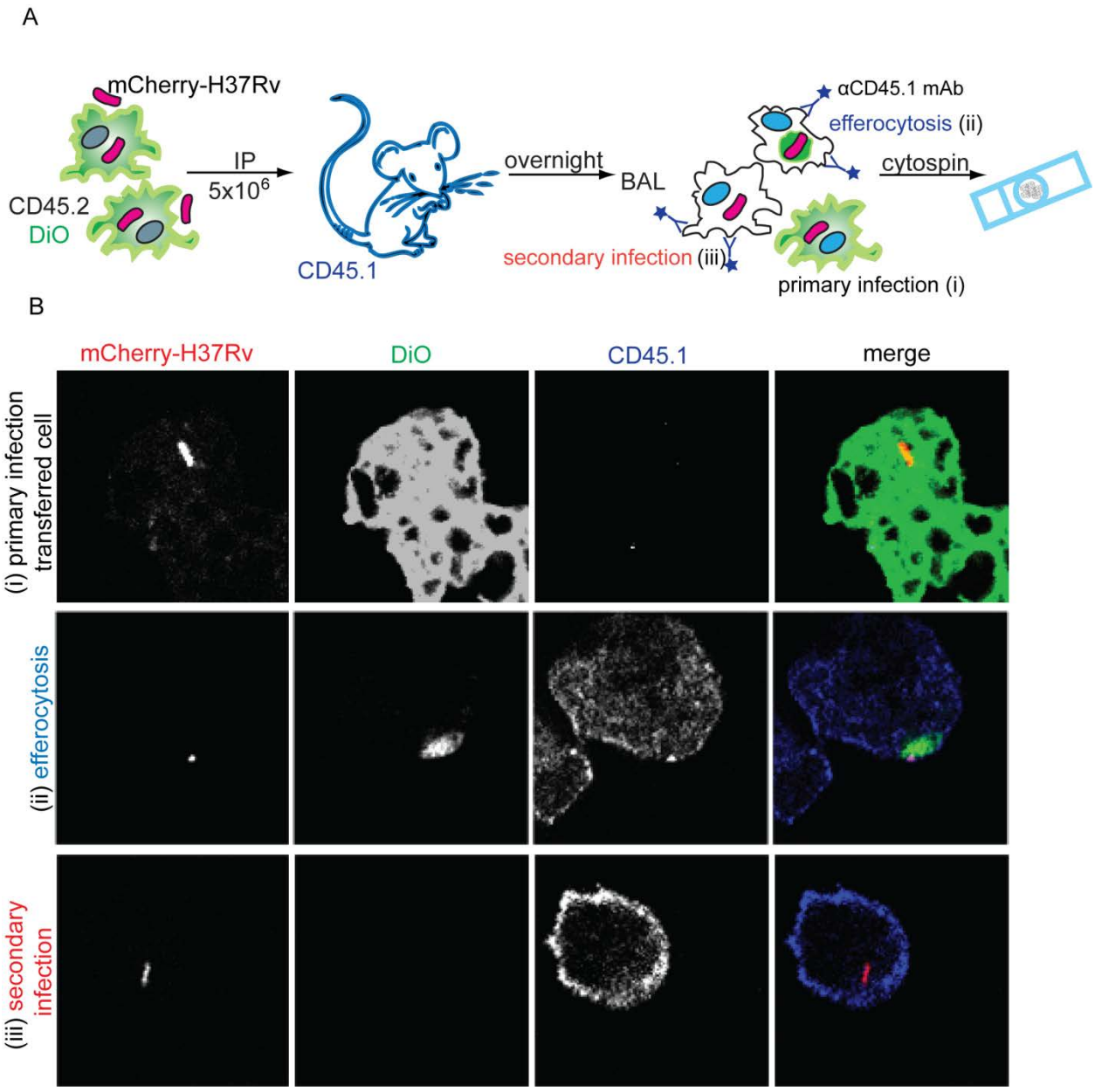

Figure 2-6. Intraperitoneal model of efferocytosis
A. Assay scheme. B. Confocal microscopy images of the three predicted outcomes. (i) Primary infected transferred cell, (ii) efferocytosis and (iii) secondary infection. Panels are Mtb (red) DiO (green- transferred cells) and CD45.1 (far red/pseudocolor blue- recipient cells). Images are representative of 3 experiments.

90% of cells retrieved from a naïve mouse lung are alveolar macrophages, this demonstrates that efferocytosis of Mtb-infected macrophages occurs in the lung by a relevant macrophage subset. Very early during Mtb infection, prior to the recruitment of inflammatory monocytes, neutrophils and dendritic cells, alveolar macrophages are the sole cell type present in the lung[31]. Thus our model more closely represents early Mtb infection. Transferred cells and secondarily infected macrophages were observed as well (Figure 2-7B). Thus, efferocytosis of Mtb infected macrophages occurs in the lung by alveolar macrophages. This result was not altogether unexpected. Dendritic cells take up apoptotic cells and blebs during Mtb infection in order to cross-prime a protective $CD8^+$ T cell response[32,33]. As dendritic cells and macrophages are both highly efferocytic and rely on similar mechanisms to recognize and engulf dying targets, efferocytosis of Mtb-infected apoptotic macrophages is thus likely to occur during infection.

Discussion

It is well understood that virulent Mtb employ necrotic cell death to escape the macrophage and disperse into surrounding macrophages. This is a major virulence strategy of the bacterium. However, what is not appreciated is that during Mtb infection a spectrum of cell death is observed. In fact, we find that apoptosis and necrosis both follow virulent Mtb infection. Such heterogeneity is worthy of study as it could lead to a better understanding of how the bacteria counters the macrophages' innate drive towards apoptotic death. As there is inherent heterogeneity on both the macrophage and bacteria side, multiple factors may be at work determining the ultimate fate of the infected cell. We chose to use TUNEL as our assay for apoptosis as it is one of the most reliable assays for apoptosis. TUNEL positivity is also an end stage marker for apoptosis and unlike other methods, TUNEL is specific for apoptosis[34]. Previous reports have focused on necrotic cell death following Mtb infection. It is true that there

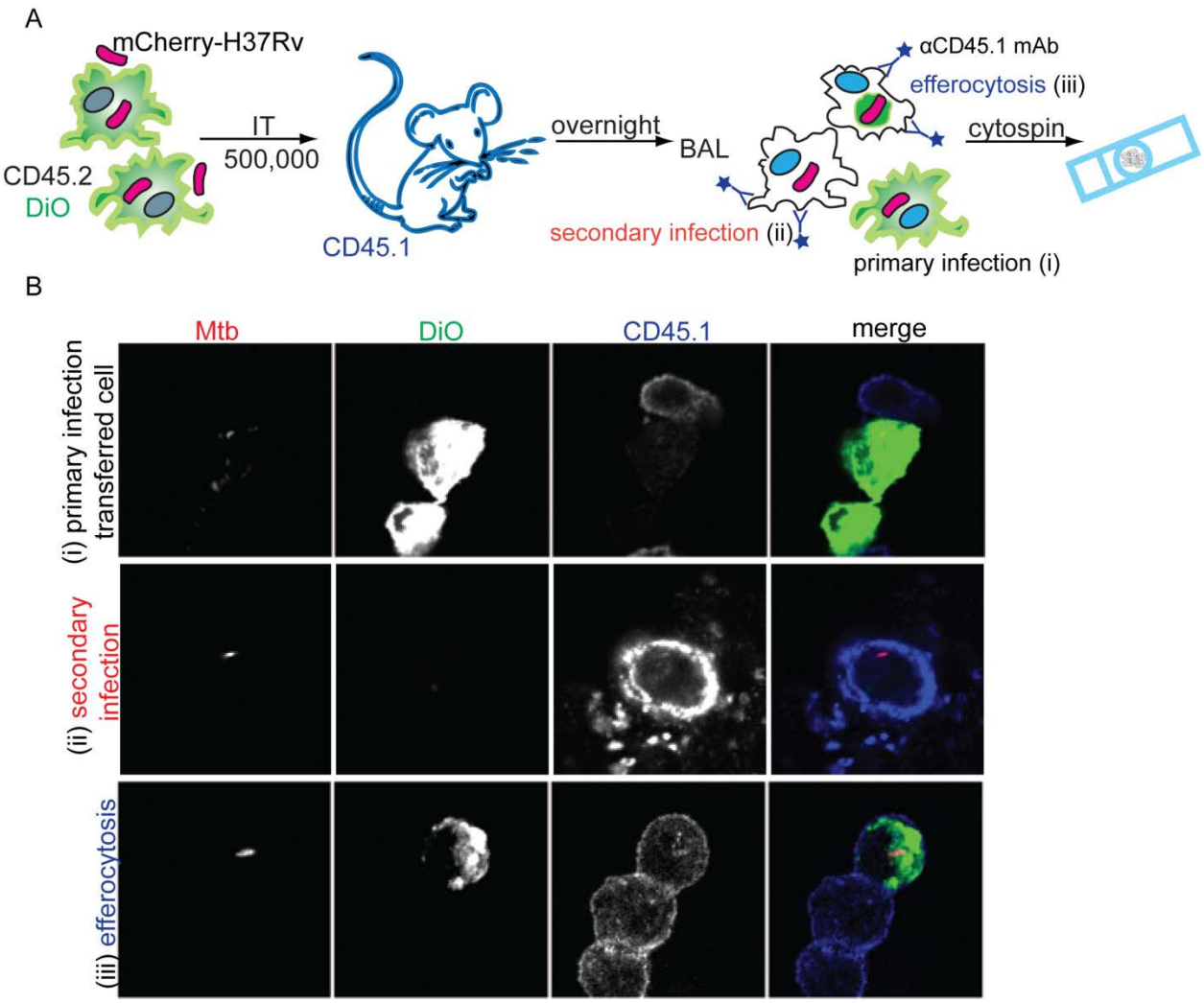

Figure 2-7 Intratracheal model of efferocytosis

A. Assay scheme. B. Confocal microscopy images of the three predicted outcomes. (i) Primary infected transferred cell, (ii) efferocytosis and (iii) secondary infection. Panels are Mtb (red) DiO (green- transferred cells) and CD45.1 (far red/pseudocolor blue- recipient cells). Images are representative of 2 experiments.

is significant necrosis, however we speculate that much of the necrosis detected in in vitro macrophage culture is the result of secondary necrosis. Secondary necrosis results following apoptosis and inefficient efferocytosis. Efferocytosis might be hampered in vitro if macrophages are cultured at too low density or if TNF production is robust.

We found that two days post infection, apoptosis is largely mediated through the intrinsic apoptosis pathway. As TNF production increases over time in in vitro culture as the infection progresses and more cells become infected, the extrinsic pathway may become more important. This is not to say that this is how apoptosis is executed in the lung during active disease. TNF is a hallmark of the granuloma, indicating that extrinsic apoptosis probably plays a bigger role in the lung[35]. However, early in infection before the recruitment of T cells and monocytes, intrinsic pathway mediated apoptosis could play a role.

In vivo apoptosis is almost always followed by efferocytosis, the process of apoptotic cell engulfment. Macrophages have tremendous efferocytic capacity, as they engulf 10^9 apoptotic cells a day. Only under some conditions are apoptotic cells not cleared by macrophages: most notably during rampant inflammation which suppresses the efferocytic machinery. Even though efferocytosis is such a common macrophage function, the consequences of apoptotic cell removal during infection have been largely ignored. As such, we developed assays that would allow us to track the fate of an infected cell after apoptosis. We find a substantial percentage (~20%) of Mtb in in vitro culture one day post infection have been efferocytosed along with dead cell material by uninfected macrophages. Efferocytosis of Mtb-infected apoptotic cells is a general phenomenon of many macrophage types, just as efferocytosis has been observed and studied by many macrophage populations.

Virulent Mtb exert a great deal of control over the macrophages they infect. As such, we asked whether Mtb could inhibit efferocytosis, as this could have implications in vivo during active disease. We found that Mtb infected macrophages are capable of ingesting apoptotic thymocytes to the same extent as uninfected macrophages. Thus, Mtb does not directly hamper efferocytosis. Pro-inflammatory cytokines have been found to inhibit efferocytosis, particularly TNF[36]. We asked whether supernatant from Mtb-infected cultures could exert an anti-efferocytic effect. However, we found no such impairment. This does not exclude the possibility that in vivo or in the presence of robust TNF that Mtb-infected apoptotic cell clearance will be impaired. Merely Mtb does not directly alter macrophage's engulfment ability. And more generally, phagocytosis is not altered in Mtb-infected macrophages. Efferocytosis might be significantly inhibited in vivo during active disease.

As apoptosis precedes efferocytosis, altering the amount of apoptosis should have a proportional effect on efferocytosis. $5LO^{-/-}$ macrophages produce PGE_2 in response to virulent Mtb infection and as such are protected from necrosis[3]. $PGES^{-/-}$ macrophages undergo more necrosis following infection. Predictably, modulating apoptosis had a proportional effect on efferocytosis. Inhibition of the intrinsic apoptosis likewise decreased efferocytosis, indicating that our assay system was reliably measuring efferocytosis.

Moving the study of efferocytosis in vivo presented numerous challenges. As most researchers who study efferocytosis do so by artificially inducing apoptosis, the biological questions they ask can be answered rapidly, as engulfment of apoptotic cells is rapid. Allowing Mtb-infected cells to die naturally entails longer time points, which restricted analysis. Fewer Mtb were found following transfer of infected cells into either the intraperitoneal space or the lungs as would be expected. Images of the infected cells taken before transfer indicated that a

high percentage of them were infected with 3+ bacteria (data not shown). Perhaps the infected macrophages homed to distal sites post transfer. Indeed, researchers who use in vivo efferocytosis models typically transfer apoptotic cells and lavage the cavity 1-4 hours later, which leaves much less time for cells to migrate out of the area. Even so, we found efferocytosis of Mtb-infected macrophages by uninfected cells using two models of in vivo adoptive transfer: intraperitoneal and intratracheal. Thus alveolar macrophages are capable of engulfing Mtb-infected macrophages and may exert tremendous influence over the course of infection.

Efferocytosis of infected cells by macrophages has been observed before. *Leishmania* species infect neutrophils and depend on their death to enter macrophages[37,38]. During *Leishmania* infection efferocytosis is crucial for the establishment of infection. *Chlamydia* infection similarly relies on neutrophil death to enter host macrophages by efferocytosis[39]. Influenza, however induces apoptosis in epithelial cells only to be engulfed and killed by responding macrophages[40,41]. It remains to be seen how Mtb responds to efferocytosis- whether it is a strategy for bacterial spread or containment. Apoptosis is known to restrict bacterial growth, however it is not known whether this is a direct consequence of apoptosis itself or if efferocytosis contributes to bacterial control. Thus further study of efferocytosis and its potential impact on bacterial growth and host survival is required.

Methods

Mice and macrophage isolation

C57Bl/6 (CD45.2) and B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1) mice were purchased from Jackson Laboratories (Bar Harbor USA), and were kept and bread using standard humane animal husbandry protocols. Mtb-infected mice were housed under BSL3 conditions. Thioglycollate-elicited peritoneal Mφs (pMφs) were lavaged from peritoneal cavities 4-5 days following 3% IP thioglycollate injection. pMφs were isolated using CD11b microbeads and magnetic columns (Miltenyi Biotec) and cultured in 24 or 96 well dishes overnight prior to infection. Resident peritoneal Mφs (rMφs) were lavaged from peritoneal cavities and selected based on $CD11b^{+}CD19^{-}$. Bone marrow-derived Mφs (BMDMφs) were differentiated from bone marrow cells 7-10 days in RPMI supplemented with 20% L929 cell supernatant containing M-CSF. Alveolar Mφs (aMφs) were lavaged from the lungs of mice with PBS.

Mtb culture and infection

mCherry-H37Rv was cultured with 0.5ug/mL Hygromycin B. Mtb infection was conducted as previously described[42] with the following modifications. Mφs were grown in 96-well plates and infected at a multiplicity of infection (MOI) of 10:1 for 4 hours. Cultures were then washed 3-5 times. Efferocytic Mφs were added at 2x the number of infected Mφs. As indicated efferocytic Mφs were pretreated with 10μM PGE_2 for 4 hours at 37^{o}C before washing and addition to infected Mφs. Caspase inhibitors (Calbiochem) (10μM). CFU were determined immediately following infection and at indicated timepoints per condition in quadruplicate by removing culture supernatant, lysing Mφs with 1% TritonX/PBS solution, plating serial dilutions on 7H11 plates and incubating at 37^{o}C/5% CO_2 for 3 weeks. All Mtb strains were confirmed by thin layer chromatography to be $PDIM^{+}$.

FACS efferocytosis assay

Apoptotic thymocytes were generated by dying thymocytes with 1μM CFSE or 1μl/mL Vybrant DiO (Invitrogen) prior to apoptosis induction with staurosporine for 6-16 hours. Thymocytes were then co-cultured with macrophages at a ratio of 5:1 for 1 hour in 24 well plate before washing, staining with antibodies directed against F4/80, fixation and analysis on a BD FACS CANTO II and FlowJo software. Heat killed *E.coli* Bioparticles (Invitrogen) were added as a control.

TUNEL assay for apoptosis

Apoptosis was assessed by TUNEL (Roche) according to manufacturer's specifications on Mφs grown on coverglass and fixed with 3.7% paraformaldehyde. 100 infected cells were counted per condition in triplicate by conventional epifluorescence microscopy using a Nikon TE2000-U inverted microscopy fitted with a SPOT-RT CCD camera.

Confocal microscopy efferocytosis assay

Mφs from CD45.2 mice were cultured in 24 well dishes in triplicate per condition upon coverglass and dyed with Vybrant DiO (Invitrogen) as per manufacturer's specifications prior to infection with mCherry-H37Rv for 4 hours. Uninternalized bacteria were washed away and uninfected CD45.1 macrophages were added at 2x the number of infected macrophages. Treatment conditions were added as appropriate. 16-24 hours later cells were washed and stained with mouse monoclonal CD45.1 (Abcam) before affixing to microscopy slides with Prolong Gold plus DAPI (Invitrogen Conditions were analyzed in triplicate and for each replicate in each condition 100-300 Mtb were counted. Images were taken using a Nikon TE2000-U inverted microscopy equipped with C1 Plus confocal laser scanner.

In vivo efferocytosis assay

Adoptive transfer of Mtb-infected macrophages was as described previously[13]. DiO-dyed CD45.2 pMφs were infected in standing culture with mCherry-H37Rv for one hour. Cells were washed and resuspended in PBS. For the intraperitoneal model $5x10^6$ macrophages in 1 mL were transferred intraperitoneally into CD45.1 mice that received 2mL 3% thioglycollate 4 days prior. 24 hours later the peritoneal cavity was lavaged, fixed with PFA, stained for CD45.1 as described above and affixed to slides by cytospin centrifugation. For the intratracheal model 3x105 macrophages in 100uL PBS was transferred into the lungs of uninfected CD45.1 mice via the intratracheal route. 24 hours later airway cells were retrieved by bronchoalveolar lavage, fixed with PFA, stained for CD45.1 and cytospun onto microscopy slides for confocal microscopy analysis.

Image Analysis

Microscopy images were analyzed using Image J[43] and colocalization analysis macros or user-generated pipelines in Cell Profiler[44]. Investigators were blinded during analysis.

Statistical Analysis

Statistical analysis and graphical output was done with GraphPad Prism. One-way ANOVA with Dunnet's post-test. * = $p \leq 0.05$

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Chapter 3: Efferocytosis suppresses growth of *Mycobacterium tuberculosis*

Parts of this chapter appears as the submitted manuscript:

"Efferocytosis is an innate antibacterial mechanism"

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Abstract

Mycobacterium tuberculosis (Mtb) subverts macrophage cell death pathways to ensure its survival and persistence. Apoptosis of an Mtb-infected macrophage is widely recognized as a mechanism of bacterial control, while necrotic death promotes infection. How precisely apoptosis mediates bacterial control is hitherto unknown. We report here that apoptosis itself is not intrinsically bactericidal, but that it is the engulfment of Mtb-infected apoptotic cells by uninfected macrophages that controls the infection. This process, known as efferocytosis thus emerges as a novel mechanism of bacterial control with profound implications for understanding the role of apoptosis in microbial infection.

Introduction

Following infection of macrophages by *Mycobacterium tuberculosis* (Mtb) the bacilli persist within arrested, immature phagosomes. Virulent Mtb inhibit macrophage apoptosis and ultimately induce necrosis, which enables the bacterium to evade host defenses, disperse and infect surrounding macrophages[1]. In contrast, attenuated Mtb strains and mutants lacking key virulence factors induce far less necrosis and instead stimulate apoptosis[2,3]. Apoptosis of Mtb-infected macrophages is associated with reduced bacterial growth[4]. It is largely assumed in the field that apoptosis is intrinsically bactericidal; however the evidence to support this is incomplete.

Molloy et al. reported decreased BCG viability in macrophages following treatment with ATP, which induces apoptosis[5]. Treatments that induce necrosis, such as hydrogen peroxide, had no effect on bacterial viability. Thus it was concluded that apoptosis alone is bactericidal. Fas ligand treatment of Mtb-infected human macrophages was also found to control the

bacteria[6]. However, in both of these studies many macrophages remained uninfected and unaffected by apoptosis treatment, leading us to speculate that the uninfected macrophages might be participating in bacterial control.

Previous work has demonstrated that adding uninfected macrophages to infected macrophage culture was associated with suppression of bacterial growth. Fratazzi and colleagues reported that the addition of uninfected macrophages to *M. avium*-infected macrophages controlled bacterial growth, but only if the infected macrophages were apoptotic[7]. Co-culture with infected alive or necrotic macrophages had no impact on bacterial growth. In a model of high-MOI induced apoptosis Lee et al found that apoptosis itself is not directly bactericidal, but that additional macrophages must be added to the culture to contribute to bacterial killing[8]. Addition of macrophages must occur shortly after infection as waiting additional days did not control bacterial growth, presumably because the infected macrophages died by secondary necrosis. These two reports stop short of speculating or studying the mechanism of how addition of uninfected macrophages controls bacterial growth. The assay used in these two reports to measure CFU control was similar to the assay we developed to study efferocytosis of Mtb infected apoptotic macrophages (Chapter 2). Thus, we asked whether conditions in which efferocytosis are observed are associated with bacterial control. Despite the substantive cell death that accompanies infection, the role of efferocytosis during infection, particularly with respect to efferocytosis of infected cells has not been investigated. Based on the known association between macrophage apoptosis and host resistance to Mtb, we hypothesized that the antibacterial role of apoptosis is mediated by efferocytosis.

We previously reported in Chapter 2 that following apoptosis, infected macrophages are efferocytosed by uninfected macrophages. Here we demonstrate that efferocytosis is capable of

controlling infection. We show that co-culture of uninfected macrophages with Mtb infected macrophages restricts bacterial replication in vitro in a variety of macrophage types. We also report that apoptosis and then engulfment of the infected macrophage is required for bacterial control. If engulfment is inhibited, secondary necrosis allows further spread of the bacteria. Thus, efferocytosis is a novel innate antibacterial mechanism capable of controlling of Mtb growth.

Results

Conditions that favor efferocytosis restrict Mtb growth

Previously we reported that following Mtb infection a substantial number of the primary infected macrophages underwent apoptosis. Using a microscopy assay we observed that the bacteria in these dying cells were engulfed along with cell debris by other macrophages. To determine whether the conditions in which we observed efferocytosis affects bacterial viability, uninfected peritoneal macrophages were co-cultured with Mtb-infected peritoneal macrophages and CFU were subsequently measured. The co-culture of uninfected thioglycollate-elicited peritoneal macrophages with virulent H37Rv- or avirulent H37Ra-infected macrophages significantly suppressed bacterial growth for a week in culture (Figure 3-1). The addition of uninfected macrophages completely suppressed Mtb growth as the CFU through day 7 post infection. The bacterial burden at day 0 was not significantly different from the CFU at day 7 in the macrophage co-culture condition, whereas Mtb grew robustly in primary infected macrophages. Uninfected macrophages controlled Mtb growth best when they were added to infected macrophages at a ratio of 1.5-2 uninfected per infected macrophage. Increasing the number of uninfected macrophages beyond 2 or 3 per infected macrophage did not contribute any additional affect, possibly because of over-crowding and faster depletion of the media.

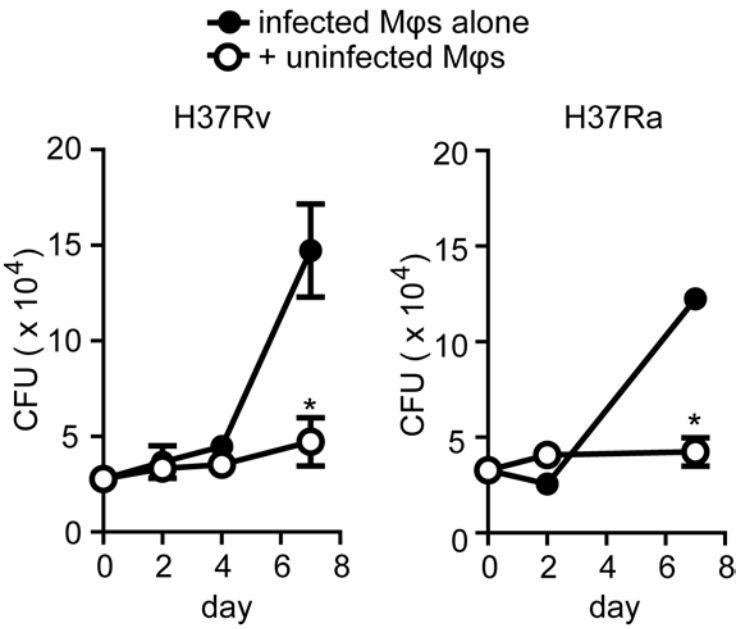

Figure 3-1. Co-culture of uninfected macrophages with Mtb-infected macrophages limits Mtb growth

Thioglycollate-elicited peritoneal macrophages were infected with a 10:1 mulitplicity of infection (MOI) of H37Rv (left) or H37Ra for 4 hours before uninternalized bacteria were washed away and uninfected macrophages were added at a ratio of 2:1. Bacterial burden was assessed at this point (day 0) and subsequent days as indicated. Data is representative of 12 experiments (left) or 4 experiments (right). Error bars ± SEM. *, $p \leq 0.05$ Student's t –test.

Thioglycollate-elicited peritoneal macrophages are highly activated due to the method of their retrieval from the mouse (unpublished observation). As such, directly ex vivo they produce a measurable amount of nitric oxide. Nitric oxide (NO) and reactive nitrogen intermediates (RNI) are potent antimycobacterial agents[9]. Because of this observation, we sought to confirm using other macrophage types that the co-culture of uninfected macrophages suppresses Mtb growth in ways other than or unrelated to NO production. In fact the antibacterial activity was a feature of all types of macrophages tested, alveolar macrophages (Figure 3-2A), resident peritoneal macrophages (Figure 3-2B), RAW264 cells (Figure 3-2C) and the A3.1A7 bone marrow derived macrophage cell line[10] (Figure 3-2D). These macrophage types are not 'pre-activated' and produce no detectable NO prior to addition to the infected macrophage culture. Thus, under conditions in which we detect efferocytosis by microscopy, bacterial growth is restricted. Uninfected macrophage co-culture is capable of Mtb control.

Macrophage activation does not boost antibacterial effect

As we found that uninfected macrophages are capable of Mtb control, we asked if activating the macrophages could enhance their antibacterial effect. Macrophages can be 'primed' to upregulate antibacterial effector functions through a variety of means. IFNγ/LPS priming increases NO production, and upregulates proteins involved in phagosome maturation. Treatment with heat-killed mycobacteria stimulates multiple TLRs and other pattern recognition receptors, ultimately activating inflammatory cytokines and readying the macrophage for bacterial encounter. TNF treatment can lead to many outcomes, but in such a situation, NF-κB activation is dominant. We activated uninfected macrophages with these three strategies prior to addition to infected macrophages as described above. Pre-activating macrophages conferred no additional antibacterial properties to thioglycollate-elicited peritoneal macrophages (Figure 3-3). Similar results were obtained priming resident peritoneal macrophages. This suggests that the

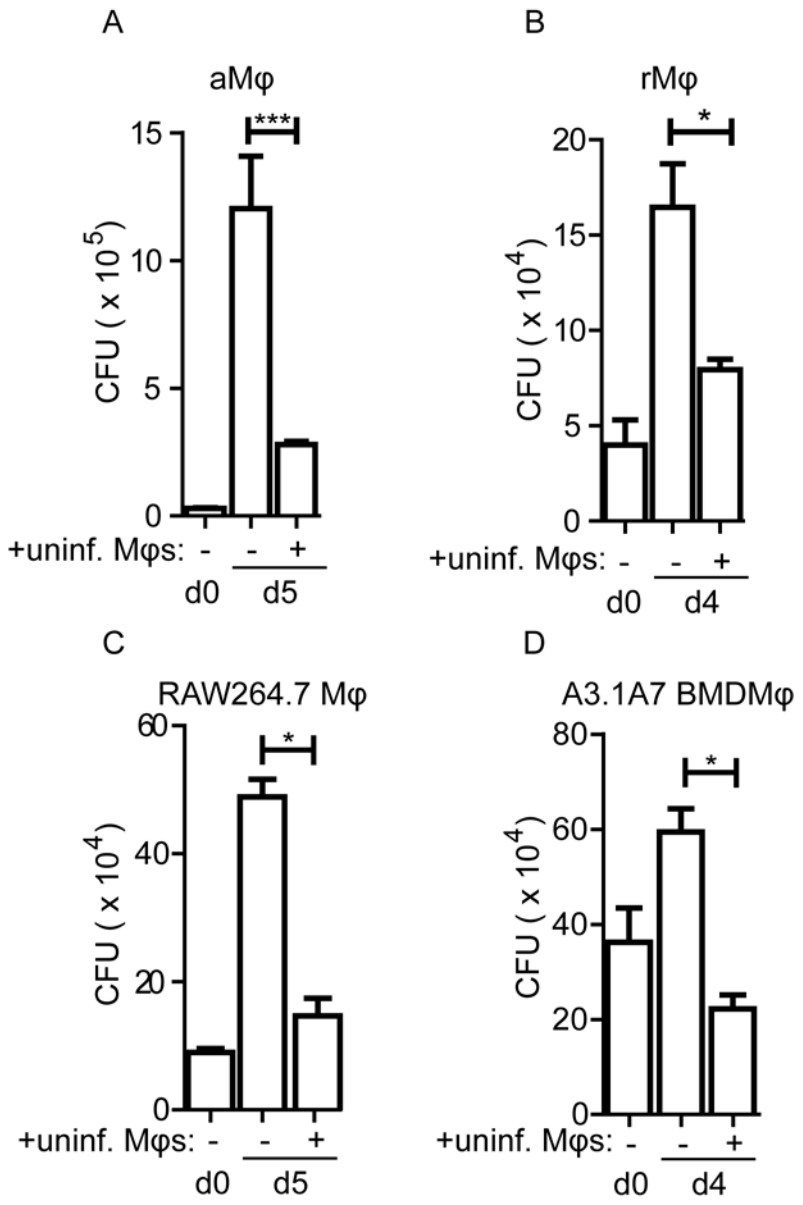

Figure 3-2. Uninfected macrophage co-culture universally limits bacterial growth

A. Alveolar macrophages (aMφs), B. resident peritoneal macrophages (rMφs), C. RAW264.7 macrophage cell line and D. A3.1A7 bone-marrow derived macrophage cell line (BMDM) were infected with an MOI of 10:1 Mtb for 4 hours before uninternalized bacteria were removed and uninfected macrophages of the same type were added. Initial bacterial burden was assessed at time 0 (d0) or after 4 or 5 days (d4,5). Data is representative of A,C, and D: 2 experiments; B: 3 experiments.. Error bars ± SEM. *, $p \leq 0.05$, ***, $p \leq 0.0005$ Student's t –test.

antibacterial effector function expressed during efferocytosis cannot be upregulated by activating macrophages via conventional means that confer protection from primary tuberculosis infection. Thus, the antibacterial function mediated by efferocytosis appears to be constitutivly expressed by all macrophages.

Apoptosis of infected macrophages is required if uninfected macrophages are to control Mtb growth

To prove that efferocytosis is responsible for the bacterial control observed and not other contact dependent or independent mechanisms, we sought to block efferocytosis itself. As efferocytosis requires the specific recognition and engulfment of apoptotic cells, we predicted that inhibition of apoptosis by caspase inhibitors would prevent efferocytosis and abrogate the CFU reduction mediated by uninfected macrophages. Conversely, if bacterial control by uninfected macrophage co-culture were independent of apoptosis, caspase inhibition would have no effect on Mtb growth. As demonstrated previously, inhibition of the intrinsic apoptosis pathway (caspase 9-dependent), but not the extrinsic pathway (caspase-8 dependent), reduced efferocytosis of Mtb-infected macrophages by uninfected macrophages. Using the microscopy assay for efferocytosis we infected DiO-dyed macrophages with mCherry-H37Rv in the presence of caspase inhibitors. Four hours later uninfected macrophages were added. Sixteen hours later cells were fixed and inspected by confocal microscopy. The percent Mtb that had been efferocytosed of total Mtb in conditions in which caspase 3 and 9 were inhibited was significantly less than in the control treated conditions or caspase 8 inhibited condition (Figure 3-4A). Next we asked if caspase inhibition would likewise have an effect on CFU in the uninfected macrophage co-culture CFU assay. Caspase 3 and 9 inhibition abrogated CFU control observed following co-culture of infected and uninfected macrophages (Figure 3-4B). Caspase

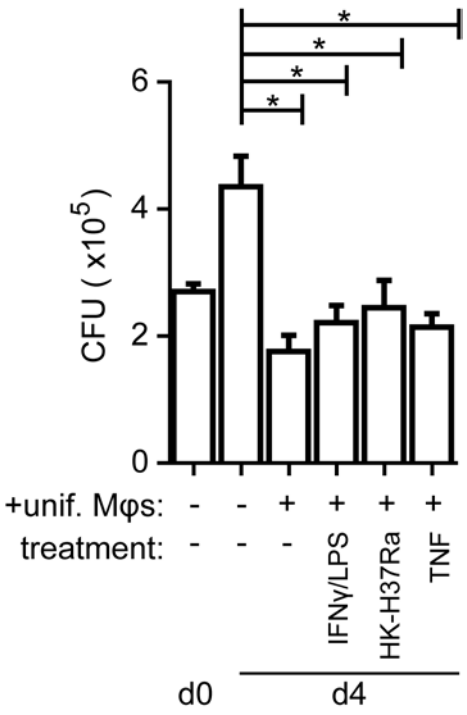

Figure 3-3. Macrophage activation confers no further antibacterial effect

Uninfected macrophages were pre-treated for 6 hours with IFNγ/LPS (10U/mL, 10ng/mL), heat-killed (HK) H37Ra sonicate (1μg/mL), TNFα (10ng/mL) or left untreated prior to addition to infected macrophages. Bacterial burden was ascertained at days 0 (d0) and 4 post infection. Data is representative of 3 experiments. Error bars ± SEM. *, $p \leq 0.05$ One-way ANOVA.

inhibition of infected macrophages, in the absence of uninfected macrophages had no effect on bacterial growth. Thus, apoptosis is a prerequisite for restricting Mtb replication in our macrophage co-culture model. As apoptosis is a prerequisite for efferocytosis, these results strongly suggest that efferocytosis is responsible for the control seen following uninfected macrophage co-culture.

Efferocytosis controls Mtb growth

Pro-inflammatory cytokines and immune mediators inhibit efferocytosis. TNF and PGE_2 are widely reported to inhibit efferocytosis. PGE_2 is known to inhibit efferocytosis in alveolar macrophages, thus we sought to inhibit efferocytosis in our infected macrophage co-culture system by this strategy[11]. First we tested whether PGE_2 was capable of inhibiting efferocytosis of apoptotic thymocytes in thioglycollate-elicited peritoneal macrophages (Figure 4-5A). PGE_2 treatment resulted in greater than 50% reduction in efferocytosis, with only a minor impact on phagocytosis of heat killed *E.coli*. Next we found PGE_2 inhibited efferocytosis of Mtb-infected apoptotic macrophages in a dose-dependent manner (Figure 4-5B). The inhibitory effect of PGE_2 is thought to be mediated by increasing intracellular cAMP, a crucial regulator of engulfment by phagocytes[12]. Thus, we showed that forskolin, a compound that causes an increase in intracellular cAMP, also inhibited efferocytosis. This translated to a dose-dependent loss of control in the uninfected macrophage co-culture model (Figure 4-5C). Uninfected macrophages that were pre-treated with PGE_2 became unable to limit bacterial growth. As the effects of efferocytosis inhibition caused by PGE_2 has been defined in alveolar macrophages we asked if PGE_2 treatment would inhibit uninfected alveolar macrophage co-culture control of Mtb growth. PGE_2 treatment of uninfected alveolar macrophages prevented their ability to restrict bacterial growth in vitro (Figure 4-5D). In several experiments co-culture of uninfected and

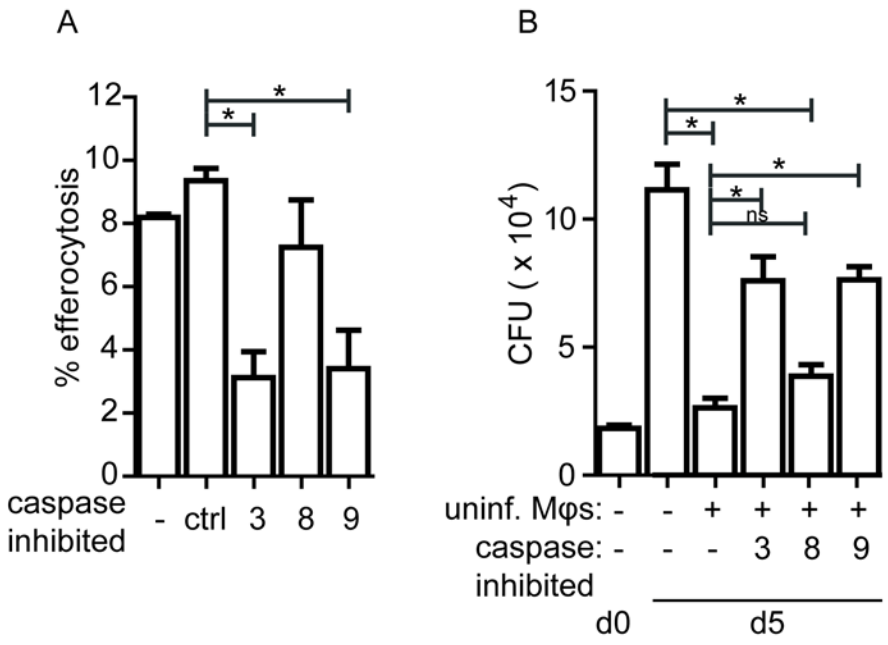

Figure 3-4. Apoptosis is required for uninfected macrophage control over Mtb growth

A. DiO-dyed macrophages were infected with mCherry-H37Rv for 4 hours before uninfected CD45.1 macrophages and the indicated caspase inhibitors (10μM) or control peptide (ctrl) was added. Cells were fixed and stained for CD45.1 16 hours later. Efferocytosis was enumerated as the percent of Mtb that had been efferocytosed of total Mtb. Data is representative of 3 experiments. B. Macrophages were infected with H37Rv as described in the presence of caspase inhibitors as indicated before addition of uninfected macrophages. Bacterial burden was determined at day 0 (d0) and 5. Data is representative of 4 experiments. Error bars ± SEM. *, $p \leq 0.05$; ns, not significant. One-way ANOVA.

infected macrophages significantly reduced the number of recovered bacteria to below the level of the initial inoculums, which is indicative of bacterial killing, a phenomenon observed in other studies[8]. Thus efferocytosis is a potent mechanism of Mtb growth suppression in alveolar macrophages.

To prove that efferocytosis was required for bacterial growth restriction, we sought to more specifically inhibit the engulfment process itself. Most macrophages express a variety of redundant receptors that mediate the recognition and uptake of apoptotic cells[13] and inhibition of any individual receptor yielded little or no inhibition of apoptotic thymocyte uptake by pMφs in a non-infectious model of efferocytosis (data not shown). An early event during apoptosis is phosphatidylserine (PS) flipping to the outer leaflet of the plasma membrane. PS is a ligand for many efferocytosis receptors and bridging molecules that aid in apoptotic cell recognition and engulfment[14,15]. Resident peritoneal macrophages rely primarily on the PS receptor TIM4 to recognize apoptotic cells and to mediate efferocytosis[16,17]. Blocking TIM4 in resident peritoneal macrophages significantly reduced efferocytosis of Mtb-infected apoptotic cells (Figure 3-6A). TIM4 blockade also significantly increased the incidence of secondarily infected cells (Figure 3-6A). This occurs presumably because the failure to clear apoptotic cells leads to more secondary necrosis and dispersal of intracellular contents including Mtb. Importantly, blocking TIM4 reduced the ability of uninfected resident peritoneal macrophages to restrict bacterial growth (Figure 3-6B). Thus engulfment of apoptotic cells specifically is responsible for the majority of the control exerted by uninfected macrophage co-culture. These data demonstrates for the first time that efferocytosis is an innate antibacterial mechanism. Apoptosis itself is not directly bactericidal as it has long been assumed, as engulfment is required for control. Additionally, in the absence of apoptotic cell clearance Mtb spread into surrounding uninfected macrophages thus

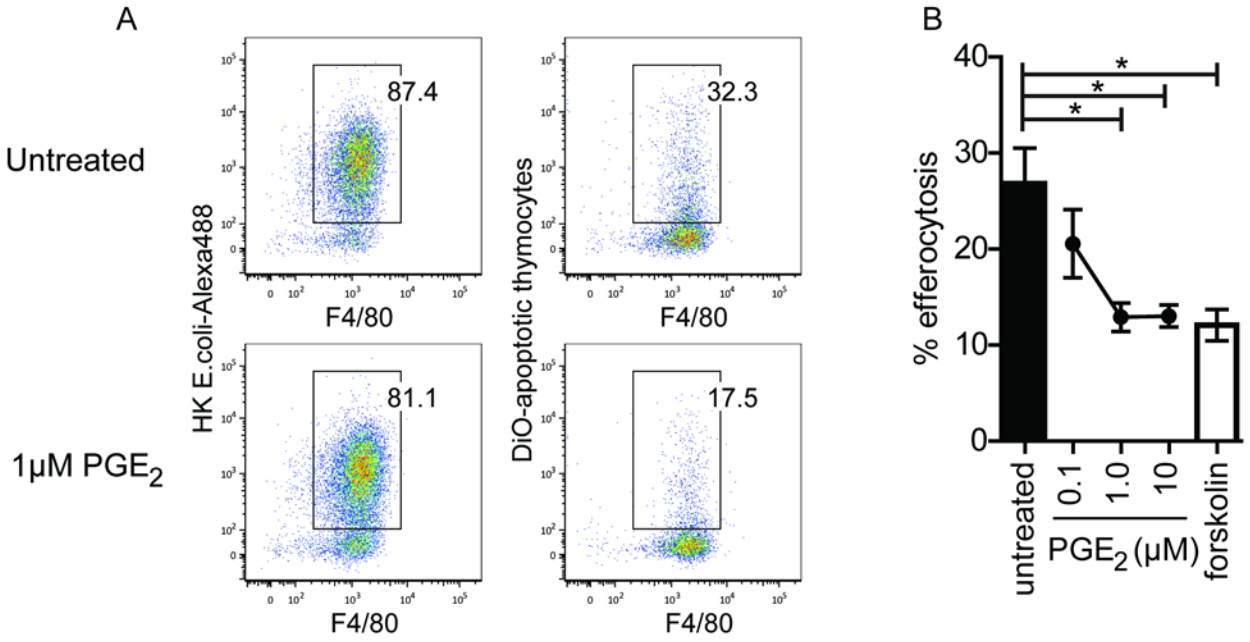

Figure 3-5. PGE_2 inhibits efferocytosis

A. Macrophages were treated with 1μM PGE_2 for 4 hours before addition of heat killed *E.coli* or DiO-dyed apoptotic thymocytes for 1 hour. Cells are gated as total cells, live cells, single cells, $F4/80^+$. Data is representative of 3 experiments. B. Uninfected Mφs were treated for 4 hours with indicated concentrations of PGE_2 or 100μM forskolin before addition to mCherry-H37Rv-infected Mφs. Efferocytosis was measured 16 as previously described. Data is representative of 3 experiments. Error bars ± SEM. *, $p \leq 0.05$ One-way ANOVA.

further broadcasting the infection. Thus, efferocytosis is an important innate immune mechanism of bacterial control and is one that can be used by alveolar macrophages to control Mtb growth or even mediate bacterial killing. Efferocytosis is a constitutive function of macrophages that cannot be primed or activated by cytokines that traditionally activate antibacterial effector functions in macrophages. This explains why we were unable to elicit more control in the infected macrophage co-culture system following IFNγ or TNF pre-treatment.

Discussion

It has long been assumed that apoptosis is capable of killing Mtb; however a mechanism for this control has never been provided. Additionally, other forms of cell death, such as necrosis hav no adverse effect on bacterial survival. In early studies the induction of apoptosis in BCG-infected macrophages lead to bacterial killing[5]. However in this study they reported efferocytosis of apoptotic BCG infected macrophages visualized by transmission electron microscopy. The investigators did not consider that efferocytosis could be contributing to control. Other reports induced apoptosis with Fas ligand and reported Mtb control[6]. In this study, a minority of macrophages following this treatment underwent apoptosis as determined by Annexin V staining. Thus uninfected macrophages could participate in control of Mtb. Previous reports indicated that addition of uninfected macrophages to Mtb-infected macrophages suppressed bacterial growth[7,8]. Based on our finding that apoptosis infected macrophages are engulfed by efferocytosis (Chapter 2), we hypothesized that efferocytosis might link apoptosis and bacterial killing, and explain the antibacterial effect of uninfected macrophages.

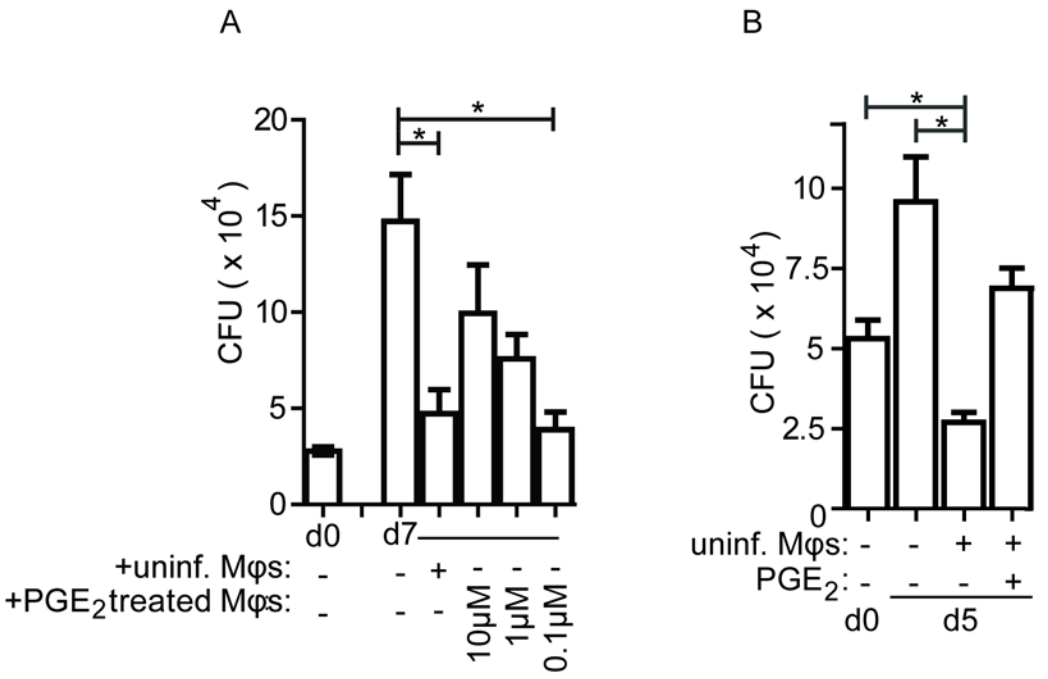

Figure 3-6. PGE_2 abrogates uninfected macrophage co-culture control of Mtb growth

A. Macrophages were infected with H37Rv before uninfected Mφs pre-treated with indicated concentrations of PGE_2 for 4 hours were added. CFU was determined at time 0 (d0) and day 7. Data is representative of 3 experiments. B. Alveolar macrophages (aMφs) were infected with H37Rv before addition of uninfected aMφs, treated with 1μM PGE_2 as indicated. CFU was determined at time 0 and day 5. Data is representative of 2 experiments. Error bars ± SEM. *, $p \leq 0.05$ One-way ANOVA.

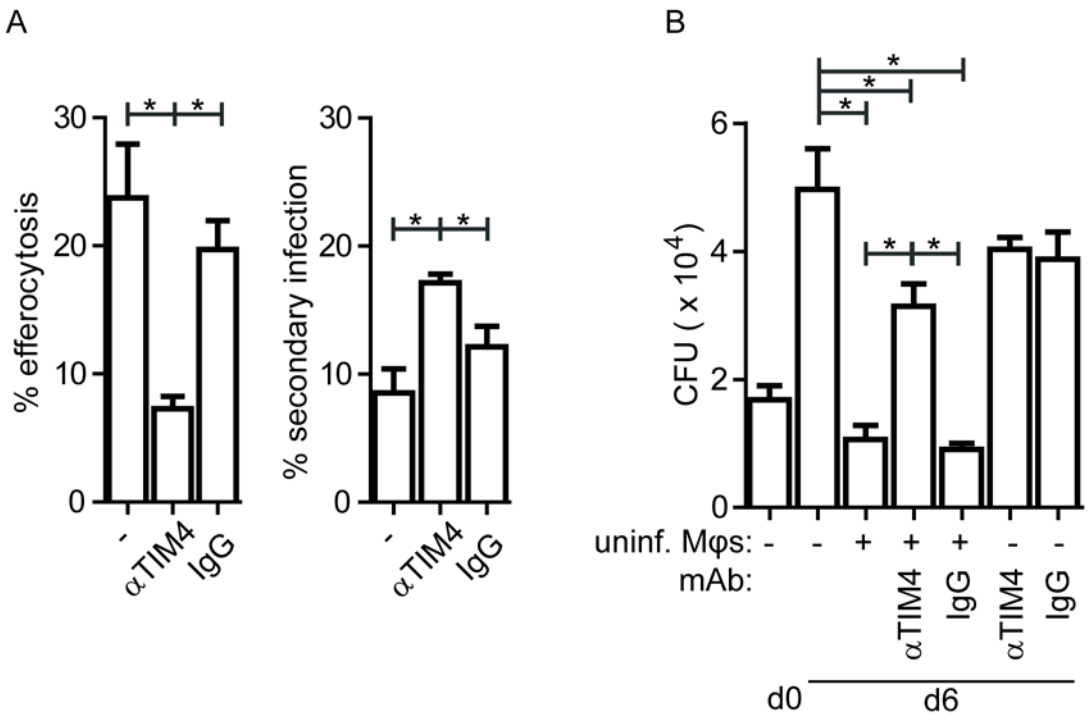

Figure 3-7. Efferocytosis controls Mtb growth

A. rMφs were infected according to the confocal microscopy assay in the presence of 10µg/mL TIM4 blocking antibody 5G3 or rat IgG1 and efferocytosis and secondary infection was enumerated as previously described. Data is representative of 3 experiments. B. rMφs were infected according to the efferocytosis assay in the presence of αTIM4 or IgG as indicated and CFU were plated in quadruplicate on days 0 and 6 post infection. Data is representative of 4 experiments. Error bars ± SEM. *, $p \leq 0.05$ One-way ANOVA.

We confirmed that the addition of uninfected macrophages in excess of Mtb-infected macrophages limits bacterial growth in vitro. We extended this observation by finding that control was uniformly observed across all macrophage types tested. We could not further enhance the antibacterial effector function of uninfected macrophages by priming and activation with stimuli such as IFNγ, LPS, or heat killed mycobacteria. This suggested to us that the antibacterial mechanism cannot be further induced, and thus might not belong to one of the known antibacterial pathways. However, this does not exclude the possibility of other untested stimuli boosting the effect. The amount of bacterial control following uninfected macrophage co-culture is similar to the level of control seen following infected macrophage treatment with IFNγ/LPS, suggesting that the effect of uninfected macrophage co-culture is just as potent as one of the paramount cytokines in protection from tuberculosis.

Our prior work implicated efferocytosis as a potential bacterial control mechanism. To confirm this, we sought to inhibit efferocytosis, which we did by targeting three mechanisms. First, inhibition of apoptosis limited efferocytosis, and also limited the ability of uninfected macrophages to control Mtb CFU in the co-culture system. Second, we inhibited efferocytosis via PGE_2 signaling. PGE_2 and other pro-inflammatory cytokines downregulate the efferocytic machinery. Specifically, high concentrations of PGE_2 cause an intracellular flood of cAMP, which inhibits the engulfment of apoptotic cells. While a non-specific effect, we found that PGE_2 limited efferocytosis, and efferocytosis-mediated control. Importantly, we demonstrated that alveolar macrophages are capable of controlling Mtb by efferocytosis. To definitively demonstrate that efferocytosis is required for control in the infected macrophage co-culture system we sought to inhibit efferocytosis specifically, which we chose to do by blocking a well-known efferocytosis receptor. TIM4. Inhibiting efferocytosis by blocking TIM4-PS interaction

abrogated control in the macrophage co-culture system, indicating that efferocytosis is a mechanism of Mtb control.

Inhibiting efferocytosis was difficult, as macrophages employ multiple receptors to mediate recognition and engulfment of apoptotic targets. Downstream of many efferocytosis receptors is Rac1, a small GTPase responsible for organizing the actin cytoskeleton during efferocytosis[18]. We attempted to target Rac1. Amiloride inhibits Rac1 activity by causing a pH increase proximal to the plasma membrane which blocks Rac1 activation[19]. In a FACS based assay we were able to inhibit macrophage efferocytosis of apoptotic thymocytes. However, amiloride is toxic over long periods, and thus we were unable to test its effects in a CFU assay. This was true of many inhibitors known to act on proteins crucial during efferocytosis. Rac1 knockdown with siRNA were never complete, potentially due to the high expression of Rac1 in most primary macrophages. Knockdown of Rac1 effectors such as Cdc42 was also unsuccessful. Thus we turned our attentions on inhibiting apoptotic cell recognition by blocking the 'efferocytosis receptors'. TIM4 was recently described as recognizing PS exposed on the surface of dying cells[16]. Using a TIM4 blocking antibody previously shown[20] to inhibit efferocytosis we demonstrated that uninfected macrophages were indeed engulfing Mtb-infected apoptotic cells. Furthermore, inhibition of efferocytosis increased secondary infection as apoptotic cells proceeded to a secondary necrotic state, spilling their intracellular contents and allowing for bacterial spread.

These data fundamentally change our view of apoptosis during intracellular infection. This is the first report of efferocytosis acting an antimicrobial effector mechanism. Our work suggests that any beneficial effect of apoptosis on host resistance to infection may ultimately be mediated by efferocytosis. This is not just limited to innate control of microbial infection. This

report has focused on the role of macrophage apoptosis as an innate response following Mtb infection. However, the killing of infected cells is an important effector mechanism of the adaptive immune response to tuberculosis[21]. $CD8^+$ T cells can induce death in target cells via the release of cytotoxic granules containing granzymes or through the engagement of surface receptors containing death domains such as CD95/95L[22,23]. Both of these pathways lead to apoptotic death in the target cells. We envision that the capacity of cytotoxic T lymphocytes (CTL) to kill infected cells restricts bacterial growth indirectly since the induction of apoptosis in the infected cells will facilitate their engulfment by uninfected macrophages recruited to inflammatory foci.

Our conclusions force one to consider whether efferocytosis is a mechanism that can be exploited or subverted by pathogens to evade host immunity during their life cycle. For example, Leishmania employ neutrophil apoptosis and subsequent efferocytosis as a mechanism of establishing infection within macrophages[24,25]. Indeed, some pathogens might rely on uptake of infected dying cells to spread the infection, as is the case in *M. marinum* infection of zebrafish embryos[26]. Efferocytosis may control or exacerbate many infectious diseases and it will be important to explore how macrophages contribute to microbial immunity in this way.

There are a few reports suggesting that dead cell debris hinders bactericidal mechanisms in macrophages. During *Francisella* infection dead cell debris stimulates arginase activity in macrophages which impairs bacterial clearance while inducing an alternatively activated "M2" phenotype[27]. In alternatively activated macrophages iNOS is downregulated in favor of arginase activity. Arginase activity is not damaging to Mtb[28]. Thus we do not believe that an alternative activation state is contributing to bacterial control in this system and we did not pursue this line of inquiry further. In another model, efferocytosis of apoptotic cells impaired macrophage-

mediated killing of *Streptococcus pneumoniae*, by perturbing inflammatory cytokine production[29]. Mtb and *S. pneumoniae* killing must proceed via different mechanisms. This is potentially due to the very different lifestyles of these two pathogens. *S. pneumoniae* is not an intracellular pathogen, and it can be killed by phagocytosis and other antibacterial effector functions of the macrophage that might be downregulated by efferocytosis[30,31].

Recently, mechanisms for increasing macrophage efferocytic ability have been described. If these mechanisms could be harnessed to increase the efficiency of efferocytosis in vivo during active tuberculosis both the spread of infection and inflammation-induced damage could both be quelled. Engulfing such a large target causes great stress to the efferocytic macrophage. Maintenance of the mitochondrial membrane potential is tied to efficient efferocytosis[32]. Perhaps modulating the mitochondrial membrane potential could enhance macrophage's efferocytic capacity that might otherwise be overwhelmed during disease. Lovastatin has been shown enhance efferocytosis as well[33]. Lovastatin inhibits RhoA, which negatively regulates Rac1[34,35]. The potential contribution of statins during active tuberculosis disease is unexplored. However, statin use does protect against influenza, an infection that is killed by efferocytosis[36-38]. Thus our work further demonstrates that how surrounding cells recognize and react to the death of an infected cell can modulate the outcome of infection. Efferocytosis emerges as a novel mechanism of microbial control.

Methods

Mice and macrophage isolation

C57Bl/6 (CD45.2) and B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1) mice were purchased from Jackson Laboratories (Bar Harbor USA), and were kept and bread using standard humane animal husbandry protocols. Mtb-infected mice were housed under BSL3 conditions. Thioglycollate-elicited peritoneal Mφs (pMφs) were lavaged from peritoneal cavities 4-5 days following 3% IP thioglycollate injection. Mφs were isolated using CD11b microbeads and magnetic columns (Miltenyi Biotec) and cultured in 24 or 96 well dishes overnight prior to infection. Resident peritoneal Mφs (rMφs) were lavaged from peritoneal cavities and selected based on CD11b^{+}CD19^{-}. Alveolar Mφs (aMφs) were lavaged from the lungs of mice with PBS.

Mtb culture and infection

mCherry-H37Rv was cultured with 0.5ug/mL Hygromycin B. Mtb infection was conducted as previously described[39] with the following modifications. Mφs were grown in 24- or 96-well plates and infected at a multiplicity of infection (MOI) of 10:1 for 4 hours. Cultures were then washed 3-5 times. Efferocytic Mφs were added at 2x the number of infected Mφs. As indicated efferocytic Mφs were pretreated with 10μM PGE_2 for 4 hours at 37^{o}C before washing and addition to infected Mφs. Caspase inhibitors (Calbiochem) (10μM), αTIM4 (10μg/mL- kind gift of Vijay Kuchroo, Harvard Medical School) or rat IgG1 (10μg/mL) were added following infection. CFU were determined immediately following infection and at indicated timepoints per condition in quadruplicate by removing culture supernatant, lysing Mφs with 1% TritonX/PBS solution, plating serial dilutions on 7H11 plates and incubating at 37^{o}C/5% CO_2 for 3 weeks. All Mtb strains were confirmed by thin layer chromatography to be PDIM^{+}.

FACS efferocytosis assay

Apoptotic thymocytes were generated by dying thymocytes with 1μM CFSE or 1:1000 Vybrant DiO (Invitrogen) prior to apoptosis induction with staurosporine for 6-16 hours. Thymocytes were then co-cultured with macrophages at a ratio of 5:1 for 1 hour in 24 well plate before washing, staining with antibodies directed against F4/80, fixation and analysis on a BD FACS CANTO II and FlowJo software. Heat killed *E.coli* Bioparticles (Invitrogen) were added as a control.

Confocal microscopy efferocytosis assay

Mφs from CD45.2 mice were cultured in 24 well dishes in triplicate per condition upon coverglass and dyed with Vybrant DiO as per manufacturer's specifications prior to infection with mCherry-H37Rv or GFP-H37Rv for 4 hours. Uninternalized bacteria were washed away and uninfected CD45.1 Mφs were added at 2x the number of infected Mφs. Treatment conditions were added as appropriate. 16-24 hours later cells were washed and stained with mouse monoclonal CD45.1 (Abcam) before affixing to microscopy slides with Prolong Gold plus DAPI (Invitrogen). Conditions were analyzed in triplicate and for each replicate in each condition 100-300 Mtb were counted. Images were taken using a Nikon TE2000-U inverted microscopy equipped with C1 Plus confocal laser scanner.

Image Analysis

Microscopy images were analyzed using Image J[40] and colocalization analysis macros or user-generated pipelines in Cell Profiler[41]. Investigators were blinded during analysis.

Statistical Analysis

Statistical analysis and graphical output was done with GraphPad Prism. One-way ANOVA with Dunnet's post-test. * = $p \leq 0.05$

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Chapter 4: Efferocytosis kills *Mycobacterium tuberculosis* by phagosome maturation

Parts of this chapter appears as the submitted manuscript:
"Efferocytosis is an innate antibacterial mechanism"
Constance J. Martin, Matthew G. Booty, Tracy R. Rosebrock, Cláudio Nunes-Alves, Danielle M. Desjardins, Iris Keren, Sarah M. Fortune, Heinz G. Remold and Samuel M. Behar

Abstract

Mycobacterium tuberculosis (Mtb) subsists within macrophages in an arrested phagosome to secure a protective niche for its survival and growth. To disseminate the infection Mtb induces the cell death of its host macrophage; however apoptotic death is associated with decreased bacterial survival. We previously reported that apoptosis is not intrinsically bactericidal, but that efferocytosis of Mtb-infected apoptotic macrophages by uninfected macrophages is responsible for suppressing bacterial growth. Efferocytosis, the engulfment of apoptotic cells, results in the destruction of the ingested target via phagosome maturation. We report here that Mtb engulfed within apoptotic cells are incapable of inhibiting phagosome maturation and lysosome fusion. Thus, following efferocytosis we find that Mtb is killed. We present here efferocytosis as a novel bactericidal mechanism.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis responsible for nearly 2 million deaths world-wide, is a facultative intracellular pathogen that inhibits phagosome maturation in the host macrophage. Typically, after ingesting a bacterium, foreign particle or apoptotic cell, the macrophage encases the target within a phagosome that successively 'matures' by fusing with, and acquiring characteristics of the lysosome. This maturation process results in a phagolysosome competent to destroy the engulfed cargo; it is acidic and contains microbicidal and hydrolytic enzymes. The macrophage is ultimately responsible for bacteria and debris removal, yet *Mycobacterium tuberculosis* (Mtb) exploits the intracellular environment for its protection and survival. From within the arrested phagosome Mtb replicates and eventually causes the death of its host macrophage as the bacteria spread to new macrophage hosts.

Mtb possesses several virulence factors that have been described as crucial for inhibiting phagosome maturation. For one, mannosylated lipoarabinomannan (ManLAM), a lipid present on Mtb's surface that is chemically similar to phosphatidyl-inositols (PI), such as PI(3)P, which is required for Early Endosome Antigen 1 protein (EEA1) recruitment and phagosome maturation [1]. ManLAM intercalates into host membrane and inhibits the PI(3)P kinase Vps34 on the phagosome membrane from depositing PI(3)P on the membrane and thus blocking EEA1 recruitment [2, 3]. SapM a lipid phosphatase, works by cleaving PI(3)P on the phagosome membrane, which similarly prevents EEA1 deposition on the membrane [4]. Trehalose dimycolate (TDM, cord factor) is a crucial cell wall lipid in Mtb that inhibits phagosome maturation [5]. TDM suppresses phagosome maturation through an unknown mechanism, although reactive nitrogen and oxygen species produced by activated macrophages can dismantle TDM and its functionality. The failure of lysosome and endosome fusion prevents the delivery of catabolic enzymes and the vacuolar ATPase (vATPase) responsible for acidifying lysosomal compartments [6]. The Mtb-containing phagosome characteristically is stuck in an altered early endosome state- decorated with GTPases of the Rab family, specifically Rab5, lacking Rab7, near neutral in pH, showing little to no Lysosomal-associated membrane protein 1 (LAMP1) and cathepsin recruitment, but accessible to the recycling endocytic pathway that delivers transferrin complexed to iron to the bacilli [7-10]. Clearly phagosome maturation arrest is a crucial aspect of Mtb's virulence strategy as the bacteria employs many methods to halt phagosome maturation and still more await discovery. Mtb is not resistant to macrophage-mediated killing once delivered to the lysosome. In fact, IFNγ treatment induces phagolysosome maturation and Mtb killing [11]. Thus, while the bacteria are uniquely capable of halting phagosome maturation, the macrophage is not entirely without defenses.

Apoptosis has been described previously to suppress Mtb growth, both in vivo and in vitro [12, 13]. While it was long assumed that apoptosis is intrinsically bactericidal, we have shown that this is not the case. The bactericidal effect of apoptosis is exclusively mediated by efferocytosis, the process of apoptotic cell engulfment by macrophages. We found that Mtb-containing apoptotic blebs are engulfed by uninfected macrophages and that this process leads to a suppression of Mtb growth in vitro. Efferocytosis is a remarkably fast process, as is the acidification of the efferocytic phagosome, the compartment that contains the engulfed apoptotic cell [14]. Efferocytosis leads to the efficient destruction of the internalized cell. This process must be fast, as macrophages may encounter several dead and dying cells in short succession, especially during development and the resolution of infection [15]. Although the receptors mediating apoptotic cell recognition are different than phagocytic receptors, once internalized, the basic machinery is the same [16]. Rab proteins are successively recruited, ensuring the fusion of lysosomes bearing degradative enzymes and ER-derived vesicles harboring the vATPase, responsible for acidification and activation of hydrolytic enzymes.

In this chapter we further define the antibacterial properties of efferocytosis. We report that the efferocytic phagosome containing Mtb is distinct from the traditional Mtb-containing phagosome. This structure is efficiently delivered to acidic compartments and acquires lysosomal markers. We find that at the single cell level following efferocytosis Mtb is killed within lysosomes. Thus we report that efferocytosis is not just capable of bacterial growth suppression, but is a novel bactericidal mechanism.

Results

Mtb and cell debris are found within vacuous phagosomes following efferocytosis

Given that efferocytosis of an Mtb-infected apoptotic cell exerts significant control over Mtb growth, we sought to understand what is unique about efferocytosis that could restrict bacterial growth. We asked whether the intracellular compartments containing Mtb differed following internalization by phagocytosis versus efferocytosis. Using transmission electron microscopy (TEM) we confirmed that one day post-infection, Mtb was contained behind a tight-fitting phagosome membrane closely juxtaposed to the bacterial membrane, as has been previously seen [17] (Figure 4-1A). By electron microscopy Mtb within macrophages have a characteristic bright white electron lucent zone surrounding the dark and electron dense bacterial body. This is a property of the bacteria's highly lipidic cell wall that does not embed optimally in the epoxy resin. The electron lucent zone does not represent 'empty space' and is an artifact of the procedure. Mtb were also intact and showed no sign of digestion. Following efferocytosis, apoptotic thymocytes within uninfected macrophages occupied very different phagosomes (Figure 4-1B). Characteristically, the efferocytic phagosome is vacuous, with fluid filling the space between apoptotic cell membrane and phagosome membrane[18]. This fluid-filled space is not the same as the electron lucent zone that surrounds the mycobacterial body. For one, the space is not bright white, indicating that the resin properly infiltrated the space. It shows the same electron density as the resin itself, as indicated by the extracellular space between macrophages in the resin. Other techniques besides electron microscopy have described a vacuous efferocytic phagosome as well[19]. Thymocytes, as well as apoptotic macrophages were found in various states of digestion and destruction of both organelle and plasma membranes (Figure 4-1B,C). Note that the cytosol for both the apoptotic thymocyte and macrophage bleb closest to the plasma membrane (PM) resembles the cytosol of the efferocytic macrophage, as these blebs are most likely newly engulfed and have yet to experience lysosomal fusion and

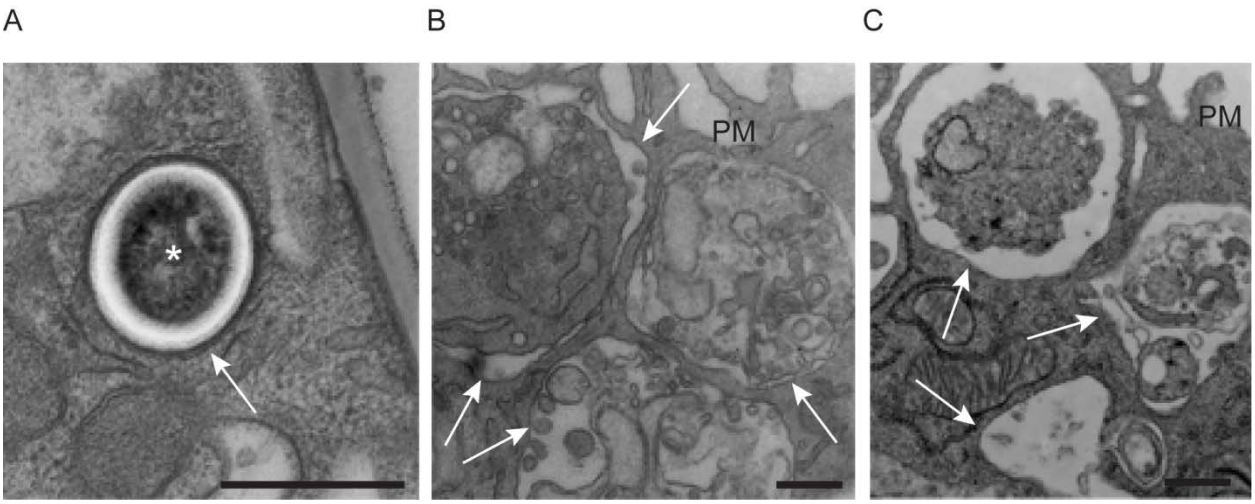

Figure 4-1. The Mtb phagosome is distinct from the efferocytic phagosome
A. Transmission electron micrograph of Mtb within a primary infected thioglycolate-elicited peritoneal macrophage (pMφ) stained with lead citrate. B. Apoptotic thymocytes co-cultured with pMφs for 1 hour prior to fixation. Note the three thymocyte apoptotic blebs in increasing stages of digestion and disintegration. C. Mtb infected pMφs co-cultured with uninfected pMφs 16 hours. Apoptotic pMφ blebs within uninfected pM. Asterisks, Mtb; arrows, phagosome membrane; PM, plasma membrane; scale bar, 1μM. Images are representative of >1000 macrophages over many non-serial 60nm sections and 2 independent experiments.

degradation. The blebs further inside the macrophages show increasing levels of digestion; both their cytosol and intracellular membranes show progressive levels of destruction and disintegration.

After overnight co-culture of Mtb-infected and uninfected macrophages, Mtb was found within efferocytosed apoptotic blebs encased within a spacious phagosome (Figure 4-2A). This spacious phagosome was very reminiscent of the efferocytic phagosomes containing apoptotic thymocytes and macrophage blebs. Note that the characteristic electron lucent zone surrounding the bacteria (two are visible, viewed head-on) is intact, indicating that the bacteria are still viable. It was difficult to determine if a phagosome membrane surrounds the Mtb within the apoptotic bleb. As the subcellular location of Mtb just prior to cell death is unknown, we chose to not investigate this observation further. Additional membranes and intact cellular components were discerned within these apoptotic blebs along with Mtb, indicating an early stage of digestion (Figure 4-2A). Thus, the efferocytic Mtb phagosome is structurally distinct from a bacterium within a primary infected macrophage. Mtb is taken up within an apoptotic bleb, and not simply amongst dead cell debris. Damaged Mtb with characteristic 'onion' morphology were also observed within vacuous phagosomes along with cellular debris, possibly indicating killed bacteria following efferocytosis[20] (Figure 4-2B). Damaged Mtb also lack the electron lucent zone characteristic of their lipidic coat, further suggesting that these bacteria are not viable. Finding damaged Mtb in the efferocytic condition was common, but never seen in the primary infected macrophage condition. This raised the possibility that bacteria efferocytosed within apoptotic macrophage blebs are processed differently than phagocytosed bacteria. In fact, damaged bacteria with the 'onion' morphology have a strong correlation with lysosomes[20]. This

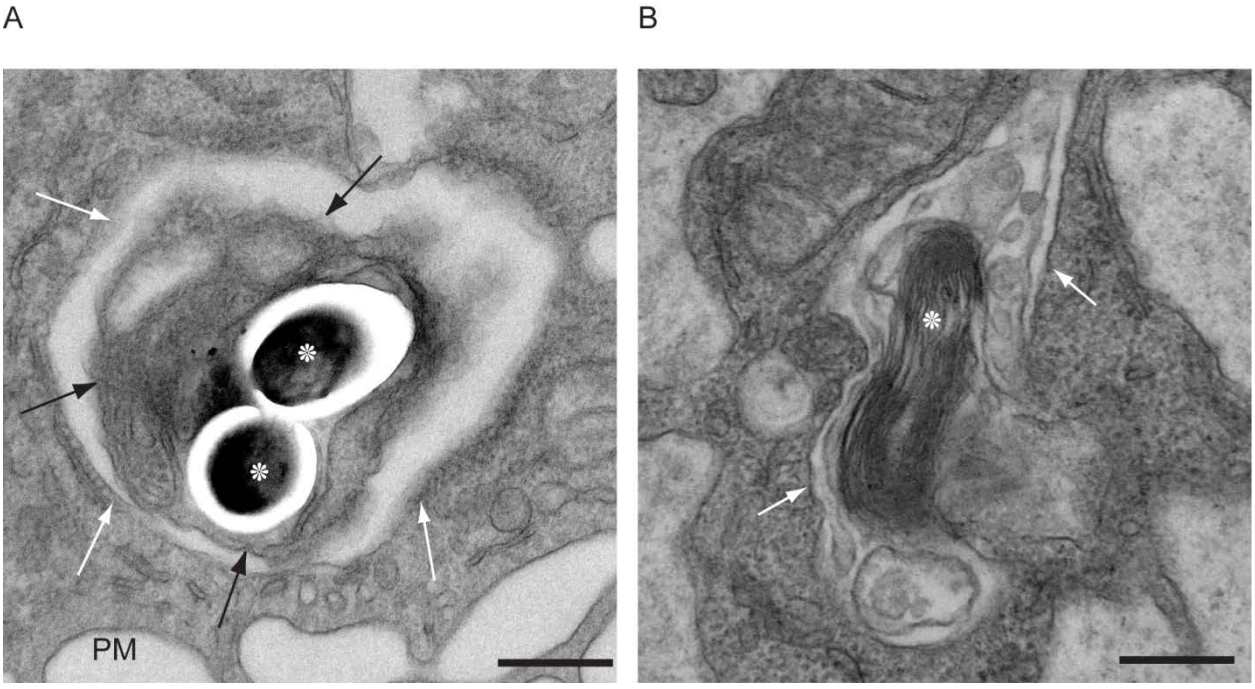

Figure 4-2. Mtb are found within vacuous efferocytic phagosomes along with cell debris following uninfected macrophage co-culture

A**.** Mtb within vacuous efferocytic phagosomes along with apoptotic bleb after the co-culture of uninfected thioglycollate-elicited peritoneal macrophages (pMφs) with infected pMφs. Note cellular structures still visible within apoptotic bleb. B. Mtb showing characteristic degraded phenotype following efferocytosis. Note the vacuous phagosome containing cell debris. Asterisks, Mtb; white arrows, efferocytic phagosome membrane; black arrows, apoptotic bleb membrane; PM, plasma membrane; scale bar, 1µM. Images are representative of >1000 macrophages over many non-serial 60nm sections and 2 independent experiments.

suggests that efferocytosis of Mtb containing blebs fuse with lysosomes more readily than Mtb phagosomes.

The Mtb-efferocytic phagosome matures and acquires lysosomal characteristics

Mtb possesses virulence factors that subvert phagosome maturation and allow it to exploit the intracellular environment of the macrophage. It is not completely understood how Mtb delivers its virulence factors to their host targets, both for the suppression of phagosome maturation and cell death induction. Even so, we reasoned that the uptake of Mtb contained within an apoptotic cell further compartmentalizes the bacteria and limits its ability to interfere with phagosome maturation. To this end we measured the co-localization of Mtb with markers of mature lysosomes following efferocytosis (Figure 4-3A). Bone marrow-derived macrophages were infected with mCherry-H37Rv and then co-cultured with uninfected CD45.1 macrophages. Sixteen to twenty hours later the cells were fixed and stained with antibodies to LAMP1, a marker of lysosomes, as well as CD45.1 to differentiate the primary infected macrophages from the uninfected added macrophages. In this assay efferocytosis cannot be distinguished from secondary infection, as our confocal microscope is limited to three colors. In all previous experiments (Chapters 2 & 3) efferocytosis outnumbers secondary infection 2-3:1, making it the dominant phenotype of Mtb within the CD45.1 macrophage population. Also, prior experience found that inhibiting efferocytosis by inhibiting apoptosis (caspase 3 inhibitor) and PGE_2 pre-treatment severely limited the amount of efferocytosis without significantly impacting secondary infection. Thus it is important to compare the amount of Mtb-lysosome co-localization in pro-efferocytosis conditions and efferocytosis inhibitory conditions. Using this assay we found more Mtb within efferocytic (CD45.1) bone marrow-derived macrophages co-localized with LAMP1 than in primary infected macrophages (Figure 4-3B). Inhibiting apoptosis (with a caspase 3

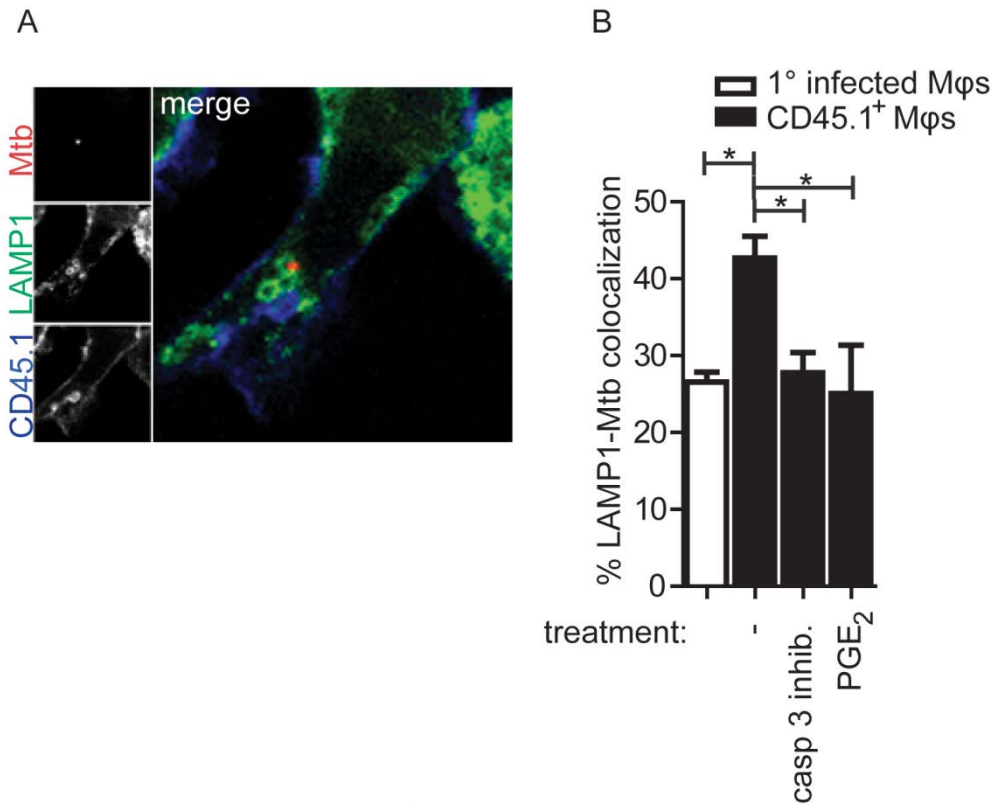

Figure 4-3. Mtb are present in LAMP1$^+$ compartments following efferocytosis
A. Mtb within a LAMP1+ vesicle. Mtb (red), LAMP1 (green), CD45.1 (far red/pseudocolor blue) and merge. Bone marrow-derived macrophages (BMDMφs) were infected with mCherry-H37Rv for 4 hours prior to uninfected CD45.1+ BMDMφ addition. 24 hours later cells were fixed and stained for LAMP1 and CD45.1 and visualized by confocal microscopy. B. Quantification of (A). Percentage of Mtb co-localizing with LAMP1 of total Mtb counted in either primary infected BMDMφs alone (clear bar) or in CD45.1+ BMDMs (filled bars) following co-culture. Caspase inhibitor (10μM) added at time of co-culture. Uninfected macrophages pre-treated with PGE_2 (10μM) for 4 hours prior to addition to infected BMDMφs. 100-200 Mtb were counted per condition in triplicate. Data is representative of 4 experiments. Error bars ± SEM, *, $p \leq 0.05$, One-way ANOVA with Dunnet's post-test.

inhibitor) or efferocytosis (with PGE_2) both reduced the Mtb-LAMP1 co-localization back to levels characteristic of primary infection.

The vacuolar ATPase is responsible for maintaining the acidic pH of the lysosomal compartment. Acidification limits bacterial growth, but more importantly activates degradative lysosomal proteins responsible for dismantling internalized bacteria and apoptotic cells alike. Mtb specifically exclude the vATPase from its phagosome to prevent acidification and lysosomal protein activation[6]. Following efferocytosis, there was greater Mtb-vATPase co-localization in efferocytic (CD45.1) macrophages than in primary infected macrophages (Figures 4-4A,B). Ultimately, low pH marks a functional, mature lysosome. Following efferocytosis more Mtb co-localize with LAMP1 and vATPase; therefore, we reasoned that Mtb would also be present within acidic vesicles as well. Thioglycollate-elicited peritoneal macrophages were infected with GFP-H37Rv for four hours prior to uninfected macrophage addition. The following day cells were incubated for two hours with Lysotracker Red, a cell permeable compound that is preferentially sequestered within acidic compartments. The Mtb-containing phagosome limits acidification; however, more Mtb were detected within acidic compartments following efferocytosis as determined by Mtb-Lysotracker co-localization (Figures 4-5A,B). We could not determine whether Mtb-Lysotracker co-localization occurred more frequently in efferocytic (CD45.1) macrophages compared to primary infected macrophages because antibody staining of Lysotracker-Red-labeled cells is not technically possible. Instead, we quantified the difference in Mtb-Lysotracker co-localization between primary infected macrophages verses infected macrophages with uninfected macrophages co-cultured (which favors efferocytosis). Many bacteria were within large acidified vesicles (arrow), reminiscent of the vacuous efferocytic phagosomes observed by confocal and electron microscopy. Blocking efferocytosis reduced the

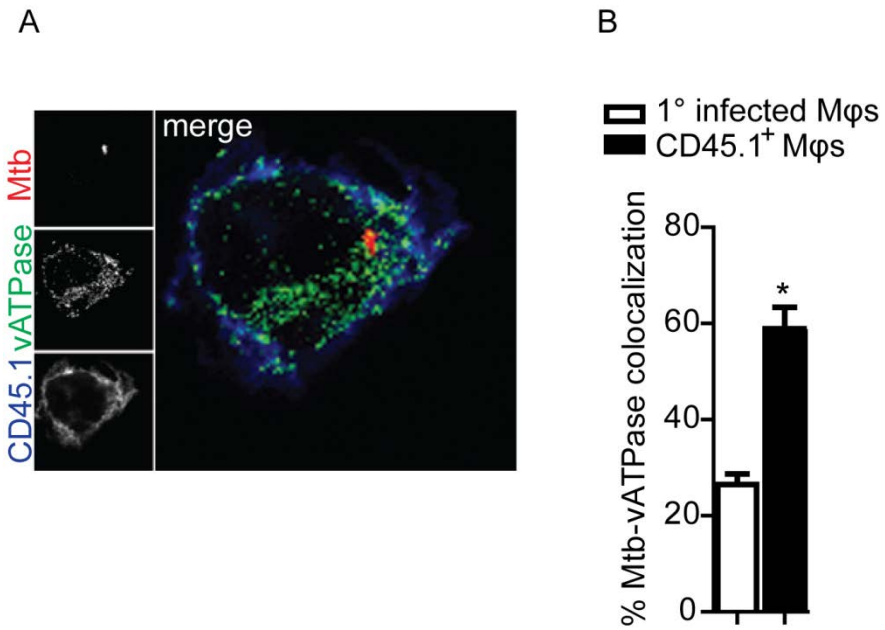

Figure 4-4. Mtb co-localizes with vacuolar ATPase following efferocytosis

A. Mtb co-staining with ATP6v1B2 subunit of the vATPase in bone marrow-derived macrophages (BMDMφs) following uninfected Mφ (CD45.1) co-culture with infected Mφ. Mtb (red), vATPase (green), CD45.1 (far red/pseudocolor blue) and merge. Co-localization was determined using co-localization analysis scripts in ImageJ. B. Quantification of (A). Percent Mtb-vATPase co-localization of total Mtb within either primary infected BMDMφs (clear bar) or $CD45.1^+$ BMDMφs (filled bar). 100 Mtb were counted per Mφ type in triplicate. Data is representative of 2 experiments. Error bars ± SEM, $p \leq 0.05$, Student's t-test.

bacteria's co-localization with acidic compartments to levels seen in primary infection. Treatment with bafilomycin, an inhibitor of the vATPase severly limited Mtb-Lysotracker co-localization and disrupted Lysotracker^{+}Mtb^{-} compartments, indicating that our staining was specific for acidified compartments. The fusion of lysosomes with the efferocytic phagosome delivers catabolic enzymes capable of destroying the bacterium. These data demonstrate that Mtb is unable to inhibit maturation of the efferocytic phagosome, exposing the bacterium to the caustic environment of degradative lysosomes.

Components of the autophagic machinery have been implicated in facilitating phagosome maturation, efferocytic phagosome maturation and Mtb control[21-23]. However, no specific enrichment of LC3 (Microtubule-associated proteins 1A/1B light chain 3/Atg8), an early marker of autophagosomes, was found on Mtb-containing phagosomes following efferocytosis (Figures 4-6A,B). Additionally, contrary to Martinez et al[22], LC3 was not found recruited to the efferocytic phagosome containing apoptotic thymocytes, ruling out autophagic machinery involvement in this model of efferocytosis as well (Figure 4-6C). Rapamycin was used as a positive control for Mtb-LC3 co-localization. Rapamycin inhibits mTOR, a complex that sits at the top of nutrient sensing within cells, and is well known to induce robust autophagy, did increase LC3 co-localization with Mtb, as did overnight serum starvation. These data suggest that autophagy plays no special role in efferocytosis of Mtb-containing apoptotic blebs.

Efferocytosis kills Mtb on a single-cell level

Phagosome maturation is capable of killing Mtb, thus we hypothesized that efferocytosis of Mtb-containing apoptotic cells does not just suppress bacterial growth, but destroys the bacteria. To determine the effect of efferocytosis on bacterial viability at the single cell level,

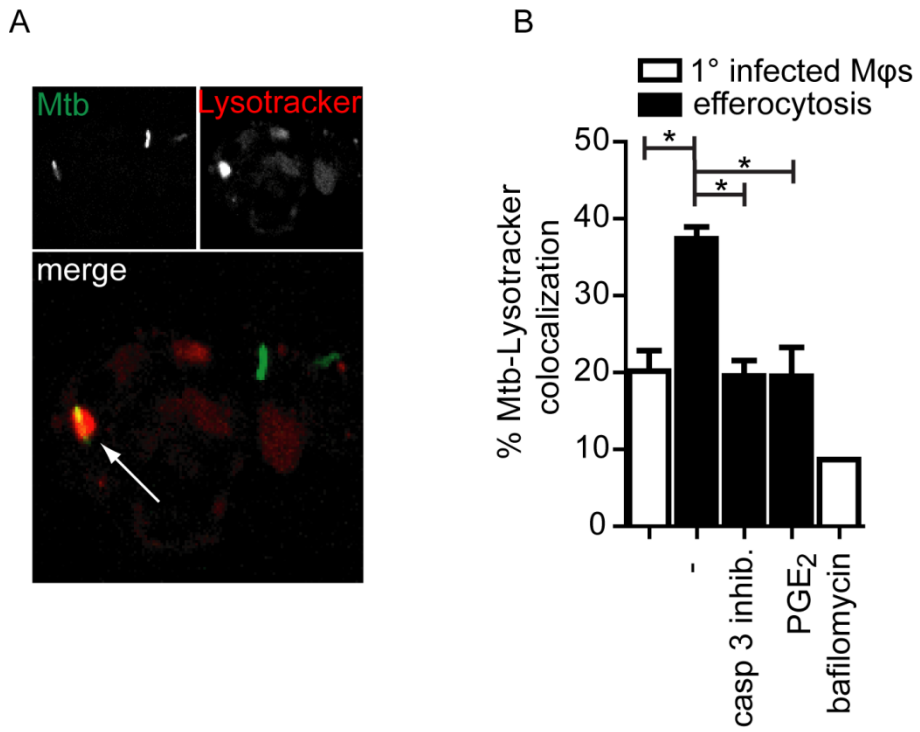

Figure 4-5. Mtb co-localizes with acidic compartments following efferocytosis

A. Mtb within an acidic compartment (arrow) in thioglycollate-elicited peritoneal macrophages (pMφs). Mtb (green), Lysotracker (red) and merge. B. Percent Mtb within acidic compartments of total Mtb counted per condition in pMφs. Efferocytosis was inhibited with 10uM caspase 3 inhibitor or uninfected macrophage PGE_2 pre-treatment. Bafilomycin (1μM) was added for 2 hours before and during the assay. 150 Mtb were counted per condition in triplicate. Data is representative of 2 experiments. Error bars ± SEM, $p \leq 0.05$, One-way ANOVA with Dunnet's post-test.

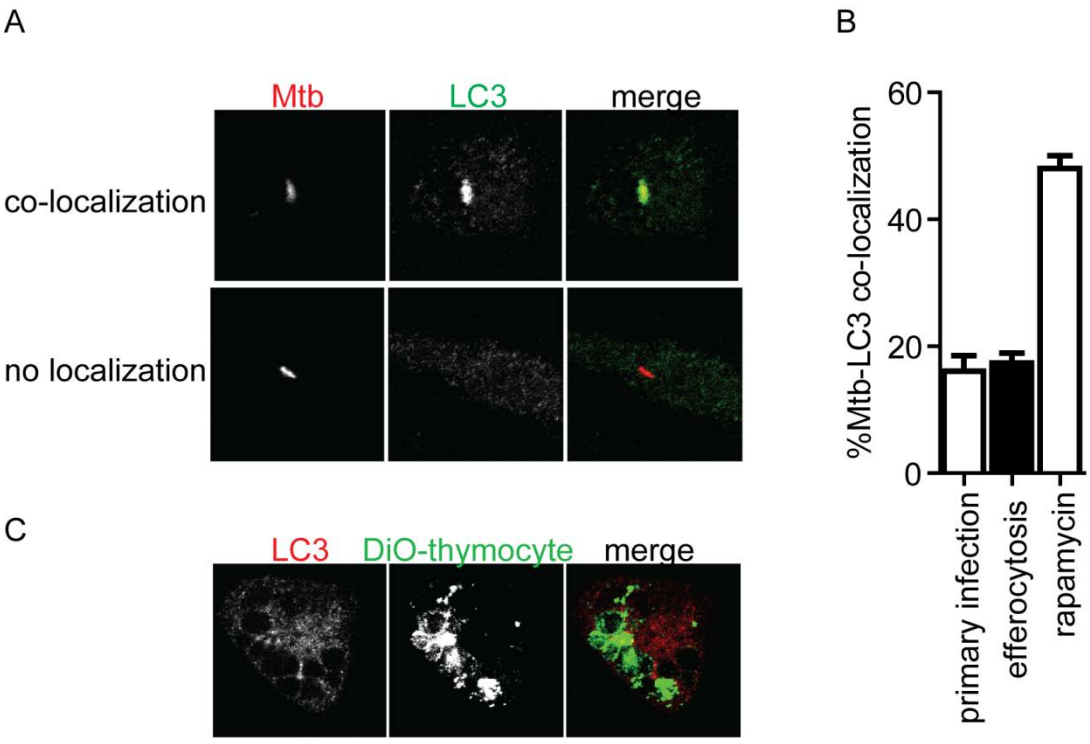

Figure 4-6. LC3 is not recruited to the Mtb-containing efferocytic phagosome

A. Co-localization and no co-localization of Mtb with LC3 in thioglycollate-elicited peritoneal macrophages (pMφs). Mtb (red), LC3 (green) and merge. B. Percentage of Mtb co-localizing with LC3 of total Mtb per macrophage type: primary infected macrophage, clear bar; CD45.1 macrophages, filled bar. Uninfected CD45.1 pMφs were added to infected pMφs and 16 hours later stained for LC3. Rapamycin (50μg/mL) was added for 4 hours prior to induce autophagy. 300 Mtb were counted per condition in triplicate. Data is representative of 2 experiments.

we infected macrophages with H37Rv that constitutively express mCherry (red) and inducibly express GFP (green) using a Tet^{ON} (tetracycline-inducible) promoter. After tetracycline induction 'live' bacteria expressed both red and green fluorescent proteins while bacteria that are dead only fluoresced red (Figure 4-7A). We confirmed that changes in viability observed using the live/dead reporter Mtb correlate with changes in CFU (Figure 4-7B). Bacteria in macrophage culture treated with rifampin caused a 20% reduction in both CFU and viability as determined by the live/dead reporter and microscopy. Thus live/dead Mtb are a valuable tool for assessing the viability of Mtb on a single cell level. However, bacterial killing is accompanied with digestion of the bacterium and the mCherry protein. With time there is a loss of signal, thus while the live/dead reporter remains a valuable tool for determining the status of Mtb within macrophages at a snapshot in time, it cannot account for any past bacterial killing.

Thioglycollate-elicited peritoneal macrophages were infected with live/dead reporter Mtb prior to addition of uninfected CD45.1 congenic peritoneal macrophages. Twenty hours later tetracycline was added to induce the GFP protein expression. Cells were fixed and analyzed by confocal microscopy twenty four hours later, approximately 48 hours post infection. Using these live/dead reporter Mtb, we find that fewer live or transcriptionally active Mtb were present within efferocytic (CD45.1) macrophages (Figures 4-8A,B). After inhibiting efferocytosis, we found the same proportion of live Mtb within the CD45.1 macrophages as in the primary infected macrophages.

Previously we reported that apoptosis is required for efferocytosis mediated control and that efferocytosis is required for subsequent suppression of Mtb growth in vitro (Chapter 3). We surmised that apoptosis itself is not intrinsically bactericidal. To further confirm these results we induced apoptotic death in live/dead Mtb infected macrophages. 80% of Mtb within apoptotic

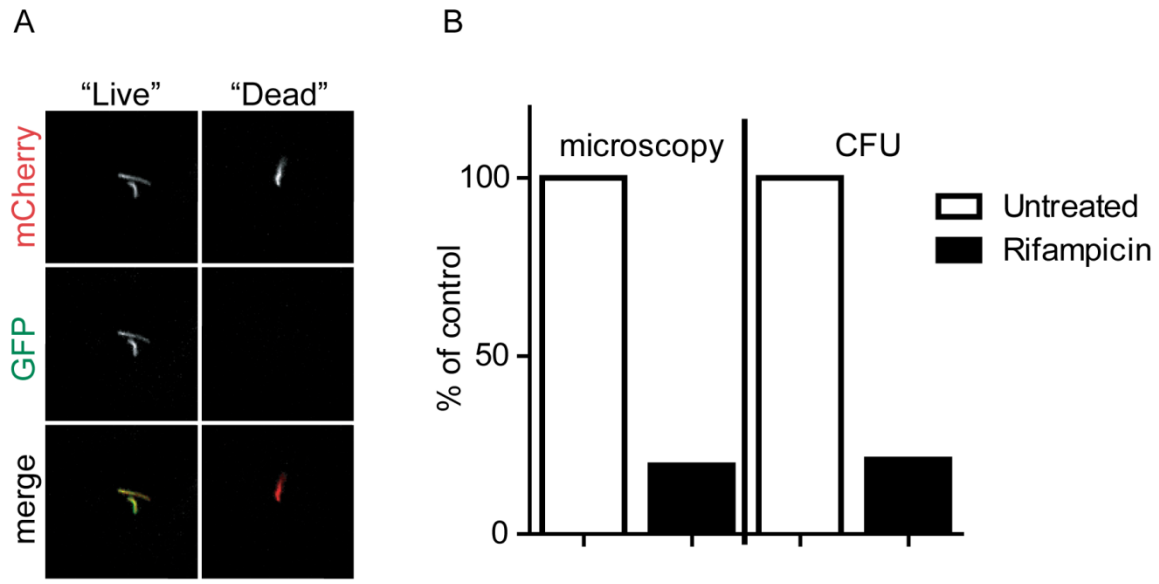

Figure 4-7. Live/Dead reporter Mtb accurately reflect the metabolic state of Mtb.
A. Live/Dead Mtb following overnight incubation with 200ng/mL tetracycline. “Live” Mtb are mCherry^{+} (red) and GFP^{+} (green) while “Dead” Mtb are mCherry^{+}. B. Primary human monocyte-derived Mφs were infected with Live/Dead H37Rv and were infected with MOI 1 for three days before treatment with rifampicin for one day. Bacterial burden was then ascertained by microscopy or serial dilution and CFU. The ratio red:green (live:dead) pixels were averaged in untreated verses rifampicin treated wells and plotted as percent ‘live’ of control untreated wells. The amount of red/green signal in the rifampicin group is 19.29% of the untreated. For CFU, rifampicin treated macrophages had 20.94% of the bacteria present in untreated wells.

macrophages, as determined by characteristic fragmented nuclei, were alive (Figure 4-9). 80% of Mtb within non-apoptotic macrophages were alive as well. Thus, the link between apoptosis and Mtb killing is not direct and efferocytosis accounts for the bactericidal effects associated with apoptosis. We also found that the majority of the dead bacteria were found within $LAMP1^+$ compartments (Figure 4-10A,B). Collectively, these data indicate that following efferocytosis, the efferocytic phagosome containing the infected apoptotic cell fuses with lysosomes, which are capable of killing Mtb.

Discussion

A key component of Mtb's virulence strategy is the ability to reside within an arrested phagosome. Typically the bacterium excludes the fusion of lysosomes from the Mtb-containing phagosome and thus avoids damage and killing. The Mtb-containing efferocytic phagosome is different. Following apoptosis we have previously reported that Mtb-containing apoptotic blebs and apoptotic cells are engulfed by uninfected macrophages through the process of efferocytosis. We have found and characterized efferocytosed Mtb-infected apoptotic blebs by TEM, thus confirming our earlier reports of efferocytosis of infected apoptotic cells. The efferocytic phagosome containing Mtb with dead cell debris is vacuous and more closely resembles ingested apoptotic thymocytes than the primary infected bacterial phagosome. Also, damaged Mtb were found along with digested cell debris, raising the possibility that the efferocytic phagosome fused with lysosomes. To point, we found increased co-localization with lysosomal markers following efferocytosis over primary infection. And using the novel live/dead Mtb reporter, following efferocytosis more Mtb is killed, and the killed Mtb are found within lysosomes, suggested that their killing and lysosomal fusion go hand- in-hand. Thus we report here that efferocytosis is a

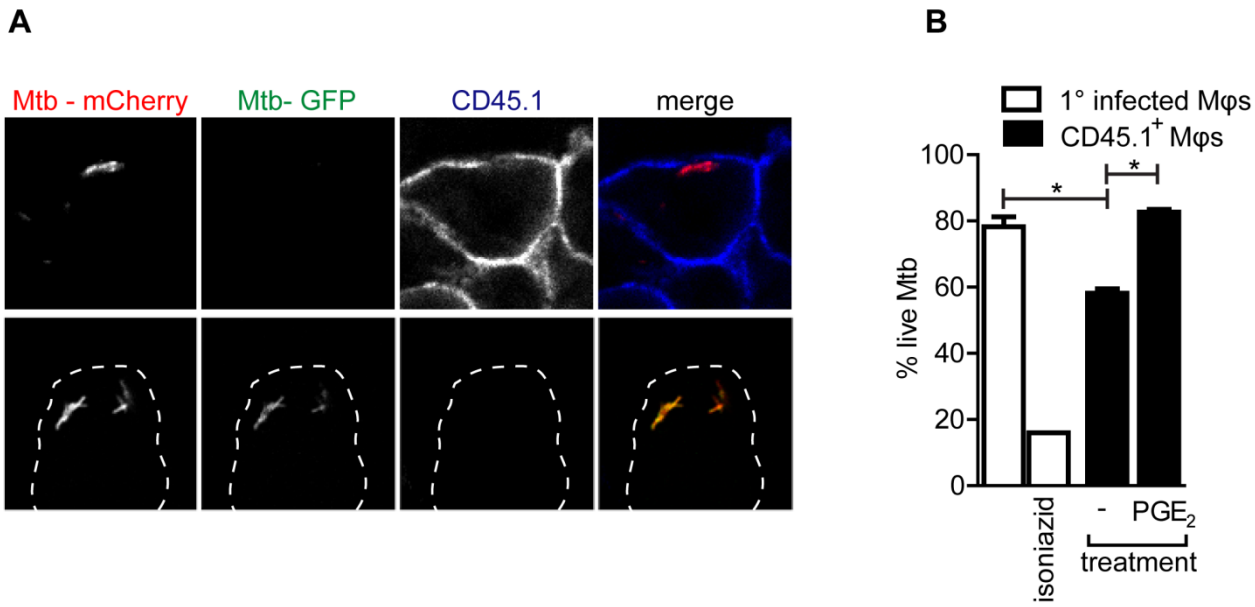

Figure 4-8. Mtb are dead following efferocytosis

A. Dead Mtb within a CD45.1 originally uninfected thioglycollate-elicited peritoneal macrophage (pMφ) and alive within a primary infected pMφ 48 hours after co-culture and 24 hours after GFP induction with 200ng/mL tetracycline. Mtb-mCherry (red), Mtb-GFP (green), CD45.1 (far red/pseudocolor blue) and merge. Dashed outline delineates CD45.2 primary infected macrophage. B. Quantification of (A). Percentage of live (mCherry+GFP+) Mtb of total Mtb (mCherry+) in indicated pMφ population. Uninfected pMφs pretreated with 10uM PGE_2 prior to addition. Isoniazid (1 µg/mL) was added immediately after co-culture. 150 Mtb were counted in triplicate per condition. Data is representative of 3 experiments. Error bars ± SEM, $p \leq 0.05$, One-way ANOVA with Dunnet's post-test.

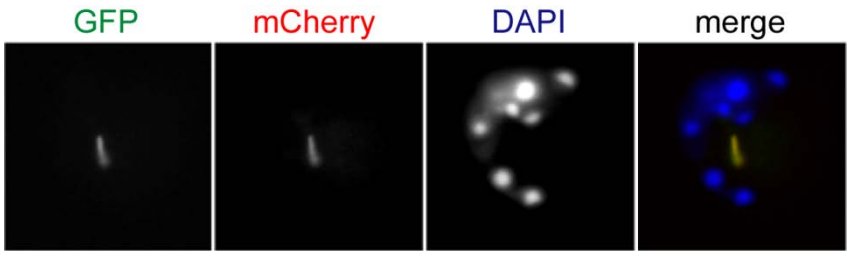

Figure 4-9. Apoptosis does not impact Mtb viability
Widefield image of live Mtb within an apoptotically dying cell. GFP (green), mCherry (red), DNA/DAPI (UV/pseudocolor blue) and merge. Thiogycollate-elicited peritoneal macrophages were infected with live/dead Mtb for 4 hours. Uninternatlized bacteria were washed away and 1µg/mL staurosporine and 200ng/mL tetracycline were added for 24 hours to induce apoptosis in the macrophages and GFP signal in the live bacteria. Image is representative of 50 Mtb-infected apoptotic (fragmented nuclei^{+}) macrophages counted.

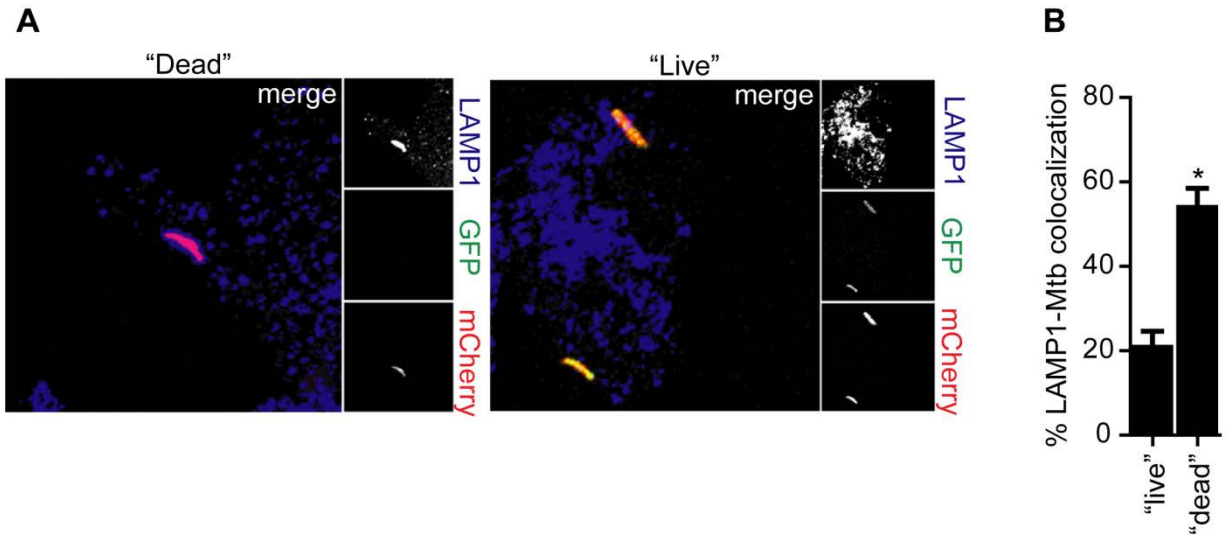

Figure 4-10. Dead Mtb are found within LAMP1$^+$ vesicles
A. "Live" (top) Mtb do not co-localize with LAMP1 as frequently as "Dead" (bottom) Mtb in bone marrow-derived macrophages (BMDMφs). LAMP1 (far red/pseudocolor blue), GFP/live (green), mCherry/Mtb (red) and merge. B. Quantification of (A). Percent LAMP1 co-localization with "Live" or "Dead" Mtb of total "Live" or "Dead" Mtb in BMDMφs. Data is representative of two experiments. Error bars ± SEM, $p \leq 0.05$. Student's T-test.

novel mechanism for bacterial killing that delivers Mtb to the caustic environment of the lysosomes.

The actual mechanism of Mtb killing outlined here, lysosomal fusion, is well described to control Mtb. IFNγ is perhaps the best known not just for iNOS upregulation, but for stimulating phagosome maturation that leads to more lysosomals fusing with the Mtb phagosome[11]. As we have reported earlier (Chapter 3) that IFNγ pre-treatment does not enhance efferocytosis-mediated control, we speculate that efferocytosis does not require an outside signal to facilitate lysosome fusion to the Mtb-containing efferocytic phagosome. We do not envision that efferocytosis evolved to be antibacterial- it is a crucial function of all multicellular organisms charged with the task of dead cell removal. In primitive organisms, such as C. elegans, effereocytosis is exclusively mediated by body cells, not 'immune-type' cells. Thus efferocytosis' primary function is dead cell removal, and microbial killing appears to be a fortunate side effect. This does not exclude the possibility that efferocytosis has since been adapted in macrophages especially as a potent means for microbial control. In macrophages especially, the rate of acidification and acquisition of lysosomal proteins to the efferocytic phagosome is faster than phagocytosis of opsonized particles[24]. Simply, Mtb is killed along with destruction of the internalized apoptotic cell material.

Until further work is done characterizing the interactions of Mtb, its virulence factors and how they interact with host proteins, we can only speculate as to why Mtb can inhibit maturation of a primary phagosome but not the efferocytic phagosome. We detected Mtb within apoptotic blebs, within the characteristic spacious efferocytic phagosome. We hypothesize that Mtb compartmentalized within an apoptotic cell within an efferocytic phagosome is further segregated from the host macrophage and phagosomal maturation machinery. While all Mtb's

effector molecules responsible for phagosome arrest are not known, it is reasonable to assume that many or all of them have limited access to their targets from behind these additional layers of membrane. For one, Mtb's lipidic effector molecules TDM and ManLAM may intercalate into the phagosome membrane to interfere with the transition from Rab5 to Rab7 and EEA1[3]. Perhaps these molecules cannot access the phagosome membrane from within the apoptotic cell. Secreted effector molecules, like SapM and proteins translocated by SecA2 may target host proteins in the cytosol[12, 25, 26]. The additional layer of membrane separating Mtb from the host phagosome membrane may act as a barrier restricting access to the cytosol. It has been proposed that Mtb requires a tight-opposing phagosome membrane to transport it's effectors into the cytosol to find their targets[27]. In this case, the vacuous efferocytic phagosome would prevent translocation of virulence factors. Not only are Mtb virulence proteins associated with phagosome arrest prevented from acting on their host targets; bacterial proteins associated cell death induction are also sequestered. It would be interesting to determine if macrophages that have taken up Mtb within efferocytic phagosomes are completely immune to other Mtb virulence strategies, such as MHC down-regulation and IFNγ unresponsiveness.

Alternatively, the proteins required for efferocytic phagosome maturation are distinct from phagosome maturation and Mtb cannot interfere with this different class of proteins. Phagosome maturation following the ingestion of either a free bacteria, particle or dead cell proceeds by exchanging Rab5 for Rab7[28]. This process, called Rab conversion, allows for the tethering and fusion of lysosomes through Rab7-RILP interaction[29]. Recently it has been described that novel Rab5 effectors, Mon1a/b and Czz1 may act exclusively on efferocytic phagosomes to link Rab5 activation with Rab7 recruitment[30]. Perhaps these proteins cannot be targeted by Mtb's secreted virulence factors. More work is required to completely understand

how efferocytosis is capable of more efficiently delivering Mtb to lysosomes. Many intracellular pathogens avoid destruction by avoiding lysosomes; either by translocation into the cytosol or by phagosome maturation arrest. Perhaps efferocytosis of these pathogens could lead to their destruction.

Experimental Procedures

Mice and macrophage isolation

C57Bl/6 (CD45.2) and B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ (CD45.1) mice were purchased from Jackson Laboratories, and were kept and bread using standard humane animal husbandry protocols. Mtb-infected mice were housed under BSL3 conditions. Thioglycollate-elicited peritoneal macrophages were lavaged from peritoneal cavities 4-5 days following 3% IP thioglycollate injection. Macrophages were isolated using CD11b microbeads and magnetic columns (Miltenyi Biotec) and cultured in 24 or 96 well dishes overnight prior to infection. Resident peritoneal macrophages were lavaged from peritoneal cavities and selected based on CD11b^{+}CD19^{-}. Bone marrow-derived macrophages were differentiated from bone marrow cells 7-10 days in RPMI supplemented with 20% L929 cell supernatant containing M-CSF. Alveolar macrophages were lavaged from the lungs of mice with PBS.

Confocal microscopy efferocytosis assay

mCherry-H37Rv and live/dead-H37Rv were grown in the presence of 0.5ug/mL Hygromycin in 7H9 media for 1 week prior to preparation as previously described[31]. GFP-H37Rv was grown in 7H9. Mφs from CD45.2 mice were cultured in 24 well dishes in triplicate per condition upon coverglass and dyed with Vybrant DiO (Invitrogen) as per manufacturer's specifications prior to

infection with mCherry-H37Rv or GFP-H37Rv for 4 hours at an MOI of 10 as described previously[31]. Bacteria were washed away and uninfected CD45.1 Mφs were added at 2x the number of infected Mφs. Treatment conditions were added as appropriate: Caspase 3 inhibitor (CalBiochem), 10μM. Uninfected macrophages were pre-treated with 10uM PGE2 for 4 hours prior to addition where indicated. 16-24 hours later cells were washed and stained with mouse monoclonal CD45.1 (Abcam) before affixing to microscopy slides with Prolong Gold plus DAPI (Invitrogen). LAMP1 (Abcam) and LC3 (MBL) staining was done 16-24 hours post infection following fixation and TritonX permeabalization. vATPase (Abcam) staining was done 16-24 hours post infection following fixation and saponin permeabalization. Lysotracker Red (Invitrogen) was added 16-24 hours post infection for 1 hour at 37^{o}C at 1uM prior to washing and fixation. Tetracycline was added 16 hours post infection/addition of uninfected Mφs at 200ng/mL and induced GFP expression for 24 hours in live/dead H37Rv-infected Mφs. Conditions were analyzed in triplicate and for each replicate in each condition 100-300 Mtb were counted. Images were taken using a Nikon TE2000-U inverted microscopy

Transmission Electron microscopy

Macrophages were grown in 6 well dishes and infected and treated as described above. 24 hours post infection cells were fixed with glutaraldehyde/paraformaldehyde/picric acid in sodium cacodylate buffer for 1 hour. Cells were postfixed for 30 minutes in Osmium tetroxide/ Potassium ferrocyanide, washed in water and incubated in uranyl acetate for 30 minutes. Cells were washed and dehydraded in alcohol, removed from culture dish in propuleneoxide, pelleted and infiltrated for 2 hours in propuleneoxide and TAAB Epon (Marivac). Samples were embedded in TAAB Epon and polymerized at 60^{o}C for 48 hours. 60nM sections were cut on a

Reichert Ultracut-S microtome, picked up onto copper grids and stained with lead citrate and examined using a TecnaiG^2 Spirit BioTWIN transmission electron microscopy and recorded with an AMT 2k CCD camera.

Image Analysis

Microscopy images were analyzed using Image J[32] and co-localization analysis macros or user-generated pipelines in Cell Profiler[33]. Investigators were blinded during co-localization analysis.

Statistical Analysis

Statistical analysis and graphical output was done with GraphPad Prism. One-way ANOVA with Dunnet's post-test. * = $p \leq 0.05$

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Chapter 5: Concluding Remarks

Apoptosis and *Mycobacterium tuberculosis* control: a perspective

The notion that apoptosis is directly mycobactericidal is deeply entrenched in the literature. This fact is so taken for granted that it is stated in the introduction to many papers and reviews, despite no specific mechanism put forth to explain how this form of cell death is detrimental to the bacteria. While no mechanism has been provided, several hypotheses exist to explain the observation that following apoptosis, *Mycobacterium tuberculosis* (Mtb) growth is suppressed. It has been speculated that Mtb cannot grow and survive within an apoptotic bleb- that the nutrients for its survival are lacking[1]. Also, it has been assumed that without the macrophage phagosome niche, the bacteria cannot grow well. Perhaps more reasonably, it has been assumed that apoptosis damages the bacteria, possibly through reactive oxygen species and nitrogen intermediates (ROS & RNI). All three of these assertions have gone untested and are in many ways antithetical to other findings. For the first instance, apoptotic blebs are rapidly removed by macrophages, both in vitro and in vivo. This process of efferocytosis is well known and has a rich history of research. Also to point, Mtb is a hardy slow-growing bacterium, and while an apoptotic bleb might not contain the full complement of Mtb's preferred nutrients, the notion that Mtb could consume them all before the bleb is efferocytosed is untested. Next, researchers have found Mtb growing outside the macrophage in caseous necrotic granulomas- so even in vivo Mtb does not need to reside within a macrophage or a phagosome. Even in vitro, extracellular growth amongst necrotic macrophage debris is observed. While the growth rates between intracellular and extracellular growth may be different, clearly Mtb can survive and grow in many different niches. Finally, the notion that apoptosis might directly harm the bacteria lacks evidence. During some forms of apoptotic cell death, reactive oxygen species are released. Then again, this is true of necrotic cell death as well. Mtb employs many strategies to

survive reactive intermediate insult, and while the bacterium is not completely protected, there is no evidence that this type for ROS/RNI is damaging. Thus we felt that the possibility that apoptosis directly damages Mtb, or limits its growth is unlikely.

The dichotomy between apoptosis and necrosis during Mtb infection is old. While current thinking dictates that necrosis is dominant, early studies favored apoptosis as the primary mode of cell death during active disease in the granuloma[2]. Following up on this observation, researchers sought to understand what the consequences of apoptosis is on the bacilli. First, BCG infected macrophages were treated with ATP to induce apoptosis and hydrogen peroxide to induce necrosis[3]. The apoptosis condition was associated with decreased bacterial growth. Furthermore, Fas ligand treatment of human macrophages infected with Mtb also suppressed bacterial growth[4]. However, in the BCG study efferocytosis of BCG-infected macrophages was observed by electron microscopy. In the Mtb study, less than half of the infected macrophages were apoptotic following Fas treatment, allowing plenty of unaffected macrophages to efferocytose the dying cells. Thus, even these oft cited studies do not completely and directly address the antibacterial mechanism of Mtb control following apoptosis. Therefore, we remained skeptical of apoptosis' bactericidal activity.

There are a few reports that hint at efferocytosis might be responsible for Mtb control following apoptosis. For one, Fratazzi et al found that M. avium growth is suppressed following apoptosis, but only if uninfected macrophages are present[5]. The authors do not speculate as to why this is true in this manuscript, but the same authors later speculate that the control exerted by uninfected macrophages could be exerted by engulfment of the Mtb-infected apoptotic cells[1]. However, no experiments were done to directly test this observation and the notion that apoptosis is bactericidal still prevailed in the literature. Additionally, it was found that addition

of uninfected macrophages to Mtb-infected macrophages suppressed growth, but only if the macrophages were added shortly after infection[6]. Waiting for 24 hours before macrophage addition had no impact on bacterial growth. And in this model, there was robust apoptosis. Presumably, waiting 24 hours prior to macrophage addition caused the apoptotic cells to succumb to secondary necrosis. Thus the inkling that efferocytosis might be responsible for Mtb control has existed for some time in the field, however it was never explicitly stated, or directly tested.

One recent finding suggested that efferocytosis is inhibited during Mtb infection, and that the control mediated by uninfected macrophage co-culture is due to a secreted small molecule[7]. A major difference between these models is the bacterial burden. We rely on infections that result in 1-5 bacteria, unclumped and in individual phagosomes inside each macrophage, while Hartman et al aimed for a much higher burden that induces lysosome-dependent atypical cell death. As many Mtb researchers will agree, the bacterial burden has a significant effect on the behavior and phenotype of the infected macrophage. Our work does not exclude the possibility of mechanisms other than efferocytosis at play during uninfected macrophage co-culture; however our evidence for efferocytosis and its consequences are robust. Thus we present for the first time, efferocytosis as a mechanism of bacterial control.

Summary of findings

Despite previous reports, we found that apoptosis is not intrinsically mycobactericidal. First, we found that there is a significant amount of apoptosis following Mtb infection. We speculate that much of the necrotic death observed by others is a consequence of the in vitro system that does not allow for efficient efferocytosis; thus apoptotic cells fall apart by secondary

necrosis and confound analysis. We then developed an uninfected macrophage co-culture model to study the fate of Mtb-infected macrophages. We found that uninfected macrophages are capable of engulfing Mtb-infected apoptotic macrophages. This was true in multiple macrophage types tested as well as in vivo, specifically in the lung. Next we found that uninfected macrophage co-culture, a condition that promotes efferocytosis, controls Mtb growth in in vitro culture. Again, this was true of all macrophage types tested, specifically alveolar macrophages. We found that apoptosis is required for this control, as inhibition of efferocytosis not only blocked efferocytosis, but also blocked uninfected macrophage co-culture control of Mtb growth. Blocking efferocytosis itself by specifically blocking the 'efferocytosis receptor', TIM4 abrogated the uninfected macrophage co-culture system's ability to control Mtb growth. Then, we asked how efferocytosis could limit Mtb growth. We found that Mtb present in efferocytic phagosomes are very different structures than traditional bacterial phagosomes. In fact, these structures fuse with lysosomes much more readily than the traditional Mtb phagosome: Mtb within efferocytic phagosomes co-localize with the vATPase, LAMP1 and are acidified. Lastly we found that Mtb is killed only after uninfected macrophages engulf the infected apoptotic cell and fuse the efferocytic phagosome with lysosomes. Thus, Mtb is destroyed along with the apoptotic cell material. Simply put, apoptotic Mtb-infected macrophages are engulfed and Mtb is killed by phagosome maturation (Figure 5-1).

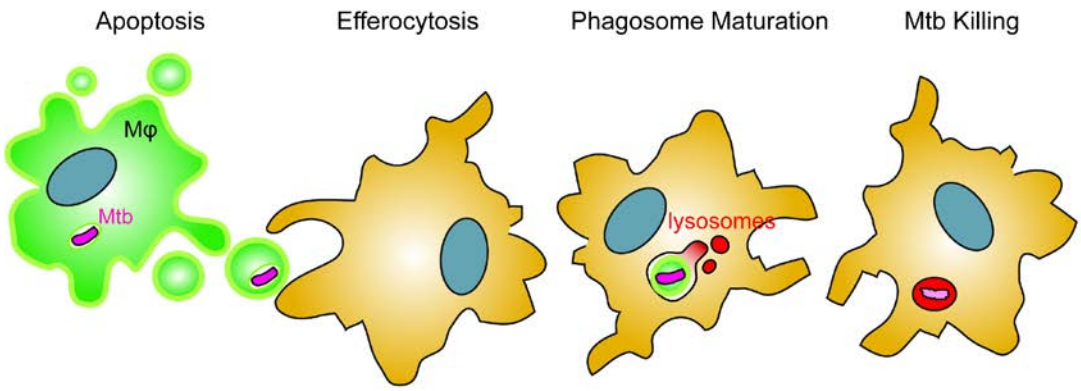

Figure 5-1. Model of efferocytosis-mediated control

The Mtb-infected macrophage dies. An Mtb-containing bleb is engulfed by a neighboring macrophage via efferocytosis. Mtb cannot inhibit the maturation of the efferocytic phagosome, as lysosomes fuse delivering their microcidal compounds. Ultimately, Mtb is killed as the apoptotic cell material is destroyed.

Implications

This report has focused on the role of macrophage apoptosis as an innate response following Mtb infection. However, the killing of infected cells is an important component of the $CD8^+$ T cell response to tuberculosis[8]. T cells can induce death in target cells via the release of cytotoxic granules containing granzymes and perforin or through the engagement of surface receptors containing death domains such as CD95/95L[9,10]. Both of these pathways lead to apoptotic death in the target cells. We envision that capacity of CTL to kill infected cells restricts bacterial growth indirectly since the induction of apoptosis in the infected cells will facilitate their engulfment by uninfected macrophages recruited to inflammatory foci. One could speculate that the benefit of CTL during infection is not to impact microbial viability by eliminating their replicative niche, but to allow for efferocytosis-mediated microbial killing.

The relative efficiency of efferocytosis will depend on the microenvironment. Some inflammatory signals, such as abundant TNF, can hamper efferocytosis[11]. This could impair efferocytosis and lead to greater secondary necrosis. If efferocytosis is inhibited during active Mtb infection, pharmacologically releasing this inhibition could benefit the infected individual in two ways. First, tuberculosis is a disease of rampant cell death, inflammation and tissue destruction. Efferocytosis would be key in maintaining proper lung tissue architecture during disease and minimizing the development of immunopathology. Second, enhancing efferocytosis may restrict bacterial replication and prevent recrudescence of infection. In contrast, efferocytosis might play an important role during the maintenance of latency or even in aborting infection before it becomes established. Additionally, once can envision that during different stages of infection apoptosis or necrosis might be more dominant, further influencing the amount

of efferocytosis. However, the changing dynamics of cell death in vivo during infection is not known.

This study focused on the effect of uninfected macrophages engulfing infected apoptotic macrophages; however we found that Mtb-infected macrophages are fully capable of engulfing apoptotic cells (Chapter 2). We did not directly test this scenario, however CD45.1 macrophages were occasionally found with evidence of both efferocytosis and secondary infection. As it is impossible to know what event happened first: efferocytosis or secondary infection, we did not follow up on this further. However, in these situations we envision that the efferocytosed Mtb will be killed via the mechanisms outlined above, while the phagocytosed Mtb will be unharmed. This is based on the fact that phagosomes are autonomous: what happens to one does not happen to the other[12,13]. Thus efferocytosis could still mediate bacterial control, even in the absence of infected macrophages at the site of infection.

Elucidating the different stages of immunity when efferocytosis is crucial for bacterial control will require experimental approaches that are capable of detecting and blocking efferocytosis in vivo. This would be exceedingly difficult, given the redundancy of 'efferocytosis receptors'. Additionally, most work on efferocytosis in vivo focuses on transferring apoptotic targets as this enables easier and faster read-outs. However, more models are under development that could facilitate in vivo study of efferocytosis during pulmonary tuberculosis infection[14,15]. It will also be important to determine whether efferocytosis is subverted by pathogens to evade immunity. For example, Leishmania employ neutrophil apoptosis and subsequent efferocytosis as a mechanism of establishing infection within macrophages [16,17]. Indeed, some pathogens might rely on uptake of infected dying cells to spread the infection, as is the case in *M. marinum* infection of zebrafish embryos [18].

Efferocytosis of influenza-infected cells is associated with viral control[19,20]. Efferocytosis may control or exacerbate many infectious diseases and it will be important to explore how macrophages contribute to microbial immunity by executing this vital 'house-keeping' function.

Future Directions

Macrophages are not the sole efferocytic cell, although they contribute most to dead cell clearance. Endothelial tissue cells and fibroblasts can engulf their dying neighbors. Perhaps most notably, dendritic cells (DCs) efferocytose apoptotic cells. Given dendritic cells' important role in altering and tailoring the adoptive immune response to tuberculosis, further study focusing on dendritic cells ability to kill Mtb following efferocytosis is needed.

Efferocytosis of apoptotic cells and cell debris by DCs is responsible for cross presentation of antigens on MHC class I normally hidden from CD8 T cell surveillance[21,22]. Apoptosis of Mtb infected apoptotic macrophages results in uptake and cross presentation by dendritic cells[23]. This results in the development of a more robust immune response as well as bacterial control[24]. However, what is not known is whether Mtb taken up by DCs via efferocytosis are killed. Dendritic cells mature their phagosomes at a much slower rate and not as robustly as macrophages[25]. Additionally, DCs possess fewer degradative enzymes in their lysosomes than macrophages[26]. Perhaps DCs are thus less equipped to kill Mtb following efferocytosis. Dendritic cells bring Mtb from the lung to the lymph node to induce T cell responses[27,28]. However, this is responsible for dissemination and spread of the infection. Whether DCs trafficking Mtb to distal sites are directly infected or have taken up Mtb via efferocytosis, is not known. Recently, the $CD103^+$ subset of DCs in the lungs, separate from the classical $CD11c^+$ subset was found to primarily engulf apoptotic cells and present antigen[15].

Discerning which DC subsets participate in efferocytosis during Mtb infection, compared with the DC subsets responsible for priming protective T cell responses could inform the design more robust vaccines. Understanding the relative merits of efferocytosis of Mtb-infected apoptotic macrophages by DCs to induce T cell responses or Mtb death is important. If there is a difference in killing capacity of DCs compared to macrophages following efferocytosis we could discern which components of the macrophage lysosomal machinery are key to Mtb killing.

Cell death is an ever expanding field, and microbe-induced cell death is incredibly complex. We speculate that efferocytosis of apoptotic infected cells allows for the further compartmentalization of Mtb, and thus more efficient lysosomal fusion and killing once in the efferocytic phagosome. And as necrosis is associated with increased bacterial growth and survival, we speculate that the uptake of a bacterium within necrotic cell debris (without an intact plasma membrane) would have a similar effect as phagocytosing free bacteria. However, early necrotic cells are described as possessing an intact membrane and can be cleared via the same mechanisms as apoptotic cells[29,30]. In fact, our data would suggest that the only requirement for efferocytosis-mediated control would be for the bacterium to be encased within an additional membrane. Thus alternative forms of cell death: pyroptosis and caspase-independent cell death might have a similar end result.

Ultimately, the study of efferocytosis of infected cells is a brand new field, with much to be learned. This study provides a mechanism for the long held observation that apoptosis attenuates tuberculosis and perhaps other pathogens as well.

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