MYCOBACTERIUM SHOTTSII INFECTION INDUCES APOPTOSIS *IN VITRO* IN PISCINE CELL CULTURE SYSTEMS

by

KATE ELIZABETH CREEVY

(under the direction of Frederick D. Quinn)

ABSTRACT

Diseases caused by *Mycobacterium* species are significant global human and animal pathogens, and are the focus of major research efforts. Recently, a novel mycobacterial species, *M. shottsii*, was identified and isolated from an epizootic in striped bass, *Morone saxatilis*, in the Chesapeake Bay. *M. shottsii* causes a chronic, wasting, granulomatous disease in its natural host, similar to the disease caused by *M. tuberculosis* in humans. Cellular and organism models of infection are required to study a novel pathogen like *M. shottsii*. The ability of this agent to induce apoptosis or necrosis upon infection is an important pathogenetic feature. In this study, we generated a cellular model for infection, and demonstratded that *M. shottsii* induces apoptosis in infected cells.

INDEX WORDS: *Mycobacterium shottsii*, Apoptosis, Necrosis
Mycobacteriosis, Tuberculosis, Fish

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DEDICATION

I dedicate this work to my mother, who was the first person in our family to earn a scientific Master's degree, and to my father, who was the first person in our family to become a doctor. The gifts of a curious mind and a strong work ethic are their legacy, and I hope that my work honors their example.

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Chapter 1. Literature Review

Mycobacterial diseases and global implications

Mycobacterium tuberculosis causes more human adult deaths each year than any other infectious agent, despite decades of active research and public health control efforts.[18,89] Worldwide, in 2005, fourteen million people were infected, and over 1.5 million died.[89] Humans are the only natural host for the disease caused by *M. tuberculosis*.[11] Human tuberculosis is a highly-contagious respiratory illness, spread easily by coughing, sneezing or even talking, and it is estimated that a new person becomes infected each second somewhere on the globe.[18,22,89] As immunocompromised people (whether due to HIV/AIDS, cancer chemotherapy, organ transplant, or comorbid chronic disease) comprise an expanding segment of the human population, the significance of severe and disseminated tuberculosis continues to grow.[18,89]

M. tuberculosis is an environmentally resistant, non-motile, facultatively intracellular Gram-positive bacillus. Since its waxy mycolic acid capsule makes Gram-staining unreliable, it is also classified as acid-fast. *M. tuberculosis* is capable of evading immune defenses and establishing

persistent asymptomatic infection for years or decades. Tuberculosis is a chronic, latent illness in most human hosts; however, at times of metabolic stress or concurrent disease, infection can become active. During activation, clinical illness becomes evident, with coincident risk of contagion. Inadequate or incomplete courses of antimicrobial therapy can facilitate microbial persistence and unsuspected transmission, and may increase development of drug-resistant strains of *M. tuberculosis*.[21,24,89]

Other species within the genus *Mycobacterium* are also significant human and animal pathogens, causing both systemic and localized disease. The *M. avium* complex (MAC) contains several important systemic pathogens. *M. avium* subspecies *avium* causes chronic wasting illness, as well as respiratory and gastrointestinal diseases in humans. Clinical disease is most prevalent in the elderly, the very young, and those whose immunity is compromised by HIV, cystic fibrosis, or other ailments. *M. avium* also causes systemic illness in many mammals, including domestic and wild dogs, birds, and ruminants. *M. avium* subspecies *paratuberculosis* is the causative agent of Johne's disease of cattle, a diarrheal disease of significant economic importance in endemic countries. This pathogen has also been implicated in Crohn's disease of humans, a recurrent painful enteritis.[57]

Leprosy is another well-known clinical illness caused by mycobacteria; *M. leprae* is the causative agent of this contagious disease of humans. Clinical disease may be limited primarily to skin and oronasal mucosa, where it is characterized by spreading nodules, ulcers and plaques. Additionally, nerve sheath involvement may be mild or extensive, causing pain, weakness, or loss of sensation in small to large regions of the body.[11] Cats show a similar clinical syndrome of spreading cutaneous nodules, without apparent neurologic disease. *M. lepraemurium* is the primary causative agent of this disease, called feline leprosy, which can be controlled by surgical resection and prolonged antibiotic therapy.[43]

Buruli ulcer (*M. ulcerans*) and fish-handlers' disease (*M. marinum*) are localized infections caused by species of *Mycobacterium* which prefer aquatic environments. While these infections seldom disseminate, they cause significant morbidity in both immunocompetent and immunodeficient people worldwide. Infections are difficult to eradicate, and are characterized by large areas of cutaneous and subcutaneous necrosis with permanent disfigurement.

Opportunistic mycobacteria have been divided into slow-growing and rapid-growing isolates, based upon their *in vitro* behavior, and include *M. chelonae*, *M. fortuitum*, and *M. smegmatis*, among many others. Following transcutaneous

inoculation, these species can cause persistent infection, leading to draining tracts and fat necrosis, and requiring extensive surgical debridement and long-term combination antimicrobial therapy to cure.[43,45,46]

The diverse genus, *Mycobacterium,* has been historically organized and classified by colony morphology, growth characteristics, and biochemical testing. More recently, Rogall and colleagues described the application of 16S rRNA sequencing for genetic classification of 23 species of a variety of mycobacteria.[69] This analysis included examples of pathogens and non-pathogens, as well as rapidly- and slowly-growing species. The framework of relationships elucidated by this work is the basis for most genetic classification structures used today.

Many mycobacterial species are non-pathogenic soil residents; *M. tuberculosis*, *M. ulcerans*, and the *M. avium* complex are somewhat unusual within the genus for their consistent ability to mount sustained and successful infections. Adaptations by these organisms enable them to evade innate and adaptive host immune defenses. These traits, and host-pathogen interactions, are major research foci in the tuberculosis field.[11]

Apoptosis and necrosis

In the face of infection, two manners of death may occur for a host cell – apoptosis, or necrosis. The causes of each, and the balance between these outcomes, are fundamental to the pathogenesis of mycobacterial inflammation.

Necrosis is catastrophic cellular failure, associated with swelling of the organelles and the entire cell, and culminating in lysis of the cell membrane. This process releases cellular contents, including the toxins or pathogens which may have led to the death of the cell, and causes inflammation. Apoptosis, conversely, is programmed cell death, carried out in a stepwise fashion. Several signal cascades of internal or external origin can induce this program, which generates blebs of membrane-bound cellular material, until the entire cellular contents are dissipated. Nearby phagocytes, including antigen-presenting cells (APC), clean up the apoptotic bodies, without inflammation or the release of potentially dangerous cellular materials. In the case of infection, APC can then process these apoptotic bodies for antigen-presentation and the induction of specific immunity. Mycobacterial species are known to cause cell death by both mechanisms.[2,19,26,31,91]

Intracellular mycobacteria and lysosome evasion

Pioneering work with *M. tuberculosis*, performed by Armstrong and Hart in the 1970s, used electron microscopy to demonstrate the presence of intact bacilli within macrophage phagosomes, but a failure of their subsequent fusion with macrophage lysosomes.[4,32] By accomplishing this feat, at least in part, they avoid the potent degradative enzymes which macrophages use to destroy most pathogens.[11,70] Alternative evidence exists that fusion may occur; however, mycobacteria can survive phagolysosomal fusion, and continue to proliferate and escape from infected cells.

The dynamic site of these mycobacterial interactions with the host cells is the granuloma, a structure which is an important determinant of chronic latency and reactivation of mycobacterial infections.[24,83] Within the granuloma, individual cells help to contain infection, by cytokine signaling, specific immune response, and coordinated morphologic changes. During granuloma formation, some cells originate the granuloma, others are recruited to the site and may remain or depart, and other cells die. [6,11,26,42]

The host cell lysosome contains hydrolytic enzymes for digestion of macromolecules. These acid hydrolases function optimally at a pH around 5.0, which is strongly acidic compared with the rest of the cellular environment. Nearly all cells

contain lysosomes for degradation of obsolete cellular elements, and foreign materials acquired by pinocytosis. However, professional phagocytes (neutrophils, macrophages and dendritic cells) also use lysosomes for the destruction of large molecules, cell fragments, or even entire cells, which have been internalized by phagocytosis. In phagocytosis, ingested material is contained within a phagosome, which is a membrane-bound organelle derived from the plasma membrane. This vesicle is trafficked into the cytoplasm where many lysosomes fuse with it, emptying their contents into the new fused organelle, now called a phagolysosome or secondary lysosome.[2] In addition to simple destruction, degraded material from pathogens can be manufactured into the fragments used for MHC Class II antigen presentation. This activity links innate immune defenses of the phagocytic cells with activated T-cells, which recognize presented antigen, thus perpetuating an antigen-specific immune response.[77]

Intracellular mycobacteria are able to persist within the host cell in part because they interfere with these normal vesicular trafficking pathways.[71] By residing within phagosomes, and preventing their fusion with lysosomes, *M. tuberculosis* avoids exposure to the noxious contents of the lysosome.[70] Normal endocytic trafficking depends upon the acquisition and clearance, in a temporally-precise fashion, of a sequence of

signals on the membranes of endocytic vesicles. Within these coordinated steps, vesicles fuse with other vesicles sequentially, as they progress toward maturation. Mycobacterial lipids, which are essential components of the bacterial cell wall, are inserted into the phagosomal membrane and can cause a disruption in the normal pathways. These lipids can mimic mammalian host phosphatidylinositols, which are key signals in the endocytic pathways. Lipoarabinomannan (LAM) is one such lipid, which prevents the acquisition of late endosomal markers, and inhibits phagosome acidification. LAM also interferes with a rise in intraphagosomal calcium levels, which are a necessary signal to proceed with the stepwise process of maturation toward eventual lysosome fusion. Another mycobacterial lipid, phosphatidylinositol mannoside (PIM), complements the activity of LAM by recycling the vesicle within the early endosomal compartments. Thus, in a two-tiered approach, PIM repeatedly signals the phagosome to fuse with other early (immature, non-acidified) vesicles, and LAM simultaneously prevents acquisition of signals which would direct the vesicle toward fusions with late (mature, acidified) vesicles.[9]

Phosphatidylinositol-3-phosphate (PI3P) synthesis inhibition is another means by which mycobacteria prevent phagosomal maturation. PI3P is a signaling molecule which must be expressed in the phagosomal membrane to promote its

maturation. PI3P is expressed in the membrane of phagosomes containing heat-killed *M. tuberculosis*, but is continually removed from the membrane of phagosomes containing live *M. tuberculosis*.[84] Maturation blockade results in minimal acidification of the phagosome lumen, and absent or deficient lysosomal hydrolases. Even partial acidification blockade will benefit the pathogen, because any hydrolases which are acquired will remain relatively inactive until the pH decreases significantly.[54]

Secretion of interferon-gamma (IFN-γ) by other host immune cells activates infected macrophages, which enable them to overcome pathogen-directed inhibition of phagolysosomal fusion.[9] To counteract the effects of macrophage activation, however, mycobacteria exhibit additional defenses. Reactive nitrogen intermediates (RNI) are important cytotoxic products of macrophage lysosomes, evidenced by the fact that mice deficient in RNI production display enhanced morbidity and mortality with acute tuberculous infection. While RNI-deficient organisms are more susceptible to *M. tuberculosis*, however, RNI-producing organisms are still unable to completely eradicate infection. Pathogen production of peroxiredoxins, which enzymatically degrade RNI, seems to be the mechanism for this second aspect of mycobacterial defense against the macrophage.[8]

M. tuberculosis may also interfere with phaogcytosis through interactions with major histocompatibility class II antigen (MHC class II) expression. Activated macrophages are known to increase surface expression of MHC class II. Using THP-1 cells as an *in vitro* macrophage model, Hmama and colleagues showed that IFN-γ upregulated MHC class II expression in control THP-1 cells, but not in cells previously infected with virulent *M. tuberculosis*. In these infected cells, MHC class II molecules were demonstrated to remain sequestered within the endocytic transport vesicles.[35] In a related study, cells were first infected with *M. tuberculosis*, and then exposed to tetanus toxoid. The reduction of MHC class II molecule expression exhibited by monocytes infected with *M. tuberculosis* decreased their response to the subsequent toxoid exposure. Infected cells were inefficient at antigen presentation and induction of a specific T-cell response to tetanus toxoid, compared with uninfected monocytes.[29] In a subsequent report, MHC class II interference required live *M. tuberculosis* bacilli. When primarily isolated, or IFN-γ activated murine macrophages were exposed to heat-killed *M. tuberculosis*, they were able to internalize the bacilli, fuse phagosomes with lysosomes, and produce and export MHC class II molecules normally.[59] These findings represent an expansion of the manners in which *M.*

tuberculosis controls macrophage endocytic transport mechanisms to preserve itself.

The tuberculous granuloma and apoptosis

The variety of self-defense mechanisms on the part of the intracellular mycobacteria results in incomplete eradication of the pathogen from the host, despite ongoing macrophage attack. In addition to their role as professional phagocytes, macrophages are a major source of cytokines necessary to recruit leukocytes, translate innate to specific immunity, and perpetuate the immune response. IFN-γ from any source activates macrophages; however, IFN-γ is also the primary secretory product of activated macrophages, creating a positive feedback cycle of activation. Macrophages can be activated by intracellular infection, or by inflammatory activation in the absence of intracellular infection.

In the pulmonary parenchyma of the human host, this cycle leads to production of a chronic, active site of immune containment, or granuloma.[25] Within this granuloma, recruited mononuclear phagocytes undergo coordinated phenotypic change, from motile single-cell macrophages to fused multinucleate giant cells and epithelioid cells.[13] Lymphocytes are also contained within the granuloma, and some researchers have identified morphology typical of secondary lymphoid follicles.[71] Being a

dynamic structure, the immune attack and mycobacterial response within the granuloma are continual; however, while contained within the granuloma, the pathogen is not causing systemic inflammation in the host. Whether this situation represents clinically silent active disease (live bacilli, but no symptoms) or true latency (dormant bacilli, host immune containment) is controversial. It is not routinely convenient to sample asymptomatic human patients, and a satisfactory laboratory model for this aspect of *M. tuberculosis* infection has not yet been created.[7] In studies where asymptomatic infection has been examined, a dormant state of the *M. tuberculosis* bacillus, characterized by altered gene expression compared with gene expression in active, acute infection, has been identified. These dormant bacilli may persist within the granuloma for decades, then reactivate under uncertain influences, at a later time.[7,71,80]

Quiescent infection, while poorly understood, is also associated with an apparent immune anergy, or hyporesponsiveness, on the part of the host. In other chronic infections known to be associated with incompletely effective host immune response, such as HIV and malaria, an imbalance exists between levels of immune activation and T-cell responsiveness. Thus despite marked, or even excessive, immune response, a paradox exists, in that mycobacterial-specific T-

cells are under represented or refractory. Furthermore, in these examples of chronic, persistent infections, infected macrophages seem to undergo apoptosis (as a means of cell death) at a relatively high rate.[83]

M. tuberculosis is the prototypical apoptosis-inducing mycobacterial pathogen. In human tuberculosis patients, apoptotic cell death predominates in infected macrophages within the granuloma. Primarily-infected macrophages are ineffective at killing the infection, but can contain it regionally, and can secrete cytokines to recruit additional immune cells to the site. Conversely, uninfected activated macrophages secrete TNF-α, which hastens death by apoptosis of *M. tuberculosis*-infected macrophages in tissue culture.[42] Thus, apoptosis may be an adaptive means by the host to contain tuberculous infection, and to strengthen the specific immune response by antigen presentation from the apoptotic bodies. Apoptosis appears to benefit the host, evidenced by the fact that virulent strains of *M. tuberculosis* were better able to evade apoptosis than less-virulent strains.[41,91]

In host cells which lack adequate resistance mechanisms, it may be beneficial for *M. tuberculosis* to kill by necrosis, allowing the escape of viable bacilli.[17] Although the primary target cell of *M. tuberculosis* infection is the macrophage, the relative occurrence of apoptosis versus necrosis has been

studied in other cells for comparison. Danelishvili and colleagues showed that *in vitro* cell lines derived from macrophages behaved differently from epithelial cell lines. When infected with virulent *M. tuberculosis*, macrophages preferentially died by apoptosis, while epithelial cells preferentially died by necrosis. Also, infection of epithelial cells with virulent *M. tuberculosis* provided a protective effect against apoptosis-induction by staurosporine.[12]

In additional research on human volunteers, monocytes were collected both from clinically healthy, purified protein derivative (PPD) skin test-positive humans, and from those with active tuberculosis. Harvested monocytes were exposed in vitro to *M. tuberculosis* and to PPD, a protein which is known to induce apoptosis. Monocytes from healthy, but PPD-positive human subjects tended to undergo only apoptosis upon exposure to either the pathogen or the PPD. Monocytes from tuberculosis patients, however, died both by necrosis and apoptosis under both exposure conditions. Again, these data support the theory that macrophages which can contain tuberculous infection (healthy, PPD-positive subjects) undergo apoptosis if they must die, as a means of control of infectious material. Conversely, macrophages which have failed to contain infection (clinically ill subjects) are those which die by necrosis, and allow dissemination of viable bacilli.[30]

Extracellular mycobacteria and necrosis

M. ulcerans, the causative agent of Buruli ulcer, is the prototypical necrosis-inducing mycobacterial pathogen. Buruli ulcer is a disease with a tropical geographic distribution, with focal areas of regional prevalence, most recently in West and Central Africa. While the disease occurs at high rates in localized areas, it is not contagious, but rather is transmitted to multiple people by common source environmental exposure. While water, or aquatic vertebrates, are suspected reservoirs for *M. ulcerans*, the exact mode of transmission has not been proven. However, once the organism gains entry to the skin of the human host, a small subcutaneous nodule forms, which persists over weeks to months, without signs of systemic disease (such as fever, weight loss or inappetance). Eventually, an ulcer forms in the skin at the site of the nodule, and the original mass dissipates. These ulcers can become extremely large, and are characterized by extensive dermal and epidermal necrosis. Massive surgical debridement with reconstructive plastic surgery have historically been the treatments of choice. Currently, systemic antimycobacterial drugs are preferred when the condition is diagnosed early, because the scarring associated with the disease is disfiguring, and frequently leads

to partial motor disability through limb contracture or circumferential scar formation.[18,74]

Histologically, *M. ulcerans* bacilli within the ulcerated areas are extracellular, and the microcolonies tend to be localized within adipose tissue. Necrosis of tissue, particularly adipose tissue, is also seen at sites distant from the bacilli themselves. In an adipose cell (SW872) culture system infected with *M. ulcerans,* bacilli remained extracellular; however, significant cell mortality was observed as early as 24 hours after infection. At culture temperature of 37°C (human body temperature), cells died by necrosis. It has also been shown that *M. ulcerans* culture filtrate (MUCF) causes cytotoxicity when applied to cell culture, implicating a secreted toxin in the pathogenesis of Buruli ulcer. Mycolactones, which are a family of polyketides first identified in *M. ulcerans*, are chiefly responsible for the induction of necrosis.[1,16]

The hallmark of toxin-induced necrotic cell death in *M. ulcerans* infections is the lack of inflammation within the acute lesion. This type of necrosis is called coagulation necrosis, or bland necrosis, to distinguish it from necrosis which occurs as an end-stage manifestation of severe inflammation. While granulomatous inflammation may occur in the tissue surrounding

the necrotic area, hypersensitivity to mycobacterial antigens, rather than the secreted toxin itself, is the cause.[1,11,18]

M. marinum is an organism which is closely related to *M. ulcerans*. The organism is acquired from contaminated water, and causes a less-virulent infection in humans than Buruli ulcer. At temperatures below 33°C, the organism can enter macrophages and replicate successfully, while at temperatures closer to human body temperature (37°C), intracellular replication is inefficient. This likely explains why the infection in humans is typically confined to the extremities (where the body temperature is slightly cooler), and is usually self-limiting. Small ulcers and/or nodules form, associated with mild local pain and tenderness, and then regress within 1-2 years. [14,18,74] Histologically, some bacilli are observed intracellularly, and small cellular aggregates, typical of *M. tuberculosis* granulomas, will be seen.

Phylogenetically, *M. marinum* and *M. ulcerans* are very close, exhibiting identical 16S rRNA sequences, despite these marked differences in pathogenesis and interaction with the host cell. The major cluster of genes where the two species differ are the plasmid-encoded genes for mycolactone production by *M. ulcerans*. The marked necrotic cell death seen with *M. ulcerans* is attributable to this genetic locus.[1,18,74]

It is clear from this variation in virulence attributes that the question of apoptosis- vs. necrosis-induction is central to any evaluation of mycobacterial infection. Mycobacterial species vary in their tendencies to induce necrosis or apoptosis. Host cell types vary in their apoptotic or necrotic responses to mycobacterial infection. Within a given cell type and mycobacterial species, virulence and viability of the pathogen affect the host apoptotic or necrotic response. Even within a given host, infected with a single mycobacterial species, relative apoptotic or necrotic outcomes can vary by stage or severity of infection. Classically, *M. ulcerans* produces infection characterized by necrosis, and *M. tuberculosis* produces infection characterized by apoptosis. Other strains of mycobacteria, or conditions of infection, can be classified against this background.

Currently available tuberculosis model systems

Although research on *M. tuberculosis* has been active for 125 years, progress has been slow in relation to that of other pathogens. Laboratory handling of the organism is hampered by its airborne, highly-contagious nature, and the lack of reliable therapy for the disease. These features classify it as a Biosafety Level 3 (BSL-3) pathogen. All investigations require the expense and technology inherent to that status.

M. tuberculosis is an organism with a complex pathogenesis, and a long-term association with its human host. Appropriate experimental systems are essential to studying this organism to elucidate its biological behavior, to discover targets for preventing infection, and for therapeutic elimination, and to test for novel means of intervention. Ideally, experimental systems should mimic the natural system as closely as possible. In the case of *M. tuberculosis*, chronic granulomatous respiratory disease would have to be created in an experimental animal, and *in vitro* assays using this model species would have to be available to clarify molecular events. Despite profound interest and need, no such ideal model system yet exists for *M. tuberculosis*.[24,55]

The first, obvious choice for an animal model has been the non-human primate. Indeed, endemic tuberculosis has historically been a problem in monkeys housed in zoos and other public displays. Several species of monkey contract tuberculosis, including cynamologous macaques (*Macaca fascicularis*), rhesus monkeys (*Macaca mulatta*), and chimpanzees (*Pan troglodytes*). One advantage to the use of primates as a study system has been that their large body size facilitates individual monitoring and therapeutic interventions. Further, their close immunologic similarities to humans has meant that study techniques, reagents and antibodies will frequently be

available. Also, the actual pathogen of interest, *M. tuberculosis*, has been the agent investigated. There are significant disadvantages to the use of non-human primates as well, including zoonotic risk to investigators, substantial expense, and increasingly negative public perception associated with their use as experimental subjects. Being dangerous to the human researcher, these studies require the use of BSL-3 conditions. Historically, it had been thought that non-human primates succumbed to acute tuberculosis far more frequently than humans, which was a disadvantage to their use in the study of chronic infection. Model systems have since been developed which create chronic, latent infection similar to that seen commonly in humans.[11,23,47]

Other mammal species have also been experimentally infected with *M. tuberculosis*, inculding guinea pigs (*Cavia porcellus*), rabbits (*Oryctolagus cuniculus*) and mice (*Mus musculus*). Again, use of the precise pathogen of interest is valuable, but also of greater expense and zoonotic risk. *M. tuberculosis* is not a natural pathogen of any of these species, however, and while experimental infection is possible, it does not closely replicate the natural disease of humans. For example, mice must be inoculated with high bacterial burdens, and commonly require simultaneous antimycobacterial drug use to create a chronic, latent infection that mimics the chronicity seen in humans.

Even when the clinical appearance of a chronic asymptomatic infection can be created in mice, bacterial numbers in the pulmonary tissue are very much higher than those in humans with a similar clinical appearance. Infected mice do not form granulomas as humans do; rather, diffuse progressive pulmonary injury is seen.[11,47,52,55] Rabbits and guinea pigs behave similarly to one another, and may be preferable to mice from the standpoint that granulomas do form with pulmonary infection. However, these species are also highly sensitive to infection; most infected guinea pigs die within 100 days postinfection. Microscopically, granulomas which initially form undergo progressive necrosis with rapid proliferation of bacilli at the center. This differs from the ongoing battle between host cell apoptosis and *M. tuberculosis* proliferation seen in the chronically infected human patient. While granuloma deterioration matches what is seen in human patients with terminal progressive tuberculosis, the more typical, stable chronic state of human disease is not observed in guinea pig or rabbit hosts. Despite these disadvantages, the guinea pig is generally considered the most rigorous and consistent model system for the study of vaccines.[11,38,47,52,78]

Even if the limitations of each experimental mammalian system could be addressed and improved, the risk and expense of working under BSL-3 conditions still applies to the study of *M.*

tuberculosis in primates, rodents, or rabbits. For this reason, interest has developed in the use of non-zoonotic organisms which are closely related to *M. tuberculosis,* and which possess similar virulence traits, as models for study. For example, *M. marinum*, the causative agent of fish-handlers' disease, is a relatively lower-virulence and low zoonotic risk pathogen which can be studied under BSL-2 conditions. While it does not cause systemic granulomatous disease in mammals, it produces granulomas in its fish and amphibian natural hosts. Chronic disease in fish can reactivate in times of stress, similar to what is observed in infected humans.[20,28] *M. marinum* is also closely related to *M. tuberculosis* genetically, as bacterial DNA sequencing projects have demonstrated, which suggests that pathogenetic mechanisms may be similar.[8,69]

Studies with *M. marinum* in zebrafish, frogs, mammalian epithelial cell lines, *Drosophila*, and amoeba have shown the utility of this model organism. Diverse experimental systems have been created for *M. marinum,* allowing investigation both *in vivo* and *in vitro*. Non-mammalian organisms have been experimentally infected (*in vivo*), and cell cultures of both non-mammalian and mammalian origin (*in vitro*) have also been utilized. Leopard frogs (*Rana pipiens*) are infected with *M. marinum* as a natural pathogen. Under experimental conditions, a chronic, nonlethal granulomatous infection can be produced in

these frogs, and subsequent immunosuppression can produce a reactivated acute fulminant infection. This course of disease mimics tuberculosis in people, and could be valuable to the understanding of progression or reactivation of latent disease.[61] Zebrafish (*Danio rerio*) are also naturally infected by *M. marinum*, and are well-suited to experimental study. The particularly valuable features of these dish are their development of systemic disease, easy maintenance within the animal laboratory, and the depth of knowledge concerning their genetic composition. Also, the transparency of macroscopic zebrafish embryos enables direct visualization of the events of granuloma formation within the whole host animal, following experimental infection.[55,58,85]

Conserved innate immune responses can also be studied using *M. marinum* infections of well-understood experimental organisms such as *Drosophila melanogaster* and *Dictyostelium discoideum*. Not useful as a model of systemic granulomatous infection, *Drosophila* instead allow the innate, phagocytic immune responses to *M. marinum* to be studied in isolation from the acquired specific response.[60] Meanwhile, as single-celled organisms with behavior similar to that of macrophages, amoeba such as *Dictyostelium* are a useful model for phagolysosomal trafficking under conditions of *M. marinum* infection.[55]

Cell lines derived from either fish or frog natural hosts of systemic *M. marinum*, or from mammalian tissues, have also been successfully infected *in vitro*. Cell culture studies have confirmed that *M. marinum* infects cultured fish monocytes, while extracellular pathogens, such as *M. smegmatis*, do not. *M. marinum* survives and replicates intracellularly, where it inhibits lysosomal fusion in these cells, validating that this organism has similar specific virulence features found with *M. tuberculosis*.[20] Mouse (J774A.1), human (HEp-2) and Chinese hamster (CHO-K1) cell lines have also been infected with *M. marinum*, and the pathogen has been shown to invade and replicate within these cell lines similarly to its behavior in its natural hosts. Decreasing the culture temperature of the infected cells from 37°C to 33°C (closer to the optimum growth temperature for *M. marinum*) improved the persistence of the organism, without adversely affecting the viability or behavior of the cultured cell lines.[62]

The use of non-human model systems for *M. tuberculosis* infection, and non-tuberculous mycobacteria in diverse experimental systems, are essential to continued research efforts directed at tuberculosis. Each model system has strengths and weaknesses pertaining both to its logistics, and to the disease features it is suited to study. Importantly, however, both *M. tuberculosis* and *M. marinum* are potential human

pathogens, and the risks to personnel are a limitation to their use in the research laboratory.

Identification of *Mycobacterium shottsii*

The Chesapeake Bay is a large estuarine ecosystem providing a significant economic recreational and commercial fishery to the Eastern United States. In the early 1980s, a population decline was noted in several resident fish species, including the striped bass (*Morone saxatilis*). Deliberate restocking, and increased regulation of the bay was undertaken by the state of Maryland, and close surveillance of the striped bass population was begun. Consequently, when a second population decline occurred in the late 1990s, several organizations (including the Maryland Department of Agriculture, Virginia-Maryland Regional College of Veterinary Medicine, Fish Health Laboratory, United States Geological Service, National Oceanographic and Atmospheric Administration, and Virginia Institute of Marine Sciences at the College of William and Mary) were poised to launch a prompt investigation. Preliminary gross examination, histopathologic and microbiologic studies of diseased and dying fish implicated systemic mycobacteriosis as the cause of the epizootic. While pathogenic mycobacterial species of fish have been recognized, mycobacteriosis had not previously been reported in the US East Coast wild fish populations.[14,33,66]

In fish, as in humans, systemic mycobacteriosis is a chronic wasting illness. Consistent with this pathology, in this new epizootic, older fish were disproportionately affected with clinical illness. Additionally, many healthy-appearing fish randomly selected for necropsy were found to have granulomas on visceral organs, suggesting a period of latent or subclinical infection.[33,66,67,68] Field isolation of the novel causative agent from fish lesions was accomplished by Rhodes and others in 2004. Initial attempts to culture the causative agent were based on protocols known to be successful for *M. marinum*, including incubation temperature of 30°C. When this failed, lower culture temperatures were attempted. Recovery was finally achieved in a period of 3 months, at 23° C, on Middlebrook 7H10 agar. By classic biochemical tests and colony description, the mycobacterial species recovered were assigned to 7 distinct groups, labelled groups A through G. Group A consisted of mycobacteria which were niacin-positive, nitrate reductase-negative, and urease-positive, and which formed non-pigmented colonies. In this study, spleens from 196 fish (both apparently-healthy, and apparently-ill) were cultured for mycobacteria, and 145 spleens (76%) yielded a positive result. Group A organisms were cultured from 109 spleens (56% of the total spleens cultured, and 75% of the spleens with positive culture results). Within these 109 spleens, group A organisms

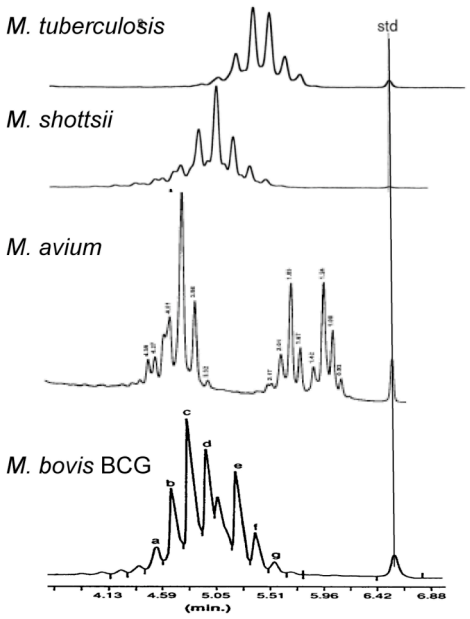
occurred alone in 62 of 109 (56%), and as part of mycobacterial polymicrobial infections in the 47 of 109 (44%). These data show that Group A mycobacteria could both be infectious agents on their own, and could contribute to a mixed mycobacterial infectious burden. When organisms from Group A occurred as part of a poly-mycobacterial infection, they always occurred at a higher colony count than the co-infectors. Group A mycobacteria were determined to be a major component of the Chesapeake Bay epizootic, and the representative isolate from the group (M175) was subsequently named *Mycobacterium shottsii.*

The species description of *M. shottsii* includes colony morphology, classic biochemical reactions, and mycolic acid profile. *M. shottsii* are acid-fast coccobacilli which may form aggregates in culture, and which form visible colonies on Middlebrook 7H10 agar in 4-6 weeks at an incubation temperature of 23°C. Colonies are negative for arylsulfatase, β-galactosidase, nitrate reductase, Tween 80 hydrolysis, and do not produce pigment after exposure to light for hours to days. *M. shottsii* does produce urease and niacin. The mycolic acid pattern is a single eight-peak cluster, which resembles *M. tuberculosis*, but elutes earlier. The 16S rRNA PCR product is a unique 1494 nt base pair, and the type strain was deposited in the ATCC as #700981.[67,68]

Simultaneous to the characterization of the prevalence and the biochemical identity of *M. shottsii*, Gauthier and others were attempting to generate experimental infections with this organism.[28] Captive striped bass were intraperitoneally inoculated with *M. shottsii*, and corresponding control bass were inoculated with *M. marinum*, or *M. gordona*. *M. marinum* produced severe diffuse mesenteric and visceral granulomas following inoculation. Fish were observed and sequentially sacrificed over a period of 45 weeks, and *M. marinum* granulomas were examined histopathologically. Over successive observation points, granulomas progressed through several characteristic phases of latency, and returned to reactivated disease in some fish. In contrast, *M. shottsii* and *M. gordonae* produced only mild mesenteric granulomas, and limited involvement of the spleen. Even without the presence of visible granulomas, both organisms could frequently be cultured from the spleen, but at far lower colony counts than found with *M. marinum*. In all cases, *M. shottsii* and *M. gordonae* granulomas resolved over time, and in no case was reactivated infection observed.[28] Given the severity of the disease in the wild striped bass population, and the frequency with which *M. shottsii* seemed to be the sole pathogen identified, this inability to reproduce disease in an experimental setting was surprising. It may be that natural infection with *M. shottsii* develops over a longer period of time

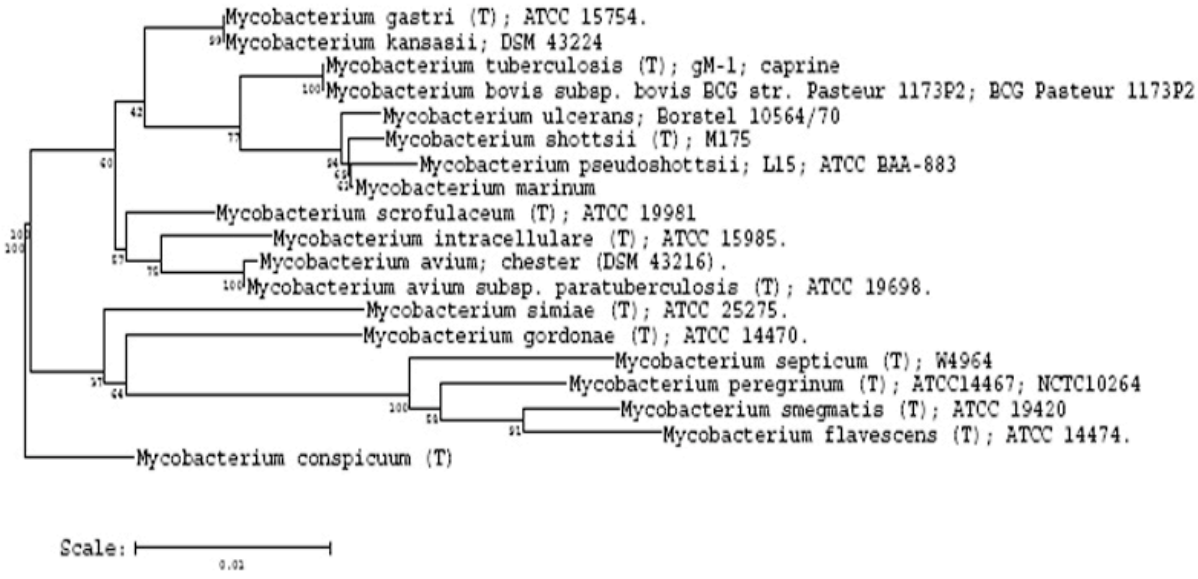
than suspected, and that 45 weeks was inadequate time for clinical disease to occur. It seems more likely, however, that captive striped bass, kept in ideal conditions of feeding, density and water quality conditions, might be more capable of eradicating infection with *M. shottsii*, than fish in wild conditions, where environmental, competitive, nutritional, or other microbial factors may contribute to stress. If true, this explanation draws great parallels between fish mycobacteriosis (due to *M. shottsii*) and human mycobacteriosis (due to *M. tuberculosis*) because of the significant contributions that host and environmental factors make to disease development following initial infection.

Rhodes and others evaluated the antigenic and genetic make-up of this novel pathogen, *M. shottsii*, both by mycolic acid high-performance liquid chromatography (HPLC) profile and 16S rRNA sequencing.[67] *M. shottsii* exhibited an eight-peak cluster on mycolic acid HPLC which strongly resembled that of *M. tuberculosis*, although its elution time was earlier.



(image courtesy of Frederick Quinn)

M. shottsii showed close 16S rRNA similarity to both *M. marinum* and *M. ulcerans*, and also to *M. tuberculosis* and *M. bovis*.



(adapted from Rhodes et al 2005)

When considering the information gleaned by both these assays, *M. shottsii* is more similar to *M. tuberculosis* than any previously-identified mycobacterial species, and is now officially a member of the *M. tuberculosis* complex, along with *M. bovis*, *M. africanum* and *M. microti*.[39]

Another group (Group F) of novel mycobacteria isolated from this same epizootic was typified by strain L15, and subsequently named *Mycobacterium pseudoshottsii*. This organism is described as an acid-fast coccobacillus which forms cell aggregates in culture and which produces pale-yellow to gold colonies in 4-6 weeks at 23°C on Middlebrook 7H10 agar. The colonies are negative for arylsulfatase, β-galactosidase, nitrate reductase, Tween 80 hydrolysis, and positive for urease production and niacin accumulation. The HPLC mycolic acid pattern is a single cluster of peaks, and the 16S rRNA, *erp* (exported repeated protein) and *hsp65* (a 65-kDa heat shock protein) gene sequences are novel among known mycobacterial species. *M. pseudoshottsii* was deposited in the ATCC as #BAA-883.[65]

M. pseudoshottsii produces a mycolactone secreted toxin, called mycolactone F. Like the mycolactone of *M. ulcerans*, this toxin can be collected from the culture medium, and the lipid fraction purififed. When this lipid fraction was applied to cell culture in a recent study, it caused a cytotoxic effect, in the absence of *M. pseudoshottsii* organisms. The predominant

pathology elicited after toxin exposure was necrosis, although mycolactone F was less potent than the mycolactones previously described in *M. ulcerans*. The production of a toxin by this organism is compatible with the pathologic behavior of *M. ulcerans*, an extracellular pathogen which causes ulcers in infected humans by mycolactone-induced necrosis.[63]

Conclusion

The novel fish pathogen, *M. shottsii,* is potentially a species of great interest in the study of *M. tuberculosis*. *M. shottsii* causes chronic granulomatous infection in its natural host, as does *M. tuberculosis*. *M. shottsii* is very closely genetically and antigenically related to *M. tuberculosis*. The use of non-mammalian model systems, and non-tuberculous mycobacterial species as surrogates for the study of *M. tuberculosis* is well-established. Importantly, *M. shottsii* would not grow until the culture temperature was reduced below 30°C, an even lower temperature restriction than that of *M. marinum*. Thus, *M. shottsii* is less likely to be capable of causing infection, even localized infection, in human research personnel.

Induction of apoptosis vs induction of necrosis categorizes novel mycobacterial species. *M. pseudoshottsii* has been shown to cause extracellular infection and host cell necrosis. The

behavior of *M. shottsii* at the cellular level has not been described. Before the potential utility of *M. shottsii* as an experimental organism can be fully exploited, much remains to be understood about culture conditions, model systems, and pathogenetic behavior of this novel pathogen.

Chapter 2. Introduction

Tuberculosis is a major global human health threat, with an urgent need for further research. While humans are the only natural host for the disease caused by *Mycobacterium tuberculosis*, several non-human animal models, and cell culture models for infection have been developed. None of these models successfully replicates all of the key features of the natural disease in humans. Additionally, all research performed with *M. tuberculosis*, regardless of the animal model system used, requires compliance with Biosafety Level 3 conditions to protect human researchers.

For these reasons, the discovery of a closely related novel fish pathogen, *Mycobacterium shottsii*, was of great interest to the tuberculosis research community. Both genetic and mycolic acid analysis demonstrate the close relationship between *M. shottsii* and *M. tuberculosis*. Furthermore, the clinical features of natural infection seen in Chesapeake Bay striped bass (*Morone saxatilis*) infected with *M. shottsii* mimic several features of tuberculosis in humans. Infected fish develop a chronic, wasting illness, which is similar to human tuberculosis patients (and which earned the human disease the nickname, "consumption"). *M. shottsii* infection also appears to be associated with an asymptomatic phase, as some fish with

clinically healthy body condition and lacking external evidence of disease were found to be infected at necropsy. Human tuberculosis is similarly associated with a prolonged latent, or subclinical phase of infection. The immune response to *M. shottsii* infection in fish results in the formation of granulomas within visceral organs, corresponding to the granulomas seen in human patients with *M. tuberculosis*. Given these strong parallels between genetic and phenotypic characteristics of the two organisms, and the similar disease manifestations of the two pathogens in their respective hosts, *M. shottsii* in fish may be a more appropriate model for the study of tuberculosis in humans than any other currently available system.

Additionally, the organism grows slowly at temperatures from 23-28°C, and does not grow at all at 30°C or above. This temperature restriction makes the pathogen much safer than *M. tuberculosis* for humans to handle in a research setting. *M. marinum* is another known fish mycobacteriosis pathogen which prefers cooler temperatures, and which has been used as a *M. tuberculosis* model. However, *M. marinum* grows at temperatures up to 33°C, and can cause localized cutaneous infection on human distal extremities, where the temperature is slightly cooler than the core temperature of 37°C. *M. shottsii* is extremely unlikely to infect humans, even at the distal extremities,

because of its lower temperature preference as compared to *M. marinum*. This feature would be a further advantage to the use of *M. shottsii* as a model system.

The use of fish as experimental animals is well established, especially given the completely sequenced genome of the zebrafish (*Danio rerio*). Catfish (*Ictalurus punctatus*) are another commonly-utilized experimental species, and cultured cell lines from fish are available. Experimental infection with *M. shottsii* has not been reported.

Pathogenic mycobacterial species cause tissue damage either by host cell necrosis or apoptosis. Necrosis occurs largely due to the release of mycolactones, and other toxins, by mycobacterial species such as *M. ulcerans*. Conversely, apoptosis is thought to be a host cell defense mechanism, seen in *M. tuberculosis*, and other mycobacterial species. *M. tuberculosis* bacilli establish infection and replicate within the host cell macrophages. Infected macrophages undergo apoptosis, in order to prevent release of the persistent viable bacilli (which could occur with necrosis). This mechanism creates membrane-bound apoptotic bodies, which eventually can be phagocytosed by uninfected macrophages, and thus present mycobacterial antigens to generate a specific immune response. Induction of necrosis versus induction of apoptosis is feature

which is used to categorize the pathogenesis of any novel mycobacterial species.

M. shottsii serves as an ideal model pathogen for M. tuberculosis as it is closely related to the target pathogen, causes similar infection in natural hosts, and is safer, cheaper, and easier to study than the target pathogen. Whether it establishes infection in controlled circumstances, or behaves similarly at a cellular level remains to be determined. To promote use of *M. shottsii* as a research tool, further work will be needed to create protocols for experimental *in vivo* and *in vitro* infection, and to establish the behavior of host cells infected with *M. shottsii*. The aims of this project were to create *in vitro* infections with *M. shottsii* in cells derived from fish, and to determine if apoptosis or necrosis occurred in these infected cells.

Chapter 3. Materials and Methods

Bacterial strains

Mycobacterium shottsii (strain M175, courtesy of Martha Rhodes, Virginia Institute of Marine Sciences) was maintained in Difco Middlebrook 7H9 Broth (Fisher Scientific, Pittsburgh, PA) with 10% OADC (BD, Inc. Sparks, MD) and 0.25% Tween 80 (Fisher Scientific, Pittsburgh, PA). *M. shottsii* M175 transformed with plasmid pFJS8 (courtesy of Stephanie Halminsky) encoding green fluorescent protein (GFP) and resistance to kanamycin (*M. shottsii* GFP), was maintained in 7H9 broth with 10% OADC and 0.25% Tween-80 plus 25 ug/mL kanamycin (Sigma, St Louis, MO). Cultures were incubated with shaking (45 rpm) in closed culture vessels with a 5-fold air:liquid ratio at 23° C. Frozen stocks were prepared by transferring a 1 mL aliquot from cultures grown to a density of $OD_{600} \approx 1.0$ into each cryovial containing 0.2 mL 50% glycerol, mixing, and storing at –80° C.

Preparation of M. shottsii for infection

To minimize bacterial clumping prior to use in infections, bead-mixing was employed. Screw-cap, skirted, 2.0 mL microcentrifuge tubes (Bio-Rad, Hercules, CA) were loaded with 0.8 g 0.1 mm Zirconia/Silica beads (Biospec Products, Inc. Bartlesville, OK) and sterilized by autoclaving. *M. shottsii* GFP culture aliquots were centrifuged (15000 g, 10 min)

decanted, resuspended in the appropriate cell culture medium (CLC medium, or RPMI-FBS, see "Infections" section) to an OD_{600} = 1.0, and vortexed to mix. Centrifugation and resuspension was repeated to remove all associated Tween-80. Aliquots of 1 mL of bacteria were added to presterilized microcentrifuge tubes containing silicon beads. Samples were vortexed for 30 seconds to disrupt bacterial clumps. Samples were allowed to set for 5 minutes, to allow the beads and residual bacterial clumps to settle out, before the supernatant (containing suspended single cells, or small clumps, of *M. shottsii*) was transferred to a new tube. To avoid disturbing the bead layer, only 850 μL supernatant was transferred from each tube. The supernatants were combined into a single 15 mL conical tube, and vortexed an additional 30 seconds. To enumerate the bacteria, 1 μL of 1% crystal violet was mixed with 100 uL of bacteria and the sample was vortexed for 10 seconds, then counted microscopically at 1000x magnification using a Petroff-Hauser counting chamber. The bacterial suspension was diluted to the desired concentration in the appropriate cell culture medium or in phosphate buffered saline (PBS, Gibco BRL, Gaithersburg, MD) just prior to inoculation.

Eukaryotic cells

Carp leukocyte cell (CLC) culture

Carp leukocyte cells (CLC, ECACC #95070628) were maintained in Eagle's modified essential medium (EMEM, Gibco BRL, Gaithersburg, MD) with 10% Hyclone fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD), 1% non-essential amino acids (NEAA, Gibco BRL, Gaithersburg, MD) and 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD) (CLC medium). Medium was 0.3 μ filter-sterilized prior to use, and L-glutamine was refreshed every 30 days. Cells were maintained in T75 tissue culture flasks (Corning Costar, Acton, MA) at 26° C with 5% CO, and passaged every 10 days, or when clumps and mounds of cells were observed. Cell passages greater than 20 were discarded and new cell cultures were initiated from frozen stocks.

To harvest CLC, the medium was aspirated from the monolayer of cells and adherent cells were gently washed with 6 mL calcium- and magnesium-free Hank's Buffered Salt Solution (CMF-HBSS, Gibco BRL, Gaithersburg, MD). Cells were washed with 1 mL 0.25% trypsin (Gibco BRL, Gaithersburg, MD) which was promptly removed. Fresh trypsin (2 mL) was added and incubated at 26° C for 3 minutes, or until cells were observed to be free-floating, by gross and microscopic evaluation. Trypsin was quenched by addition of 6 mL room temperature CLC medium, and the cellular suspension was vortexed for 5 seconds to mix. Viable cells were

counted, using trypan blue (Gibco BRL, Gaithersburg, MD) to enumerate dead cells. Cells were collected by centrifugation (175 g, 10 min) resuspended to the desired concentration in fresh CLC medium.

Prior to infection studies, plain or collagen-coated glass-bottom microwell dishes (MatTek, Inc., Ashland, MA) were preloaded with 2 mL room temperature CLC medium for 2 hours. Two mL aliquots of CLC at 5 x 10^5 to 5 x 10^6 cells/mL were seeded into the microwell dishes and incubated at 26°C and 5% CO_2 for up to 7 days to allow attachment. To encourage adherence, some dishes were also treated with 2 ng/mL rhGM-CSF (Endogen, Woburn, MA). Also prior to infection studies, 5 mL aliquots of CLC at 5 x 10^6 cells/mL were seeded into T25 flasks (Corning Costar, Acton MA) and incubated for 72 hours, at 26°C and 5% CO_2 to allow attachment. Dishes and flasks (with adherent cells) were washed once with 3 mL CMF-HBSS before being infected with bacteria.

THP-1 cells

THP-1 cells (ECACC #88081201) are an established human monocytic macrophage cell line which proliferate in suspension and are grown in RPMI-FBS medium. Catfish blood donors (see below) were a limited resource in this project, therefore, THP-1 cells were used as a proxy cell line to develop the protocol for *M. shottsii* infections in cellular suspensions, and evaluation by flow cytometry.

THP-1 cell cultures were recovered from stock frozen at 1 x 10^7 cells/mL, by adding 1 mL cells to 25 mL room temperature RPMI-FBS medium in a T75 flask. Cells were incubated in 5% CO_2 at 37° C for seven days, and then split into five new T75 flasks, each containing 5 mL cells and 20 mL fresh RPMI-FBS, and incubated for another 7 days prior to use.

Catfish peripheral blood leukocytes

CLC had shown a tendency to clump during cultivation which interfered with their utility for both adherent and suspension studies. For this reason, a protocol was developed that utilized primary cultivation of catfish monocytes freshly isolated from peripheral blood.

For collection of blooc, catfish (*Ictalurus punctatus*) were anesthetized by addition of tricaine (MS-222, 150 mg/L, Western Chemical, Inc., Ferndale, WA) to a holding tank and blood was collected from the caudal vein into heparinized or plain syringes. Heparinized blood was transferred to plain Vacutainer® tubes (BD Diagnostics, Franklin Lakes, NJ). Non-heparinized blood was transferred to di-potassium EDTA Vacutainer® tubes, or plain Vacutainer® tubes. Fish were allowed to recover in a fresh holding tank containing unmedicated water. All procedures were performed in accordance with protocols approved by The University of Georgia

Institutional Animal Care and Use Committee (IACUC) (Animal Use Project #A2004-10158-C2).

Supplemented RPMI medium was used for cultivation of the fish monocytes. It was made using 450 mL RPMI (with phenol red, with 2 mM L-glutamine, without HEPES buffer) (Sigma, St Louis, MO) and 50 mL FBS (RPMI-FBS). Fresh L-glutamine was added every 30 days.

Three techniques were evaluated for the isolation of monocyte-like cells from catfish peripheral blood. Based on published studies, Percoll (Sigma, St Louis, MO) gradients were used to separate catfish mononuclear cells. A discontinuous gradient was created by carefully layering 2 mL of each Percoll dilution (specific gravities = 1.080, 1.070, 1.065, 1.060 created with CMF-HBSS) into a 15 mL conical tube (BD Falcon, Franklin Lakes, NJ). Anticoagulated catfish blood was diluted with 2 parts CMF-HBSS, and 5 mL diluted blood was carefully added to the Percoll gradient. Tubes were centrifuged (25°C, 300 g, 20 min) and allowed to stop gradually with the brake turned off. Layering was incomplete, therefore two superficial layers were harvested and plated separately in microwell dishes (1 mL), or in T25 flasks (5 mL) to allow further selection for adherent cells. Cells were incubated in 5% CO_2, 26° C. On the third day, medium was aspirated. Monolayers were washed 3 times with CMF-

HBSS, covered with RPMI-FBS medium, and incubated an additional 2 days.

As fish erythrocytes have also been reported to lyse in EDTA, peripheral blood collected into di-potassium EDTA Vacutainer® tubes was incubated at room temperature for one hour after collection, and centrifuged (400 g, 10 min) prior to decanting the supernatant. While the supernatant was red due to hemolysis, visual inspection showed many intact erythrocytes remaining in the pellet with the leukocytes. These cells were resuspended in CMF-HBSS and layered onto a discontinuous Percoll gradient as described above.

The third condition investigated for isolation of catfish leukocytes from blooc was the use of ACK lysis buffer (Invitrogen, Carlsbad, CA). Anticoagulated catfish blood (1.5 mL) was added to 10 mL ACK lysis buffer, and incubated for 15 min at room temperature in 15 mL conical tubes. Tubes were centrifuged (400 g, 15 min) and the supernatants aspirated. Pellets were resuspended by pipetting in 10 mL fresh ACK lysis buffer, and cells were incubated an additional 15 min at room temperature. Tubes were centrifuged (400 g, 15 min) and the supernatants aspirated. Pellets were resuspended in 10 mL CMF-HBSS, and washed three times, using centrifugation (400 g, 15 min). Cells were resuspended in 10 mL CMF-HBSS, counted using trypan blue as described above, and resuspended in RPMI-FBS

medium at 5 x 10^7 cells/mL. This higher cell concentration was used because residual erythrocytes, and leukocytes other than monocyte-like cells, were not expected to persist in subsequent steps, thus the cell count was expected to decrease. The tendency of monocyte-like cells to adhere to surfaces was used to further select these cells from the total leukocyte population recovered above. For this, glass-bottom dishes (MatTek, Inc., Ashland, MA) were preincubated with 2 mL room temperature RPMI-FBS medium for 2 hours. Medium was aspirated and 2 mL catfish leukocytes in RPMI-FBS medium at 1 x 10^7 cells/mL were added to each dish. Cells were incubated in 5% CO_2 at 26° C for 3 days. Cells were inspected on the third day, and fed with 1 mL fresh RPMI-FBS medium. On the sixth day, cells were inspected for adherence, and old medium and non-adherent cells were aspirated. Monolayers were gently washed twice with 2 mL CMF-HBSS, and 1 mL fresh RPMI-FBS medium was added in preparation for use in infections. This technique proved most successful for the isolation of viable, adherent monocyte-like cells, and was used for subsequent experiments.

Zebrafish coelomic macrophages

Zebrafish (*Danio rerio*) are a widely-used laboratory species, and are potentially an attractive *in vivo* model system for *M. shottsii* infection. For that reason, *in vitro* infection

was evaluated in this species as well, to characterize the cellular mechanisms of infection.

Zebrafish were maintained at 23°C in a 30 L aquarium, containing tap water dechlorinated with 13% sodium thiosulfate solution, and were fed a commercial fish ration daily. To recruit macrophages to the coelom for infection and harvest, zebrafish were anesthetized by addition of tricaine (MS-222, 200 mg/L, Western Chemical, Inc., Ferndale, WA) to a small holding tank, and 30 μL mineral oil was injected into the coelom of each fish. Fish were allowed to recover and were maintained in a 4 L holding tank for 72 hours, at which point infections were carried out as described below. All procedures were performed in accordance with protocols approved by The University of Georgia Institutional Animal Care and Use Committee (IACUC) (Animal Use Project ##A2006-10087-c1).

Infections

Adherent cells

Carp leukocyte cells (CLC)

For infections in adherent CLC, *M. shottsii* M175 or *M. shottsii* GFP (uninfected control, CLC medium) was added to microwell dishes at multiplicities of infection (MOIs) of approximately 1, 5 or 10. *M. shottsii* GFP was added to the adherent CLC cultures and incubated for 4 - 48 hours at 26° C and

5% CO_2. A fixed volume of bacterial inoculum (200 μL) was added to each microwell dish. CLC were also seeded at 5 x 10^6 cells/mL in T25 flasks (Corning Costar, Acton, MA), and subsequently harvested to create a cellular suspension.

Catfish peripheral blood leukocytes

For infections in adherent catfish cells, *M. shottsii* GFP (uninfected control, RPMI-FBS) was added to microwell dishes at estimated MOIs of 0.1, 1 and 10. Infection proceeded for 3 days in 5% CO_2 at 26° C. On the third day, supernatant containing non-adherent cells and free-floating bacteria was removed by aspiration. Monolayers were washed gently once with 3 mL CMF-HBSS in preparation for staining.

Suspended cells

Carp leukocyte cells

For infections of suspended CLC, cells were first infected in as adherent monolayers in T25 flasks and in microwell dishes, as described above. (This is because CLC naturally clump and quickly form free-floating rafts in suspension.) After 48 hours, supernatants were aspirated and adherent cells gently washed with CMF-HBSS (2 mL/flask, 1 mL/ dish). Harvest conditions to create a cellular suspension were studied. Infected CLC were harvested with trypsin or TrypLE Express (Gibco, Carlsbad, CA) which is a purified, non animal-derived, microbially-produced, cell dissociation enzyme. Cells were

washed with CMF-HBSS (2 mL/flask, 1 mL/dish), and trypsin or TrypLE Express was added (0.5 mL/flask, 200 uL/dish) and incubated for 3 minutes. Trypsin or TrypLE Express was quenched with room temperature CLC medium (3 mL/flask, 2 mL/dish) and cells were aspirated, and counted, using trypan blue to exclude dead cells. Cells were washed with 3 mL CMF-HBSS, centrifuged (175 g, 3 min) decanted and resuspended at 2 x 10^6 cells/mL in CMF-HBSS. The presence of clumps was recorded.

Suspended CLC (1 mL) from each growth environment were aliquoted into tubes containing differing volumes of trypsinizing enzyme; 100 μL trypsin, 10 μL trypsin, 100 μL TrypLE and 10 μL TrypLE. Cells were incubated at 26°C for 3 minutes, and vortexed for 10 seconds. The four trypsinizing conditions from each growth environment were divided into two further categories. From each tube, one half of the cells (500 μL) was promptly fixed in 500 μL 5% formalin for 20 minutes. The other half of the cells from each tube (500 μL) was quenched with 1 mL room temperature CLC medium, washed with 2 mL CMF-HBSS, centrifuged and decanted. Quenched cells were resuspended in 500 μL CMF-HBSS and observed for clumping, then fixed in 500 μL 5% formalin for 20 minutes. Fixed cells from both experimental categories were washed three times in 2 mL CMF-HBSS and observed for clumping, and counted using trypan blue to exclude dead

cells, prior to resuspension at a concentration of 2 x 10^6 live cells/mL.

THP-1 cells

On the day of infection, THP-1 cells were harvested and counted with trypan blue to enumerate live and dead cells, and adjusted to a concentration of 1 x 10^7 live cells/mL with fresh RPMI-FBS medium. Separate 15 mL conical tubes were created for five conditions: infected, infected with apoptosis blocked, uninfected, uninfected with apoptosis induced, and uninfected with necrosis induced (all described below).

M. shottsii suspension (control, RPMI-FBS) was added to designated tubes at an MOI of 1. Tubes were incubated in 5% CO_2 at 37°C on an orbital shaker (45 rpm), and aliquots were removed for analysis at 6-24 hour intervals for 3 days. Staining was carried out as described below.

Catfish peripheral blood leukocytes

After the protocol was developed using THP-1 cells, catfish cells were collected for flow cytometry studies. Blood was drawn and ACK lysis buffer was used to eliminate erythrocytes as described above, cells were counted using trypan blue, and cells were resuspended in RPMI-FBS at 1 x 10^7 cells/mL. (No adherence step was performed to further isolate monocyte-like cells.) Separate 15 mL conical tubes were created for five conditions: infected, infected with apoptosis blocked, uninfected,

uninfected with apoptosis induced, and uninfected with necrosis induced (all described below under "Flow Cytometry"). Aliquots were collected from each condition at intervals of 6-24 hours for 3 days, and stained as described below under "Flow Cytometry." Aliquots were also collected for electron microscopy as described below under "Electron Microscopy."

Zebrafish coelomic macrophages

Infections in zebrafish were carried out after macrophages had been mobilized into the coelom for 72 hours. Zebrafish were anesthetized by addition of tricaine (MS-222, 200 mg/L, Western Chemical, Inc., Ferndale, WA) to a small holding tank. One mL aliquots of *M. shottsii* GFP at 1 x 10^7 bacteria/mL (control, PBS) were centrifuged (15000 g, 2 min), resuspended in 30 μL PBS, and injected into the coelom of each fish.

After 24 hours of infection, zebrafish were euthanized by placement into a small holding tank containing tricaine (MS-222, 300 mg/L) until 10 minutes after cessation of all opercular movement. The contents of the coelomic cavity were harvested by coelomic lavage with 300 μL RPMI-FBS. Continuous Percoll gradients (45% Percoll in PBS) were created in 15 mL conical tubes, and harvested cells were carefully layered onto the Percoll. Samples were centrifuged (25°C, 1250 g, 10 min) and allowed to stop gradually with the brake turned off. The buffy coat (containing infected macrophages) was carefully aspirated

from the gradient. Cells were washed with PBS, centrifuged (175 g, 3 min) decanted and resuspended in RPMI-FBS.

Cellular quantities sufficient for flow cytometry were not obtained from zebrafish, so suspended cells were stained as described below in "Fluorochromes," and slides were prepared for examination by fluorescence microscopy.

Fluorochromes and fluorescence microscopy

RH 414, a lysosomal stain (Molecular Probes, Carlsbad CA, Ex = 532 nm, Em = 716 nm), was used in initial infections. Fresh RH 414 was prepared each time, using a stock of 5 mg/mL adjusted to a final concentration of 5 μg/mL with CMF-HBSS containing 1% bovine serum albumin fraction V (BSA, Fisher Scientific, Pittsburgh, PA). Two hundred μL of the fluorochrome was added to each microwell, either before, or 24 hours after, *M. shottsii* GFP infection, and incubated for 1 hour, in the dark at 26°C. Plates were washed 3 times with 3 mL CMF-HBSS and examined by fluorescence microscopy.

Annexin V-PE (phycoerythrin, BioVision, Mountain View, CA, Ex = 488 nm, Em = 578 nm, Annexin) was used as a marker of early apoptosis in adherent and suspended infections, in CLC and catfish cells. This protein has a strong natural affinity for phosphatidylserine, which is translocated from the inner to the outer face of the cell membrane as initiation of apoptosis

occurs. For adherent infections, supernatant was aspirated from microwell dishes after 48 hours of infection, and adherent cells were gently washed with 3 mL CMF-HBSS. Annexin V Binding Buffer (500 μL) was applied to each dish, followed by Annexin (5 μL). Cells were incubated for 5 minutes, in the dark, at 26° C, and washed with 3 mL of CMF-HBSS prior to examination by fluorescence microscopy. For suspended infections, 500 μL cells were aliquoted into snap-cap 5 mL polystyrene tubes (PS tubes, BD Falcon, Franklin Lakes, NJ), centrifuged (175 g x 5 min), decanted, and resuspended in 500 μL Binding Buffer. Annexin (5 μL) was added to each tube, and cells were incubated at 26°C, in the dark, for 5 minutes. Cells were washed once in 1 mL CMF-HBSS, and resuspended in 500 μL flow buffer, incubated on ice, in the dark, for 30 minutes to 24 hours, and read by flow cytometry.

CaspGLOW Green Caspase-8 Staining Kit and CaspGLOW Red Caspase 8 Staining Kit (BioVision, Mountain View, CA, sulforhodamine Ex = 540 nm, Em = 570 nm) were used as markers of late apoptosis, in adherent and suspended infections. These kits use green or red fluorescently-labeled caspase inhibitor IETD-FMK, which is cell permeant, and non-toxic, to bind irreversibly to activated caspase-8. Each kit also contains the unlabeled caspase inhibitor Z-VAD-FMK to be used at the start of the infection, as a negative control for induction of apoptosis.

At the time infection was initiated, a negative control plate or tube was created by adding CaspGLOW Z-VAD-FMK (1 μL/mL of medium) to the cells. For adherent infections, supernatant was aspirated from each microwell dish after 48 hours of infection, and cells were gently washed with 3 mL CMF-HBSS. Fresh medium (1 mL) was added to each dish. CaspGLOW (Red or Green) IETD-FMK (1 μL) was added to each dish and cells were incubated for 2 hours, in the dark, at 26° C, then washed twice with 0.5 mL of CaspGLOW Wash Buffer prior to examination by fluorescence microscopy. For suspended infections, 300 uL cells were aliquoted into PS tubes, 1 μL CaspGLOW (Red or Green) IETD-FMK was added and cells were incubated at 26°C, in the dark, for 2 hours. Cells were washed twice with 500 μL CaspGLOW Wash Buffer, resuspended in 300 μL flow buffer, incubated on ice, in the dark, for 30 minutes to 24 hours, and read by flow cytometry.

Lyso-Tracker DND-99, an acidotropic fluorochrome which is freely permeant to cell membranes, and which concentrates in acidic organelles, was also used for adherent infections (Molecular Probes, Carlsbad, CA, Ex = 577 nm, Em = 590 nm). Fresh Lyso-Tracker DND 99 (LT) was prepared for each use by diluting 5 μL of stock solution in 5 mL CLC medium or RPMI-FBS. For CLC, a fixed volume (200 μL) of LT (control, CLC medium) was added to each microwell dish. Cells were incubated for 1 hour in the dark, then LT was removed and replaced with an equal

volume of CLC medium. Cells were incubated an additional 30 minutes in the dark, then washed 3 times with 3 ml CMF-HBSS, and examined by fluorescence microscopy. For zebrafish coelomic macrophages, 10 μL LT (control, RPMI-FBS) was added per 1 mL of cellular suspension and cells were incubated for 1 hour in the dark, prior to preparation of slides for examination by fluorescence microscopy.

To enable visualization of cytoskeletal structures, phalloidin-TRITC was used in combination with LT in adherent infections. Phalloidin is a derivative of *Amanita* spp phallotoxin, which is water-soluble, and binds at nanomolar concentrations to F-actin from a wide variety of plant and animal species. As such, phalloidin fluorochromes can label the cytoskeleton and aid in the microscopic evaluation of localization of organelles within the cell. After infection and LT staining, dishes were gently washed twice with 3 mL CMF-HBSS. Cells were fixed with 5% buffered formalin, 3 mL/dish, and incubated for 20 minutes. Dishes were washed three times in 3 mL CMF-HBSS. Cells were permeabilized with 0.1% Triton-X100 for 20 minutes, then washed three times with 2 mL CMF-HBSS. Fresh stain was prepared for each use, by adding 20 μL of phalloidin-TRITC stock (0.5 mg/mL, Molecular Probes, Carlsbad, CA) to 2 mL PBS. Working dilution of phalloidin-TRITC (0.5 μg/mL) was applied

to microwell dishes (200 μL/dish) and incubated for 20 minutes, in the dark, at 26° C, then washed twice with 2 mL CMF-HBSS.

To minimize photobleaching and optimize imaging quality of fluorescence microscopy, mounting agents and sealants were evaluated. Residual liquid after the final washing step was aspirated with a fine-tip pipette until dishes were dry. Vecta-Shield (Vector Laboratories, Burlingame, CA) was applied to dishes to fill the microwells. ProLong Antifade (Invitrogen, Carlsbad, CA) was reconstituted according to package insert directions and applied to dishes to fill the microwells. As a control, CMF-HBSS was also applied to dishes to fill the microwells. An additional mounting agent was evaluated for use with catfish cells. Trolox-DAPI was made by adding 3 μL Trolox 0.1 M prepared in ethanol to 30 mL CMF-HBSS, and 30 μL DAPI (25 mg/mL).

Coverslips were applied carefully, to minimize formation of air bubbles, and were sealed with clear acrylic nail polish or VALAP. VALAP is an equal mixture by volume of vaseline, lanolin, and paraffin, melted together over a hotplate, and applied to the edges of the coverslip while in a molten state. Cells were evaluated by fluorescence microscopy.

All dishes that were to be removed from the laboratory for analysis were treated by formalin fixation. After most staining was complete (but before permeabilization for phalloidin

staining) 3 mL 5% formalin was added to each dish, and cells were fixed for 20 minutes. Formalin was aspirated and discarded, and plates were washed three times with 2 mL CMF-HBSS before mounting and sealing as described.

Fluorescence microscopy was carried out on a Zeiss Axiovert 200M microscope with fluorescence optics, using Axiovision software (Carl Zeiss, Inc, Thornwood, NY).

Flow cytometry

Flow buffer used in all flow cytometry studies was 1% BSA in PBS, made ahead of time and stored at 4°C. In some cases, 1x propidium iodide (PI) (50 μL) was added to flow buffer (4.95 mL). Stock was made in advance and 500 uL aliquots were stored at -20°C.

A method of cell fixation was desired because of the use of live cells and bacteria in the study. However, ethanol fixation introduced artifacts into the analysis, as did attempted glutaraldehyde fixation, formalin fixation, and paraformaldehyde (PF) fixation, because all changed the scatter pattern of the cells. It was decided not to fix the cells prior to flow cytometric evaluation, because it was possible to disinfect the cytometer by bleaching after use.

Apoptosis induction was attempted with 1 μM and 100 μM dexamethasone (Sigma, St Louis, MO), 2 μg/mL and 4 μg/mL

staurosporine (BioVision Inc., Mountain View, CA), and 0.02 μg/mL camptothecin (BioVision Inc., Mountain View, CA). These agents were added to control cell cultures (instead of bacteria) at the time that infection was initiated. A combination of camptothecin (0.02 μg/mL) and staurosporine (4 μg/mL) was determined to be the most successful means of inducing apoptosis (cam/staur).

Necrosis induction was accomplished by incubation in 1% paraformaldehyde (PF) for 12-24 hours. PF was freshly made for each use. To prepare a 1% solution, 0.25 g paraformaldehyde was dissolved in 22.5 mL deionized distilled water (ddH_2O) with 4 μL 10 N NaOH, in a 55°C water bath, then 2.5 mL 1X PBS was added, and the final solution was filter-sterilized.

After staining as described, catfish peripheral blood leukocytes were gently washed twice with 500 μL CMF-HBSS, or CaspGLOW Wash Buffer as indicated, and resuspended in 300 μL flow buffer, with or without 1x PI and incubated on ice for 30 minutes. Cells were centrifuged at 175 g x 3 minutes and brought up in 300 uL flow buffer for storage until evaluation by flow cytometry.

It was subsequently determined that use of PI well in advance of flow cytometric evaluation was allowing non-specific diffusion and binding of PI over the period of delay. Therefore, the protocol was modified to add PI at a lower

concentration of (0.1x) 30 minutes prior to evaluation. This did not resolve the non-specific binding of PI ,thus its use in the flow buffer was discontinued. PI (0.1x) was used in the necrosis-induced condition and was added immediately prior to evaluation.

The flow cytometer used was a CyAn ADP (Dako, Fort Collins, CO), using the blue diode laser (excitation, 488 nm). Data were analyzed using FlowJo Flow Cytometry Analysis Software (Tree Star, Inc., Ashland, OR).

Electron microscopy

Some catfish peripheral blood leukocytes from suspended infections were also used for electron microscopy. The tissue culture medium was removed, 2% glutaraldehyde fixative was added, and the cells were incubated at 4°C for 1 hour. Glutaraldehyde solution was removed and ice cold Collidine buffer (Electron Microscopy Sciences, Fort Washington, PA) was added before the cells were stored at 4°C for an additional 6-24 hours. Cells were then pelleted and fixed with 2% osmium tetroxide, dehydrated in ethanol, passed through propylene oxide, and embedded in maraglas resin. One micron sections were cut and stained with uranyl acetate and lead citrate. Sections were observed by transmission electron microscopy using a

Philips 410 electron microscope (The Philips Co., Amsterdam, The Netherlands).

Chapter 4. Results

M. shottsii infects CLC in microwell culture

An *M. shottsii* strain containing the gene for GFP had been previously created within our laboratory. Phenotypically, the bacilli exhibited a stable constitutive green glow under fluorescence microscopy that was maintained through every subculture utilized (Fig. 1).

CLC were cultured, passaged and plated into microwell dishes as described. From the beginning of the experiments, it was clear that CLC readily formed clumps and mounds in cell culture, even before achieving a confluent monolayer (Fig. 2A, 2B). Cells would also break free of the microwell surface and float free in the medium in large rafts and sheets (not shown). In an attempt to encourage the formation of a confluent monolayer, the conditions were adjusted. Attempted measures included varying the cell concentration used to seed the microwell dishes, varying the dishes themselves using both plain and collagen-coated microwells, increasing the time of incubation prior to infection, and using rhGM-CSF when seeding the dishes, which has been reported to facilitate adherence. None of these measures promoted formation of a confluent monolayer, or eliminated the free-floating clumps of cells.

When medium was aspirated and adherent cells were washed for staining steps, these free-floating cells were lost.

Regions of the CLC plates did contain individual cells, and these regions were evaluated by fluorescence microscopy. Phalloidin-TRITC was used to identify cytoskeletal structures (Fig. 3). LysoTracker was used to identify acidic organelles, which chiefly includes lysosomes (Fig. 4). Both phalloidin-TRITC and LysoTracker exhibit red fluorescence with the rhodamine filter (Fig. 5). While LysoTracker stains lysosomes red, when colocalized with GFP (for example, in *M. shottsii* GFP), the merged structures appear yellow.

Plated CLC were infected with *M. shottsii* M175 and *M. shottsii* GFP as described. Wild-type *M. shottsii* were used with green fluorochromes (such as CaspGLOW Green) on the CLC, while *M. shottsii* GFP (green bacilli) were used with CLC stained with red fluorochromes (such as Annexin PE). Using the *M. shottsii* GFP, the association of the bacilli with the CLC could be convincingly and repeatedly demonstrated (Fig. 6)

Within individual cells, *M. shottsii* GFP could be seen to colocalize with red LysoTracker, within the lysosomal compartment of CLC. The colocalization of GFP and LysoTracker appears yellow (Fig. 7, Fig. 8) Together, these findings show that CLC, despite some tendency to clump and float away from the

surface of the microwell dishes, can be used as an experimental host cell for infection with *Mycobacterium shottsii*.

Infection with *M. shottsii* induces apoptosis in CLC

M. shottsii M175 (which expresses no fluorescence) was used to infect CLC. The non-fluorescent wild-type bacilli enabled the use of green fluorochromes to study host cell behavior. CaspGLOW Green is a marker of the late events of apoptosis. It labels caspase 8, one of the steps in the cytoplasmic caspase cascade, which is initiated by internal signals within the infected cell. Plates with *M. shottsii*-infected CLC showed cells with green fluorescence, consistent with apoptosis induction by infection (Fig. 9).

M. shottsii GFP was used to infect CLC. The fluorescent bacilli appeared green, and the host cell was stained with a red fluorochrome to study its behavior. Annexin is a marker of early apoptosis, which is complexed with phycoerythrin (PE), a red fluorochrome. It labels phosphatidylserine, which inverts from the internal to the external surface of the cell membrane as one of the earliest steps of apoptosis. Plates with *M. shottsii* GFP-infected CLC which had been stained with Annexin-PE showed colocalization of green bacilli and cells with red fluorescence, consistent with apoptosis induction by infection (Fig. 10).

M. shottsii infects zebrafish coelomic macrophages

Macrophages were mobilized into the coelomic cavity of zebrafish, and subsequently infected with *M. shottsii* GFP as described above in "Infections.". Infected macrophages in suspension were successfully harvested with this system, but in relatively small numbers. While there were insufficient cells for flow cytometric analysis, cells could be stained with fluorochromes and plated onto slides for fluorescent microscopic examination.

The small size of zebrafish coelomic macrophages is apparent in the image (Fig. 11). The panels of the fluorescent image show the green of *M. shottsii* GFP, the blue of DAPI (which labels DNA and identifies the nucleus of the cell), and the red of LysoTracker (which labels lysosomes). The merge shows yellow fluorescence which indicates the colocalization of green *M. shottsii* within the red lysosome. This confirms trafficking of the pathogen to the lysosome, as CLC studies indicated.

M. shottsii infects catfish leukocytes in microwell culture

Because CLC clumping in cell culture and the low numbers of zebrafish coelomic macrophages harvested made these systems ineffective for flow cytometric studies, primary isolation of catfish leukocytes was undertaken. A protocol was developed for

the collection of peripheral blood from catfish, and enrichment of monocyte-like cells by adherence in microwell dishes (Table 1).

Catfish cells were plated and adhered to the microwell dishes singly and in confluent regions, without forming mounds and clumps (Fig. 12). Phalloidin-TRITC and LysoTracker were effective at demonstrating cytoskeletal elements and acid organelles, although the smaller size of the catfish leukocytes compared with CLC made these intracellular structures harder to appreciate (Fig. 13).

Catfish peripheral blood leukocytes can be evaluated by flow cytometry

A protocol was also developed for the collection and infection of catfish peripheral blood leukocytes in suspension (Table 2). Suspended cells were evaluated by flow cytometry and exhibited a repeatable scatter pattern in forward-scatter vs. side-scatter pseudocolor dot plots, even when different individual fish blood donors were used (Fig. 14).

Paraformaldehyde (PF) is a common fixative used in mycobacterial infection studies, and it was evaluated in the catfish peripheral leukocyte suspensions. Propidium iodide (PI) is also commonly used in flow cytometry to identify necrotic cells or cell fragments. However, when used in the catfish

peripheral leukocytes, PF and PI greatly shifted the forward-scatter and side-scatter characteristics of the cellular population. Based on this significant shift in cellular distribution, PF and PI were not used in subsequent experiments (Fig. 15).

Once the PF and PI were removed from the protocol, uninfected catfish leukocytes were evaluated for autofluorescence. In the GFP filter channel, or the PE filter channel, the cells exhibited minimal background fluorescence (Fig. 16).

M. shottsii infection induces apoptosis in catfish peripheral blood leukocytes

Catfish peripheral blood leukocytes were collected and maintained in suspended culture, while they were infected with *M. shottsii* GFP. Infection was successful as evidenced by green fluorescence (GFP filter channel) exhibited by approximately 20% of the leukocytes (Fig. 17).

Infected catfish peripheral blood leukocytes were stained with Annexin PE, a red fluorochrome which marks an early event of apoptosis. The infected cells were gated by selecting the GFP-positive events, and then were plotted for fluorescence in the PE channel. After the first hour of infection, eighty-five percent of infected cells were PE-positive, indicating that the

predominant effect of infection of catfish peripheral blood leukocytes with *M. shottsii* is apoptosis (Fig 18, A). To confirm that this effect was not simply due to the maintenance of leukocytes in culture conditions, the uninfected cells were gated by selecting the GFP-negative events, and then were plotted for fluorescence in the PE channel. None of the uninfected cells were PE-positive, confirming that apoptosis was not occurring in the uninfected cells (Fig. 18, B). A four-quadrant graph of PE (Annexin) vs. GFP confirms that most GFP-positive (ie, infected) cells are also PE-positive (ie, apoptotic). Importantly, no uninfected (GFP-negative) cells are apoptotic (PE-positive) (Fig. 19). Additionally, at the 24-hour time point of infection, it remained true that only GFP-positive cells were PE-positive, again confirming that infection, and not simply maintenance in culture, was the true cause of apoptosis (Fig. 20).

To confirm that green fluroescence truly represented intracellular, versus epicellular, *M. shottsii* GFP, infected cells were examined by electron microscopy. Bacilli were seen within catfish leukocytes (Fig. 21). Within infected cells, cytoplasmic blebbing, nuclear condensation, and formation of membrane-bound apoptotic bodies could be seen, confirming the apoptosis induction documented with flow cytometry (Fig. 22).

Results Figures and Tables

Fig 1. *M. shottsii* M175 transfected with GFP-expression plasmid PJF58, showing constitutive green fluorescence. Original magnification, 400x.

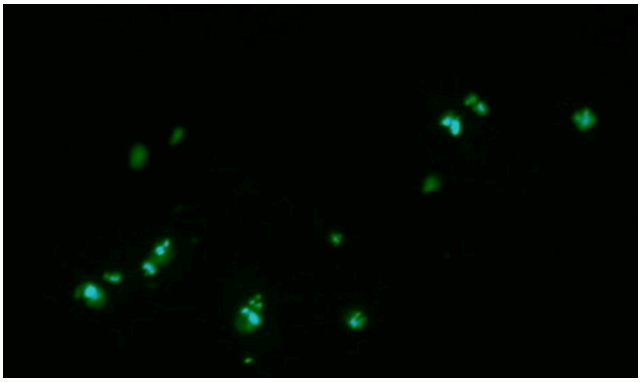
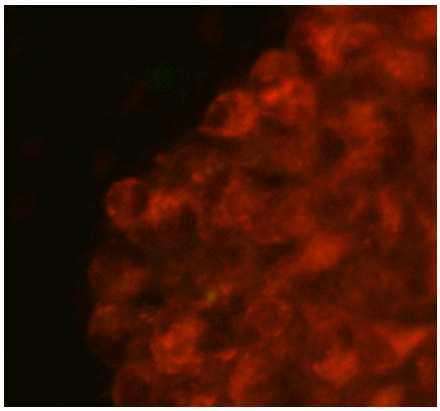
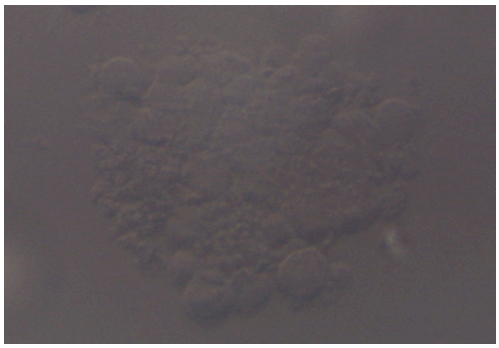


Fig 2A and 2B. CLC culture in microwell dishes, stained with RH414 (A) and under DIC (B), showing tendency of cells to form clumps and mounds. Original magnification, 400x.



A



B

Fig 3. CLC culture stained with phalloidin, showing cytoskeleton. Original magnification, 400x.

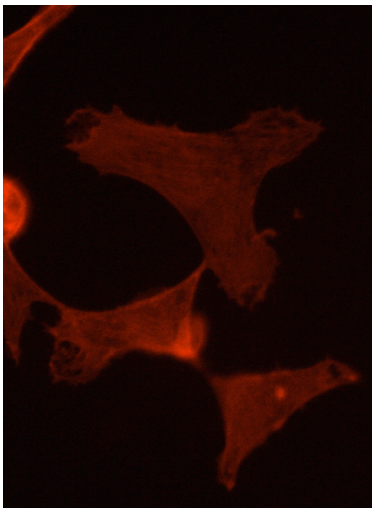


Fig 4. CLC culture stained with LysoTracker, showing acidic organelles (lysosomes). Original magnification, 400x.

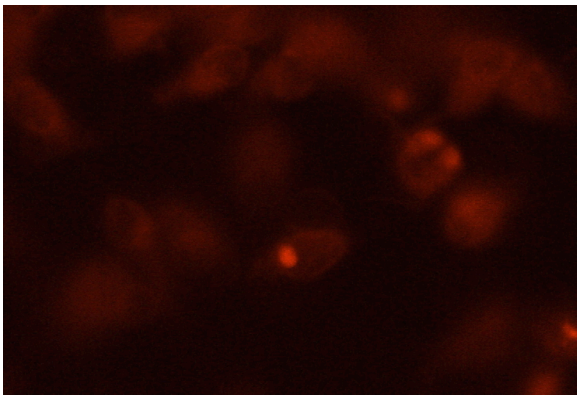


Fig 5. CLC culture infected with *M. shottsii* GFP, stained with phalloidin and Lyso-Tracker, showing both fluorochromes with the rhodamine filter, again exhibiting clumping. Original magnification, 400x.

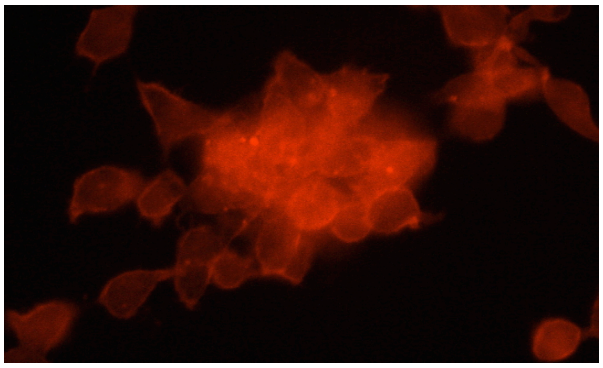


Fig 6. Same CLC culture infected with *M. shottsii* GFP, stained with phalloidin and Lyso-Tracker, showing merge with GFP filter as well, demonstrating *M. shottsii* GFP associated with CLC. Original magnification, 400x.

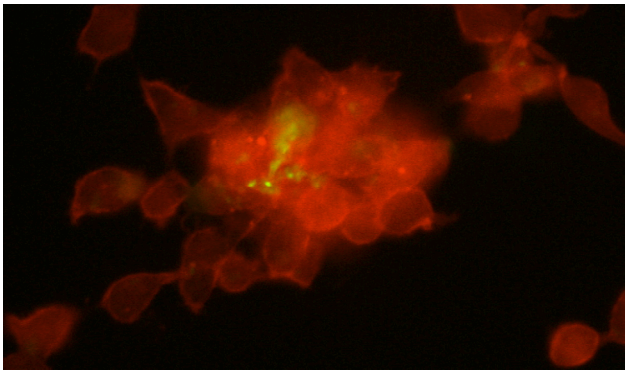


Fig 7. CLC infected with *M. shottsii* GFP, stained with phalloidin and LysoTracker, showing red cytoskeleton, green bacilli, and yellow (arrows) where the bacilli colocalize with the lysosomes. Original magnification, 400x.

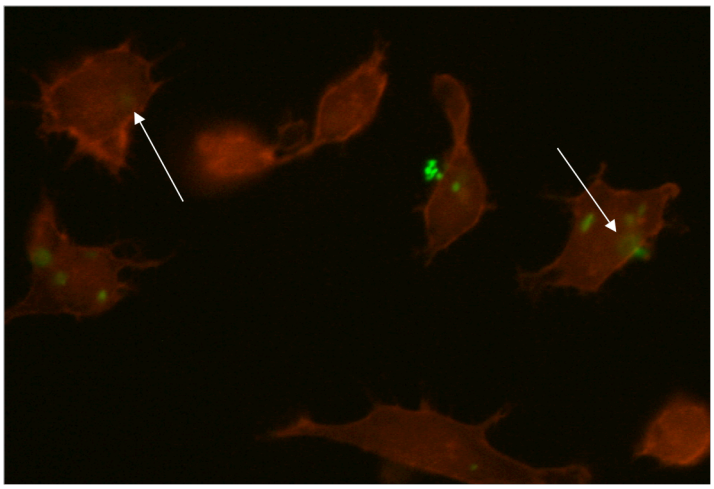


Fig 8. CLC infected with *M. shottsii* GFP, stained with phalloidin and LysoTracker, showing red cytoskeleton, green bacilli, and yellow (arrows) where the bacilli colocalize with the lysosomes. Original magnification, 400x.

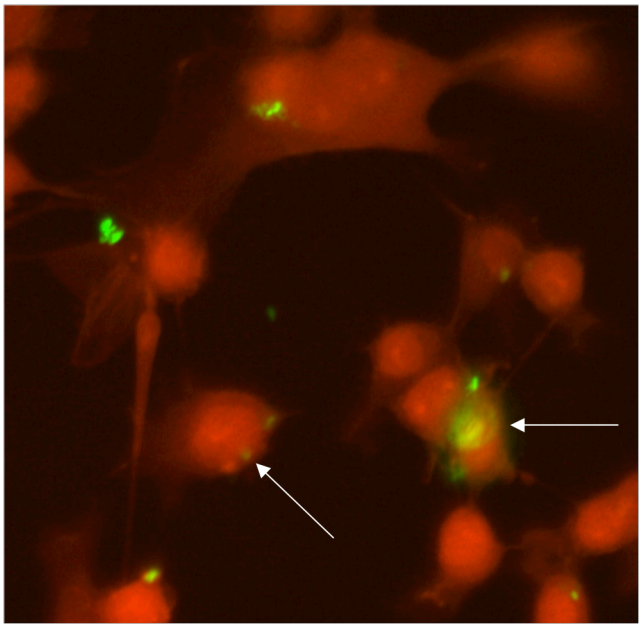


Fig 9. CLC infected with *M. shottsii* M175 and stained with CaspGLOW green as a marker of late events of apoptosis, showing a cluster of CLC with a pair of CaspGLOW-positive cells. Original magnification, 400x.

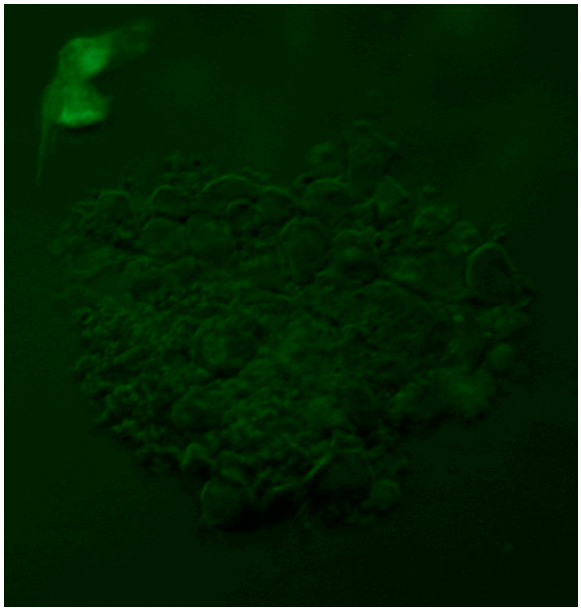


Fig 10. CLC infected with *M. shottsii* GFP and stained with Annexin PE as a marker of early events of apoptosis, showing green *M. shottsii* associated with red CLC. Original magnification, 400x.

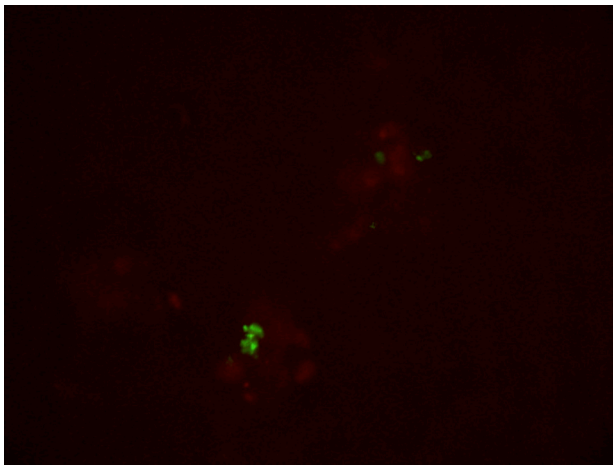


Fig 11. Zebrafish cells, infected with *M. shottsii* GFP, and stained with DAPI and LysoTracker. Yellow fluorescence (arrows) shows the colocalization of green bacilli with red lysosomes. Panel A shows GFP only, Panel B shows DAPI only, Panel C shows LysoTracker only and Panel D is the merge. Original magnification, 400x.

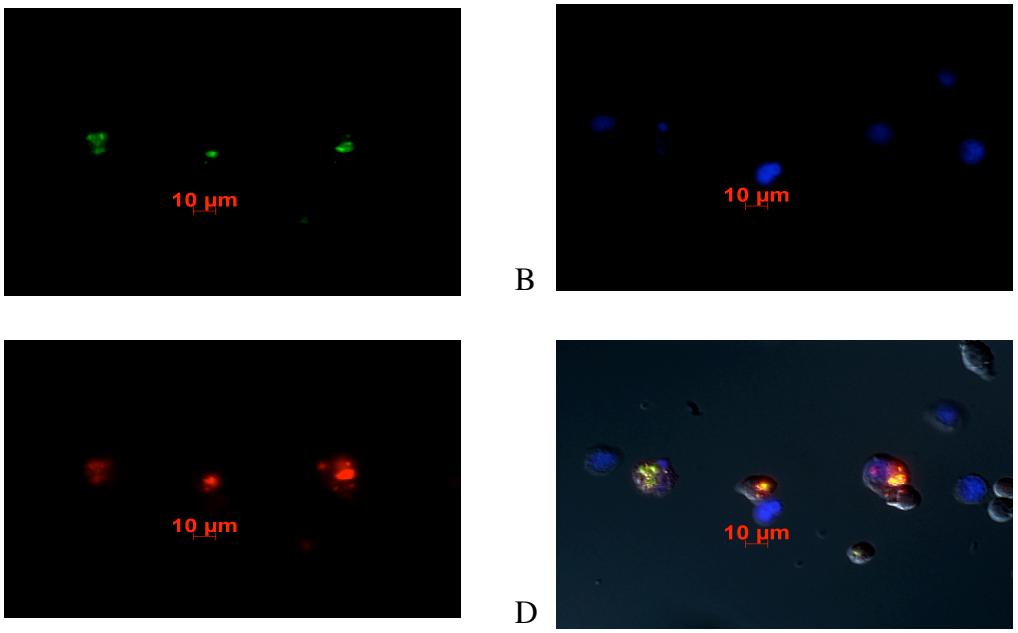


Table 1. Protocol for the collection, isolation and plating of peripheral blood leukocytes from catfish.

Cell preparation:

1 - Collect blood (1% body weight) from Tricaine-anesthetized catfish
- **a** 1 mL of blood will contain roughly 1-3 x 10^7 white cells
- **b** place into heparinized tubes

2 - ACK lysis
- **a** add 10 mL ACK lysis buffer to 1 mL of blood
- **b** incubate 15 minutes at room temperature
- **c** centrifuge at 400 g for 10 minutes
- **d** aspirate (do not decant) supernatant
- **e** repeat 10 mL ACK lysis buffer per 1 mL blood, room T x 15 min
- **f** centrifuge at 400 g for 10 minutes
- **f** aspirate supernatant and RBC debris
- **g** wash cells 3 x in 10 mL CMF-HBSS
- **h** prepare slide for inspection of residual RBC amount

3 - Count cells
- **a** count cells on hemacytometer
- **b** exclude dead, but do not exclude RBC
- **c** bring up to 1 x 10^7 cells/mL in RPMI w/ 10% FBS

Prepare plated cells

1 - Use glass-bottom MatTek microwell plates
- **a** label plates
- **b** preload 6 plates with 2 mL prewarmed medium for 2 hours
- **c** aspirate wells to empty
- **d** seed 1 mLs of cells at 1 x 10^7 per mL in each plate
  well holds 200 uL
  goal 1 x 10^6 cells per well
- **e** for delayed infections, add 1 mL RPMI w/10% FBS to each plate

Fig 12. Catfish leukocyte culture in microwell dishes, showing small size under DIC. Original magnification, 400x.

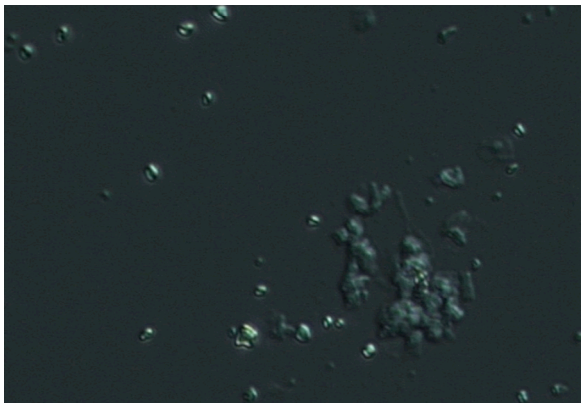


Fig 13. Catfish leukocyte culture, stained with phalloidin and LysoTracker. Original magnification, 400x.

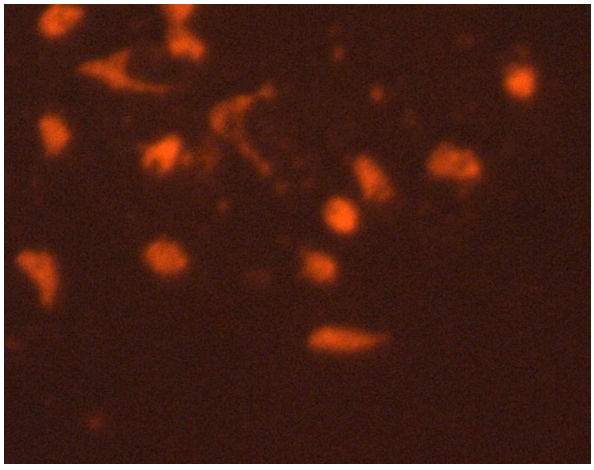


Table 2. Protocol for the collection, isolation and infection of catfish peripheral blood leukocytes in suspension.

Cell preparation steps 1-3 as described in Table 1

4 - Create tubes for infection

A infected

B infected, apoptosis blocked

C uninfected

D uninfected, apoptosis induced

***M. shottsii* GFP infection**

1 - To create MOI of 10

a use *M shottsii* GFP

b centrifuge 5 mLs culture at 15000 g (3000 rpm max) x 10 min

c decant supernatant

d bring up in equal volume RPMI w/ 10% FBS

e spin, decant, and repeat once

2 - Bead beating

a add 1 mL bacteria to 0.4 mL pre-sterilized silicon beads

b vortex for 30 sec

c allow to settle for 5 minutes to clear beads

d aspirate bacteria, in supernatant (b/w 600-800 uL recovered)

e combine bacterial s/n in sterile 15 mL conical tube

f count on Petroff-Hauser chamber

g bring up in RPMI w/ 10% FBS to 1 x 10^8 bacteria/mL

3 - Infect cells

a add equal volume bacterial suspension to tubes A, B

b add equal volume RPMI w/ 10% FBS to tubes C, D

c new cell concentration will be 5 x 10^6 cells/mL

d new bacterial concentration will be 5 x 10^7 / mL (ie MOI=10)

e add Z-VAD-FMK to tube B

f add cam-staur cocktail to tube D

g incubate at 27 C, on shaker, 5% CO2 for 3 days

Fig 14. Forward scatter vs. side scatter pseudocolor dot plot of catfish peripheral blood leukocytes. Two separate fish blood donors are shown. A single-cell suspension of catfish leukocytes is suitable for flow cytometric analysis.

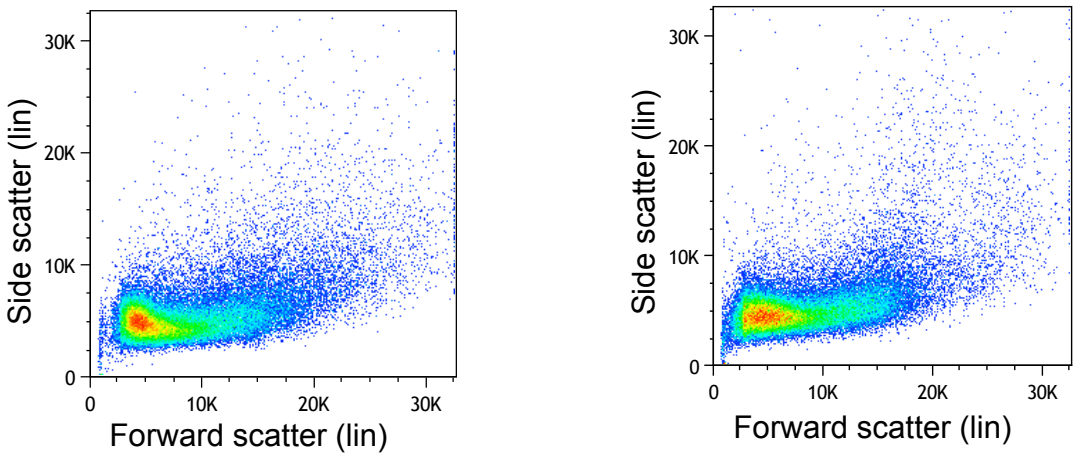


Fig 15. Forward scatter vs. side scatter pseudocolor dot plot of catfish peripheral blood leukocytes. Panel A shows untreated cells, Panel B shows paraformaldehyde-fixed, propidium iodide stained cells. Use of paraformaldehyde and propidium iodide markedly changes the scatter characteristics of uninfected, unstained catfish leukocytes. Gated area highlights different scatter characteristics.

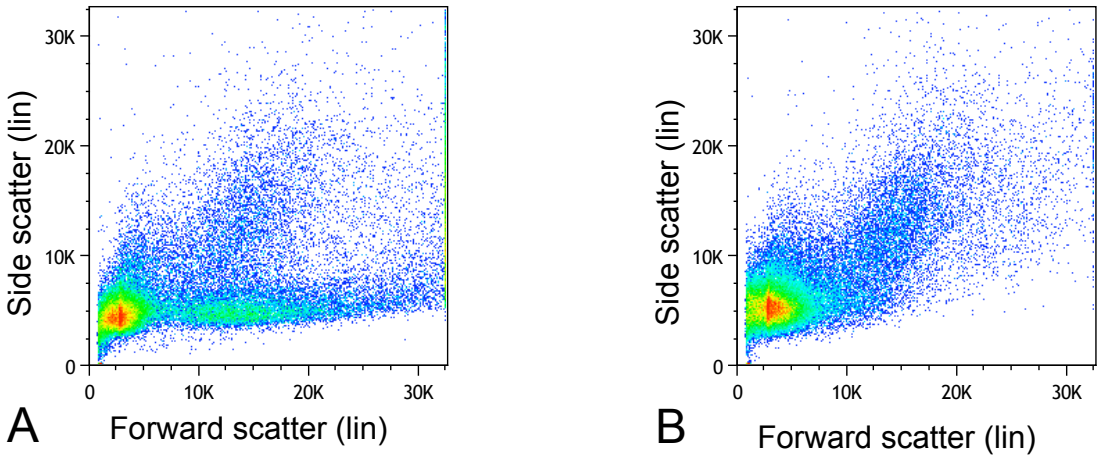


Fig 16. Forward scatter vs. fluorescence intensity pseudocolor dot plot of catfish peripheral blood leukocytes. Uninfected catfish leukocytes have minimal background GFP (Panel A) or PE (Panel B) fluorescence by flow cytometry.

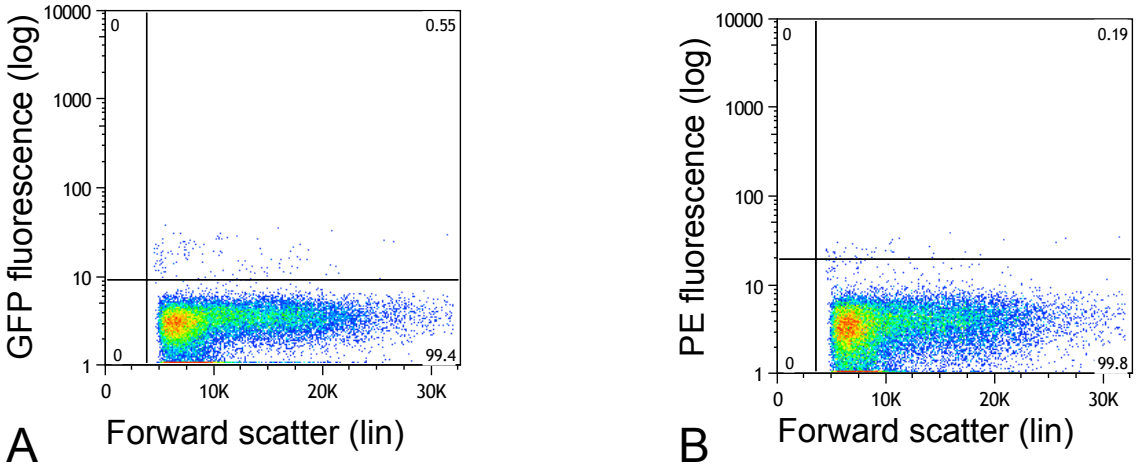


Fig 17. Forward scatter vs. GFP fluorescence intensity pseudocolor dot plot of *M. shottsii* GFP-infected catfish peripheral blood leukocytes. Infected catfish leukocytes show green fluorescence, indicating association with *M. shottsii* GFP.

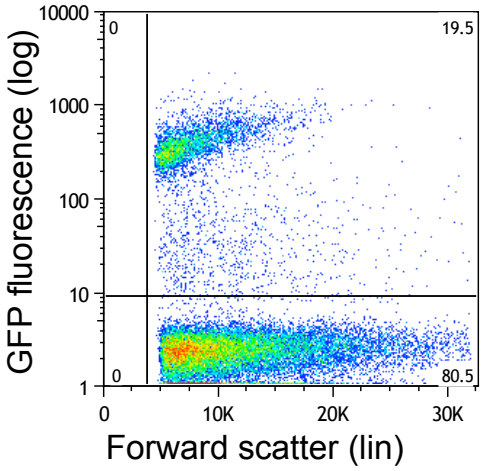


Fig 18. Forward scatter vs. PE fluorescence intensity pseudocolor dot plot of *M. shottsii* GFP-infected catfish peripheral blood leukocytes. The GFP-positive (infected) subset of catfish leukocytes also shows PE fluorescence, indicating Annexin labeling, and the occurrence of apoptosis, in Panel A. The GFP-negative (uninfected) subset of catfish leukocytes does not show PE fluorescence, indicating that apoptosis is not occurring, in Panel B.

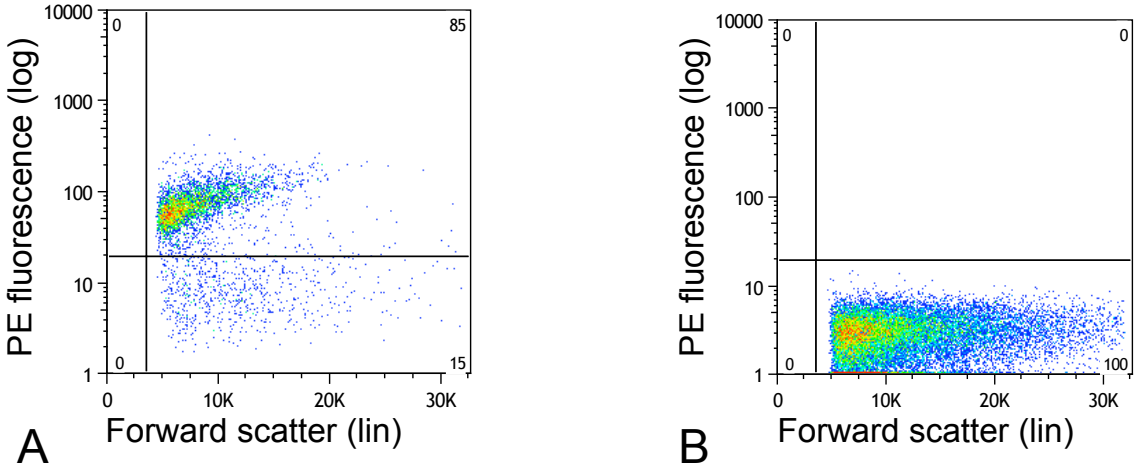


Fig 19. PE vs. GFP fluorescence intensities pseudocolor dot plot of *M. shottsii* GFP-infected catfish peripheral blood leukocytes, at one hour post infection. PE fluorescence indicates Annexin binding and early apoptosis. While 2.82% of cells are infected and PE negative (ie, not undergoing apoptosis), 16.7% of the total cell population is both infected and apoptotic. Of note, 0% of cells are uninfected and apoptotic.

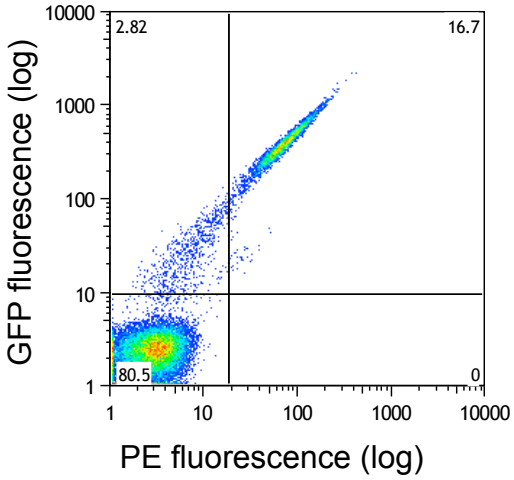


Fig 20. PE vs. GFP fluorescence intensities pseudocolor dot plot of *M. shottsii* GFP-infected catfish peripheral blood leukocytes at 24 hours post infection. Apoptosis induction by *M. shottsii* GFP infection occurs quickly, but uninfected cells remain viable over time, indicating the cell harvest and culture conditions are successful.

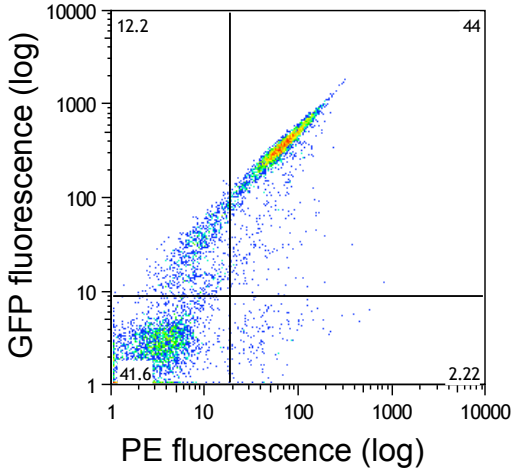


Fig 21. Electron microscopy shows that *M. shottsii* are contained within the catfish leukocyte. Green arrow shows the *M. shottsii* organism. Original magnification 44100x.

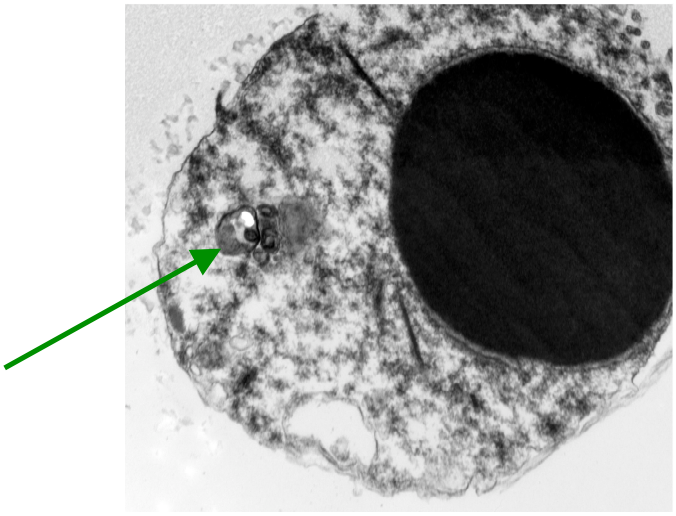
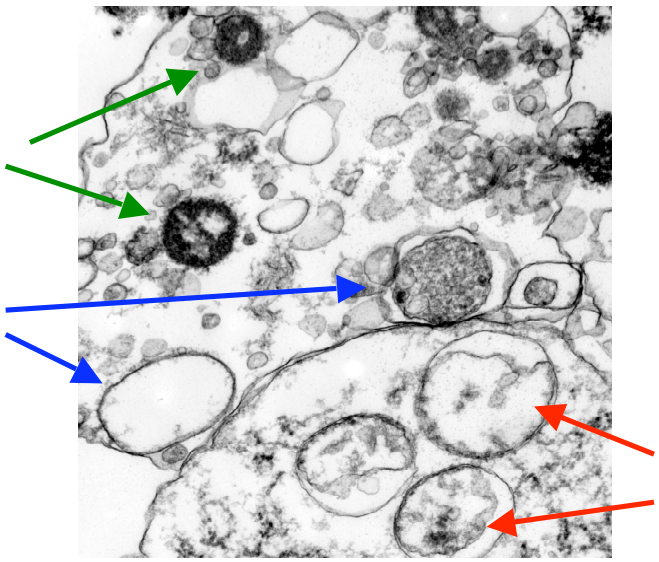


Fig 22. Electron microscopy shows that *M. shottsii*-infected cells undergo apoptosis. Arrows show *M. shottsii* bacilli (green), nuclear condensation (red), and formation of membrane-bound apoptotic bodies from the cytoplasmic components (blue). Original magnification 44100x.



Chapter 5. Discussion

Mycobacterium shottsii is a novel pathogen of striped bass (*Morone saxatilis*) in the Chesapeake Bay, with close genetic and phenotypic similarity to *Mycobacterium tuberculosis*. *M. tuberculosis* is the causative agent of tuberculosis in humans, a disease responsible for considerable human morbidity and mortality. The identification of additional, and perhaps superior, model systems for the study of pathogenic mycobacteria would be invaluable to the research community.

The goal of our project was to evaluate *M. shottsii* infection *in vitro*, to determine whether its primary mode of pathogenesis was the induction of apoptosis (like *M. tuberculosis*) or necrosis (like *M. ulcerans*). For this to be accomplished, several criteria must be met. First, the bacterium must be isolated, and cultured in a laboratory setting. In our project, it was also deemed helpful to generate a transfected strain which produced green fluorescent protein, facilitating identification of the bacilli within infected cells. These aims had been met prior to phase of the project presented in this manuscript.

Next, a suitable cell culture system must be identified and cultivated for use with the study pathogen. As *M. shottsii* is a temperature-restricted pathogen of estuarine fish from a cool

climate, fish cells which are tolerant of cool-temperature culture conditions were sought. Initially, carp leukocyte cells (CLC) were selected, because they are an established cell line from an estuarine cool-climate fish species. Infection was successfully generated in this cell line, and this model system proved useful for fluorescence microscopy evaluation. Immortalized cells are generally simple to maintain, and this was true of CLC. Immortalized cells are also commonly very much larger than the native cells from which they are derived, which improves their visualization with techniques such as fluorescence and confocal microscopy. CLC had this advantage as well. Fluorescence microscopy showed that bacilli were localized within lysosomes, and showed apoptosis of *M. shottsii*-infected CLC.

However, fluorescence microscopy techniques are best used as proof of principle, as it is difficult to generate quantifiable, objective, statistically significant data with this research tool. As such, flow cytometric evaluation was intended to parallel the microscopy studes. CLC proved unacceptable for this study technique. CLC formed rafts, clumps and mounds in all conditions tested. Whether cultured in microwell dishes, or in suspension on an orbital shaker, the cells stuck to one another tightly. This created minor problems in microwell culture, as a true confluent monolayer was

difficult to achieve. Free-floating rafts of cells formed in every condition tested, and these cells were washed away, unanalyzed, during the staining steps. More importantly, this tendency to clump was an insurmountable obstacle to the use of CLC in flow cytometry. If a single-cell suspension cannot be created, the events recorded by the cytometer are meaningless. Despite many attempts to find conditions that would create a single-cell suspension, no suitable protocol could be found. Variations in media, manner of shaking, cell concentration, and timing of infection were ineffective. Trypsinization (and use of a synthetic trypsin-like reagent) of the cells prior to infection were unsuccessful. An attempt was also made to culture in microwells initially, wash away the free-floating rafts, then trypsinize and harvest the cells which had adhered singly to the dish; this also resulted in clumping of the harvested CLC.

An alternate model system which was evaluated was the zebrafish (*Danio rerio*). While zebrafish are a truly tropical species, preferring water temperatures warmer than the ideal culture conditions for *M. shottsii*, they can be maintained at 23° C, (although they are unlikely to reproduce at such temperatures). This species is an extremely versatile laboratory resource, because of its small size, simple maintenance, sequenced genome, and the plethora of reagents and

protocols which have been developed for it. Mobilization of macrophages to the coelom has been described in zebrafish, which provided a ready source for the target cells of *M. shottsii* infection.[86] Infection was successfully carried out within the coelom of the zebrafish, and then mobilized, infected cells, which were expected to be predominantly individual macrophages, were harvested.

The zebrafish model was successfully infected, and infected cells were successfully harvested. Despite the relatively smaller cell size of zebrafish macrophages compared with CLC, fluorescence microscopy was rewarding, confirming the lysosomal localization of *M. shottsii* bacilli in this species. However, the small size of the zebrafish limited the total numbers of macrophages which could be acquired. A sample size sufficient for meaningful flow cytometric analysis was not achieved. Thus, our data remained largely qualitative.

For purposes of flow cytometry, an alternate model system was required. Channel catfish (*Ictalurus punctatus*) are also estuarine cool climate fish, which are commonly used in laboratory settings. They are relatively easily maintained, and are large enough to use for repeated phlebotomy, given time to recover between blood collections. Given the problems encountered with the immortalized CLC line, and the low cell numbers obtained from zebrafish, use of primarily isolated

monocyte-like cells from catfish was deemed desirable. Scientific literature supports the ability to collect, harvest and maintain such cells.[10,49,50,73,80] Anecdotally, catfish seem resistant to systemic infection by pathogenic mycobacteria; however, our studies were not directed at the organism level, and this species seemed a suitable candidate for a cellular *in vitro* model. *M. shottsii* infection of primarily-isolated catfish peripheral blood monocyte-like cells was succcessful in microwell culture. Infection of primarily-isolated catfish peripheral blood leukocytes was also succesful in suspension.

Single-cell suspensions for flow cytometry were easily created and maintained with the catfish peripheral blood leukocyte system. Analysis of infected cells by flow cytometry confirmed the results suggested by fluorescence microscopy; infection of catfish peripheral blood leukocytes with *M. shottsii* induced apoptosis. Further inspection by electron microscopy of infected catfish leukocytes from suspended culture substantiated the interpretation of the quantitative data; intracellular bacilli and apoptotic bodies were seen.

The finding of apoptosis induction is an important criterion which could be used to advocate the use of *M. shottsii* as a research model for *M. tuberculosis*. Mycobacterial species classically cause either apoptosis or necrosis as a principal virulence effect. *M. tuberculosis* causes chronic wasting

granulomatous disease, characterized by apoptosis of infected macrophages. An appropriate model system would have these same features. The early studies which identified *M. shottsii* described clinical signs in fish, consisting of visceral granulomas and wasting disease; now, our study has shown that apoptosis is the mechanism of death of infected cells.

Additional aspects of this experimental study system remain to be perfected. Attempts are ongoing to independently confirm apoptosis induction by the use of gel electrophoresis to identify the characteristic DNA pattern of apoptotic cells. Also, fluorescence-activated cell sorting will be used to harvest the infected subset of catfish peripheral blood leukocytes to further characterize their phenotype, outcome, and ultrastructure by electron microscopy.

Related projects within our lab are expanding upon this information. Zebrafish are now being evaluated as a possible model system for systemic infection with *M. shottsii*. In addition to the valuable features described above, they have a shorter life span than that of striped bass. This should facilitate the study of a pathogen which seems to require long-term infection, and may require environmental co-stressors for maximal virulence. Thus far, intra-peritoneal inoculation of zebrafish with *M. shottsii* has induced granulomatous infection. Current studies are directed at infection by more physiologic

means, such as water or food contamination, and histopathologic description of the nature and location of granulomas formed in infected zebrafish.

With this project, we have established three separate culture and infection systems for the study of *M. shottsii*, a pathogen extremely similar in genetic background, phenotypic characteristics, and biologic behavior, to *M. tuberculosis*. We have demonstrated that CLC can been used to generate microwell infections for study by fluoresence microscopy, and that apoptosis is induced in infected cells. We have shown that *M. shottsii* bacilli localize to the lyosome in infected coelomic macrophages from zebrafish. We have also shown that primarily-isolated peripheral blood leukocytes from catfish can be used for microwell or suspended culture and infection. As such, they can be studied by microscopy or flow cytometry, and apoptosis induction of infected cells is confirmed in both modalities. This work provides a strong foundation for further studies of this economically, and experimentally important novel fish pathogen.

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