The immune response of the goldfish (*Carassius auratus* L.) to natural mycobacterial pathogens

by

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Abstract

Disease caused by pathogens of the genus *Mycobacterium* is of serious consequence to many animals, not least of which is *M. tuberculosis*, the etiological agent of tuberculosis. The ongoing efforts to characterize the immune response during mycobacterial infection have rendered a complex yet incomplete version of events, although molecules, cells, and effector functions central to antimycobacterial immunity have been highlighted through investigation. The ability of mycobacterial pathogens to evade and persist within host phagocytes, co-opt and benefit from granuloma formation, and remain dormant in the majority of immunocompetent individuals is a serious health concern, where an estimated one third of the world's population is infected by *M. tuberculosis*. Recently, the use of non-traditional models to study host defense mechanisms, including work in teleosts, has provided new insights into this area of research.

My doctoral research focused on the immune response of goldfish to natural mycobacterial pathogens, *M. marinum*, *M. chelonae* and *M. fortuitum*, utilizing these infection models that vary in overall pathogenicity. I characterized the cytokine-induced alternative functional phenotype of primary goldfish macrophages and the relative antimicrobial states of effector phagocytes in response to pathogenic challenge. I measured the expression of cytokines that are central to the control of mycobacterial immunity in higher vertebrates in goldfish infected with each pathogen. This graded level of host protection and bacterial load allowed for the assessment of each immune parameter and the corollary infection outcome.

Similar to mammals, goldish macrophages are broadly capable of polarization into classically (M1) and alternatively (M2) activated macrophages. I characterized the cytokine-driven polarization of goldfish primary kidney macrophages into an M2 phenotype, marked by the increase in arginase activity and the corresponding dampening of nitric oxide production. I utilized the established effector functions for primary kidney leukocytes to detail them in response to exposure to natural mycobacterial pathogens. *M. marinum* demonstrated the capacity to evade nitric oxide production and promoted arginase activity. Moreover, a varying capacity of neutrophils to respond to each pathogen was observed, with generally less responsiveness to *M. marinum*. Additionally, growth and bacteremia of *M. marinum* in goldfish spleen and kidney increased through the first 4 weeks of infection, while bacteremia of *M. chelonae* and *M. fortuitum* diminished at every observable time point. Together, these findings suggest mycobacterial evasion strategies that contribute to a less effective immune response to *M. marinum*.

Preface

This thesis is the original work of Jordan Hodgkinson and was conducted under the supervision of Dr. Miodrag Belosevic. This thesis was part of a broader research project which received ethics approval from the University of Alberta Research Ethics Board, under the project "Innate Immunity in Bony Fish", protocol # AUP00000069. The animals contributing to this thesis were housed in the research facility according to guidelines set by Canadian Council of Animal Care (CCAC).

Portions of chapter 2, 4, 5 and 7 of this thesis have been published. The manuscripts are: (1) Hodgkinson, Jordan W., Chad Fibke, and Miodrag Belosevic, 2017. Recombinant IL-4/13A and IL-4/13B Induce Arginase Activity and down-Regulate Nitric Oxide Response of Primary Goldfish (*Carassius Auratus* L.) Macrophages. Developmental and Comparative Immunology, 67: 377–84; (2) Hodgkinson, Jordan W., Jun-Qing Ge, Barbara A. Katzenback, Jeffrey J. Havixbeck, Daniel R. Barreda, James L. Stafford, and Miodrag Belosevic, 2017. Development of an *in vitro* Model System to Study the Interactions between Mycobacterium Marinum and Teleost Neutrophils Developmental and Comparative Immunology 53: 349–57; (3) Hodgkinson, Jordan W., Jun-Qing Ge, Leon Grayfer, James Stafford, and Miodrag Belosevic, 2012. Analysis of the Immune Response in Infections of the Goldfish (*Carassius Auratus* L.) with Mycobacterium Marinum. Developmental and Comparative Immunology 38: 456–65. Chapter 5 is a chapter in preparation for publication.

The research in this thesis is the first comprehensive analysis of the immune response of goldfish to the most prominent natural mycobacterial pathogens. It is also the first functional report of IL-4/13 with regards to fish macrophage function, the first report of a novel primary neutrophil *in vitro* assessment of effector cell function to mycobacterial pathogens, first *in vitro* assessment of macrophage effector function challenged with mycobacteria, and first comprehensive cytokine analyses for each infection course. Grayfer, L. assisted with infection and tissue collection, Fibke, C. assisted with Q-PCR expression studies, Jun-Qing Ge assisted with infection, tissue collection and paraffin embedding, Katzenback, B. assisted with neutrophil RNA isolation, Havixbeck, J.J assisted with flow cytometry. Stafford, J. was the co-supervisor aiding in writing and conceptual discussions. Belosevic, M. was the supervisory author and guided discussion of concepts and helped with writing and editing of the manuscripts.

To my darling Christina, Darwin and my son on the way

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List of Abbreviations

ANOVA	analysis of variance
α	alpha
Arg	arginase
ASC	Apoptosis-associated speck-like protein containing a card domain
β	beta
BCG	Bacille de Calmette et Guérin
CCL	CC chemokine ligands
CCM	cell conditioned medium
CCR	chemokine receptor
CCR	CC chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDS	coding sequences
CFU	colony forming units
DAMPs	danger associated molecular patterns
DC	dendritic cells
DNA	Deoxyribonucleic acid
Dos	dormancy survival regulator
γ	gamma
GFP	green fluorescent protein
HBSS	hank's balanced salt solution
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HIV	human immunodeficiency virus
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible Nitric oxide synthase
IVDKM	*in vitro* derived kidney macrophages
kDa	kilodaltons
LAM	lipoarabinomannan
LPS	lipopolysaccharide
LXA4	lipoxin
LXA4	lipoxin A4
M1	classically activated macrophages
M2	alternatively activated macrophages
MDR-TB	multidrug-resistant TB
MMP	metalloproteinase

mRNA	messenger ribonucleic acid
MW	molecular weight
MyD88	Myeloid differentiation primary response 88
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
NK	natural killer cells
NLR	Nod-like receptors
NO	nitric oxide
O.D	optical density
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PDIM	phthiocerol dimycoceroserate
PGE2	prostaglandin E2
PKM	primary kidney macrophages
PMA	phorbol myristate acetate
PRR	pattern recognition receptors
Q-PCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
RD1	region of difference
RNA	Ribonucleic acid
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS	suppressor of cytokine signaling
SOD	superoxide dismutase
TB	tuberculosis
TDM	trehalose 6,6’-dimycolate
TGF	transforming growth factor
Th	T helper cell
TIR	Toll/Il-1 Receptor
TLR	Toll-like receptors
TNF	tumor necrosis factor
Tregs	T regulatory cells
XDR-TB	extensively drug-resistant TB

Chapter I: General Introduction

1.0 Introduction

Comparative biology is a field aimed at the understanding of lower vertebrates and invertebrate organisms, contrasted with those of higher vertebrates. The seminal experiments of Metchnikoff in 1882 using a starfish larvae comparative model led to his discovery of phagocytic leukocytes, thereby establishing the branch of cellular immunology [1]. Over a century later, the use of various models in comparative immunology have flourished, often providing unique advantages and bolstering the evolutionary context of the ancient mechanisms that give rise to cellular and humoral components of higher vertebrates. Teleosts, or bony fish, represent the most expansive class of vertebrates, with over 30 000 identified species [2]. Additionally, they are one of the lowest classes of vertebrates with innate and adaptive cellular components that are functionally equivalent to mammals [3], offering insight into the evolution of metazoan immune systems and providing a number of useful organisms for modeling human disease.

Bacterial pathogens are major contributors to disease in fish in wild and farmed populations. In marine ecosystems there are currently 67 well defined agents of disease, 34% of which are pathogenic bacteria [4]. Fish possess various non-specific humoral factors (bacterial inhibitors of growth, lysins), specific humoral factors (antibodies), non-specific cell mediated mechanisms (phagocytosis, reactive intermediates) and specific cell mediated mechanisms (T cell activation of phagocytes) [5]. Generally, bacterial pathogens can be classified as extracellular, facultative intracellular or obligate intracellular, although many of pathogens employ

various strategies during stages of infection [6]. The most prevalent bacterial pathogens include members from the genus *Aeromonas*, *Yersinia*, *Edwardsiella* and *Vibrio* and *Mycobacterium*, each with specific immune evasion strategies to subvert the host immune system.

A significant emphasis on the identification and functional characterization of teleost immune mediators over the last few decades has consolidated our understanding of the immune response of fish, which exhibits remarkable conservation to that of higher vertebrates. During my thesis work I cloned and characterized IL-4/13 gene duplicates, the proposed ancestor to the type 2 cytokines IL-4 and IL-13 in mammals. I demonstrated the ability of IL-4/13A and IL-4/13B to induce arginase activity in goldfish primary macrophages, akin to the mammalian function of inducing an M2-type "repair" phenotype. I utilized the *in vitro* primary kidney macrophage culture system to measure the variable effector function following exposure to *M. marinum*, *M. chelonae* and *M. fortuitum,* the main natural mycobacterial pathogens of fish. I also examined the effects of mycobacterial exposure on primary kidney neutrophils and measured the expression of immune mediators central to antimycobacterial function shown in mammalian studies. A demonstrable variation in bacteremia and pathological outcome during infection of each mycobacterial species provided a basis for the comparison of immune function.

1.1 Thesis objectives

The main objective of my thesis was to study the immune response of goldfish to natural mycobacterial pathogens, with an emphasis on phagocyte effector function. The specific aims of my research were:

1) To measure tissue bacteremia and cytokine expression levels during *in vivo* infection of natural mycobacterial pathogens.
2) To examine the function of goldfish phagocytes exposed to natural mycobacterial pathogens *in vitro.*
3) To identify and characterize the cytokine-driven regulation of an alternative activation phenotype of macrophages in goldfish.

1.2 Thesis Outline

This thesis consists of 8 chapters. Chapter I is the general introduction. Chapter II is a literature review that focuses on the immune response during infection with *M. tuberculosis,* and the relative contributions in each section of fish infected with *M. marinum*. Chapter III are the materials and methods used to conduct the research in this thesis. Chapter IV is the *in vivo* assessment of fish infected with *M. marinum*, *M. chelonae* and *M. fortuitum*. Chapter V is the *in vitro* assessment of primary goldfish neutrophils exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*. Chapter VI is the *in vitro* assessment of primary kidney monocytes/macrophages exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*. Chapter VII is the cloning and functional characterization of IL-4/13A and IL-4/13B. Lastly, chapter VIII is a

general discussion on the findings in this thesis, in addition to ideas for future directions of research.

1.3 Significance

The study of host defense in teleost fish, especially in the context of mycobacterial infection, has emerged as a useful strategy for elucidating the consequence of infection. Here, the description of immune function following exposure to mycobacterial pathogens provides insight into the fate of infection, as well as potential modulation of the immune response by mycobacterial pathogens. The understanding of molecular and phagocytic effector regulation is during mycobacterial infection is critical in paving the way for the development of novel therapeutic intervention, both in aquaculture settings and as a model for human disease.

Chapter II: Literature Review[1]

2.0 Introduction

The currently recognized *Mycobacterium* genus consists of over 190 species with a broad range of ecological niches, ranging from serious human pathogens like *M. tuberculosis* and *M. leprae*, to more innocuous soil-dwelling organisms. *Mycobacterium spp.* are characterized as Gram-positive, acid-fast staining, non-motile, non-spore forming bacilli that are classified further by growth rate (rapid or slow growing) and pigmentation [7]. Mycobacterial disease has plagued humans since ancient times, the most serious of which, *M. tuberculosis*, has afflicted humans for approximately 70 000 years and it is thought to have killed more persons than any other microbial pathogen [8]. Tuberculosis (TB) was well known in classical Greece as 'phthisis' or 'consumption', and was described by the Hippocratic school as the most common disease in the world [9]. Prior to the late 1800s, TB was considered to be a heritable disease in Northern Europe, although it was at the same time considered infectious and contagious in Southern Europe, first proposed in 1790 by Benjamin Marten, who suggested the disease was caused by "some certain species of *animalcula*" [10]. In 1865 Jean-Antoine Villemin, a French military surgeon, first demonstrated the infectiousness of TB when he inoculated a rabbit with lung tissue from an individual that had died of tuberculosis [8]. Eighteen years later, Koch

[1] A portion of this chapter has been published: Hodgkinson, Jordan W., Leon Grayfer, and Miodrag Belosevic. "Biology of Bony Fish Macrophages." *Biology* 4, no. 4 (November 30, 2015): 881–906.

identified the bacillus responsible for TB and conceived of his famous four postulates, setting the gold standard for demonstrating etiology of infectious disease [11], [12].

It was initially thought that the breakthrough by Koch would soon lead to a cure for TB, but nearly 40 years later, a partially effective vaccine was manufactured in 1921, the Bacille de Calmette et Guérin (BCG) vaccine. BCG was developed by serial passage of *Mycobacterium bovis*, reducing the risk of acquiring TB by around 50% [13], although there are varying reports of effectiveness depending on region, genetic factors, exposure to other pathogens, and BCG culturing practices [14]. In 1944, the first effective antibiotic against TB was introduced by Selman Waksam. Currently, there are a number of antibiotic therapies with a relatively high treatment success (~85%) [15], though the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) create new and urgent challenges in antibiotic therapy [16].

Despite the efforts of vaccination and the efficacy of antibiotic therapy, it is estimated that one-third of the world's population is currently infected with TB, the majority of which are asymptomatic, with approximately 9 million cases of active TB and 1.3 million deaths are reported annually [15], [17]–[19]. The ability of mycobacterial infections to infect and exist latently, where 5-10% of *M. tuberculosis* infections lead to eventual active TB disease, represents a unique public health challenge of an asymptomatic reservoir for the disease [20], [21]. Lack of an effective vaccine, coinfection with human immunodeficiency virus (HIV), inherent resistance

to antibiotics, and emergence of drug resistant strains compound the challenges faced in controlling TB moving forward [22], [23].

In addition to serious human disease, the *Mycobacterium* genus has the capacity to infect a wide range of domestic and wild animal species, including ruminants (*M. bovis*/*M. paratuberculosis*), rodents (*M. microti*), birds (*M. avium*) reptiles (*M. smegmatis*) amphibians and fish (*M. marinum*) [24]. Each of the natural pathogens are exquisitely adapted to evade and co-opt host immune responses, allowing them to infect, replicate, persist and transmit to new hosts. Disease typically manifests as granuloma formation, a hallmark of mycobacterial infection, where aggregates of immune cells contain pathogenic mycobacteria [25]. In the case of *M. marinum*, relative genetic relatedness [80% of total coding sequences (CDS), which share 85% amino acid similarity, in addition to 99.3% 16S rRNA sequence similarity] has rendered fish models of infection useful for study of the tuberculosis-like disease [26]. I am using the goldfish model system to study host-pathogen interactions with the aim to compare immune response during infection with *M. marinum*, *M. chelonae* and *M. fortuitum*, natural fish pathogens with demonstrated varying levels of pathogenicity in goldfish. Reports in adult goldfish infected with these pathogens have shown bacterial growth patterns and granuloma formation in tissues, although it has not been fully elucidated what constitutes an effective host immune response for control of these infections [27]–[29].

This review aims to summarize the immune response to mycobacterial pathogens in mammalian and non-mammalian models, which guide our mechanistic knowledge base of the host-pathogen interactions that influence infection outcome.

The focus is on mammalian models infected with *M. tuberculosis* as well as contributions of fish models infected with *M. marinum*.

2.1 *Mycobacterium* spp.

Mycobacteria range in length between 1-10 μm long and 0.2-0.6 μm in diameter [30], [31]. Mycobacteria are considered Gram positive, although their envelope shares features of Gram negative organisms, including an outer impermeable layer that acts like an outer membrane [32], [33]. The peptidoglycan layer is covalently linked to branch chain fatty acids termed mycolic acids, which are α-alkyl, β-hydroxyl long-chain fatty acids [34]. Mycolic acids are the most abundant lipid in mycobacterial cell walls, making up 60% of the dry weight. This layer provides a protective waxy and relatively impermeable coat compared to more prototypical Gram-positive cell wall structures. Outside of this mycomembrane, there is a capsule comprised of polysaccharides, predominantly glucan and arabinomannan, as well as protein and lipid, predominantly lipoarabinomannan (LAM) [35]. The unique nature of the cell wall renders Gram staining ineffective. Mycobacteria are characterized as 'acid-fast', as they resist the acid decolorization stain used in typical staining methods. Instead, the Ziehl-Neelsen staining procedure is readily employed for positive identification of mycobacterial organisms [36].

Composition of mycolic acids are diverse within the *Mycobacterium* genus and are used in conjunction with genomic approaches for identification of species and strains; crudely, fast growers are considered species that can form colonies in a week or less, while slow growers take longer than a week to form colonies on solid media.

Molecular phylogenetic analysis has been used to further distinguish and categorize species, which suggests a molecular basis for the growth speed divisions [30]. Slow growing species include prevalent pathogens like *M. tuberculosis*, *M. marinum*, *M. leprae* and *M. avium*. Faster growing mycobacterial species are generally saprophytic, although *M. fortuitum* and *M. chelonae* have been shown to be pathogenic in fish and frog species or in nosocomial settings [30], [38], [39].

2.2 Morphology and growth

M. marinum are considered long rods and grow with cross-barring, clumping or cording [40]. Colonies grown on solid media are non-pigmented if grown in the dark but turn bright yellow when grown in the light, a trait known photochromogenicity [34]. *M. marinum* grows slowly and has a doubling time of 4-6 h, rendering visible colonies in roughly 5-7 days. Optimal growth occurs between the temperature range of 28°C and 35°C, although variations have been reported in strains of *M. marinum* capable of infecting warm-blooded animals, which can withstand warmer temperatures [42]. *M. chelonae* and *M. fortuitum* have been reported as having short rods, long rods or coccoid-like cellular morphology, and colonies are smooth, creamy, and non-photochromogenic. The optimum growth temperature range for *M. chelonae* is between 25 and 40°C, but growth can occur up to 43°C, while *M. fortuitum* grows optimally between 30-37°C [43]. Both *M. chelonae* and *M. fortuitum* are fast growing, with an approximate doubling time of between 2-4 h and visible colonies in 48-96 h [44]. By contrast, *M. tuberculosis* are slender and beaded rods that clump or cord, with an extremely slow growth rate,

doubling once every 24 h and rendering observable colonies between 4 to 6 weeks post-cultivation. Colonies are non-photochromogenic and grow optimally at 37°C [45], [46].

2.3 Tuberculosis

Tuberculosis is predominantly a lung disease, which accounts for 70% of infection cases [47]. TB can be divided into two major patterns, primary and post primary TB, often bridged by long stretches of dormancy. Primary TB is defined as the initial infection that is typically controlled by an immunocompetent individual, although serious disease can manifest in children and immunocompromised individuals [48]. The lesions in the lung that develop during primary tuberculosis often heal, but despite a minimizing pathology, the infection is rarely sterilized and bacteria persist for up to several decades in dormancy [49]. Post-primary TB occurs following first infection events, where systemic immunity has already been established due to re-emergence of infectious bacilli that multiply and produce cavities in the lung [50]. While the majority of post primary infections spontaneously recover, 80% of the estimated 9 million yearly clinic cases of TB are the result of post primary infections [49]. Ironically, immunocompetent individuals between the ages of 15-40 are most likely to die from acute post primary TB [51]–[53]. Further, survivors of post primary TB have an increased risk in contracting the disease again, rendering no immunity to previous bouts of the disease [54], [55]. Symptoms of active pulmonary TB are chest pain, coughing (with or without blood), difficulty breathing, excessive sweating, fatigue, fever, weight loss, wheezing, cachexia [19].

Active TB can also manifest as miliary disease (widespread dissemination), meningitis, abdominal disease and sepsis [56].

2.4 Modelling tuberculosis

Despite the fact that *M. tuberculosis* can infect most warm-blooded animals, infection models in the laboratory do not transmit the disease by aerosol exposure like in humans. Moreover, virtually all mammalian models develop acute primary TB, but fail to recapitulate post primary infection and disease, which is the predominant manifestation of disease in humans [57]. As a result, many animal models exist for the study of mycobacterial disease, including non-human primates, mice, rabbits, guinea pigs, and non-mammalian models, including fish and frogs. The lack of a standardized model and the proliferation of multiple systems has obfuscated our understanding and generated results that are non-reproducible across models; however, the relative strengths for each model aids in helping to complete the scope of studying the immune response to mycobacterial pathogens. For example, many mouse models do not generate necrotic (caseous) granulomas when infected with *M. tuberculosis*, whereas guinea pigs develop caseous granulomas [58]. The overwhelming majority of TB research is generated in the mouse model system due to a number of advantages, including the expansive repertoire of reagents and genetic malleability [59].

2.5 Fish mycobacteriosis

Three *Mycobacterium* spp. have been shown to elicit disease most commonly in fish: *M. marinum*, *M. chelonae* and *M. fortuitum*, although an increasing number of species have been isolated from fish in recent years [30]. Piscine mycobacteriosis typically presents as chronic disease, which may or may not produce clinical signs both externally or internally. Fish infected with mycobacterial pathogens can display symptoms externally by non-specific scale loss and dermal ulceration, pigmentation changes, spinal defects, lethargy, ascites and emaciation [31], [60]. Internal pathology includes enlargement of the kidney, spleen and liver, as well as grey or white nodules on internal organs [30]. Similar to disease in mammals, granulomatous inflammation is the classical hallmark of infection. Granulomas tend to be found in multiple organs and tissues, and are highly variable in size and organization [31]. *M. marinum* infection in humans, known as fish tank granuloma, occurs when *M. marinum* enters the extremities through abrasions on the hand or arms resulting in granulomatous nodes [61], and the pathology of fish tank granuloma is indistinguishable from cutaneous tuberculosis. The relative relatedness to *M. tuberculosis*, as well as the remarkably similar pathology in fish infections has rendered *M. marinum* a popular surrogate pathogen. The current natural model of choice is the zebrafish embryo, where optical transparency and genetic tractability allows for visualization of the early events of infection and granuloma formation [62]. Other fish models, such as the goldfish, allow for the study of primary immune cells and immune parameters in adult fish [63].

2.6 Transmission

Tuberculosis is transmitted through an aerosol route by airborne particles roughly 1-5 μm in diameter. Infected individuals generate infectious droplets from talking, coughing or sneezing, which can remain suspended in the air for several hours depending on environmental conditions [64]–[66]. Inhalation of droplets containing *M. tuberculosis* leads to infection, where pathogenic bacilli gain entry into the upper respiratory tract and reach the alveoli of the lungs where they are internalized by resident and infiltrating phagocytes.

Transmission of fish tuberculosis is not well understood although it is thought to be transmitted by contaminated food, either by cannibalism of infected fish or environmental sources [70], [71], including soil, water and detritus, where bacterial cells can remain viable for many years [30]. Lesions that form in the gut or on the skin may also enable shedding of mycobacteria in to the water, but bacteria from internal organs is thought to only be liberated upon death and decomposition. Environmental sources of mycobacteria may also gain entry through cuts or abrasions [70]. Due to the ability of piscine mycobacteria to infect a variety of other animals, including frogs, snakes, turtles and snails, it is likely that these animals may also contribute to a source of infection and/or act as a reservoir [30].

2.7 Initial infection events

Following the inhalation of *M. tuberculosis*, a variety of host phagocytes act to internalize the pathogen, including resident macrophages, dendritic cells and infiltrating neutrophils [72]. Internalization of the bacilli is mediated by a diverse

group of receptors including the mannose receptor, complement receptors, and scavenger receptors [73]. Following internalization, mycobacteria are either neutralized by innately activated phagocytes which act as the first line of defense through a variety of antimicrobial mechanisms (i.e. respiratory burst, nitric oxide production, phagosomal maturation), or evade these responses and persist within the phagocyte [74], [75]. Evasion of these effector responses results in persistence within the phagocyte itself, and either development of latent infection or acute, active disease [76]. Generation of an effective immune response during early events play a critical role in determining the fate of infection, either by protective innate immunity or initiation of a protective adaptive response, resulting in active tuberculosis, latent infection or clearance of the pathogen [76].

The early stages of infection events have been well documented in *M. marinum* fish models and is especially advantageous in the transparent zebrafish embryo model, where fluorescent modifications can be readily observed. Similar to mammalian models, resident fish macrophages internalize *M. marinum* [77] and neutrophils rapidly migrate to the site of infection and uptake the pathogen [78], [79], although neutrophil phagocytosis of *M. marinum* is largely dependent on the site of infection [80]. Study in the zebrafish has also demonstrated that virulent forms of *M. marinum* can influence the recruitment of a permissive phenotype of macrophages, while less virulent forms attract macrophages capable of producing reactive nitrogen intermediates (RNI) following phagocytosis [81].

2.8 Pattern recognition receptors

Host recognition of mycobacteria is through a number of specific pattern recognition receptors (PRR), Toll-like receptors (i.e. TLR2, TLR4 and TLR9), C-type lectin receptors (dectin-1, mannose receptor, and DC-SIGN), complement receptors and Nod-like receptors (NLR) [73], [82], [83]. Not surprisingly, polymorphisms in PRR genes have been shown to affect recognition of *M. tuberculosis,* corresponding to a disruption in recognition events, as well as disease outcome [84]. MyD88 and CARD9, master adaptors for TLRs and NLRs, are essential for host protection to *M. tuberculosis* [85]–[89]. Mice deficient in MyD88 lose resistance to *M. tuberculosis*, marked by reduced IL-12, TNF, Th1 cytokine production and iNOS expression [92]. Similarly, *Card9*$^{-/-}$ mice have impaired host resistance to *M. tuberculosis* and heightened bacterial burden [89], as well as mice deficient in NOD-2 [93]. Perhaps not surprisingly, hyper-virulent forms *M. tuberculosis* have been demonstrated to augment TLR signalling to their advantage. It was recently shown that the virulent Beijing (Bj) lineage of *M. tuberculosis* preferentially signals through TLR-2 as opposed to TLR-4 that is seen in less virulent strains, leading to the induction of a less protective cytokine profile [86].

In fish, recognition of *M. marinum* pathogen-associated molecular patterns (PAMPs) by PRRs recognize can induce the reactive oxygen and nitrogen based defense mechanism in macrophages, which is attenuated in *M. marinum* containing the RD-1 virulence locus [94], or in *M. marinum* absent cell wall phthiocerol dimycoceroserate (PDIM) lipids [81]. These findings emphasize both the importance of TLRs to initiate appropriate immune function, as well as evasion techniques that

M. marinum possess by disruption of these interactions. Impairment of MyD88 in zebrafish can also accelerate granuloma formation [95], although this is likely also due to the loss of IL-1 signalling, since both IL-1 and TLRs signal through the Toll/Il-1 Receptor (TIR) domain, which is MyD88 dependant. Damaged host molecules have also been shown to be important to antimycobacterial immunity in fish, where TLRs in zebrafish have been shown to mediate autophagy by binding DNA damage-regulated autophagy modulator (DRAM1) [96], demonstrating the importance of TLR signalling in later stages of infection. The implication of NLR signalling has also been suggested in expression studies of goldfish primary kidney leukocytes following stimulation with *M. marinum* [97]. Early recognition of PAMPs by PRRs results in the production of a number of innate cytokines, most critically in the context of mycobacterial protection, tumour necrosis factor (TNF), interferon (IFN)-γ, major IL-1 family cytokines (IL-1α, IL-1β, IL-18 and IL-33), IL-12, IL-17 and IL-23 [78], [98]–[100]. Orthologous cytokines of the fish have also been studied in early response to mycobacterial infections, although not extensively. Concomitant increases of innate cytokines in BCG vaccination in Japanese medaka has shown early increases of innate cytokines including IL-1β, IL-6, IFN-γ and TNFα, akin to mammalian observations, and this early cytokine response has been observed in a number of other fish species including goldfish and adult zebrafish [78], [81], [101].

2.9 Phagosome maturation

Pathogenic mycobacteria have evolved a number of evasion strategies to subvert the hostile environment following professional phagocytic internalization.

Typically, after a microbe is recognized, it is engulfed into a vacuole or vesicle, known as the phagosome, which is remodelled continuously by fusion events with endosomes and lysosomes [102]. Membrane bound proteins of the phagosome aid in recruiting and fusing with endosomes and lysosomes and eventual transformation into a highly acidic, hydrolytic and bactericidal compartment [103]. Pathogenic *Mycobacterium* spp. are particularly adept at interfering with the maturation of the phagosome, which seems not to acquire late endosomal or lysosomal characteristics [70]–[72], and retains markers derived from the plasma membrane, thought to allow for nutrient acquisition [107], [108]. A number of mycobacterial factors have been identified that interfere with phagosomal maturation and lysosomal fusion. The mycobacterial cell wall PDIM has been demonstrated to contribute to both gaining entry into host cells through receptor-dependant phagocytosis, as well as impair phagosome maturation and acidification, compared to PDIM mutants [109], [110]. Trehalose 6,6'-dimycolate (TDM) and LAM have also been shown to play an important role in arresting of normal phagosome processing [111], [112]. More recent findings suggest pathogenic mycobacteria actually escape the phagosome by translocating to the cytosol, although this may only occur at later stages in infection at the level of the granuloma [79]–[81].

M. marinum infection models have uncovered and corroborated interference with phagosome maturation findings in mammalian models. Circumvention of the phagosome and lysosome has been demonstrated by *M. marinum* in trout and carp macrophages [116]–[118], and the pathogen has also been shown to localize in non-acidified phagosomes [118]. Studies in the zebrafish embryo model has shown

internalization of fluorescent *M. marinum* by macrophages that are incapable of eradicating them, suggesting resistance to normal phagosome maturation [78]. Interestingly, *M. marinum* has also been demonstrated to escape the phagosome and enter the cytosol, where it has the ability to propel itself via actin-based motility by inducing actin polymerization using host cytoskeletal factors, which has not been observed in *M. tuberculosis* infected phagocytes [119]. The factors contributing to interference of phagolysosomal fusion have been narrowed to the ESX secretion system of *M. marinum* and *M. tuberculosis*, which is present in a wide variety of Gram-positive bacteria [114], [120], [121].

2.10 Respiratory burst

The fate of internalized particles depends heavily on the activation state of the phagocyte. Macrophages influence a wide range of physiological outcomes through functional plasticity, including maintenance of homeostasis though tissue repair and removal of debris and apoptotic cells. These processes occur in a non-inflammatory manner, where internalized particles are preferentially degraded in lysosomes rich in proteolytic and lipolytic enzymes [122], [123]. Stimulation with pro-inflammatory cytokines or TLR agonists, shifts the activation state of macrophages to an effector cell, thereby increasing antimicrobial activity and antigen presentation [124]. Respiratory burst is a central defense mechanism of macrophages that requires nicotinamide adenine dinucleotide phosphate (NADPH or NOX2), a multicomponent enzyme that assembles at the plasma and phagosomal membranes, resulting in production of superoxide anion [125]. The superoxide anion converts into potent

reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and hypochlorous acid (HOCl) [126], [127]. These highly reactive chemicals readily interact with intracellular macromolecules, including nucleic acids, lipids and proteins, culminating in microbicidal activity [128]. As such, the potential toxic effects of these products are tightly regulated in order to limit the damage to the host tissues.

Increased risk of mycobacterial diseases is observed in humans with chronic granulomatous disease, where defective NADPH oxidase is incapable of producing ROS in phagocytes [129]. However, controversy over the role of ROS exists, as it has been shown that mice deficient in NOX2 are relatively resistant to mycobacterial infection [98]–[100]. Although the role of ROS in mycobacterial defense is not well understood, *M. tuberculosis* possess a number of resistance mechanisms for neutralizing reactive oxygen products, including *katG* catalase-peroxidase, an enzyme that neutralizes H_2O_2 into H_2O and O_2. *M. tuberculosis katG* mutants (*Mtb*Δ*katG*) cannot grow in wildtype and iNOS$^{-/-}$ macrophages, but grow inside NOX2 deficient mice (gp91phox$^{-/-}$) [133]. Additionally, *M. tuberculosis* possesses two superoxide dismutase genes, *sodA* and *sodC*, which catalyze the conversion of superoxide anions to hydrogen peroxide and water. These enzymes are critical for virulence in several other pathogens, including *Helicobacter pylori*, *Salmonella typhimurium* and *Yersinia enterocolitica* [134]. Deletion of *sodC* has led to increased susceptibility to superoxide and killing in IFN-γ-activated, but not TLR-activated murine macrophages, which may correspond to functional importance at later stages of infection [135]. *Sod*A seems to play a complementary role to *SodC*, and it has been

shown to protect against ROS in TLR-activated macrophages during infection [136], where *Sod*A mutants were attenuated in mouse infection models [137]. Despite the controversy of the relative contribution, ROS clearly play an important role in mycobacterial protection, evidenced by the redundant virulence mechanisms. It is likely that infection timing, host model system, and strain specificity confound findings, though generally it appears respiratory bust is a necessary component of antimycobacterial immunity.

Fish possess orthologous NADPH oxidase machinery and are similarly capable in producing toxic reactive oxygen intermediates from both TLR and cytokine activation [63]. *In vitro* and *ex vivo* studies of the *M. marinum* goldfish infection model have demonstrated attenuation of IFN-γ and TNF-α-stimulated ROS production by kidney macrophages [98], [101]. In the zebrafish model, ROS in macrophages has been shown to kill intracellular *M. marinum*, although excessive activation of infected macrophages by TNF led to induction of programmed necrosis (necroptosis), resulting in the release of bacteria into the more growth permissive extracellular milieu [138]. More recently, *M. marinum* cell wall component hypoxanthine-guanine phosphoribosyltransferase (HGPRT) has been shown to enhance bacterial survival by inhibition of oxidative stress and autophagy pathways [139]. These findings highlight the importance of fine tuning the regulation of ROS,

where both inhibition and excessive activation enhances bacterial growth and disease pathology.

2.11 Nitric oxide response

In addition to respiratory burst, activated macrophages express inducible nitric oxide synthase (iNOS/NOS2), enzymes that catalyze the conversion of L-arginine to L-citrulline, resulting in the production of a potent antimicrobial compound, nitric oxide (N [140]. iNOS has become the hallmark of classically activated macrophages, which are effector cells with a "kill" phenotype, as opposed to the homeostatic "repair

phenotype of alternatively activated macrophages [141]. Stimulation of macrophages with PAMPs (e.g. LPS) or inflammatory cytokines (e.g. IFN-γ) leads to activation of iNOS and production of large amounts of nitrate (NO_2^-) and nitrite (NO_3^-), known as reactive nitrogen species (RNS) [142], [143]. Parallel production of superoxide and NO can also result in the formation of peroxynitrite ($ONOO^-$), a potent antiparasitic/antimicrobial agent [144]. Like ROS, RNS can modify DNA, proteins and lipids, resulting in antimicrobial function, but can also damage host cells [145]. Activation of iNOS is essential for the destruction of intracellular pathogens, including mycobacteria [146], [147]. Indeed, iNOS knockout studies have demonstrated uncontrolled bacterial replication, dissemination, tissue destruction and mortality [148].

Members of the *M. tuberculosis* complex possess a number of virulence factors to combat host RNS. Exposure to NO can trigger a transition to dormancy,

leading to persistence of infection [149], [150]. The sensing of NO is accomplished through a three-component dormancy survival regulator (DosR/S/T), which shifts the bacteria from aerobic to anaerobic metabolism to enter dormancy [151]. Other virulence factors involved in denitrification include TpX, a thiol peroxidase that reduces peroxynitrite [152], AhpC, a catalase peroxidase [153], PknE, a serine/threonine kinase E, an inhibitor of NO-mediated apoptosis [154]. Forrellad et al. recently published a comprehensive review on mycobacterial virulence factors that combat oxidative and nitrosative stress [155].

Infections of *M. marinum* in fish have yielded corroborative insight into the role of iNOS in mycobacterial immunity. In zebrafish, MyD88-dependent TLR recognition of *M. marinum* resulted in NO production, although virulent strains containing the region of difference (RD1) locus, a well characterized region of genome in *M. marinum* and *M. tuberculosis* that confers virulence, were capable of attenuating the response [94]. Interestingly, *M. marinum* has recently been shown to preferentially recruit permissive macrophage phenotypes during early infection, which do not produce nitric oxide upon internalization [81]. This evasive selective screening of macrophages is accomplished using cell-surface-associated PDIM lipids that mask underlying PAMPs and recruit permissive macrophages through a host chemokine receptor 2 (CCR2)-mediated pathway [81]. In PDIM deficient *M. marinum*, TLRs stimulation leads to recruitment of macrophages with microbicidal potential through reactive nitrogen intermediate-based mechanisms. Further reports using the zebrafish model demonstrated that the enhancement of iNOS reduced bacterial burden, while impaired iNOS increases host susceptibility [78], [99], [156],

[157], and the Dos dormancy survival regulon is also seemingly conserved [158]. *In vitro* and *ex vivo* dampening of cytokine-induced nitric oxide production has also been observed in goldfish macrophages, suggesting conserved mechanisms for attenuating iNOS function in teleosts [98], [101].

2.12 Tryptophan degradation

The IFN-γ-elicited expression of macrophage indoleamine 2,3-dioxygenase (IDO) is another marker of classical macrophage polarization, which oxidizes L-tryptophan to N-formyl-kynurenine [159]. Expression of IDO can be induced by either IFN or TLR pathways, leading to depletion of L-tryptophan which halts the growth of pathogens that depend on hosts as a source [160]. The catabolism of L-tryptophan results in production of metabolites known as kynurenines that promote a broad spectrum of downstream effects, including immunotolerance and suppression of T cell proliferation [161]. While the roles of macrophage IDO are contentious and remain poorly understood, it is generally accepted that IFNγ-mediated activation of IDO deprives supply of tryptophan to pathogens, limiting growth and persistence [133]–[136].

Pathogenic mycobacteria are capable of *de novo* L-tryptophan biosynthesis, rendering a deprivation strategy by IDO ineffective. Moreover, the downstream effects of kynurenine may actually interfere with T cell activation, a critical component of antimycobacterial immunity. Indeed, increased IDO expression has been shown to correlate with severity of pathology in *M. tuberculosis* infected individuals [166]. Due to the disruption of a productive adaptive immune response,

interference of IDO has been proposed as a potential therapeutic target, in addition to the L-tryptophan biosynthesis machinery in mycobacteria, anthranilate synthase (TrpE) [167], [168].

Fish IDO orthologues (proto-IDOs) appear to have less efficient tryptophan degradative capacities as compared to the mammalian IDOs, suggesting the possible presence of alternative fish IDO substrates that are yet to be identified [169]. To date, only a few expression analyses of proto-IDO orthologues has been studied in the context of mycobacterial infection. Goldfish macrophages infected with *M. marinum* up-regulated proto-IDO gene expression and exposure of macrophages to live *M. marinum in vitro* and induced substantially greater proto-IDO mRNA levels than the heat-killed bacteria, suggesting a possibly similar tryptophan metabolism strategy seen in mammalian hosts pathogen infection models [98]. Similarly, large increases in IDO in *M. marinum* infected spleen and kidney tissue has been observed [101].

2.13 Macrophage apoptosis

Regulated cell death by infected cells is an important mechanism to contain pathogen replication and spread. The importance of apoptosis to an effective immune response is underlined by the various pathogenic virulence factors that inhibit the process [170]. Apoptosis in *M. tuberculosis* infected macrophages is generally regarded as host protective, where intact bacteria encased in plasma membrane are internalized by phagocytes in a process called efferocytosis [171]. Sequestration of mycobacteria inside of the apoptotic body is thought to disrupt the interference of phagolysosomal fusion following phagocytosis, thereby delivering the bacilli to

lysosomal components and facilitating degradation [172]. Apoptosis was first recognized as an important line of defense against mycobacteria when attenuated *M. tuberculosis* strains such as *M tuberculosis* H37Ra exhibited reduced viability and increased apoptotic turnover [173], whereas virulent *M. tuberculosis* strains induced less apoptosis and persisted intracellularly [174]. Pro-apoptotic mutants have led to the discovery of virulence factors that impair apoptotic function, including the *sec*A2 gene, a secretion system that secretes mycobacterial superoxide dismutase, a strong superoxide scavenger [175]. Contradictory reports suggest apoptosis can also promote mycobacterial spread [176] although this is likely due to contrasting stages of infection, species/strain differences and immunological context.

Fish models have also demonstrated the importance of apoptosis in the control of mycobacterial infection. Recently, PDIM deficient *M. marinum* was shown to increase the level of apoptosis in early infection compare to wild type bacteria in the zebrafish embryo model, although these roles were reversed in the granuloma [177]. The increased apoptosis in the granuloma leading to the expansion of infected macrophages has previously been demonstrated in zebrafish larval granulomas infected with *M. marinum* possessing the RD1 virulence locus [178], reiterating the immunological context of these processes as paramount.

2.14 Macrophage necrosis

Recent insight into death by necrosis has added a nuanced view of what was originally considered ‘un-controlled cell death’. This original definition holds as partially true, where unfavorable chemical or physical conditions results in

unregulated cell death. However, a number of controlled states of necrosis have been defined as controlled means of necrotic death [179]. Accordingly, the means of mycobacterial control by necrotic cell death is complicated. The well acknowledged role of TNF for host defense has also been shown to exacerbate disease outcomes through programmed necrosis, by excessive ROS production [138], [180], [181]. Eicosanoids have been shown to regulate the axis of cell death, where induction of lipoxin (LXA4) and inhibition of prostaglandin (PGE2) by virulent strains of *M. tuberculosis* led to programmed necrosis and mycobacterial spread [182]–[185]. Mice deficient in PGE2 are more susceptible to mycobacterial infection due to enhanced necrosis, while LXA4 mutations enhance apoptotic response, leading to less susceptibility [186].

Fish models have been critical in advancing our understanding of necrotic cell death with regards to mycobacterial disease, and caseating necrosis has been observed in zebrafish, goldfish and tree frog granulomas, commensurate with heightened pathology [29], [157], [189]. The discovery of polymorphisms in *ltah* (leukotriene A4 hydrolase) was originally observed in the zebrafish and was later demonstrated in susceptible human populations [180], [190]. Host mechanisms of control influencing the development of a necrotic core in the granuloma has been elegantly demonstrated in the zebrafish model. Restriction of macrophage recruitment to the granuloma resulted in necrosis of core macrophages, and was highlighted as a tipping point where fresh macrophage recruitment was exceeded by the demand within the granuloma [25]. Virulence products of the ESX-5 secretion system have also been identified as influencers the necrotic/apoptotic balance in fish, where

knockouts drive a hyper-virulent necrotic response in the center of the granuloma, though the mechanism underpinning this observation is unclear [99].

2.15 Role of neutrophils

The role of neutrophils during mycobacterial infection is controversial, although neutrophils are infected by mycobacteria, and are the predominant infected cell type during active tuberculosis [191], [192]. Numerous reports suggest a negative role for neutrophils in tuberculosis, where respiratory failure and death are associated with elevated blood neutrophil levels, although it is not clear whether this is causal or a result of unfettered pathology [193], [194]. Further reports have shown neutrophils to facilitate delivery of mycobacteria to dendritic cells, thereby aiding in the subsequent activation of $CD4^+$ T cells [195]. Similar to what is seen in macrophages, apoptosis of neutrophils is inhibited by pathogenic mycobacteria, leading to the delayed priming of $CD4^+$ T cells, concomitantly resulting in a higher bacterial load per cell [196]. The contribution to pathology and/or host protection is likely due to host/pathogen genetic factors, as well as the stage of disease and immune context. Indeed, early events that accompany infiltration of neutrophils to the infection site influence activation states differently that at later stages in the granuloma. Early protective effects of neutrophils has been observed [79], [184], [197], while depletion of neutrophils at later stages of infection has been shown to reduce the bacterial load [201]. Therefore, it may be possible that early protective responses and promotion of T cell activation by neutrophils renders later immune contribution unnecessary, and disruption of this tightly controlled process may lead to neutrophil pathology at later

disease stages. Indeed, it has been suggested that neutrophilia during active disease is an indication of failed Th1 immunity [202].

In fish models, recent interest in neutrophils in *M. marinum* infection has helped to underline their importance in protection. Initially, it was thought that macrophages, but not neutrophils internalize *M. marinum*, as this was observed following injection of bacilli into the bloodstream or hindbrain of zebrafish embryos [80]. Subsequent reports of internalization of *M. marinum* by neutrophils in subcutaneous infection studies suggest that site specificity of infection exists, and is more likely to represent the correct role for neutrophils in early infection due to a closer resemblance of natural infection [203]. Further, a recent study in zebrafish using confocal laser scanning methods illustrate that neutrophils may contribute more to the dissemination of bacteria than macrophages, due to their high mobility post infection [203]. Clarification of the role of neutrophils in early infection has been greatly aided in the adult zebrafish model, where death by bacteremia is associated with neutropenia, further suggesting a protective response by neutrophils [204]. This is corroborated in transgenic zebrafish expressing truncated chemokine receptor Cxcr4 leading to retention of neutrophils in hematopoietic compartments and an increase in the bacterial burden [80], [205]. Interestingly, enhancing nitric oxide production by neutrophils prior to infection by manipulation of hypoxia-inducible factor (Hif-α) signaling mediated a protective response [206]. At the granuloma, neutrophils have been shown to internalize apoptotic bodies of macrophages, scavenging and killing internalized bacilli via oxidative mechanisms [80], [203]. In

this manner, neutrophils play an important protective role at the later stages of infection in fish models.

2.16 CD4+ T cells

2.16.1 Th1

Adaptive immunity is first detectable between 3-8 weeks post mycobacterial infection [207], which is widely accepted as being delayed compared to other bacterial infections [208], [209]. This adaptive response to mycobacterial infection is highly dependent on T helper cells, although the evidence is highly correlative, suggested by the increased susceptibility to TB in HIV coinfection [210]. Following initial infection and internalization events, antigen presenting cells, predominantly dendritic cells, traffic to a nearby lymph node and stimulate CD4+ T cell expansion [79], [116], [211], although inhibition of MHC class II peptide presentation by *M. tuberculosis* has been observed as a proposed evasion mechanism [211]. The delay in T cell expansion may in part be due to delayed migration of dendritic cells that may internalize apoptotic bodies of infected macrophages or neutrophils [186], [196]. However, once migrated, it has been observed that infected dendritic cells are capable of releasing intact bacterial antigens that are then taken up and presented by the uninfected dendritic cells, optimizing T cell priming [212]. Following DC migration and T cell activation, further delays in effector function has been observed. Interestingly, even fully differentiated pathogen-specific Th1 cells that are transferred to a naïve host prior to infection do not provide protection against *M. tuberculosis* for

7 days post infection [213], demonstrating that even following T cell activation there is inhibition of movement and downstream effects.

Antigen-driven differentiation of T cells results in the capacity of CD4+ (and to a lesser extend CD8+) to produce essential Th1 cytokines, particularly IFN-γ, which is well established in protection against mycobacterial pathogens [214]. These cytokines are critical for activation of the antimycobacterial function of macrophages, including phagosome maturation, reactive nitrogen intermediates and antigen presentation (described above) [215], [216]. Indeed, cessation of bacterial growth correlates strongly to the arrival of CD4+ T cells at the infection site or granuloma [212], [213], [217], although the mechanisms have yet to be fully understood. Evidence for the protective capacity multifunctional CD4+ T cells, subtypes that produce IL-2, IFN-γ and TNFα, suggest the balanced combination of cytokine levels to be an important factor [218]. Development of multifunctional Th cells seems to depend heavily on antigen presentation of dendritic cells, as well as proper cytokine stimulation of a well-orchestrated innate immune response, but maintenance of the response is not fully elucidated.

The adaptive components of the immune response to mycobacterial infection is not well established in fish. This is partially owing to the relative lack of reagents in teleost systems, although T cells (TcR and CD4-related genes), as well as B cells have been identified in bony fish [219], [220]. Recently, antigen induced cytokine production of CD4+ T cells was observed in adult zebrafish, where *M. marinum* infected fish showed a collection of Th cells surrounding the granuloma, similar to that of mammalian infection models [221]. Importance of T cells for the control of

mycobacterial infection was corroborated in fish, where depletion of CD4+ T cells corresponded to granuloma disruption and dissemination of *M. marinum* [222]. However, most of the research in the zebrafish has been in the embryonic system which predates development of lymphocytes [62], so the mechanisms of control of *M. marinum* by CD4+ T in teleosts is largely unstudied, although the lack of T cell immunity in this system corresponds to the inability of embryos to control infection. In adult fish models, correlative importance of Th cells in mycobacterial control has been studied with regards to IFN-γ expression studies. Increases in IFN-γ mRNA expression has been observed in adult goldfish and zebrafish *in vivo* [101], [221], [223] and in goldfish primary cultures [98]. IFN-γ-primed macrophage effector function was also shown to be attenuated by *M. marinum*, suggesting effector evasion even with IFN-γ stimulation [98].

2.16.2 Th2

CD4+ T cells can functionally polarize as Th2 cells, which generally produce IL-4, IL-5, IL-10, and IL-13, stimulating a strong antibody response and inhibiting antimicrobial macrophage activation [224]. Because a productive Th1 response is generally regarded as host protective, a Th2 response, known to cross-regulate and inhibit Th1, may be counterproductive in mycobacterial control. Relatively little research has been conducted with regards to Th2 responses in mycobacterial infections, although it seems that chronic helminth infection decreases the immunogenic response of BCG [225] and impairs a productive Th1 response in concurrent *M. tuberculosis* infections [226], [227]. Interestingly, IL-4Rα$^{-/-}$ deficient

mice with a helminth infection, exhibit improved ability to combat mycobacterial infection, suggesting that a possible mechanism of interference is the alternative activation of macrophages, a functional state that naturally down-regulates iNOS function [228].

Not surprisingly, fish also possess a Th2/M2 functional state in response to parasitic infection, which has been exhibited by increases in arginase activity in macrophages [229]. Fish also possess Th2-type cytokines capable of a homologous regulatory and anti-inflammatory role, as was demonstrated in goldfish macrophages exposed to recombinant forms of IL-4 [230]. Zebrafish Th2-like cells have been characterized in response to *M. marinum* infections [221], and interestingly, adequate Th2 gene expression levels are necessary for well-controlled latency [231]. It is likely the case that the timing and regulation of Th2 response is important in mycobacterial infection as well as containment in fish as well as mammals.

2.16.3 Tregs

T regulatory cells (Tregs), characterized as CD4+ Foxp3+, are critical in the regulation of immune responses to self-antigens and in promoting homeostasis. Tregs are generally immunosuppressive through a number of mechanisms, including cytokine production (TGF-β, IL-10, IL-35), induction of effector cell apoptosis, and increasing IDO expression [232]–[235]. Regulatory T cells have been shown to accumulate at the lymph node and granuloma at a similar rate of effector T cells during *M. tuberculosis* infection in mice [236]. Accumulation of Tregs has also been shown to prevent eradication the of *M. tuberculosis* by suppression of a Th1 response

in an IL-10 independent manner, where depletion of Tregs also resulted in reduced bacterial load [237]. Despite the likelihood of an impairment of an effective antimycobacterial response role of Tregs is still relatively unclear in different stages of infection, where they likely function in minimizing pathology by controlling inflammation.

Treg cell markers Foxp3 and Gata3 are present in teleost genomes and Tregs have been identified as a functionally conserved cell type in puffer fish [238] sea bass [239] and zebrafish [240]. A few studies regarding the contribution of Tregs to the immune response in *M. marinum* infections have been completed in fish. In adult zebrafish, reactivation of latent *M. marinum* infections was correlated with increased *foxp3* transcription levels, suggesting a role for Tregs in this process [231]. Further research is required in both fish and mammalian model systems to determine the relative contribution of Tregs to host protection/disease pathology during the course infection.

2.16.4 Th 17 cells

Th 17 cells may play a role in mycobacterial protection, as they are known to have significant pro-inflammatory effects on intracellular pathogens [210]. During TB infection, Th 17 cells have been shown to accumulate at the granuloma but seem to be counteracted by IFN-γ producing CD4+ T cells. This inhibition of IL-17 producing T cells was demonstrated to limit the neutrophilic accumulation and survival, which may decrease inflammation and limit pathology [202]. It has also been shown that IL-17 response is dispensable with sufficient IL-12p70 production

[241]. Still, partial protection has been reported following transfer of antigen-specific Th 17 cells in to naïve hosts [217]. The variety of roles for IL-17 and Th 17 cells is likely due to genetic variability in host and pathogen models, and more work is necessary to understand the role of Th 17 cells in mycobacterial host defense. Very little has been established in fish regarding IL-17 following *M. marinum* infection, although expression levels have been observed in zebrafish, where heightened IL-17 levels were related to a lesser bacterial burden [242].

2.17 CD8+ T cells

Although relatively less studied, CD8+ T cells have been described during TB infection. Antigen specific CD8+ T cells are found at the site of active disease but seem to possess less cytotoxic activity compared with latently infected individuals [243]. Decreases in IFN-γ^+ TNF^+ IL-2^+ trifunctional CD8+ T cells has been observed in active disease states [244] as well as cellular dysfunction in individuals with a high bacterial load [245], marked by a higher proportion of pro-apoptotic markers and diminished proliferative capacity [246]. It has been suggested that during active disease, *M. tuberculosis*- specific CD8+ T cells are arrested in an intermediate point in differentiation with a reduced capacity for cytotoxicity and proliferation [207]. Inhibition of T cell function is exacerbated in TB patients taking anti-TNF therapy for auto-immune disorders [247], which may mean that impaired upstream activation events (i.e. macrophage activation of Th1) could lead to arrested CD8+ function.

In fish, T cells expressing CD8 have been characterized and have homologous cytolytic function [248]. CD8+ activation has been implicated in fish immunity

towards *Edwardsiella tarda* [249], although to date, there is no contribution from fish models on the research of CD8+ T cells during mycobacterial infection.

2.18 B cells

B cells and antibody production are vital for a protective response and vaccination to numerous infectious agents, although their contribution to mycobacterial protection is not well understood. It was originally conceived that B cells do not contribute meaningfully toward protection due to the intracellular nature of *M. tuberculosis* [250], although they likely play a role beyond what was previously thought [251]. B cells have been observed at the granuloma site [252] and they have been shown to influence inflammatory progression and bacterial containment [253]. Despite the implication of B cells in host protection, reports in B cell deficient mice show delayed pathology, limited involvement, or no contribution, which may be due to the genetic variability of hosts and pathogens [251].

Fish and mammalian B cells share many similarities, including the generation of hyper-specific antibody repertoires by somatic gene rearrangement, and heavy chain isotypes IgM and IgD. However, fish possess a unique antibody isotype profile, IgT/IgZ, and lack IgG/IgE, although the exact isotype picture remains controversial [254]. In fish, antibody production following infection with *M. marinum* has been observed, although a definitive link to host protection is yet to be established [156], [255].

2.19 Cytokines

2.19.1 TNF

TNF is produced primarily by macrophages but can also be produced by dendritic cells (DCs), neutrophils and T cells [256] and is the regulator of a broad range of functions, many of which involve propagation of inflammatory and host protection type responses [257]. TNF has long been recognized as critical for protection against mycobacterial infection. The importance of TNF signaling was first revealed by blocking with anti-TNF receptor antibodies, which increased bacillary load within the granuloma and expedited host death [258], [259]. Moreover, interference with TNF signaling during latent infection resulted in reactivation, suggesting an important role in maintaining a latency programme and fending off active disease [139], [260], [261]. Impaired TNF function in mycobacterial infection reduces iNOS and increases IL-10 production, while IFN-γ and IL-12 remain unchanged [262]. It has been suggested that TNF is required for the generation and maintenance of granulomas [259], [261], although this has been shown to not necessarily be the case, where various infection models have demonstrated granuloma formation in the absence of TNF [258], [263], [264].

Experiments in fish have revealed the importance of a balanced TNF response in the control of mycobacterial infection. Similar to mammalian models, TNF induces a mycobacterial killing response by stimulating effector RNS and ROS in macrophages [98], [138]. TNF has been shown to be induced in early infection events in zebrafish, leading to restriction of the growth of *M. marinum* [78]. Unsurprisingly, morpholino knockdown of TNF receptor (TNFR) led to increased bacterial growth and decreased

containment [265], although importantly, hyper-expression of TNF is also unfavorable to the host, enhancing inflammation and necrosis [138], [181]. The temporal duality in outcome of TNF signaling has been clearly described in the zebrafish embryo system [138]. Interestingly, chemical inhibition of necrosis in high TNF fish have been shown to uncouple the positive and negative effects, and is a possible means of mycobacterial treatment [138].

2.19.2 The IL-12 family

IL-12p70 is made up of a heterodimer encoded by two separate genes, IL-12p35 and IL-12p40. Production of IL-12p70 is primarily by macrophages, DCs, neutrophils and B-cells following antigenic stimulation through TLR2 and TLR9 [85], [185]. *M. tuberculosis* is a potent inducer of IL-12 subunits, and deficiency in IL-12 signaling leads to increased susceptibility to infection in humans [266]. It is widely accepted that IL-12-p70 is essential for mycobacterial control, as it drives the generation of IFN-γ producing T cells [267], where absence of IL-p70 signaling corresponds to a reduction in antigen specific T cells [268]. Additionally, IL-12p40 can also act as a homodimer (IL-12p80) in the absence of IL-12p35, and has been shown to mediate the migration of infected dendritic cells to lymph nodes to initiate the adaptive immune response [269]. IL-23, a heterodimer of IL-12p40 and IL-23p19, is induced following NOD2/TLR2 ligation, and may be important in temporal regulation of inflammation following mycobacterial infection [270], although experiments in IL-23p19$^{-/-}$ mice had no reduction in functional IFN-γ producing T cells [241]. It appears that absence of IL-12p70 can be partially but insufficiently

compensated by IL-23, suggesting a redundant role of IL-12p70 and IL-23 in T cell stimulation [241]. Indeed, otherwise healthy subjects with mutations in IL-12/IL-23 have what is referred to as Mendelian susceptibility to mycobacterial disease (MSMD), where BCG vaccine develops into progressive infection [271]. Together, members of the IL-12 family are a cornerstone of resistance in mycobacterial immunity by initiating an effective Th1 response.

Relatively little has been studied about the contribution of IL-12 to host protection in fish models. Expression analyses have shown increases in IL-12p35 and IL-12p40 in goldfish infected with *M. marinum* [101], as well as adult zebrafish [272], [273], and these increases have been shown to be dose dependant [274]. Increases in IL-12p35 and IL-12p40 subunits have also been described in goldfish macrophages exposed to *M. marinum in vitro* [98].

2.19.3 The IL-1 family

The IL-1 family of cytokines is comprised of 11 members. Predominantly IL-1α, IL-1β and IL-18 have been studied in the context of mycobacterial infections. The importance of IL-1α and IL-1β for mediating inflammation during these infections is well substantiated and has been repeatedly demonstrated in a number of animal models [91], [275], [276]. Due to a shared adaptor of MyD88, animals deficient in MyD88 were originally thought to be susceptible due to impaired TLR signaling [277], although IL-1R deficient mice are indistinguishable in their high susceptibility to mycobacterial infection, suggesting that IL-1 signaling is the essential MyD88 component for resistance [278], [279]. At the site of infection,

mycobacteria are strong inducers of IL-1α and IL-1β. IL-1α requires no post-translational activation, while IL-1β requires processing to an active form [280]. Identification of the inflammasome, a multiprotein oligomer that processes IL-1β and IL-18, raised questions to whether mycobacterial pathogens interfere with the maturation of these cytokines. Indeed, macrophages deficient in inflammasome components Apoptosis-associated speck-like protein containing a card domain (ASC) and (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3) showed diminished ability to secrete mature IL-1β [278], [281], [282], although these impairments to mature IL-1β are not observed *in vivo* [283], [284]. It is possible that the contrasting findings on inflammasome processing are due to redundant mechanisms of IL-1β processing. Despite the fact that IL-1 signaling is essential for host protection, the mechanism by which IL-1α and IL-1β confer resistance is not well understood, but it seems to be independent of Th1 responses [278]. IL-18, by contrast, has yet to be strongly implicated in host protection during mycobacterial infection [277].

The role of IL-1 in fish protection is not well established, although expression analysis confirms *M. marinum* as a strong inducer of IL-1β. Increases in IL-1 expression levels has been documented in adult zebrafish, goldfish and Japanese flounder following infection with mycobacteria [101], [274], [285]. An elegant study in zebrafish embryos demonstrated that macrophages increased IL-1β expression levels specifically following *M. marinum* internalization [78]**.** Similar to mammalian

models, more work is necessary to determine to role of IL-1 in fish host protection to mycobacterial pathogens.

2.19.4 IFN-γ

It is well established that IFN-γ is essential for protective function towards intracellular pathogens, including mycobacteria [286]. As previously mentioned, CD4+ Th cells are the predominant source of IFN-γ during mycobacterial infection. CD8+ T also produce IFN-γ, although they do not compensate for a lack of CD4+ cells [287]. Early transient sources of IFN-γ from NK cells, NK T cells and γδ T cells has also been observed which is thought to tide over a protective response until adaptive sources are generated, and these sources seem to be more prominent during infection with hyper virulent *M. tuberculosis* infection [288]. As previously discussed, antimicrobial mechanisms of the macrophage are critically important in eradicating intracellular mycobacteria. IFN-γ is largely responsible for macrophage activation following expansion of antigen specific T cells, thereby exerting numerous downstream effects through a suite of transcriptional programs [289], activation of reactive oxygen production and iNOS transcription [148], autophagy, endosome maturation [290], and production of antimicrobial peptides [75]. Mice deficient in IFN-γ are unable to control even low dose infections of *M. tuberculosis*, where they fail to produce reactive intermediates and bacteria replicate unabated [291], [292]. Moreover, mutations in cognate receptor IFN-γR1 has been implicated in fatal BCG infection [293]. Despite the role in controlling infection, IFN-γ levels are correlated with the severity of disease, where excessive and seemingly ineffective levels of IFN-

γ are seen in patients with severe TB [294]. Following productive therapy, these levels are reduced [295].

Not surprisingly, interferon signalling is similarly necessary in host protection of fish towards *M. marinum*. Expression analysis confirms increases in interferon expression levels following *M. marinum* infection in goldfish immune tissues [101] and primary cultures [98]. Interestingly, *M. marinum* was shown to impair the IFN-γ primed respiratory burst and nitric oxide response in cultured leukocytes [98]. In adult zebrafish, IFN-γ levels have corresponded to a partially protective BCG vaccination [223] as well as protective iNOS activation [274].

2.19.5 IL-10

IL-10 is produced by a variety of immune cells, including macrophages, neutrophils, B cells, DCs and T cells [296] and provides critical regulatory feedback of inflammation to prevent immunopathology [210], [288], [297]. Similar to IFN or TNF, IL-10 production during infection can act as a double-edged sword, where a precise level of control is imperative to immune function. Overproduction during mycobacterial infection has been shown to contribute to chronic infection, while excessive inflammation and pathology occurs with insufficient production [296]. Accordingly, mycobacterial pathogens have been shown to dampen host defense by tweaking the IL-10 response, where IL-10 has been implicated in impaired immune response to mycobacteria. Mice deficient in IL-10 exhibit increased protection to mycobacterial infection [298], [299] and the blocking of IL-10R signalling using antibodies has resulted in decreased bacterial loads, enhanced T cell proliferation and

IFN-γ production, and host survival [300]. Interference of immune activation by IL-10 is through a variety of mechanisms, including diminished antigen presentation, limited development of a Th1 response (and subsequent IFN-γ production), leading to inhibition of TNF, and prevention of iNOS expression [301], [302]. Increased IL-10 by infected macrophages has also been shown to arrest phagosome maturation in *M. tuberculosis*, which was reversed by blocking IL-10 antibodies [303]. The relative pathogenicity of mycobacterial strains seems to correlate with IL-10 production, where hypervirulent strains of TB (HN878), characterized by the presence of a phenolic glycolipid, have been shown to induce an early IL-10 and arginase-1 expression via CD4+CD25+FoxP3+CD223+ regulatory T-cells [304]. Coinfection with complementary strains demonstrate a virulence mechanism utilized by *M. tuberculosis* HN878 to exploit host immune systems through induction of IL-10, which is presumed to be an immune evasion strategy [305].

M. marinum induced expression of IL-10 has been observed in goldfish primary kidney monocytes and macrophages [98], although no apparent increases of IL-10 were observed in spleen or kidney tissues during *in vivo* infection of goldfish [101] or zebrafish [180], [205] and introduction of recombinant IL-10 did not alter the viability of *M. marinum* within goldfish phagocytes [98]. While the data in mammalian models and humans are more or less settled with regards to IL-10 impairing the ability to eradicate mycobacteria infection when in excess, studies in fish have not reached this same consensus.

2.19.6 MMP9

Metalloproteinase 9 (MMP9) has been identified as a critically important chemotactic factor for recruiting macrophages and monocytes to the granuloma, and has been shown to be modulated by an RD1 factor, ESAT-6 [306]. Interestingly, MMP9 is not produced by infected cells at the granuloma, but rather nearby adjacent epithelial cells. Production is initiated by the virulence factor ESAT-6 and seems to be independent of macrophages, although the mechanism has yet to be defined [306]. The generation of MMP9 by epithelial cells is thought to attract naïve macrophages and monocytes while simultaneously allowing for the dampened antimicrobial response in infected phagocytes in the granuloma [307]. Similar to RD-1 knockout *M. tuberculosis*, mice deficient in MMP9 have decreased phagocyte recruitment and reduced bacteria load compared with wildtype mice. Increased MMP9 in human cerebrospinal fluid is associated with more severe TB disease [307]–[309].

The role of MMP9 was corroborated in the zebrafish model that offers some insight site specificity of granuloma formation. If a granuloma seeds to form in muscle it generally does not grow, and it is proposed that the proximity of MMP9-producing epithelial cells is an important factor in determining the prospective site of a granuloma [306]. Due to the relative distance of epithelial cells from muscle, it is possible that insufficient local MMP9 generation prohibits granuloma formation. MMP9 induction by ESAT-6 and subsequent granuloma formation was first discovered in the zebrafish embryo [306]. Disruption of MMP9 function in the zebrafish model led to inhibited bacterial growth, reduced granuloma formation and

greater host survival [306]. More recent reports of sustained increases in MMP9 during infection with a virulent form of *M. marinum* has also been observed in adult zebrafish [99].

2.19.7 CCL2

C-C motif chemokine ligand 2 (CCL2) is a strong chemoattractant of monocytes, basophils and CD4+ T cells [311]. Following engulfment of mycobacteria, macrophages produce various chemokines including CCL2 [312], which remains significantly upregulated during infection with TB. CCL2 has been demonstrated as host protective, where deletions lead to larger bacterial burden, although expression levels are also associated with TB severity and pathology [313][313]. Recruitment of inflammatory cells for granuloma formation has been shown to be crucially depend on CCL2 [314]. In humans, polymorphisms in the CCL2 allele have been identified with increased risk of developing active TB in Asian and Hispanic populations [315].

In fish, increased expression of CCL2 following infection with *M. marinum* has been observed and was linked to cell wall associated phenolic glycolipids (PGL). Contrary to mouse studies, increases in CCL2 and signaling through CCR2 was shown to increase host susceptibility in zebrafish embryos [81]. It has been suggested that higher inoculum studies have failed to reveal the increased host susceptibility with clinically relevant low inoculums that are exploited through the CCL2-CCR2 signaling [316]. Moreover, while CCL2 is generally regarded as proinflammatory due to its ability to recruit macrophages, it has been shown to shift macrophage

polarization to anti-inflammatory [317]. Together, reports in fish and humans of CCL2 being linked to host susceptibility may necessitate further investigation in mammalian models.

2.19.8 TGF-β

TGF-β is a multifunctional cytokine that is involved in a number of immunological processes, including suppression of T cell responses, deactivation of macrophage antimicrobial responses, and wound healing. The immunosuppressive nature of TGF-β has been implicated in TB, which has been shown to be induced by *M. tuberculosis*, as well as purified protein derivative (PPD) [318]. Other studies have demonstrated that cell wall component LAM preferentially induces TGF-β, and not TNF-α in monocytes, suggesting a regulatory bias [318]. Disruption of TGF-β activity in mice has shown to promote activity of mycobacterial immunity [319]. Moreover, abrogation of TGF-β by neutralizing antibodies increases the IFN-γ production and T cell response during *M. tuberculosis* infection [320]. In humans, monocytes from patients with active TB constitutively express TGF-β [321].

In fish, TGF-β seem to exert similar immunosuppressive effects as is seen in mammals [322], [323]. Goldfish and carp recombinant TGF-β1 has been shown to down regulate macrophage and monocyte antimicrobial responses [324], [325]. TGF-β1 expression levels have been studied in tilapia, striped bass, goldfish and zebrafish, where variable increases have been observed following infection with *M. marinum* [101], [222], [242], [326], [327]. The degree to which TGF-β plays a role in infection or pathology has yet to be fully elucidated.

2.19.9 SOCS3

Suppressor of cytokine signaling (SOCS) molecules are comprised of 8 family members which dampen the activation of signal transducer and activator of transcription (STAT) molecules, critical for the control of the inflammatory process [328]. SOCS3 is a negative regulator of inflammation that results in inhibition of STAT3 activation, and is a critical factor for growth, apoptosis and transcription of inflammatory genes [329]. Induction of SOCS3 has been shown to contribute significantly to evasion of macrophage activation during infection [329], and it has been demonstrated in cells, animals and patients infected with *M. tuberculosis* [328].

Relatively little has been studied in fish with regards to SOCS3. Our lab has demonstrated that that both *in vivo M. marinum* infections in goldfish as well as *in vitro* exposure to *M. marinum* has resulted in increased expression of SOCS3 [98], [101]. More work is necessary to determine the role of SOCS3 during mycobacterial infection in fish.

2.20 Lipid mediators

Balance of the eicosanoids prostaglandin E2 (PGE2) and lipoxin A4 (LXA4) plays a major role in the outcome of mycobacterial infection, which can be altered by virulent strains as a survival strategy [178]–[181]. Less virulent mycobacterial infections, including the BCG vaccine, preferentially increase PGE2, leading to apoptosis in macrophages and ultimately bacterial containment [186]. Deficiencies of PGE2 in mice have resulted in increased bacterial loads and host susceptibility [187], [188], although deficiencies in LXA4 show increased resistance [182], [297]. As

previously mentioned, the balance of PGE2 and LXA4 govern whether macrophages undergo apoptosis or necrosis, which is an important determinant in host protection during infection [330].

In fish, polymorphisms in *ltah4* led to increased LXA4 production that resulted in greater host susceptibility to *M. marinum* infection [180]. These polymorphisms were later confirmed to affect human susceptibility [331]. Despite this impactful funding, there has been relatively little research of eicosanoids and prostaglandins in fish infection models.

2.21 The granuloma

The granuloma is an organized cellular aggregate that can for in the presence of ineradicable infectious or non-infections stimuli [332]. The most prominent known cause of granulomas (or tubercles) are those generated in response to pathogenic mycobacterial infections, giving rise to the name TB due to the hallmark pathology [307]. At the granuloma macrophages can undergo a variety of transformations: they can tightly interdigitate with other macrophages and take on a epithelioid morphology known as epithelioid cells; they can turn into foam cells, which are rich in lipids, thought to be due to the interference of lipid metabolism by internalized mycobacteria; they fuse into multinucleated giant cells; and they can create caseous centers in the granuloma by necrotic cell death, mediated by hypoxia [50], [62], [333]. The accumulation of macrophages in these heterogeneous forms, as well as infected neutrophils, are surrounded by lymphocytes and fibroblasts that create a

fibrotic encapsulation [334]. A wide range of chemokine, cytokine and adhesion molecules orchestrate the formation of granulomas, as reviewed in [334].

The granuloma was historically viewed as a generally protective feature, where the host could effectively encase material that it could not destroy. This was partly due to autopsies revealing healed granulomas with no live bacteria, suggesting the granuloma was effective at controlling an infection [335]. Furthermore, multiple forms of deficiencies, such as IFN-γ, TNF, IL-12 or MyD88 prevent the development of an organized granuloma, corresponding to hyper-susceptibility [262], [287], [297], [336], [337], although these deficiencies affect a number of other critical effector functions, such as macrophage antimicrobial capacity. Paradoxically, acute disease is also marked by the existence of granulomas, meaning granulomas often fail at controlling bacterial proliferation, resulting in the classical view of the granuloma as an immune response that is attempting, but failing, to control the infection [307]. More recently, the "trying but failing" function of granulomas has been called in to question, where the organized structure may actually be more beneficial to the pathogen. This is largely due to the contributions of teleost immunology, specifically the transparent and genetically tractable zebrafish embryo model, where visualization of granuloma formation and bacteria movement is challenging the established dogma. It was previously thought that generation of granulomas requires adaptive immunity, coinciding with slower growth of mycobacteria [338], [339], however, granuloma formation in zebrafish embryos infected with *M. marinum* occurs in the absence of adaptive immune components, which are not present developmentally [77]. Visualization studies in the zebrafish revealed that nascent granulomas continually

accept infiltrating macrophages and monocytes, responding to a chemokinesis gradient, into the structure until they are heavily infected, and infected macrophages can leave established granulomas to seed new sites of formation [77], [178]. Interestingly, the influence of chemokine production has been isolated to the RD-1 locus, where RD-1 deficient *M. marinum* attract fewer monocytes and macrophages to the nascent granuloma, and bacterial expansion is much slower by comparison [178]. Further, the attraction of macrophages and monocytes to the granuloma by virulent mycobacterial strains results in an average of 2.3 fresh phagocytes internalizing dead cell components containing live bacteria, thereby multiplying the number of host cells [62]. Therefore, RD-1 deficient mycobacteria are capable of infecting and multiplying intracellularly, but do not expand further, likely due to the impaired recruitment of myeloid cells to the granuloma. While the kinetics of monocyte and macrophage recruitment with regards to RD-1 have not been confirmed in mammalian models, it appears there may be conserved function in mice [340], and RD-1 deficient mycobacterial infections in mice also demonstrated poorly formed granuloma structures [157], [341].

Further characterization of granuloma function has been elucidated in the zebrafish embryo system, implicating the role of granulomas in disseminating infection. Until recently, granulomas were viewed as static structures where bacteria were contained and controlled. Visualization in the zebrafish model demonstrate the ability of infected macrophages to efflux and seed new granuloma sites, by entering the blood stream or through tissue parenchyma [178]. Moreover, the onset of granuloma formation has been shown to accelerate the proliferation of virulent

mycobacteria, and attenuated versions of *M. tuberculosis* were incapable of initiating the assembly of granulomas, impairing bacterial growth [342]. Together, these data suggest an alternate role of the granuloma which are traditionally thought of as host protective structures, as one that generally favours the pathogen. However, it is not the case that observations in the embryonic model translate to adult organisms. For example, *M. marinum* deficient in ESX-5 is attenuated in zebrafish embryos, but hyper-virulent in adult zebrafish [99]. Therefore, validation of embryonic studies in adult fish is necessary due to unwind those observations that are embryonic specific, or those that are conserved in adult models despite vast developmental differences

2.22 Summary

In this review, I have summarized critical components of the immune response required for control of the prominent pathogen *M. tuberculosis*, and the relevant contributions of natural fish: *M. marinum* infection models. Various immune molecules and mechanisms have been established as central for host protection while many others are either controversial or poorly defined. Despite the historically robust research of immunology and host evasion mechanisms, *M. tuberculosis* remains a deadly pathogen responsible for millions of deaths annually. A consensus of macrophage effector function, as well as activation of a cell mediated adaptive response remains central to limit susceptibility, though the culmination of these responses depends on the orchestration of a panoply of events that remain to be fully elucidated. This thesis is dedicated to the natural infection model of goldfish and

mycobacterial pathogens, which allows for observing the interactions of primary immune cell cultures, as well as natural infection events in an adult host.

Chapter III: Materials and methods

3.0 Cell culture

3.0.1 Fish

Goldfish (*Carassius auratus* L.) were purchased from Aquatic Imports (Calgary, AB) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 17 °C in a flow-through water system on a simulated natural photoperiod and fed to satiation daily with trout pellets. Each fish was acclimated to this environment at least 3 weeks prior to use in experiments. All of the fish ranged from 10-15 cm in length, and whenever possible, an equal number of both sexes were used.

3.0.2 Carp serum

Carp serum was obtained by bleeding carp (*Cyprinus carpio* L.). Fish were anesthetized with tricaine methane sulfonate (TMS) (40-50mg/L) and bled from the caudal vein using 3 mL syringes with 23G needles. Carp were maintained in the aquatic facility as blood donors and were bled every 4-8 weeks. Collected carp blood was pooled and allowed to clot overnight at 4°C. After clotting, blood was then centrifuged at 1000 x *g* for 25 min, the serum was removed with a pipette, and was heat inactivated at 56°C for 30 min. The serum was filter sterilized (0.22 μm) and stored at -20°C prior to use in generating media.

3.0.3 Goldfish leukocyte culture medium

Medium for the culture of goldfish leukocytes (MGFL-15) has been previously described [343]. GFL-15 components are listed in Table 3.1, MGFL-15 components are listed in Table 3.4 and complete MGFL (CMGFL-15) components are listed in Table 3.5.

3.0.4 Isolation of goldfish leukocytes

Fish were sedated using ~40-50mg/L TMS (Syndel Laboratories, Canada) and killed by cervical dislocation. Kidneys were aseptically removed and placed in a 2.5 mL FACS tube containing 1 mL of ice-cold homogenizing solution as listed in Table 3.6. Tissues were gently pressed through sterile mesh screens with a 3cc syringe plunger, whilst rinsing with a total volume of 12 mL of homogenizing solution. Tissue particulates were allowed to settle, and the cell suspension was layered on 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 min at 4ºC. Cells suspended on top of 51% Percoll were isolated for establishment of PKM cultures, while pelleted cells comprised of granulocytes (neutrophils) and red blood cells were utilized for neutrophil isolation.

3.0.5 Goldfish primary kidney macrophage cultures

Isolation of goldfish kidney leukocytes was performed as described (Section 3.0.4). Kidney leukocytes from the suspended layer were washed twice with MGFL-15 and cultured in 15mL CMGFL-15 supplemented with 5 mL conditioned medium

from previous PKM cultures in 75cm^2 tissue culture flasks (Corning). PKM cultures consisted of a dynamic population of progenitor cells, monocytes and macrophages as previously determined by flow cytometry, morphology, cytochemistry and function [344]. Two-to-4 day old cultures were predominantly monocytes, while 6-8 day old cultures were predominantly macrophages [344], [345].

3.0.6 Cell-conditioned medium

Establishment of quality PKM cultures is highly dependent on collection of cell-conditioned medium from prior proliferative cultures. Goldfish PKMs aged 6-8 days (in proliferative phase) were centrifuged at 230 x *g* for 10 min at 4ºC, and the resulting cell-conditioned medium (CCM) was pooled, filter sterilized (0.22µm) and stored at 4ºC prior to use in subsequent cultures.

3.0.7 Goldfish primary kidney neutrophil cultures

Isolation of goldfish kidney leukocytes was performed as described (Section 3.0.4). Following initial centrifugation, cells that passed through the 51% Percoll solution were decanted and resuspended in residual Percoll (~70-100 µL). 1 mL ACK Lysis Buffer (BioWhittaker) was immediately added to cells and incubated at room temperature for 3 min in order to lyse contaminating red blood cells, after which 9mL of MGFL was added to stop lysis. Cells were centrifuged at 230 x *g* for 5 min at 4ºC and subsequently washed twice with 10mL MGFL-15 at 230 x *g* for 5 min at 4ºC. The pelleted cells were decanted, and the layer of lysed cells/membrane was carefully removed using a pipette. Cells were resuspended in 10 mL MGFL-15 containing 10%

carp serum and were incubated overnight 25cm^3 tissue culture flasks (Corning). Non-adherent cells (neutrophils) were utilized for experiments the following day.

3.0.8 Isolation of goldfish splenocytes

Spleen tissue from individual fish was aseptically removed, passed through stainless steel screens and with 10mL homogenizing solution. Cells were centrifuged at 230 x *g* for 10 min, decanted, and resuspended in residual homogenizing solution (~70-100 μL). 1mL ACK red blood cell lysis buffer (BioWhittaker) was added at room temperature for 3 min to lyse remove red blood cells, after which 9mL of MGLF-15 was added to stop lysis. Cells were washed twice in MGFL-15 medium (230 x g for 10 min) and harvested for use in experiments.

3.0.9 Isolation of goldfish peripheral blood leukocytes

Individual fish were bled from the caudal vein and 2 mL of whole blood was diluted in 10 mL of MGFL-15 medium containing heparin 50 U/mL (Sigma) and 100 U/mL penicillin/100 μg/mL streptomycin. The peripheral blood leukocytes PBL were pelleted by centrifugation at 230 x *g* for 10 min and the red blood cells were removed by resuspension in 1 mL ACK red blood cell lysis buffer (BioWhittaker) for 3 min. When necessary, a second round of RBC lysis was performed The PBL suspensions were washed twice in MGFL-15 medium (230 x *g* for 10 min) and lysed cells/membrane fragments were removed prior to use in experiments.

3.1 Mycobacteria

3.1.1 Mycobacteria growth conditions

Mycobacterium marinum (strain ATCC 927) *M. chelonae* (strain 9532) and *M. fortuitum* (ATCC 6841) were grown either still or by shaking at 30°C as a dispersed culture in 7H9 broth (Difco, Detroit, Michigan) supplemented with 0.5% glycerol and 10% albumin- dextrose complex. *M. marinum* was harvested after approximately 7-10 days of cultivation, while *M. chelonae* and *M. fortuitum* were harvested approximately after 2-5 days of cultivation. Before all experiments, bacterial cultures were dispersed by 10-15 passages through a 25 - gauge needle and were washed and re-suspended in PBS + 0.05% Tween 80.

3.1.2 Enumeration of mycobacteria *M. marinum*, *M. chelonae* and *M. fortuitum*

To determine the growth kinetics of *M. marinum*, *M. chelonae* and *M. fortuitum*, colonies were grown by streaking from frozen glycerol stock on Middlebrook 7H10 agar (Difco) at 30°C in a humidified chamber until colonies were present. A single colony was used from each species was used to inoculate Middlebrook 7H9 broth supplemented with OADC and 0.05% Tween 80. 1mL samples of culture were taken every 12 or 24 h to measure optical density at 600 nm (Fig. 3.1) and for enumeration of colony forming units (Fig. 3.2). Prior to measurement of OD, bacteria were passed through a 25-gauge needle 10-15 times to disperse clumps into a uniform solution.

3.1.3 Live *M. marinum*, *M. chelonae* and *M. fortuitum*

M. marinum, (strain ATCC 927 – fish isolate) was generously donated by Dr. Lourens Robberts, School of Public Health, University of Alberta. *Mycobacterium chelonae* (strain 9534 – fish isolate) and *Mycobacterium fortuitum* (strain ATCC 6841 – fish isolate) were a kind gift from Dr. James Talbot (Provincial Laboratory of Public Health, University of Alberta). Glycerol stocks of bacterial species were used to streak Middlebrook 7H10 agar (Difco). Plates were incubated at 30°C in a humidified chamber until colonies were present. Colonies were stored in the dark 4°C. Single colonies were used to inoculate 6 mL Middlebrook 7H9 broth supplemented with OADC and 0.05% Tween 80 and 1 mL was scaled into 20 mL of broth prior to stationary phase. Cultures were harvested at mid-log phase (OD ~ 0.5 – 0.7) and were washed twice in sterile PBS + 0.05% Tween 80.

3.1.4 Heat-killed *M. marinum*, *M. chelonae* and *M. fortuitum*

Bacterial cultures were generated using the same technique described above and were harvested in mid-log growth and enumerated using a standard curve. Bacteria were washed twice and resuspended in 1 X PBS+ 0.05% Tween 80, pH 7.4. Prior to killing, a sub-sample of each culture was serially diluted and plated on 7H10 media to confirm CFUs. Killing of bacteria was accomplished with incubation at 80°C for 30 min. Loss of viability was confirmed by plating resulting cultures on 7H10 media and observing no growth following 7 days of incubation. Heat-killed bacteria preparations were kept at -20°C until used.

3.1.5 Transformation of *M. marinum* with GFP plasmid

M. marinum plasmid 30173 (Addgene) constitutively expressing GFP was kingly made available by Dr. Ramakrishnan (Department of Medicine, University of Cambridge). To transform bacteria, 100 mL cultures of *M. marinum* were grown to mid-log phase from frozen stock in Middlebrook 7H9 broth supplemented with 10% OADC and 0.05% Tween 80. Bacteria were washed 3 times with ice-cold 10% glycerol, following resuspension in 1 mL ice-cold 10% glycerol. 100 µg of plasmid was mixed with 100 µL of bacteria and was placed in a 0.2-cm-gap electroporation cuvette on ice for 5 min. Bacteria were pulsed with 2.5 kV, 25 mF and 800 W (Bio-Rad electroporator), after which 1 mL of Middlebrook 7H9 broth was added to the cells in the cuvette and incubated for 5 h on Middlebrook 7H10 agar supplemented with 50 µL hygromycin B (Sigma) per mL and were grown at 30°C in a humidified chamber until colonies were formed (~5 days). Colonies with constitutive GFP expression were scaled up and were used to generate glycerol stock cultures. A modified version of this protocol is available [346].

3.1.6 Preparation of single cell suspensions

Due to the clumping nature of *M. marinum*, single cell suspensions of GFP expressing bacteria were generated [347]. Briefly, 250 mL of culture grown to the mid-log phase was centrifuged for 15 min at 4000 x *g* at room temperature. The pellet was resuspended in 7H9 media, and 200 µL was aliquoted into mini-centrifuge tubes, in addition to 1 mL fresh media. The bacteria were passed through a 27-gauge needle 10 times and were centrifuged at 100 x *g* for 1 min. 1 mL of supernatant was

collected, and the passage/centrifugation step was repeated 3 more time. Pooled supernatants were passed through a 5 μm filter (Millex), and the filtrate was centrifuged at 8500 x *g* for 10 min to collect single cell bacteria. 1:1000 dilutions in pure Milli-Q H_20 were used to enumerate bacteria with a hemocytometer on fluorescent microscope. Single cell *M. marinum* suspensions were stored at -80°C until use in experiments.

3.1.7 Fish infections and analysis of bacterial load

Goldfish were infected by intra-peritoneal injection of 10^6 CFU of mycobacteria diluted in 1X PBS + 0.05% Tween 80. CFU/fish were determined after 7, 14, 28 and 56 days post infection (dpi) by plating 10-fold dilutions of weighed tissue homogenates on Middlebrook 7H10 agar (Difco) supplemented with 0.5% glycerol and 10% albumin-dextrose. Gentamicin (10μg/mL), nalidixic acid (20ug/mL), carbenicillin (50ug/mL), cycloheximide (500ug/mL) and amphotericin B (10μg/mL) were added to OADC supplement and dissolved prior to mixing with warm 7H10 agar and pouring plates, in order to inhibit background microbial growth. Colonies were confirmed using a combination of colony morphology, acid-fast staining using staining procedures as suggested by the manufacturer (BD Diagnostics, NJ), as well as by sequencing of rRNA subunits.

3.2 Cytochemical staining

3.2.1 Acid fast staining of bacteria

Acid fast (Ziehl-Neelsen) staining was performed to confirm purity and identify bacteria in fish tissues. Carbol fushcin is retained in waxy hydrophobic cell wall and is a positive indicator for mycobacteria. Colonies were added to 50 µL Milli-Q H_20 and mixed thoroughly, and cultures of *M. marinum*, *M. chelonae* and *M. fortuitum* cultures were placed on a glass slide and allowed to air dry. Cells were then heat fixed by passing the slide quickly though a flame 10 times. Smears were stained using BD BBL TB Stain Kit K (Becton Dickinson, NJ). Slides were mounted with Permount® and observed at 400X magnification (Fig. 3.3).

3.2.2 Preparation of histological sections

Spleen and kidneys were dissected from goldfish 28 days following PBS or *M. marinum* infections. Tissues were fixed in 10% neutral buffered formalin for 48 h at room temperature and embedded in paraffin wax after processing using Tissue Processor TP1020 (Leica Biosystems, Wetzlar, Germany). Five µm sections were prepared using a rotary microtome and were adhered to slides overnight at 37°C.

3.2.3 Acid fast staining of histological sections

Slides were de-waxed, hydrated to water using toluene and progressively more diluted ethanol (100% - 50%) in 2 min intervals. Slides were stained in freshly filtered Carbol Fuchsin (5% phenol, 10% ethanol, 1% w/v Basic Fuchsin (BioRad, USA) for 10 min, and washed under running water for 3 min. Slides were dipped in

Acid Alcohol (1% concentrated HCl in 70% ethanol) until color stopped running and were rinsed by running water for 10 min. Slides were then counterstained with Methylene Blue (0.5% Methylene Blue w/v, 0.5% glacial acetic acid) [348] and acid-fast (Ziehl-Neelsen) for 8 dips, and rinsed with water, and were subsequently dehydrated to toluene. Following dehydration, slides were coverslipped with DPX, which was allowed to cure overnight. Stained tissue sections were observed at 400X magnification.

3.3 Molecular identification of goldfish immune genes

3.3.1 Transcriptome

Transcriptome sequencing was performed at Beijing Genomics Institute (BGI-Hong Kong, China) according to the manufacturer's instructions (Illumina). Using template cDNA from *M. marinum* infected spleen tissue, *de novo* assembly of transcriptome was carried out using Trinity program [349]. This method assembles reads with overlapping nucleic acid sequences generating contigs, which were further annotated as unigenes with requisite redundancy. Paired-end reads were realigned to known contigs, present on the corresponding zebrafish transcripts. For validation and annotation of the assembled unigenes, sequence similarity search was conducted against the Nr database, the COG database, the Swiss-Prot protein databases and the KEGG database with an E-value threshold of 10^{-5}. Together, 122,614 (66.9%) unigenes showed similarity to genes that encode known proteins in the queried databases. A more in-depth description of transcriptome development can be found in Jiasong Xie's doctorate thesis (2016).

3.3.2 Primers

Primers used in this thesis for pJET1.2 specific sequencing are shown in Table 3.8. Primers used in homology-based PCR and Rapid Amplification of cDNA ends (RACE)-PCR and full sequence transcriptome validation are shown in Table 3.9.

3.3.3 RNA isolation with Trizol®

RNA was isolated from goldfish cells ($\geq 10^6$) or goldfish tissues using Trizol® (Invitrogen) according to the manufacturer's instructions. Briefly cells washed in PBS and decanted, or tissue was placed in RNAse free micro-centrifuge tubes and lysed in 1 mL Trizol® reagent by continual passage through 18G and 25G needles. Following homogenization, samples were incubated for 5 min at room temperature. 0.2 mL chloroform was added and samples were vortexed for 15 s and allowed to settle for 2 min at room temperature. Samples were centrifuged at 12000 x g for 15 min at 4°C, separating the lower phenol-chloroform phase from the upper aqueous phase. The upper aqueous phase containing RNA was carefully removed into a fresh micro-centrifuge tube containing 0.5 mL isopropyl alcohol. 3 µL of 3 M sodium acetate was added to cell samples to aid in precipitation. Tubes were inverted several times and incubated at room temperature for 10 min and were centrifuged at 12,000 x *g* for 10 min at 4°C. Supernatants were aspirated and the RNA pellet was washed with 1 mL 75% reagent grade alcohol and centrifuged at 7400 x *g* for 5 min. The washing procedure was repeated a second time and following aspiration, samples were allowed to air-dry for 5-10 min. RNA pellets were resuspended in nuclease-free

water and were treated with DNAseI (Ambion) contaminating genomic DNA according to manufacturer's instruction. Nucleic acid concentration was quantified using a Nanodrop® at an absorbance of 260 nm. Samples were also read at absorbances of 230 nm and 280 nm to determine phenolic and protein contamination.

3.3.4 cDNA synthesis

cDNA synthesis from RNA was performed using either the Superscript II or Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's instruction. RNA levels were quantified (above) and standardized prior to generation of cDNA. In all cDNA synthesis reactions, Oligo $(dT)_{18}$ was used as the primer.

3.3.5 RT-PCR

Target cDNA sequences were amplified using 0.25 µL cDNA template, 2.5 µL 10X PCR buffer (1 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin), 0.5 µL of 20 mM primer solution, 0.5 µL of 10 mM dNTP solution and 0.125 µL *Taq* DNA polymerase (0.75 U), and nuclease-free water up to 25 µL. Reactions were amplified in an Eppendorf Mastercycler themocycler. A general thermocycling program of initial denaturation (94°C for 2 min) followed by 25-30 cycles of: 94°C for 30 s (denaturing); 50-55°C for 20 s (annealing), 72°C for 2.5 min (extension). Extension time varied but was generally set at 1 min per kb of the expected amplicon. PCR products were run on a 1% agarose gel at 130V, stained with ethidium bromide and visualized under UV light.

3.3.6 Cloning into pJET2.1 vector

Bands of interest were excised from gels and purified using a Gel Extraction Kit (Qiagen) according to manufacturer's specifications. All DNA products were eluted in 30 µL of provided elution buffer. 1 µL of elution product was added to 5 µL of the reaction buffer provided, and 0.5 µL vector, 0.5 µL ligase and nuclease-free water (up to 10 µL) was added. Reactions were incubated for 30 min at room temperature, after which, NEB 5-alpha electrocompetent *E.coli* were thawed on ice for 5 min, and 1 µL of the ligation reaction was added to thawed cells with gentle stirring. Cells were incubated on ice for 5 min, heat shocked at 42°C for 30 s and were placed back on ice for 1-2 min. Two hundred µL of SOC medium (from manufacturer) was added to the cells, and they were incubated with shaking at 37°C at 230 rpm for 1 h. Cells were plated on LB agar containing 100 µg/mL ampicillin and were incubated overnight at 37 °C. The following day, validation of inserts was assessed by colony PCR using vector specific primers.

3.3.7 DNA sequencing and *in silico* analysis

Colonies positive for inserts were added to LB with 100 µg/mL ampicillin and shaken overnight. Plasmids were isolated using QIAspin Miniprep kit (Qiagen) according to manufacturer's protocol and was sequenced using an ET terminator cycle sequencing dye and PE Applied Biosystems 377 automated sequencer. Single pass sequences were analyzed using 4peaks software for mac (mekentosj.com).

3.3.8 DNA sequencing of goldfish interleukin 4/13A, arginase – 1 and arginase -2

Goldfish IL-4/13A, arginase-1 and arginase-2 were obtained from the transcriptome database described above. Gene specific primers for were generated against regions of transcriptome sequences and amplified by PCR before cloning into pJET 1.2 (ThermoFisher). Constructs were then sequenced for molecular verification and transcriptome sequence validity. In all three cases, the transcriptome sequences were found to be identical to the cloned fragments.

3.3.9 DNA sequencing of goldfish interleukin 4/13B

Primers were designed against the zebrafish IL-4/13B sequences and were used to obtain a partial sequence. Subsequent primers were designed to satisfy the requirements of the SMART RACE PCR system (Clonetech). PCR was performed according to manufacturer's specifications, and both 3' and 5' amplicons of expected sizes were cloned into pJET1.2 and sequenced. Overlapping sequences in each fragment was used to generate an open reading frame, and primers were generated to confirm the validity of the full-length sequence of the transcript.

3.3.10 *In silico* analysis of goldfish molecules

All nucleotide and protein alignments were performed using the Clustal W software (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was conducted using a neighbor joining method with Mega 7.0. All nucleotide and protein sequences in this thesis have been submitted to GenBank. Predicted protein sequences, conserved motifs, a secretion signal cleavage site, transmembrane domains and cytoplasmic

domain were predicted using programs from the ExPASy website (http://ca.expasy.org/)

3.3.11 QPCR primers

All QPCR primers in this thesis were designed using Primer Express software (Applied Biosystems) and are shown in Table 3.10. Primers for QPCR were validated using 1:2 serial dilutions of cDNA and creating a standard curve, which was used to determine R^2 value, y-intercept and primer efficiency using 7500 Fast software. All primer forward and reverse sets were chosen with an R^2 value of 0.995 or higher, a y-intercept of -3.0 - -3.2 and an efficiency of 90% or higher. Melt curves were also analyzed to ensure a single melting peak. QPCR products were sequenced to ensure the correct sequence was being amplified.

3.3.12 Quantitative PCR conditions and analysis

Quantitative expression of goldfish genes was performed using SYBR reagents and Applied Biosystems 7500 Fast Real Time PCR Instrument. Elongation factor 1 alpha (EF-1α) was used as an endogenous control as has been established previously in our laboratory. Thermocycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A melting curve step was also added at the end of this protocol. Data were analyzed using 7500 Fast software (Applied Biosystems) and is represented as the average with standard error. Fold difference of gene expression was determined using the ΔΔCt method (2-(ΔCt,

experimental sample - ΔCt, reference sample)). ΔCt = Ct (target gene) – Ct (endogenous control).

3.3.13 Quantitative PCR analysis of IL-4/13A and IL-4/13B in healthy goldfish tissues

Gill, liver, muscle, intestine, kidney, brain, spleen and heart were isolated from individual fish (n=5) and were flash frozen in liquid nitrogen and stored at -80°C until processing. RNA was isolated from tissues using TRIzol (Invitrogen) and reverse transcribed into cDNA using Superscript III cDNA synthesis kit. Triplicate plates were run and analyzed using 7500 Fast software. Average fold differences to the lowest expressing tissue was calculated.

3.3.14 Quantitative PCR analysis of IL-4/13A and IL-4/13B in goldfish immune cells

FACs sorted kidney macrophages, sorted monocytes, peripheral blood leukocytes, kidney neutrophils and splenocytes were harvested from individual fish (n=5). Isolation was performed as previously described, flash frozen in liquid nitrogen and stored at -80°C until processing. RNA was isolated using Trizol® and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit. Triplicate plates were run and analyzed using 7500 Fast software. Average fold differences to the lowest expressing immune cell was calculated.

3.3.15 Quantitative expression of immune genes in activated primary kidney macrophage cultures

Day 7 PKM cultures from individual fish (n=5) were enumerated and adjusted to a concentration of 1 x 10^6 cells in 500µL complete MGFL-15 medium. To each sterile 2.5 mL FACs tube, 500 µL of cell suspension was added. Ten µL of PBS negative control, or 10 µL of *M. marinum*, *M. chelonae* and *M. fortuitum* at a bacterium to macrophage ratio of either 0.5:1 or 5:1. Tubes were sealed and incubated at 20°C for 24 h in the absence of CO_2. Cells were harvested by centrifuging at 230 x *g* for 8 min, washed with ice-cold 1X PBS, and were collected by centrifugation. Supernatants were removed, and cells were used for RNA isolation using Trizol® extraction and was reverse transcribed into cDNA using the Superscript III cDNA synthesis kit. Triplicate plates were run and analyzed using 7500 Fast software. Average fold differences to untreated cells was calculated.

3.3.16 Quantitative expression analysis of arginase-1 and arginase-2 expression in activated goldfish macrophages

Day 7 primary kidney macrophages (1x10^6) were untreated or stimulated with 50ng/mL of recombinant IL-4/13A, IL-4/13B or IFNγ. Cells were collected at 6, 12, 18 and 24 h post exposure and washed twice with PBS. RNA was isolated using Trizol® and cDNA was synthesized using Superscript III cDNA synthesis kit. Triplicate plates were run and analyzed using 7500 Fast software. Average fold differences to untreated cells at 6 h were calculated.

3.3.17 Analysis of immune gene expression in activated goldfish neutrophils

Neutrophils from individual fish (n=5) overnight cultures were enumerated and adjusted to a concentration of 2 x 10^6 cells in 900 µL of complete MGFL-15 medium. To each well of a 12-well tissue culture plate, 900 µL of neutrophil cell suspension was added. Treatments consisted of 100 µL of 1X PBS (negative control), or 100 µL of heat-killed or live *M. marinum* at a bacterium to neutrophil ratio of either 0.5 bacterium to 1 neutrophil, or 5 bacteria to 1 neutrophil. Plates were sealed with Parafilm® and incubated at 25°C for 24 h in the absence of CO_2. Cells were harvested by transferring to a 1.5 mL tube and centrifuging at 230 x *g* for 10 min. Supernatants were removed and cells were used for RNA isolation. RNA was isolated from the neutrophils using Trizol® method according to manufacturer's specifications and was reverse transcribed using the Superscript II cDNA synthesis

3.4 Prokaryotic expression of goldfish recombinant cytokines

3.4.1 Cloning of goldfish cytokines into pET SUMO vectors

The production of goldfish cytokines (rgIFNγ, rgTNFα2, rgIFNγrel, rgIL-4/13A and rgIL-4/13B) has been previously described [230], [350]–[352]. Briefly, pET SUMO vectors (Invitrogen) encoding the mature, signal cleaved rgIFNγ, rgTNFα2, rgIFNγrel, IL-4/13A and IL-4/13B were transformed into BL21 One Shot *E. coli* (Invitrogen). The pET SUMO vector encoded a His^6 tag on the N-terminal domain of the recombinant molecules.

3.4.2 Pilot expression studies

Pilot expression studies were performed to determine optimum production conditions for each recombinant molecule. Transformed bacterial cultures from frozen stock were grown overnight at 37°C. 20µL of culture was added to fresh LB broth containing kanamycin and was grown to an OD 600 of ~0.6. Cultures were inducted with 0.1mM IPTG and sampled every 2 h up to 6 h. Following the sampling period, bacterial lysates and membrane fractions were resolved by SDS-PAGE and visualized by western blotting against His^6 N-terminal tags on the recombinant proteins. Optimum induction time, as well as cellular location of recombinant molecules (i.e. lysate or inclusion bodies) was determined. Recombinant molecules found in inclusion bodies required isolation in denaturing conditions during lysis in scale-up production (below). Refer to Table 3.11 for a list of optimal induction conditions as determined by preliminary studies. Recombinant molecules rgIFNγ, rgTNFα2 and rgIFNγrel were previously optimized [350]–[352].

3.4.3 Scale-up production of recombinant cytokines

Following pilot expression studies, transformed BL21 overnight starter cultures from frozen stock were scaled into 500mL of LB with kanamycin until reaching an OD of 0.6-0.8. From a 1M IPTG stock solution, cultures were induced to a final concentration of 0.1 mM IPTG and allowed to grow for their optimal times, as indicated in Table 3.11. After induction, *E. coli* were pelleted by centrifugation at 8,500 x *g* for 10 min, decanted and frozen at -20°C overnight to aid in subsequent lysis steps. Bacteria were resuspended lysed in 5mL 10X FastBreak cell lysis reagent

(Promega) in 45mL wash buffer (100 mM HEPES, 10mM imidazole, pH 7.5) or denaturing wash buffer (same components including 7M Urea) and allowed to lyse for 15 min by rocking at room temperature. Lysates were pelleted by centrifugation at 16,000 x *g* for 10 min at 4°C. Resulting supernatants were incubated with MagneHis Ni-Particles (Promega) for 30 min at room temperature, and washed 4X with either non-reducing or reducing wash buffer detailed above. Purified recombinant cytokines were eluted using elution buffer (100mM HEPES, 250 mM imidazole ± 7M Urea) in 4, 1 mL fractions. The proteins were re-natured in 10 volumes of re-naturation buffer (4 mM reduced glutathione, 2 mM oxidized glutathione, 50 mM sodium borate, 5 mM EDTA, 5 mM EDTA) overnight and dialyzed against 1X PBS and concentrated against polyethylene glycol flakes (10 MW cutoff). Recombinant proteins were passed through Pierce™ High Capacity Endotoxin Removal Spin Columns (ThermoFisher) to remove trace endotoxin, which was confirmed by Limulus Amebocyte Lysate (LAL) Endotoxin Quantification (ThermoFisher).

3.4.4 Western blot analysis

Following purification of cytokines was confirmed by both western blot and were developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.) and Commassie brilliant blue stained SDS-PAGE and the identity of proteins was confirmed by mass spectrometry. Protein concentrations were measured using a Micro BCA Protein Assay Kit (Pierce).

3.5 Goldfish neutrophil/monocyte/macrophage bioassays

3.5.1 Assessment of chemotactic response to *M. marinum*, *M. chelonae* and *M. fortuitum* by goldfish neutrophils

The chemotaxis assay was performed using blind-well chemotaxis chambers (Nucleoprobe) as previously described [353]. *M. marinum, M. chelonae* and *M. fortuitum* were washed twice and re-suspended in MGFL-15 supplemented with 10% carp serum. Bacteria were added to the bottom chamber of the chemotaxis apparatus at ratios of 0.5:1, 5:1, and 50:1 (bacteria to neutrophils) and the upper chamber contained 2 x 10^5 neutrophils from individual fish in 200 µL of complete MGFL. Upper and lower chambers were separated by a 5 µm pore size polycarbonate membrane filter (Neuroprobe). Medium alone in the lower chamber served as a negative control, and 10 ng/mL N-formyl-methionyl-leucyl-phenylalanine (fMLP) was a positive control. The chemokinesis control had equal concentrations of fMLP in both the upper and lower chambers. Following 1 h incubation, duplicate filters were fixed and stained using Gills Solution 3 and chemotaxis assessed by counting 20 random fields of view under oil immersion (100X) (n=4).

3.5.2 Assessment of *M. marinum* phagocytosis and surface binding by goldfish neutrophils

Single cell suspensions of *M. marinum*-GFP in Middlebrook 7H9 broth were washed twice and re-suspended in 1X PBS containing 0.05% Tween 80. Bacteria were added to 1 x 10^6 kidney neutrophils at a ratio of 5:1 or 50:1 and incubated for 2 h at 20°C. Cells were fixed in 1% formaldehyde in PBS at 4°C overnight. Nuclei

were stained with DraQ5 (Biostatus), and data were acquired using ImageStream multi-spectral imaging flow cytometer (Amnis) followed by analysis with INSPIRE software [354]. Minimum of 10,000 cells were analyzed from each of 5 individual fish (n=5) in two separate experiments.

3.5.3 Assessment of respiratory burst production of goldfish neutrophils following mycobacterial exposure

Goldfish neutrophils from eight fish were seeded individually into 96 well plates at a density of 2 x 10^5 cells per well in 200 µL of MGFL supplemented with 10% carp serum in triplicate. To each well, 0.5:1, 5:1, and 50:1 CFUs per neutrophil of live *M. marinum* or heat-killed *M. marinum* were added to the cells and incubated for 2 h at 20°C. PBS alone was added as a negative control, and treatment with phorbol ester (PMA) at a final concentration of 100 ng/mL served as a positive control. Following incubation, 2 mg/mL Nitroblue tetrazolium (NBT) was added to cells and incubated for 30 min at 20°C [351]. The plates were centrifuged at 400 x *g* for 5 min, supernatants were removed, and the cells were fixed using ice-cold 70% methanol. Non-reduced NBT was removed by washing subsequent times with 70% methanol. Reduced NBT was dissolved by adding 120 µL of 2M KOH followed by 140 µL of DMSO. Plates were read at 630 nm using a microplate reader (n=4).

3.5.4 Assessment of killing of mycobacteria by goldfish neutrophils

Neutrophils (5x10^6) in 250 µL MGFL+10% carp serum were exposed to mycobacterial pathogens at 50:1 and 5:1 bacteria to neutrophil ratios. After 4 h, cells

were lysed in sterile, 2x concentrated Triton X- (0.5% in PBS). As a reference, bacteria were incubated in MGFL+ 10% serum alone. Lysates were serially diluted 10-fold in PBS + 0.05% Tween 80 and plated on sterile 7H10 Middlebrook agar plates (Difco). Plates were incubated at 30°C and were counted when colonies became visible, roughly 2 days for *M. chelonae* and *M. fortuitum*, and 7 days for *M. marinum*.

To assess the ability of neutrophils to destroy internalized pathogens upon activation, neutrophils were incubated for 2 h, washed 3X in PBS to remove extracellular bacteria, and were treated with 100 ng PMA, a known agonist for goldfish neutrophils. After 30 min, cells were lysed and plated on Middlebrook agar using the same methods described above. Colonies were enumerated when visible.

3.5.5 Neutrophil viability following exposure to mycobacteria

Goldfish kidney neutrophils were isolated as described above, and 1 x 10^6 cells in 500 µL MGFL supplemented with 10% carp serum, were incubated for 2, 4, 6 and 24 h with 0.5:1, 5:1, 50:1 of *M. marinum* to neutrophils at 20°C in sterile 5 mL round bottom Falcon FACS tubes (BD). Cells were washed twice and re-suspended in 100 µL of Annexin V binding buffer (BD Pharmingen). Annexin V FITC (eBioscience) was added to each sample according to manufacturer's protocols, and propdium iodide (Sigma) was added to a final concentration of 4 µg/mL. The suspensions were incubated in the dark for 30 min at room temperature. Data were acquired using a FACS Calibur flow cytometer (BD), using neutrophils from six individual fish. Cells single positive for annexin staining were considered to be

apoptotic, while cells staining for PI and annexin or PI alone were considered to be necrotic. Viability was determined by the ratio of cells negative for both annexin and PI staining over the total cells gated.

3.5.6 Assessment of respiratory burst production of PKMs following mycobacterial exposure

Respiratory burst activity following exposure to *Mycobacterium* spp. was determined using a nitro blue tetrazolium (NBT) assay. 3-5-day old kidney-derived macrophages from six individual fish were seeded in duplicate into flat bottom, 96 well plates at a density of 3 x 10^5 cells/well in 200 µL complete MGFL medium. Cells were either incubated alone, or in the presence of live/heat killed *M. marinum*, *M. chelonae*, or *M. fortuitum* at the MOI shown, and incubated for 4 h. Following incubation, NBT (Final concentration 400 µg/mL, Sigma) and PMA (final concentration, 100 ng/mL) in 1X PBS was added to the wells, along with NBT in PBS alone for background control. Samples were incubated for 20 min at 20°C, and were centrifuged for 8 min at 300 x *g*. After removing supernatants, cells were fixed in ice-cold 70% methanol. Non-reduced NBT was removed by subsequent washed in ice-cold 70% methanol. 120 µL 2M KOH was added to dissolve NBT followed by 140 µL DMSO to allow dissolution and induce colorimetric conversion. Plates were read at 630 nm, and readings from cells alone without PMA were subtracted from the treatment values to factor in background NBT reduction.

3.5.7 Assessment of nitric oxide production following mycobacterial exposure by goldfish PKMs

Day 7 kidney macrophage cultures from 6 individual fish were seeded in duplicate into 96-well plates at a density of 5 x 10^4 cells/well in 200 µL complete MGFL medium. Live and heat-killed *M. marinum*, *M. chelonae* and *M. fortuitum* were added to the cells in PBS + 0.05% Tween 80 in either 0.5:1, 5:1 or 50:1 bacteria to macrophage rations. PBS + 0.05% Tween 80 alone was added to control macrophages. Cells were incubated for 48 h at 20°C and nitric oxide was measured using the Greiss reaction. Briefly, after centrifugation for 5 min at 300 x *g*, 75 µL of supernatants were added to a fresh 96-well plate, along with a standard curve was generated with sodium nitrite in MGFL. 100 µL of each reagent A (0.1% naphthylethylenediamine HCl) and reagent B (1% sulfanilamide in 5% H_3PO_4) was added to the supernatants. Absorbances were measured at 540 nm and the concentration of nitrite was determined using a nitrite standard curve.

3.5.8 Assessment of PKM arginase activity following mycobacterial exposure

Arginase activity was performed as previously described [355], [356]. Day 7 PKMs were exposed to either 5:1 or 0.5: ratios of *M. marinum*, *M. chelonae* and *M. fortuitum* to macrophages and incubated at 20°C for 18 h. Cells were washed in PBS and lysed in 50 µL of 0.1% Triton X-100 containing 5 µg antipain (Invitrogen), 5 µg aprotinin (Invitrogen) and 5 µg pepstatin (Invitrogen) for 30 min at room temperature with rocking. Fifty µL of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 was added, following incubation at 55 °C to activate the enzyme. Fifty µL of this lysate was

added to 50 µL of 0.5 M L-arginine, pH 9.7, and incubated for 1 h at 37 °C. The reaction was stopped with 400 µL acid solution containing a 1:3:7-part mixture of H_2SO_4, H_3PO_4 and H_20, and 25 µL 9% α-isonitrosopropiophenone in 100% ethanol. Samples were incubator for 45 min at 100 °C, cooled in the dark for 10 min, and read at 540 nm. Arginase activity (mU/5 × 10^5 cells; mU = nmol urea formed per min) was calculated using a urea standard curve. Two separate experiments of 3 fish were combined for analysis (n=6).

3.5.9 Viability of PKMs following mycobacterial exposure

Day 7 PKMs were cultivated as described above, and 1 x 10^6 cells in 500 µL complete MGFL medium. Cells were incubated for 24 h with 5:1 *M. marinum*/*M. chelonae*/*M. fortuitum*: bacteria at 20°C in sterile 5 mL round bottom Falcon FACS tubes (BD). Cells were washed twice and re-suspended in 100 µL of Annexin V binding buffer (BD Pharmingen). Annexin V FITC (eBioscience) was added to each sample according to manufacturer's protocols, and propdium iodide (Sigma) was added to a final concentration of 4 µg/mL. The suspensions were incubated in the dark for 30 min at room temperature. Data were acquired using a FACS Calibur flow cytometer (BD), using neutrophils from six individual fish. Cells single positive for annexin staining were considered to be apoptotic, while cells staining for PI and annexin or PI alone were considered to be necrotic. Viability was determined by the ratio of cells negative for both annexin and PI staining over the total cells gated.

3.5.10 Assessment of gfIFNγ, gfIL-4/13A and gfIL-4/13B to elicit goldfish macrophage arginase activity

Arginase activity was performed as described above Following treatment of either cytokines or 0.5mg/mL cyclic AMP (Sigma A6885**), c**ells were washed in PBS and lysed in 50µL of 0.1% Triton X-100 containing 5µg antipain (Invitrogen), 5µg aprotinin (Invitrogen) and 5µg pepstatin (Invitrogen) for 30 min. at room temperature with rocking. Fifty µL of 10mM $MnCl_2$, 50mM Tris-HCl, pH 7.5 was added, following incubation at 55°C to activate the enzyme. Fifty µL of this lysate was added to 50 µL of 0.5 M L-arginine, pH 9.7, and incubated for 1 h at 37°C. The reaction was stopped with 400 µL acid solution containing a 1:3:7-part mixture of H2SO4, H3PO4 and H20, and 25 µL 9% α-isonitrosopropiophenone in 100% ethanol. Samples were incubator for 45 min. at 100°C, cooled in the dark for 10 min., and read at 540 nm. Arginase activity (mU/ 5 x 10^5 cells; mU = nmol urea formed per min) was calculated using a urea standard curve (n=4).

3.5.11 Flow cytometry analysis of primary kidney macrophages

Primary kidney macrophage cultures were analyzed on a FACS Calibur flow cytometer based on forward scatter (size) and side scatter (internal complexity). Flow cytometer parameters were previously established in our lab and were as follows: forward side scatter: E0, AmpGain 1.05, side scatter: voltage 455V, AmpGain 1.00.

3.6 Statistical analysis

Data from quantitative-PCR, chemotaxis, nitric oxide, arginase activity, annexin/PI and reactive oxygen production assays were analyzed using one-way ANOVA with a Dunnett's multiple comparison post hoc test or Tukey's post hoc test (Graphpad Prism)**.** Data from phagocytosis and killing assays were analyzed using paired Student's T-test (Graphpad Prism). Significance was set at $P < 0.05$.

Table 3.1. Constituents of GFL-15

Leibovitz's L-15 medium	1 package dry powder
Dulbecco's modified eagle medium	1 package dry powder

Table 3.2. Hank's balanced salt solution (10X) in 500mL

KCl	2g
KH_2PO_4	0.3g
NaCl	40g
Na_2PO_4 H_2O	0.45g
D-glucose	5g
Phenol Red	0.05g

Table 3.3. Nucleic acid precursor solution in 100mL

Adenosine	0.067g
Cytidine	0.061g
Hypoxanthine	0.034g
Thymidine	0.061g
Uridine	0.061g

Table 3.4. MGFL-15 (pH 7.4) medium in 2L

HEPES	7g
KH_2PO_4	0.688g
K_2HPO_4	0.570g
NaOH	0.750g
$NaHCO_3$	0.340g
10X HBSS	80mL
MEM amino acid solution	25mL
MEM non-essential amino acid solution	25mL
MEM sodium pyruvate solution	25mL
MEM vitamin solution	20mL
Nucleic acid precursor solution	20mL
2-mercaptoethanol	7μL
GFL-15 medium	1L

Table 3.5. CMGFL-15 (pH 7.4) medium

MGFL-15	100mL
Newborn calf serum	10mL
Carp serum	5mL
Pen/Strep (5000ug/mL)	2mL
Gentamycin (50µg/mL)	200µL

Table 3.6. Constituents Homogenizing solution

MGFL-15	15mL
Pen/Strep (10 000ug/mL)	150uL
Heparin (10 000 U/mL)	75uL

Table 3.7. Identified goldfish (*Carassius auratus* L.) immune gene cDNAs

GENE	ACCESSION NO.
goldfish IL-4/13A	KX574594
goldfish IL-4/13B	KX574595

Table 3.8. Vector specific primers

Primer	Vector	Sequence (5'-3')
SUMO Forward	SUMO	AGATTCTTGTACGACGGTATTAG
T7 Reverse	SUMO	TAGTTATTGCTCAGCGGTGG
FWD sequencing	pJET2.1	CGACTCACTATAGGGAGAGCGGC
REV sequencing	pJET2.1	AAGAACATCGATTTTCCATGGCAG

Table 3.9. RT-PCR and recombinant protein expression primers

Primer	Sequence (5'-3')
Homology based primers	
ARG1 sense	ATC CCC AAT GAT GAG CCA GTT GGC A
ARG1 antisense	ATG ACT TTT GGC CAT TCA CAT G
ARG2 sense	ACT ACG CTG TGC ATG ATT TTG GA
ARG2 antisense	CAT CTC TGA GGC CAA TGT AGA CCA G
IL-4/13A sense	ATG AAG ACT ATA CTA CTG CTC AC
IL-4/13A antisense	TTA TGA CTT TTG GCC ATT CA
IL-4/13B sense	TTG AAA CAT AAA GCG GGA CCA ATC
IL-4/13B antisense	ATT CAC TTG TTT TTT CTC ATC TGG
RACE PCR primers	
RACE IL-4/13B sense	GCG GGA CCA ATC CTC CTA ATG GAA
RACE IL-4/13B antisense	GCA TGC TGT AGC TTA GCT CTG TGT
SUMO primers	
SUMO IL-4/13B sense	ATG AGG ACT TTC ATG CTG TTG GT
SUMO IL-4/13B antisense	ATT CAC TTG TTT TTT CTC ATC TGG
SUMO IL-4/13A sense	ATG AAG ACT ATA CTA CTG CTC ACT
SUMO IL-4/13A antisense	TTA TGA CTT TTG GCC ATT C

Table 3.10. Quantitative PCR primers for goldfish genes

Primer	Sequence (5'-3')
QPCR ARG1 sense	ACT GGA AAC ATT CAC GGA CA
QPCR ARG1 antisense	GAC AAT GTC CTT AGC TGC GA
QPCR ARG2 sense	TCC TCT GAC GTC ACC TTC TG
QPCR ARG2 antisense	TCT GAG GCC AAT GTA GAC CA
QPCR IL-4/13A sense	GGA GGT TTC TGG ACG GTC TG
QPCR IL-4/13A antisense	GAG CAG GTT TGC AGG TTT CC
QPCR IL-4/13B sense	GGA CCA ATC CTC CTA ATG GA
QPCR IL-4/13B antisense	CAT CAT GAC CAT TGC TGC TT
CCL-1 sense	AAG GTC ACC GAA CCC ATC AG
CCL-1 antisense	TCG TCA CAT GAT GGC CTT CA
CXCL-8 sense	CTG AGA GTC GAC GCA TTG GAA
CXCL-8 antisense	TGG TGT CTT TAC AGT GTG AGT TTG G
gp91 phox sense	CCC ATC ACC TGT TCA TCG TCT T
gp91phox antisense	TAG TCT GGC CTC GCA CGA TA
IDO sense	CCA TCT CTG CGC AGC TTT G
IDO antisense	GCG AGC CAC ACA CAG ATC AA
IFNγ sense	GAA ACC CTA TGG GCG ATC AA
IFNγ antisense	GTA GAC ACG CTT CAG CTC AAA CA
IFNγrel sense	TGT CGG AGC CAG ACT TCC A
IFNγrel antisense	GAC TCG ATT TTT TCT CGT ACG TTC T
ARG1 sense	ATC CCC AAT GAT GAG CCA GTT GGC A
ARG1 antisense	ATG ACT TTT GGC CAT TCA CAT G
ARG2 sense	ACT ACG CTG TGC ATG ATT TTG GA
ARG2 antisense	CAT CTC TGA GGC CAA TGT AGA CCA G
IFNGR1-1 sense	TTT TAC GAC TGC CCA CAT GCT
IFNGR1-1 antisense	GGG TCC GTA ACT ATC TAC CGT ATC T
IFNGR1-2 sense	CAG TAA CCC AAC TGA ACA GAC GAA
IFNGR1-2 antisense	CAC TGT TTG GGA AGG ACT TTC AT
IL-1β-1 sense	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 antisense	GTG ACA CAT TAA GCG GCT TCA C
IL-1β-2 sense	GAT GCG CTG CTC AGC TTC T
IL-1β-2 antisense	AGT GGG TGC TAC ATT AAC CAT ACG
IL-10 sense	CAA GGA GCT CCG TTC TGC AT
IL-10 antisense	TCG AGT AAT GGT GCC AAG TCA TCA
IL-12 p35 sense	TGT TTT ACG TGC ATT CCT TTG G
IL-12 p35 antisense	GGC GCC TGA AAA AAA TAC GA
IL-12 p40 sense	CTT CAG AAG CAG CTT TGT TGT TG
IL-12 p40 antisense	CAG TTT TTG AGA GCT CACCGA TAT C
IL-4/13A sense	GGA GGT TTC TGG ACG GTC TG
IL-4/13A antisense	GAG CAG GTT TGC AGG TTT CC
IL-4/13B sense	GGA CCA ATC CTC CTA ATG GA
IL-4/13B antisense	CAT CAT GAC CAT TGC TGC TT
iNOS A sense	TTG GTA CAT GGG CAC TGA GAT T
iNOS A antisense	CCA ACC CGC TCA AGA ACA TT

iNOS B sense	CAT CTT CCA TCC GAC CCT AGT G
iNOS B antisense	AAA GCT ACG GAA GGG AGC AAT
NRAMP sense	TCT GCC CTG CTC TCC ATC AC
NRAMP antisense	GAC GCC CAG AGC GA T CAG
p22phox sense	TGG ACC CCT GAC CAG AAA CT
p22phox antisense	AAC ATG AAC CCC CCT GGA A
p40phox sense	TCC AAG AGC GGG AAT CAT G
p40phox antisense	GTC GAT GCC CTC TGG CTG TA
p47phox sense	CCA GGA ATG GGA CAC GAT CT
p47phox antisense	GAG GAG AGC CTG AGT TTG CAA
p67phox sense	TGC CTG GCA ACA TTG TCT TC
p67phox antisense	CCC GCT TCT CAT TGA AAA CAA
SOCS-3 sense	CGA GTC GGG CAC CAA GAA
SOCS-3 antisense	AAG CTC TGG AGT CCG TCT GAA
TGFβ sense	GTA CAC TAC GGC GGA GGA TTG
TGFβ antisense	CGC TTC GAT TCG CTT TCT CT
TNFα1 sense	CAT TCC TAC GGA TGG CAT TTA CTT
TNFα1 antisense	CCT CAG GAA TGT CAG TCT TGC AT
TNFα2 sense	TCA TTC CTT ACG ACG GCA TTT
TNFα2 antisense	CAG TCA CGT CAG CCT TGC AG
TNFR1 sense	GCC CCC TGA CTC AAA AGA AAT
TNFR1 antisense	GCC AGC AAC GTC AGG AAA
TNFR2 sense	CCA AAA CAA CCG CGT GAA T
TNFR2 antisense	CA GAGA TGT GGT GAA GGT CGT ATC
	CGA GTC GGG CAC CAA GAA

Table 3.11. Recombinant protein isolation conditions

Protein	Induction time	Lysis conditions
rgIFNγ	4h	denaturing
rgIFNγrel	2h	non-denaturing
rgTNFα2	4h	denaturing
rgIL-4/13A	4h	non-denaturing
rgIL-4/13B	4h	non-denaturing

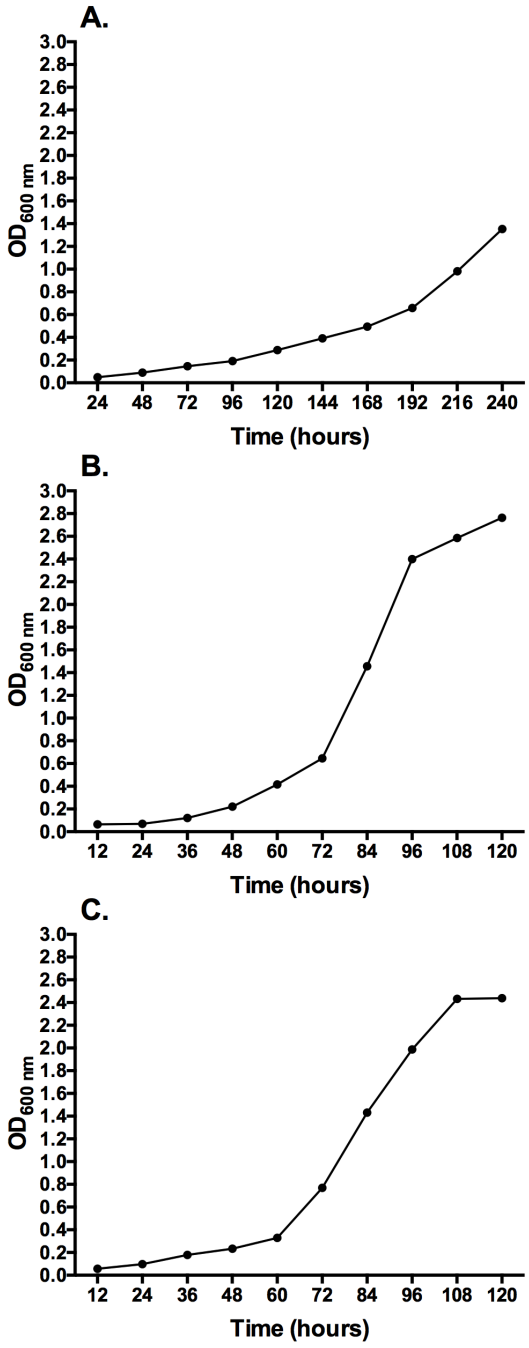


Figure 3.1. Growth curve of *Mycobacterium marinum*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*.
Mycobacterium spp. were grown at 30°C while shaking at 230 rpm (A) *Mycobacterium marinum* was grown for 240 h and a sample was taken every 24 h to measure the optical density (OD) at 600 nm. (B) *Mycobacterium chelonae* and (C) *Mycobacterium fortuitum* was grown for 120 h and a sample was taken every 12 h to measure OD at 600 nm.

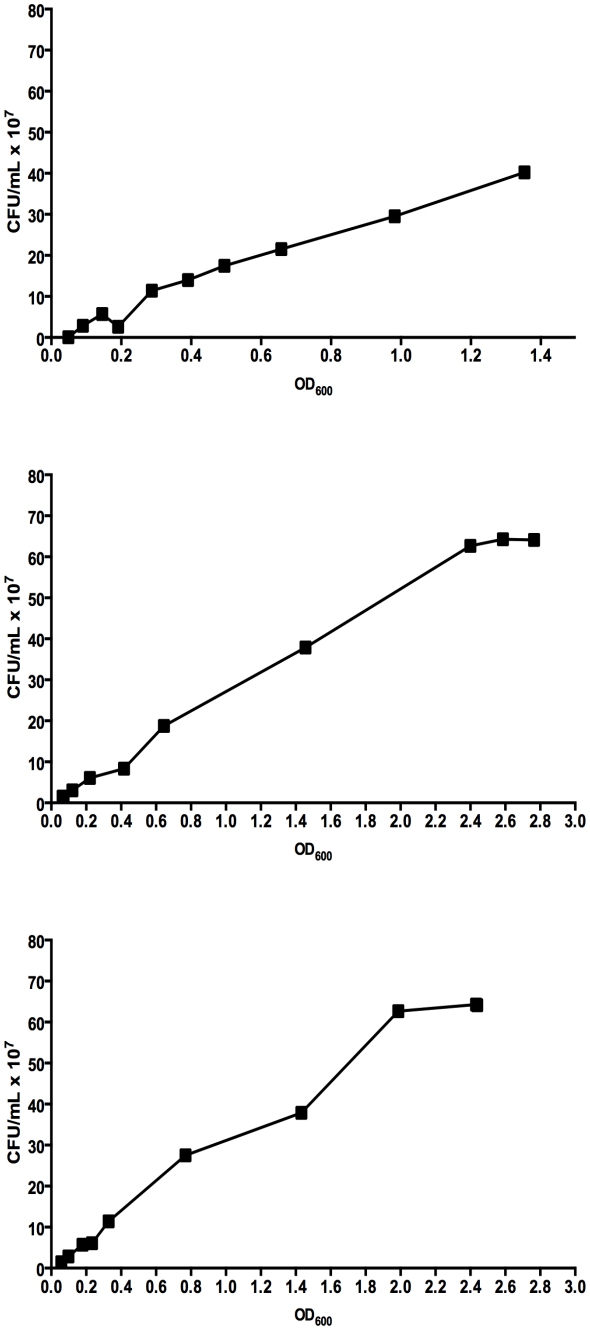


Figure 3.2. CFU growth curve of *Mycobacterium marinum*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*.
Mycobacterium spp. were grown at 30°C while shaking at 230 rpm (A) *Mycobacterium marinum* was grown 240 and a sample was taken every 24 h to measure the optical density (OD). (B) *Mycobacterium chelonae* and (C) *Mycobacterium fortuitum* was grown for 120 h and a sample was taken every 12 h to measure OD at 600 nm. Colony forming units from each sample were observed by serial dilution on Middlebrook 7H10 media supplemented with OADC.

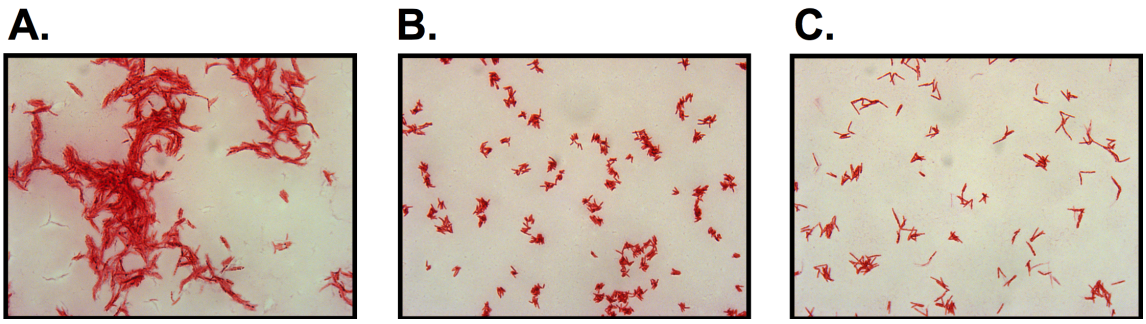


Figure 3.3. Ziehl-Neelsen staining of *M. marinum*, *M. chelonae* and *M. fortuitum*. A) *M. marinum*, B) *M. chelonae* and C) *M. fortuitum* were grown at 30°C while shaking at 230 rpm. 10 µL of bacterial suspension was placed on a glass slide and allowed to air dry. Smears were heat fixed by passing the slide quickly though a flame, and we were stained using BD BBL TB Stain Kit K (Becton Dickinson, NJ). Slides were mounted with Permount® and observed at 400X magnification.

Chapter IV: *In vivo* cytokine regulation of goldfish infected with mycobacterial pathogens[2]

4.0 Introduction

Fish mycobacteriosis is caused predominantly by *M. marinum*, *M. fortuitum*, and *M. chelonae* [30]. While it is estimated that a small percentage of many natural fish populations are infected with pathogenic mycobacteria, crowding and associated stress in aquaculture settings leads to greater incidence of infection, which can result in devastating loss of stock [357], [358]. Further, these natural fish pathogens can cause opportunistic infections in immunocompromised individuals, and are gaining attention as etiological agents of zoonotic disease [359]. A large amount of information has been generated regarding the host-pathogen interaction of mycobacterial pathogens, particularly *M. marinum*, due to its close genetic relationship and similar pathology to *M. tuberculosis*, though this relationship is not yet fully understood. Relatively less is known concerning host response to more distantly related, faster growing fish pathogens *M. fortuitum* and *M. chelonae*, despite the relative contribution to disease.

Development of animal models that functionally reproduce the pathology and disease characteristics observed in humans has been an emphasis in research for several decades. Emergence of fish mycobacterial infection models due to relative ease of infection, cost, biosafety stringing levels and likeness of disease pathology have complemented established mammalian models. In the zebrafish *M.*

[2] A portion of this chapter has been published: Hodgkinson et al. *Developmental and Comparative Immunology* 38, no. 3 (November 2012): 456–65.

marinum model, conservation of crucial virulence factors and immune host factors involved in human *M. tuberculosis* pathogenesis has been demonstrated [157], [265], [342], [360]–[364]. Similarly, goldfish have been utilized as an *in vivo* model bacterial growth, lethality, immunity, granuloma formation in body tissues [27], [365], [366]. Furthermore, fish models have been utilized to study the role of cytokines during mycobacterial infection [101], [138] Here, I expand on the current knowledge of bacterial growth and pathology and address molecular regulation during infection, with emphasis on cytokine and inducible antimicrobial gene expression.

In this chapter, I measured the expression levels of genes that have been implicated in anti-mycobacterial immune function or pathology. Broadly, genes were categorized by pro-inflammatory (CCL-1, CXCL-8, IFNγ, IFNγrel, IL-12p35, IL-12p40, IL-1β1, IL-1β2, TNFα1 and TNFα2), anti-inflammatory (IL-10, TGFβ and SOCS-3), Th2/M2-type (IL-4/13A, IL-4/13B, arginase-1 and arginase-2) and antimicrobial (iNOS A, iNOS B) functions. My working hypothesis was that severity of mycobacterial infection would be linked to Th1/Th2 balance; inducing anti-inflammatory and T2-type cytokines could thereby evade the Th1 and antimicrobial response.

4.1 Results

4.1.1 Enumeration of colony forming units in goldfish tissues and granuloma formation

Goldfish were infected by intraperitoneal injection with 10^6 CFUs in 100 μL PBS + 0.05% Tween 80. At 7, 14, 28 and 56 days past infection, spleen and kidney

tissues were removed aseptically and homogenized in PBS with 0.02% SDS and plated by 10-fold dilution on 7H10 agar containing antibiotics [10 µg/mL gentamicin, 20 µg/mL nalidixic acid, 50 µg/mL carbenicillin, 500 µg/mL cyclohexaminde, 10 µg/mL amphotericin B] to inhibit background growth. *M. marinum*. CFU burden increased over the first 4 weeks of infection and peaked at 28 dpi, while *M. chelonae* and *M. fortuitum* decreased in mean CFU over the infection course. *M. marinum* had the highest bacterial burden of each infection type at every time point (Fig. 7.1). Acid-fast (Ziehl-Neelsen) staining of kidney and spleen sections at 28 dpi showed mycobacterial granuloma structures, where purple staining, indicative of acid-fast bacteria, was observed in the centers of these granuloma (Fig. 4.1B).

4.1.2 Pro-inflammatory gene expression in kidney and spleen tissue of fish infected with *M. marinum*, *M. chelonae* and *M. fortuitum*

Patterns of gene expression were similar in spleen and kidney tissue of *M. marinum* infected fish. Increased expression levels of pro-inflammatory genes were observed in the early stages of infection (7 dpi), where IFNγ, IL-12p25, Il-12p40 and IL-1β1 were upregulated in both tissues, followed by a return to baseline gene expression levels by 14 dpi (Fig. 4.2Aii/Fig 4.2.Bii). At 28 and 56 dpi, the pro-inflammatory genes assayed were largely down-regulated with the exception of IL-1β1 which was increased at both time points (Fig. 4.2Aiii/iv and Fig. 4.2Biii/iv). In contrast, different patterns of gene expressions were observed in *M. chelonae* infected fish tissues. A general up regulation of pro-inflammatory mRNA levels

throughout the course of infection was observed in both spleen and kidney tissues (Fig. 4.3AB). Unlike *M. marinum*, no down-regulation of genes at 28 and 56 dpi was observed; rather, increased pro-inflammatory gene expression was measured at these time-points (Fig. 4.3Aiii/iv and Fig. 4.3Biii/iv). *M. fortuitum* showed sporadic pro-inflammatory gene expression during the course of infection (Fig. 4.4). At 7 dpi, the expression of genes encoding CCL-1, IFNγ and TNFα2 decreased compared to control fish in kidney tissue but increases in pro-inflammatory cytokine gene expression was observed in spleen tissue (Fig. 4.4Ai and Fig.4.4Bi). Sustained pro-inflammatory gene expression was measured in spleen tissue at 14, 28 and 56 dpi (Fig. 4.4B), expression in kidney tissue showed similar pro-inflammatory levels to PBS-injected fish, with the exception of IFNγ and IL-1β2 at 14dpi (Fig. 4.4Bi), CCL-1, IFNγ and TNFα2 at 28 dpi (Fig. 4.4Aiii), and CCL-1 and IL-12p40 at 56 dpi (Fig. 4.4Aiv).

4.1.3 Regulatory gene expression in kidney and spleen tissue of fish infected with *M. marinum*, *M. chelonae* and *M. fortuitum*

Expression of regulatory genes IL-10, TGFβ and SOCS-3 were also measured throughout the course of infection. In *M. marinum* infected kidney tissue, SOCS-3 gene expression increased early in infection (7 dpi), followed by up-regulation of all 3 genes examined at 14 dpi (Fig. 4.5A). On days 28 and 56 dpi a down regulation of the regulatory gene expression was observed. Spleen tissue showed similar gene expression patterns, with the exception of early down-regulation of TGFβ and IL-10 at 7 dpi (Fig. 4.5B). Expression levels of regulatory genes in *M.*

chelonae infected fish displayed an up regulation of SOCS-3 in both tissues through the course of infection. In spleen tissue, IL-10 was also up-regulated at 28 and 56 dpi (Fig. 4.6). *M. fortuitum* infected spleen tissue showed significant down-regulation of regulatory genes 7, 14 and 28 dpi, followed by significant up regulation at 56 dpi (Fig. 4.7B). Kidney tissue expression levels of IL-10 and SOCS-3 were increased at 28 dpi, and TGFβ gene expression was significantly increased at 56 dpi (Fig. 4.7A).

4.1.4 M2-type gene expression in kidney and spleen tissue of fish infected with *M. marinum*, *M. chelonae* and *M. fortuitum*

The IL-4/13A, IL-4/13B, Arginase-1 and Arginase-2 were also measured over each course of infection. A general up regulation of M2-type genes was observed at each time point in *M. marinum* infected spleen and kidney tissue (Fig. 4.8AB). Conversely, increased IL-4/13A and IL-4/13B was seen in *M. chelonae* infected fish at days 7, 14 and 28 dpi in kidney tissue, while only IL-4/13A was increased in spleen tissue. Levels of all 4 genes were comparable to control fish at 56 dpi (Fig. 4.9). In *M. fortuitum* infected kidney tissue the mRNA levels of IL-4/13B and Arginase-2 at 14 dpi, Arginase-1 decreased on 28 dpi, while an increase in mRNA levels was observed for Arginase-2 at 56 dpi (Fig. 4.10). Sporadic expression of these genes was observed in spleen tissue following infection, with decreases of Arginase-1 through the first 28 dpi, an increase of Il-4/13A at 14 dpi and increases of IL-4/13B and Arginase-2 at 56 dpi (Fig. 4.10).

4.1.5 Antimicrobial gene expression in kidney derived leukocytes of fish infected with *M. marinum*, *M. chelonae* and *M. fortuitum*

Inducible iNOS genes (M1 putative markers) that have been highly correlated with mycobacterial resistance were also measured in infected fish at 7 and 56 dpi in kidney-derived primary leukocytes *ex vivo*. Robust increases of iNOS A and iNOS B isoforms were observed in all infections at both time points (Fig. 4.11).

4.2 Discussion

The focus of this chapter was to examine the molecular regulation of critical immune genes over the course infection with mycobacterial pathogens *M. marinum*, *M. chelonae* and *M. fortuitum*. The course of infection in terms of CFU growth reported in this thesis is consistent with previously reported infections in fish mycobacteriosis [27], [365], [366]. Contrasting growth rate in tissues suggest graded levels of evasion and/or interference of immune regulation, which should manifest in cytokine dysregulation. It is widely accepted that protective immunity depends largely on initiation of strong inflammatory immune responses as well as activation of cell-mediated adaptive components. From early production of cytokines following initial infection of macrophages to later cytokine production by adaptive immune cells, the balance of cytokine signaling sets the threshold for inflammation, and therefore mycobacterial replication [91], [276], [367]–[370]. In this chapter, I assessed expression levels of critical cytokine and macrophage effector genes, broadly grouped into pro-inflammatory (Th1-type), regulatory, pro-humoral (Th2/M2-type) and iNOS genes.

An effective inflammatory response that culminates in an efficient cell-mediated response is widely accepted as critical to host defense to mycobacterial infection. Initial recognition of mycobacteria by pattern recognition receptors has been shown to elicit protective, pro inflammatory responses or more permissive, anti-inflammatory response, dependent on a number of pathogen or host specific factors. For example, mycobacterial cell wall components lipomannan (LM) and lipoarabinomannan (LAM) are robust immunomodulatory lipoglycans [371]. Addition of mannosyl (ManLAM) or phosphoinositide residues (PILAM) to LAM molecules alters recognition but the immune system; PILAM is a potent TLR2 stimulator identified on nonpathogenic mycobacterial species [372] while ManLAM has powerful anti-inflammatory properties [373]. Therefore, it seems critical that innate protection hinges on early activation of inflammatory response, culminating in cytokine production. Signaling of IL-1β [91], [276], [367], [368], TNFα [259], [264], [374], [375] is essential for mounting a protective response, and to a lesser degree CXCL-8 [376], [377], whereas CCL1 has been shown to enhance host susceptibility to infection [378]. Cell-mediated immune responses during mycobacterial infections are initiated by the emergence of protective CD4+ T cells that secrete cytokines, importantly IL-12 and IFNγ, which have both been shown to be critical in mammalian models [292], [379]. In fish, cytokine regulation during mycobacterial infections has been less studied, although commonalities of the role of IL-1β, TNFα and IFNγ have been observed in zebrafish embryos and goldfish primary kidney macrophage culture infected with *M. marinum* [78], [98], [265]. Here, I conducted a comprehensive analysis of pro-inflammatory cytokine

expression in three different piscine natural mycobacterial pathogen models, focusing on infected spleen and kidney tissue. The mRNA levels of pro-inflammatory molecules were measured for each mycobacterial infection at early stages of infection, during primarily innate protection phase of the infection, and during the persistent infection. For *M. marinum*, *M. chelonae* and *M. fortuitum* infections, early increases in pro-inflammatory genes were generally observed, although in some cases these increases were tissue specific. Increases in IL-1β1, TNFα2 and IL-12 subunits were observed 7 dpi, in addition to modest increases of IFNγ. Interestingly, *M. chelonae* infections induced very large increases in IFNγ-rel at 7 dpi, which may have aided in protective immune function, as it has been shown in our laboratory that IFNγ-rel potently activates antimicrobial activities in goldfish macrophages, and aids in priming macrophages to kill *M. marinum in vitro* [98]. Later stages of infection are marked by an increase in bacterial loads of *M. marinum* infected fish, and a relative decrease bacterial load in *M. chelonae* and *M. fortuitum* infected fish. Contrasting patterns of expression for pro-inflammatory genes was also observed for *M. marinum* infected fish, a reversion to levels of uninfected fish was observed at day 14, followed by marked down regulation of pro-inflammatory cytokines compared with control fish, apart from IL-1β1, which was increased throughout the infection course. A general persistent up-regulation of pro-inflammatory genes was observed in *M. chelonae* infected fishes, including IFNγ-rel, IL-1β1 and IL-12 subunits, most likely contributing to the diminishing CFU load in infected tissues. Modest increases in pro-inflammatory cytokines during infection with *M. fortuitum* was observed. TNFα2, a cytokine critically important to

macrophage activation and mycobacterial control was increased at all time points in spleen tissue. Taken together, the lack of activation of pro-inflammatory genes in *M. marinum* infected fish, and the sustained inflammatory cytokine activation seen in *M. chelonae* and *M. fortuitum*-infected fish may contribute to the control of bacterial load in the tissues.

The delicate balance of the inflammatory process is regulated by anti-inflammatory mediators to limit the collateral damage to the host. Anti-inflammatory cytokines IL-10 and TGFβ have been shown to down-regulate the clearance of pathogenic mycobacteria, with proposed mechanisms of interference with T helper cell maturation and interferon signaling [300], [300], [320], effector cell function and polarity [380] [381], and decreasing overall resistance to mycobacterial infection [299], [382]. In this study, I measured the mRNA levels of TGFβ and IL-10 during infection with each mycobacterial species, along with SOCS3, a suppressor of STAT3 activation and thus interferon signaling, that has been implicated in control of infection in *M. tuberculosis* infected individuals [328]. *M. marinum* infected fish showed initial increases in anti-inflammatory gene expression, but interestingly were down-regulated in the later stages of infection. In *M. chelonae* infected fish, SOCS-3 gene was up-regulated at all time-points measured, but levels of IL-10 and TGFβ were largely unaffected, with the exception of modest increases in IL-10 at 28 and 56 dpi in spleen tissue. Alteration in the expression of the regulatory genes most likely influences the outcome of infection, as has been demonstrated with lack of killing capacity in our laboratory [35]. Further analysis and an expansion of gene repertoires should be conducted, or alternatively the use of knockout mutants (re zebrafish)

models. Additional complexity with teleost mRNA with whole-genome and/or gene duplications may further complicate these type of analyses [383].

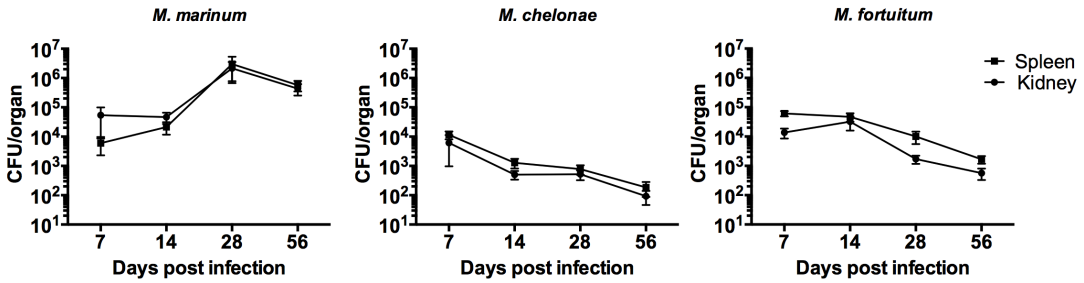
Recent insight into Th2/M2-type cytokines in teleosts have suggested a conserved mechanism of T helper cell and macrophage phenotype polarity to that seen in higher vertebrates [230], [356], [384], [385]. It has been proposed that a lack of protective immunity is linked to not only the robustness of a Th1 response, but also Th2 activity [386]. Reports of increases in IL-4 have been shown in active mycobacterial infections with increased pathology, and it is thought to be the result of inappropriate macrophage activation [387] or inhibition of iNOS and eventual production of reactive nitrogen intermediates, which is fundamental in a protective anti-mycobacterial response [388]. M2-like gene regulation was assessed for in each infection trial. *M. marinum* infected fish resulted in a general increase of IL-4/13 and arginase genes. Modest increases of IL-4 genes was also seen in *M chelonae* infected fish in the first 28 dpi, although arginase genes were unchanged. In *M. fortuitum* infected fishes, modest down- regulation in arginse-1 was seen in the first 28 dpi, followed by modest up regulation of arginase at 56 dpi. Taken together, it is possible that IL-4 production and arginase activity is playing a role in limiting immune defense of fish.

I found significant up regulation of iNOS isoforms A and B at both 7 and 56 dpi in the kidney leukocytes examined. It has been established that a significant NO response in mammals [389]–[391] and fish [392], [393] is critical for protection of mycobacterial infection. Interestingly, large increases in mRNA was seen for each infection type, although it is not clear whether infected cells or uninfected cells

contribute to the heightened expression levels. Moreover, pathogenic mycobacteria have been shown to prevent localization of iNOS to phagosomes by interfering with actin scaffolding proteins, rendering the response ineffective in infected cells [394], [395]. Additionally, evidence of low levels of NO production has been shown to enhance intracellular mycobacterial growth [396]. Although we observe increases in iNOS isoforms, further work determining iNOS cellular localization in mycobacterial infected fish is required.

I reported for the first time a comprehensive analysis of expression of genes that encode pro-inflammatory, anti-inflammatory and Th2/M2-type cytokines, as well as antimicrobial gene regulation, during both acute and sub chronic phases of mycobacterial infections in fish. My findings indicate that pro-inflammatory, regulatory and M2- type genes may play a significant role in the progression of host immune responses in fish mycobacteriosis.

A



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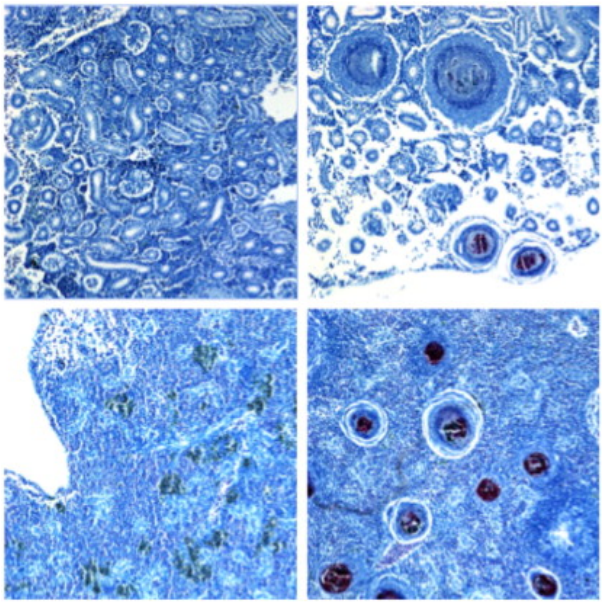


Figure 4.1. CFU of mycobacteria from goldfish spleen and kidney and granuloma formation. (A) The kidney and spleen of infected fish were homogenized and colony forming units were determined by plating serial dilutions on Middlebrook 7H10 agar containing antibiotics. The data are mean ± CFU/organ of five fish (n=5). (B) Kidney and spleen were removed from mycobacteria infected fish and stained using the Ziehl-Neelsen carbol fuchsin staining protocol. The left panels are representative images of spleen and kidney tissues. The right panels are representative images positive for granulomas following mycobacterial infection, as determined by dark red staining in the center of organized, spherical structures. Tissues were visualized at 400X.

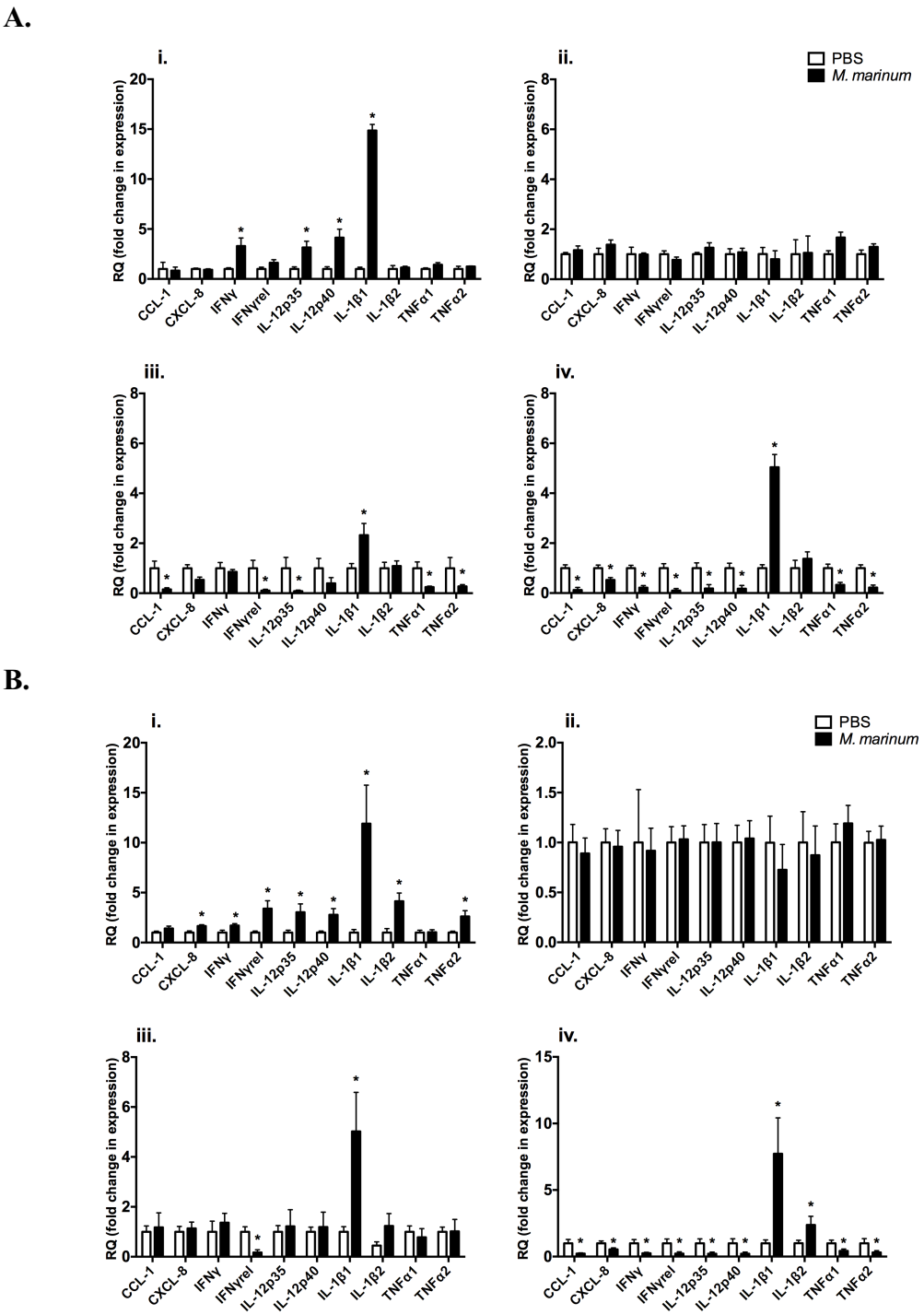
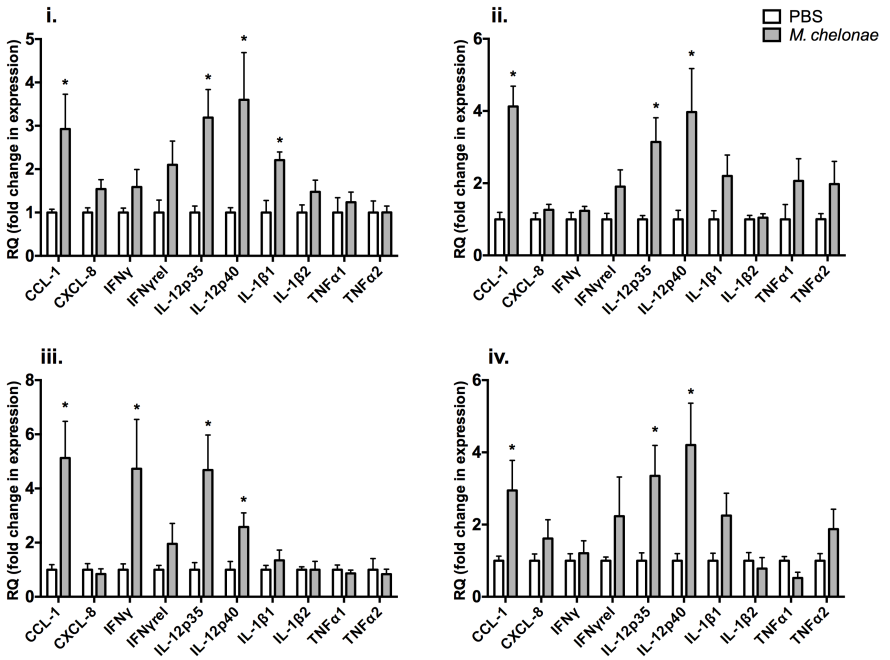


Figure 4.2. Pro-inflammatory gene expression during *M. marinum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

A.



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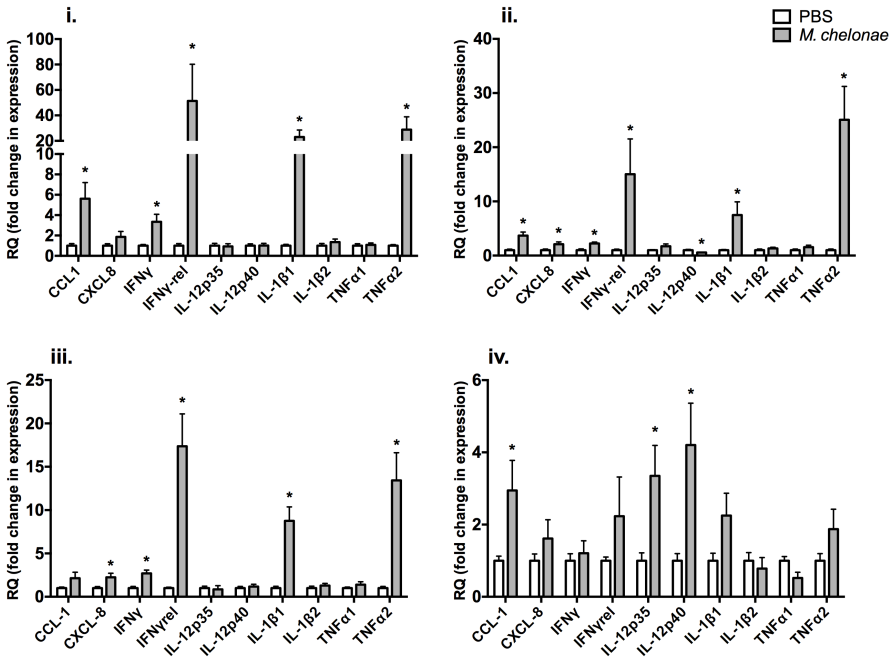
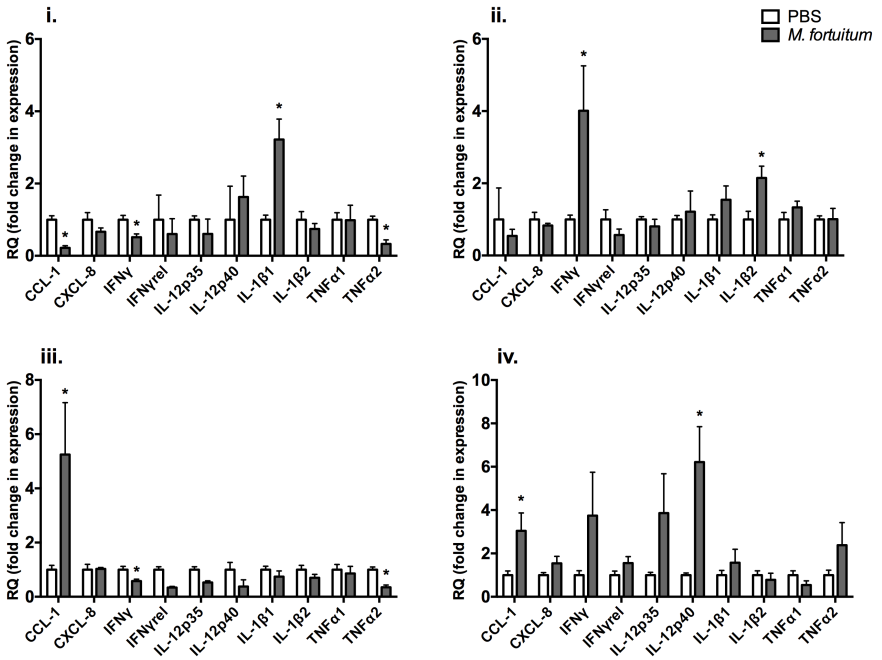


Figure 4.3. Pro-inflammatory gene expression during *M. chelonae* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

A.



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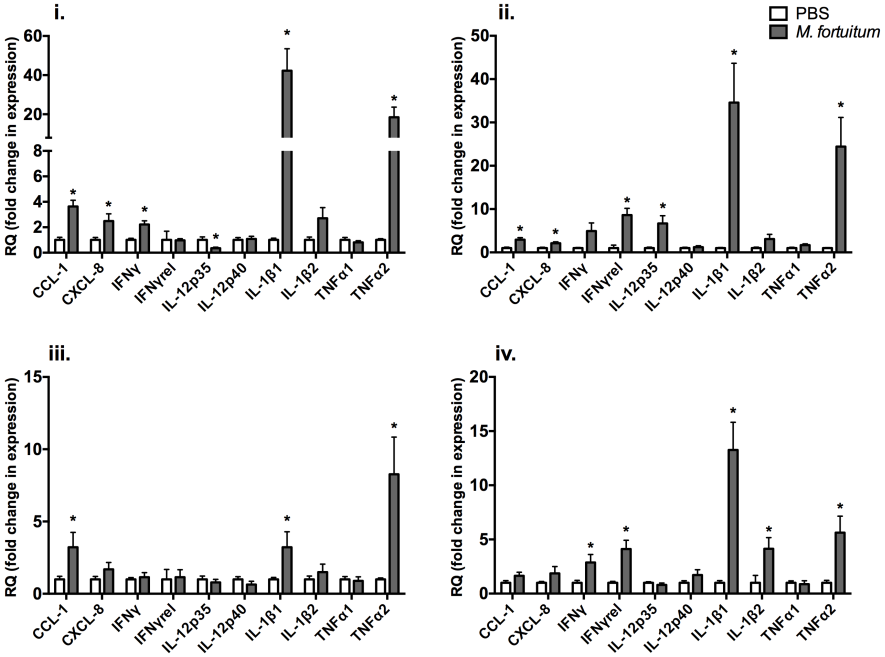

Figure 4.4. Pro-inflammatory gene expression during *M. fortuitum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

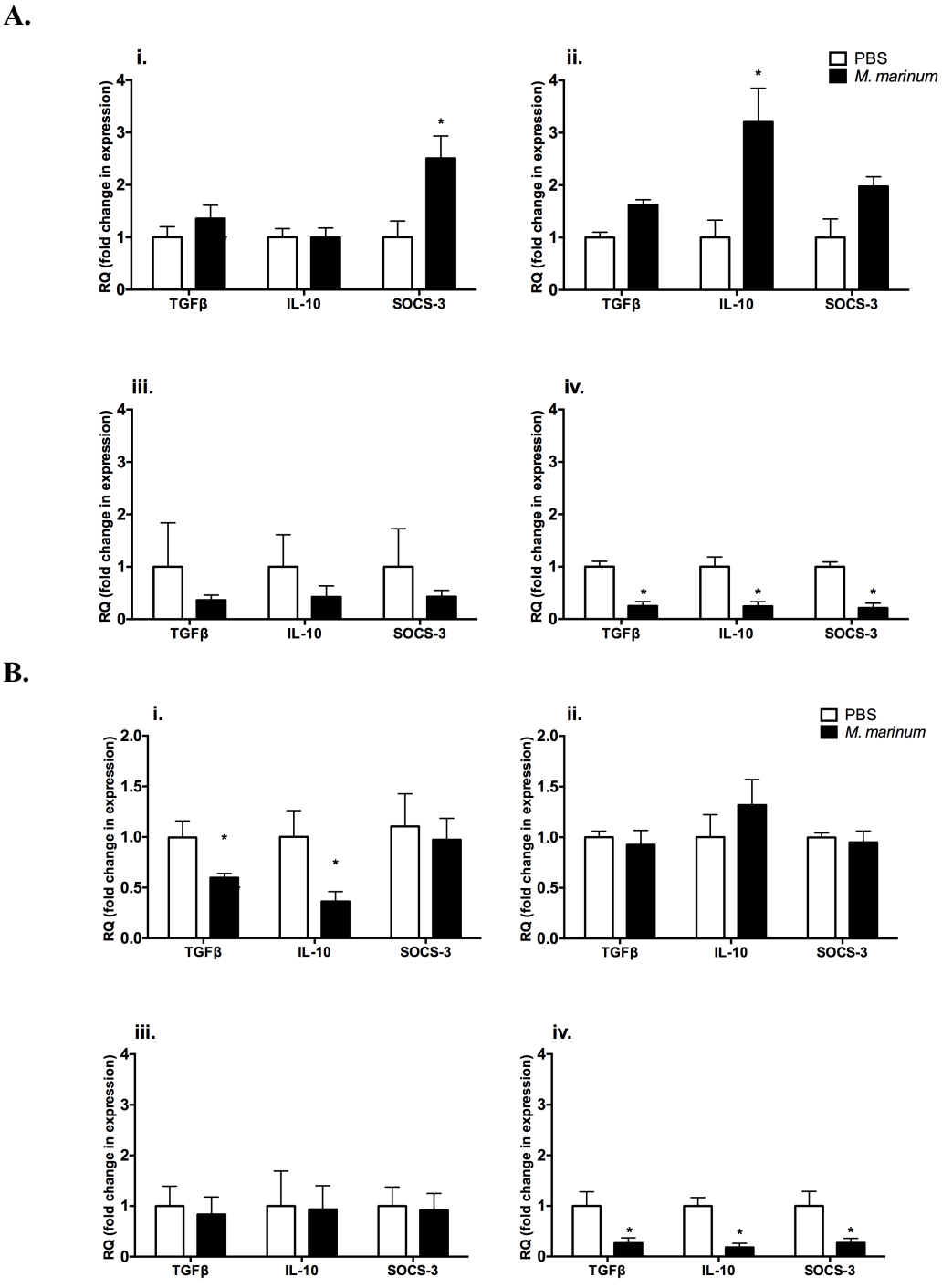

Figure 4.5. Regulatory gene expression during *M. marinum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

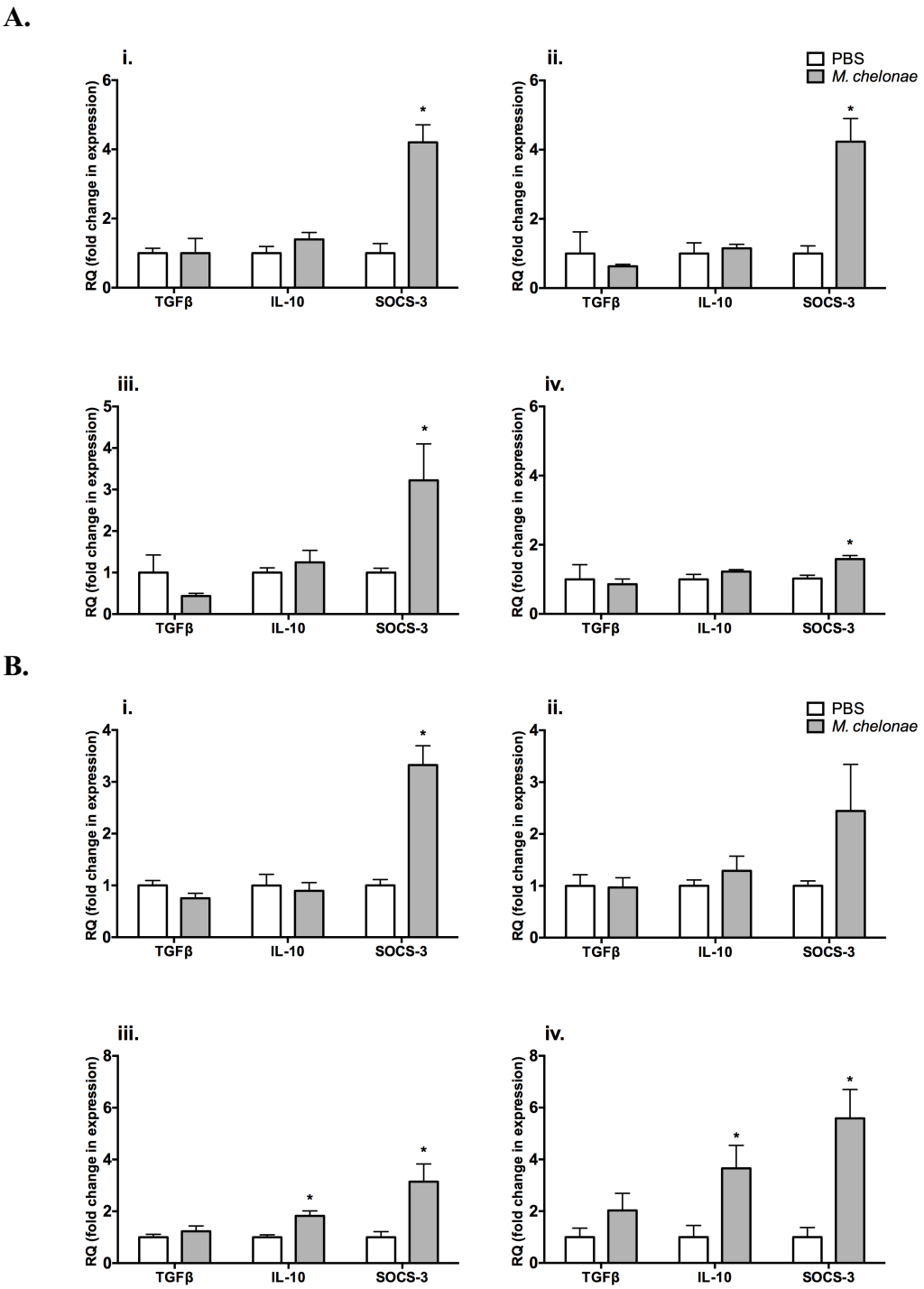

Figure 4.6. Regulatory gene expression during *M. chelonae* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

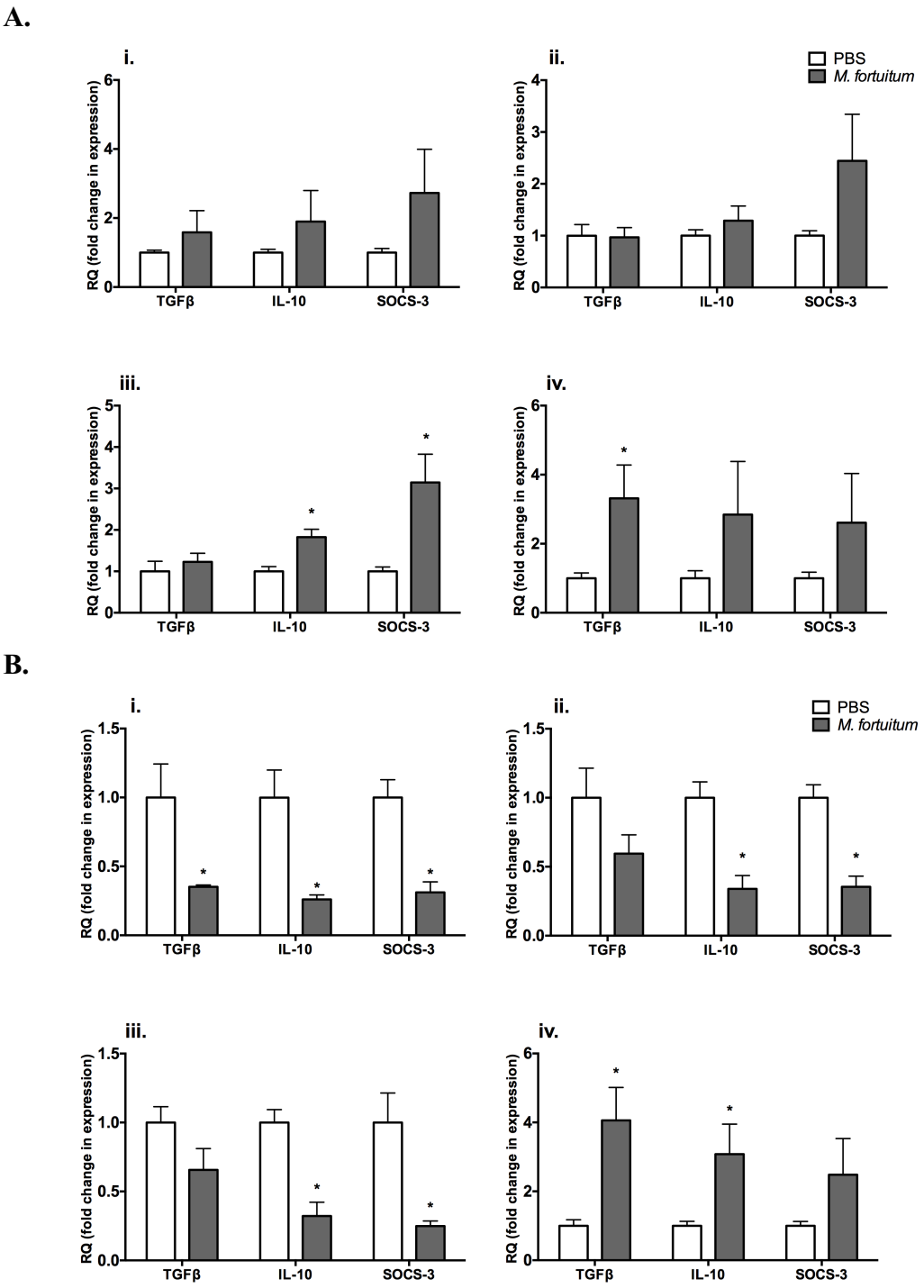

Figure 4.7. Regulatory gene expression during *M. fortuitum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

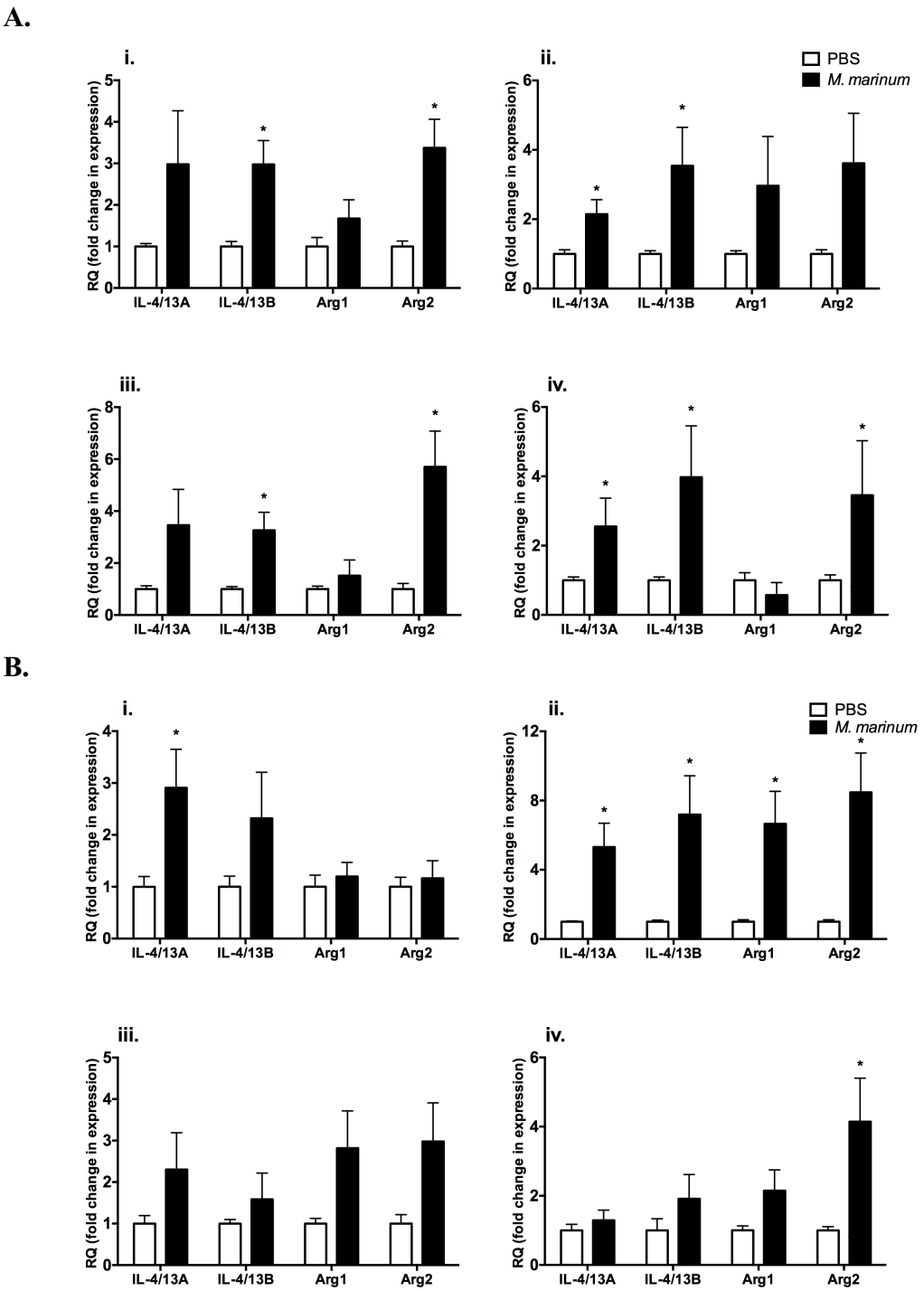

Figure 4.8. M2-gene expression during *M. marinum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

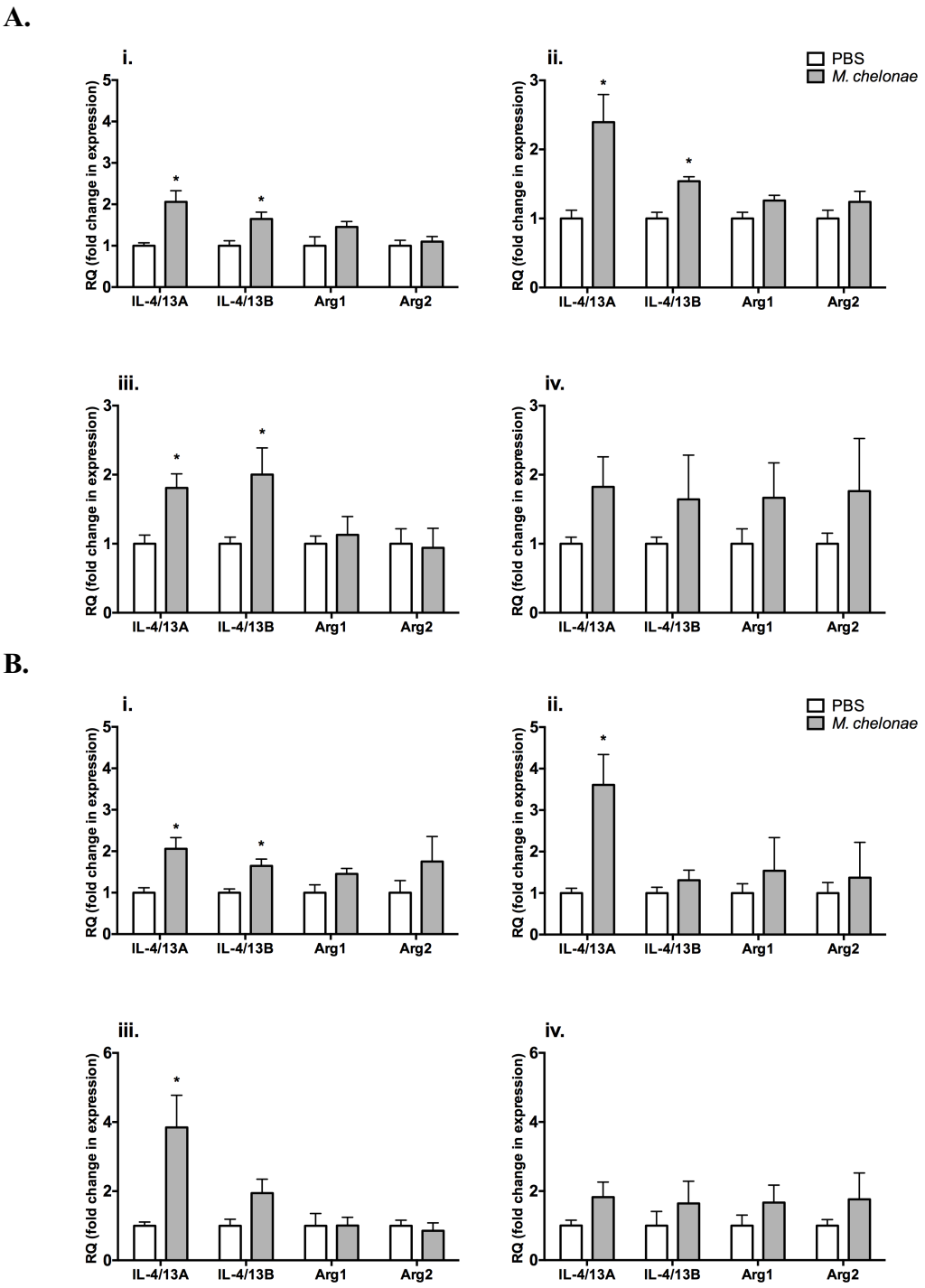

Figure 4.9. M2-gene expression during *M. chelonae* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

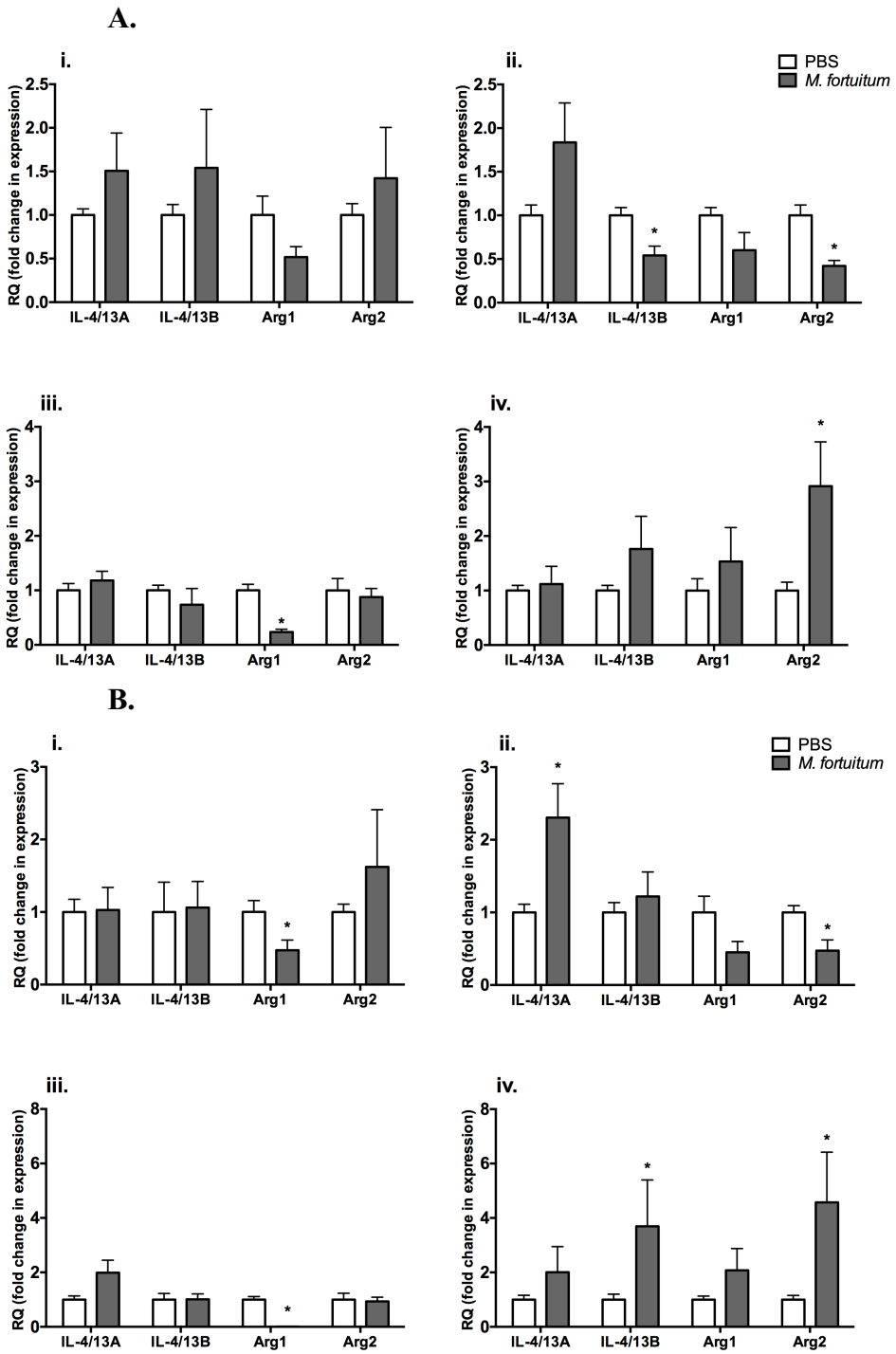

Figure 4.10. M2-gene expression during *M. fortuitum* infection. (A) Kidney and (B) spleen tissues were harvested at (i)7, (ii) 14, (iii) 28 and (iv) 56 days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

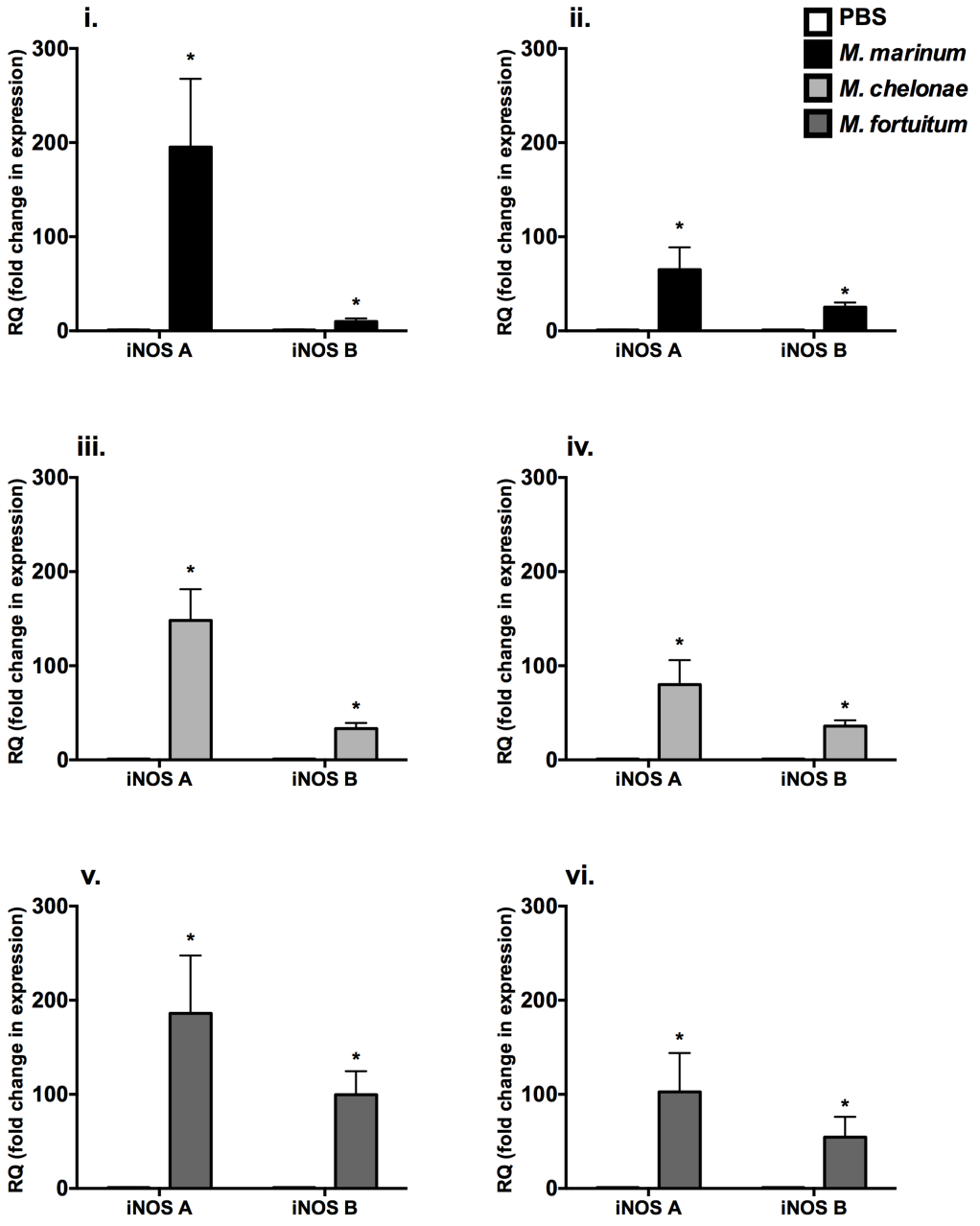

Figure 4.11. Antimicrobial gene expression following *M. marinum*, *M. chelonae* and *M. fortuitum* infection. Kidney leukocytes were isolated at 7 (i/iii/v) and 56 (ii/iv/vi) days post infection. The expression data were normalized against PBS-injected controls. Results are means ± SEM of RQ values from 5 fish (n=5). Data were analyzed using a paired t-test. (*) indicate significant different from control fish ($P < 0.05$).

Chapter V: Characterization and functional analysis of goldfish kidney neutrophils exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*[3]

5.0 Introduction

Neutrophils are rapidly recruited to sites of infection where they defend against pathogens through a variety of oxidative and non-oxidative mechanisms [397]. Prolonged establishment of mycobacterial infections is heavily dependent on a microbe's ability to withstand or subvert the antimicrobial response of neutrophils and resident macrophages during acute infection. The defined role of neutrophils in mycobacterial infections remains unclear, despite the fact that neutrophils are the predominantly infected cell type during active tuberculosis [191], [192], and infected patients exhibit a neutrophil-driven interferon transcriptional signature [398]. Studies describing neutrophil responses in mycobacterial infections suggest a prominent early killing response [200], [399]–[403], although contradictory reports indicated a lack of neutrophil killing response [198], [404]. Neutrophils may also exacerbate pathology in mycobacterial infections [202], [398], [405], [406], and it was proposed that they could aid in mycobacterial dissemination by shuttling bacilli to different host tissues [79], [407]. The role of neutrophils at the granuloma level is a hallmark of mycobacterial infections, and it suggests a protective role mediated by toxic reactive oxygen intermediates (ROI) and neutrophil extracellular traps (NETs), which entangle bacilli. NETs have also been shown to activate nearby macrophages [80],

[3] A portion of this chapter has been published Hodgkinson et al. Developmental & Comparative Immunology 2015; 53: 349–57

[192], [408]. Discrepancies in the literature regarding early neutrophil-mycobacterial interactions are likely due to: i) the model system employed; ii) host and mycobacterial genetic factors; and iii) the difficulty in working with mammalian neutrophils *ex vivo,* as they are short lived in culture [407]. There are also practical disadvantages of studying the human pathogen *Mycobacterium tuberculosis* since it grows very slowly (24 h doubling time), yielding colonies after 30 days incubation, and requiring stringent Level 3 biosafety facilities. In contrast, the use of fish hosts and their much faster-growing natural pathogen *Mycobacterium marinum* has served as reliable and convenient surrogate model system to study *Mycobacterium*-host interactions. For example, the genetic tractability and transparency of zebrafish larvae have contributed significantly to mycobacterial research, challenging entrenched dogma regarding the formation and function of mycobacterial granulomas as structures that augment pathogen survival, rather than being strictly host-protective response to infection [178], [409]. This model has also been critical for the identification of molecules that contribute to host protection [265], [410], and helping elucidate the role of neutrophils at the level of mycobacterial granulomas [80], [411], [412]. Moreover, study of faster-growing and less conventional natural mycobacterial pathogens in fish, such as *M. chelonae* and *M. fortuitum* provide a comparative axis of immune regulation and disease pathology.

Several fish species, including zebrafish, goldfish, and medaka have been used as *in vivo* model systems to elucidate the pathology caused by mycobacteria [101], [157], [365], [366], [413], as well as *in vitro*, using primary kidney macrophages to assess their anti-mycobacterial responses [98]. More recently, our

group isolated and characterized goldfish primary kidney neutrophils that remained viable in culture for several days and were also functionally similar to mammalian neutrophils in that they were highly motile, exhibited degranulation and produced a robust respiratory burst response, [353]. To date, this is the first study of fish neutrophil interaction with mycobacterial pathogens *in vitro*.

In this chapter, I describe the functional response of goldfish neutrophils to *M. marinum*, *M. chelonae* and *M. fortuitum in vitro.* The objectives of this chapter were to: (1) measure the chemotactic response of goldfish neutrophils towards the mycobacterial pathogens; (2) quantify the level of respiratory burst activation following mycobacterial exposure; (3) examine the expression levels of key immune genes, as well as subunits of the NADPH oxidase complex; (4) determine whether goldfish neutrophils are capable of internalizing mycobacteria; (5) measure the killing response of naïve and activated neutrophils; and (6) examine the viability of goldfish neutrophils following mycobacterial exposure.

5.1 Results

5.1.1 The chemotactic response of kidney neutrophils to *M. marinum*, *M. chelonae* and *M. fortuitum*

Neutrophils from four individual fish were set up in duplicate (n=4). In the presence of fMLP, a known chemoattractant, neutrophils migrated towards the bottom well of the chamber significantly more than medium alone (Fig. 5.1). Migration towards mycobacteria was observed using higher concentrations of bacteria (5:1 and 50:1), however, only *M. fortuitum* at the lowest bacteria

concentrations (0.5:1) induced chemotactic response of neutrophils (Fig. 5.1). Furthermore, migration of neutrophils was not random as the chemokinesis control, consisting of fMLP in the upper and lower chambers of the blind-well chemotaxis apparatus, was not significantly different from medium control. At both 5:1 and 50:1 bacteria to neutrophil concentrations significantly increased migration was observed for *M. chelonae* and *M. fortuitum* groups compared to *M. marinum* at the same concentration ($P<0.05$; one-way ANOVA) (Fig 5.1).

5.1.2 Reactive oxygen intermediate production of goldfish neutrophils to *M. marinum*, *M. chelonae* and *M. fortuitum*

The induction of neutrophil activation by *M. marinum, M. chelonae* and *M. fortuitum* was determined by measuring respiratory burst response using NBT reagent. Neutrophils from 4 individual fish were cultured overnight, and the cells used in the assays. This experiment was adapted from assessment of macrophage respiratory burst, where test macrophages are triggered with PMA, in order to induce activation of neutrophil antimicrobial responses. When treated in the sequential manner neutrophils rapidly produced reactive oxygen intermediates providing a suitable positive control for their activation state.

Kidney neutrophils were exposed to varying concentrations of mycobacteria at 0.5:1, 5:1 and 50:1 to bacteria: neutrophil ratios with NBT, as well as medium only and PMA control groups. All concentrations of *M. chelonae* and *M. fortuitum* induced significant NBT reduction, while only the highest level of *M. marinum* (50:1) induced ROI production. Heat killed *M. marinum* did not induce significant ROI at

all concentrations tested, when compared to *M. chelonae* and *M. fortuitum* that did (Fig. 5.2). As expected, PMA treated neutrophils produced large amounts of ROI (Fig. 5.2A).

We previously reported that one of the strategies that *M. marinum* employed to persist in goldfish macrophages was to down-regulate macrophage ROI response [98], although the necessity of ROI down regulation by mycobacteria is contentious in the literature. In an effort to see if *M. marinum*, *M. fortuitum* or *M. chelonae* has the ability to down-regulate ROI production, varying concentrations of each bacteria was co-incubated with goldfish neutrophils for 2 hours prior to treatment with PMA in the presence of NBT. In contrast to the reported down-regulation of ROI response of macrophages exposed to mycobacteria, no differences in the production of ROI were observed among any treatment group compared with PMA-alone treated cells, suggesting no apparent down-regulation of ROI by mycobacteria of PMA-stimulated neutrophils (Fig. 5.2B).

5.1.3 Immune gene expression of goldfish kidney neutrophils exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*

To examine the response of goldfish neutrophils following *M. marinum, M. chelonae* and *M. fortuitum* exposure, I measured mRNA levels of *il1β* (isoforms 1 and 2), *tnf*α (isoforms 1 and 2), *il12 p35* and *il12 p40* subunits, *cxcl8* and *tgfβ* by quantitative reverse transcriptase PCR (qRT-PCR). Significant increases in mRNA levels of *il-1β1, il-1β2, tnf*α*-1* and *tnf*α*-2* were observed after exposure to 5:1 (bacteria: neutrophil) live mycobacteria, whereas the mRNA levels of *il-12p35* and *il-*

12p40 subunits were similar to controls (Fig. 5.3). Heat-killed mycobacteria at 5:1 (bacteria: neutrophil) increased *il-1β1, il-1β2, tnfα-1* and *tnfα-2* mRNA levels, albeit to a lesser extent than those induced by live bacteria, and also did not significantly impact *il-12p35* and *il-12p40* subunits mRNA levels. *cxcl8* gene expression was unchanged compared to medium control groups, and a modest down-regulation in *tgfβ* gene expression following exposure of 5:1 *M. marinum* and *M. chelonae* was observed (Fig. 5.3).

5.1.4 Expression of genes that encode NADPH oxidase components p40phox and p91phox in neutrophils exposed to mycobacteria

The mRNA expression of NADPH oxidase components p40phox and p91phox was also determined following mycobacterial exposure. Neither 0.5:1 or 5:1 live or heat-killed *M. marinum* induced p40phox or p91phox expression compared to control cells. Conversely, both *M. chelonae* and *M. fortuitum* significantly increased both p40phox and p91phox mRNA levels. This was observed for both live and heat-killed experimental groups (Fig. 5.4).

5.1.5 Killing of *M. marinum*, *M. chelonae* and *M. fortuitum* by goldfish kidney neutrophils

The capacity of goldfish neutrophils to kill *M. marinum*, *M. chelonae,* and *M. fortuitum* was assessed, in both non-stimulated and PMA-treated kidney neutrophils. Bacteria were incubated with and without goldfish neutrophils at a MOI of 5:1, and CFUs were determined after 6 hours of cultivation. Goldfish neutrophils failed to kill

M. marinum, but did kill *M. chelonae* and *M. fortuitum*, since significantly lower CFUs were recorded compared to medium alone bacteria controls (Fig. 5.5A). I then wanted to examine whether PMA-activated neutrophils were capable of killing internalized bacteria. To measure this, I incubated neutrophils for 4 hours with *M. marinum, M. chelonae* or *M. fortuitum*. After incubation, the cells were washed 3X to remove extracellular bacteria and then treated with PMA for 30 minutes. Lysates were plated on Middlebrook 7H10 and the number of CFUs were determined. PMA-stimulated neutrophils significantly reduced the number of internalized CFUs for all species of bacteria, indicating a killing response by activated neutrophils (Fig. 5.5B).

5.1.6 Phagocytosis and surface adhesion of *M. marinum* by goldfish kidney neutrophils

The ability of goldfish neutrophils to engulf *M. marinum* was examined using GFP-expressing *Mycobacterium marinum*, since this was the only GFP-expression bacterium that we possessed. Phagocytosis was measured using imaging flow cytometry that allowed for discrimination between internalized and surface bound bacteria (Fig. 5.6, upper panel). Following a 2 h exposure, approximately 2% of neutrophils engulfed mycobacteria, while 6% of cells had surface bound *M. marinum* (Fig. 5.6, upper panel). At higher bacteria to cell ratio (50:1), 10% of neutrophils engulfed mycobacteria, while 20% of neutrophils had surface bound *M. marinum* (Fig. 5.6, upper panel). The internalization of GFP-expressing mycobacteria was analyzed using Amnis® single cell flow cytometry apparatus (Fig. 5.6, lower right panel).

5.1.7 Apoptosis and necrosis of goldfish neutrophils following exposure by *M. marinum*

Induction of apoptosis or necrosis of host neutrophils by mycobacteria is thought to play an important role in the outcome of infection [407]. We examined whether *M. marinum* induced either apoptosis or necrosis of goldfish kidney neutrophils. After exposure of neutrophils for 24 h to live mycobacteria none of the neutrophils were viable (Fig. 5.7A), while 50% of the neutrophils were viable after exposure to heat-killed bacilli (Fig. 5.7D). The exposure of neutrophils to live mycobacteria at a ratio of 50:1, induced significant apoptosis as early as 2 hours after exposure, and the number of cells undergoing apoptosis increased at subsequent time intervals (Fig. 5.7B). At lower ratios of live bacteria to neutrophils, significant apoptosis was observed only after 24 h of exposure (Fig. 5.7B). Significant apoptosis of neutrophils exposed to high numbers of heat-killed mycobacteria was only observed after 24 h of exposure (Fig. 5.7E).

A small number of neutrophils (< 5%), when exposed to high (50:1) ratios of live or heat-killed mycobacteria, exhibited necrotic cell death as indicated by PI staining (Fig. 5.7C and Fig. 5.7F). When exposed to live bacilli, significant necrosis was observed early (2 and 4 hours of exposure) (Fig. 5.7C), which differed from progressive increase in necrosis with time of incubation (4 to 24 h) for neutrophils exposed to heat-killed mycobacteria (Fig. 5.7F). The exposure of neutrophils at lower ratios (0.5:1 or 5:1) did not induce significant necrosis (Fig. 5.7C and Fig. 5.7F).

5.2 Discussion

Teleost fish have neutrophils that are morphologically and functionally similar to those of higher vertebrates, attesting to their essential role in host defense throughout evolution. The contribution of neutrophils to host defense against mycobacterial infection remains unclear, in part due to the difficulty of working with mammalian neutrophils *in vitro* (short life-span), leading to contradictory reports in the literature. Unlike mammalian neutrophils, whose viability rapidly decreases when cultured *in vitro*, goldfish kidney neutrophils remain viable for several days [414], enabling the assessment of their antimicrobial responses against mycobacteria.

As first responders to the site of infection, neutrophils express and secrete cytokines and chemokines that are essential for initiation of a robust pro-inflammatory response [197]. The expression of genes that encode cytokines and chemokines in neutrophils exposed to mycobacteria has been observed in various mammalian model systems [200], [415]–[418], but to our knowledge, has not been comprehensively examined in fish. I demonstrated that *M. marinum, M. chelonae* and *M. fortuitum* induced robust and significant expression of pro-inflammatory genes that encode cytokines IL-1β1, IL-1β2, TNFα-1 and TNFα-2 and small but significant increase in the mRNA levels of chemokine CXCL8. Production of pro-inflammatory cytokines aids in further recruitment of inflammatory cells, in addition to promoting activation of resident and infiltrating immune cells to elicit protection against invading pathogens [197]. The robust increases in the cytokine transcripts in neutrophils exposed to the natural mycobacterial pathogens suggest their protective role by mediating a proinflammatory response, similar to that seen in mammals.

Paradoxically, it has been reported that the early antimicrobial responses of phagocytes (neutrophils and macrophages) was incapable of completely eliminating pathogenic mycobacteria [101]. Therefore, an increased influx of phagocytes to the inflammatory site may provide increased numbers of host cells for mycobacteria to infect and reside within, resulting in enhanced dissemination of the bacilli to different tissues and eventual granuloma development [80], [366], [419].

The trafficking of neutrophils to the site of mycobacterial infection has been demonstrated in mammals *in vivo* [79], [80], [407], [420], [421], as well as *in vitro* [422], [423], although this appears to be pathogen-specific. For example, *M. ulcerans* has been shown to cause minimal attraction of neutrophils to the infection sites, thereby limiting a robust innate immune response against mycobacteria and exacerbating disease [423]. Trafficking of neutrophils to the site of infection was shown in zebrafish larvae, but direct interaction between neutrophils and *M. marinum* following neutrophil infiltration was not evident. Subsequent reports of internalization of *M. marinum* by neutrophils in subcutaneous infection studies suggest that site specificity of infection exists in zebrafish [203]. At the level of the granuloma, a oxidative-dependent protective role for neutrophils have been established in zebrafish [80].

My results indicate that goldfish neutrophils are attracted to the mycobacteria and that the migration of the cells toward mycobacteria is dose-dependent. The mycobacteria-derived soluble molecules promoting neutrophil chemotaxis *in vitro*, and the cell receptor(s) that they bind to, are unknown at present and require further study. Factors that mediate neutrophil migration to the site of mycobacterial infection

in vivo remain to be elucidated in teleosts but are likely a combination of soluble bacterial products and pro-inflammatory cytokines secreted by resident tissue innate immune cells.

Goldfish kidney neutrophils bound to and internalized mycobacteria *in vitro*. In other mammalian models, it is well established that neutrophils internalize both opsonized (i.e. complement receptor- mediated) and non-opsonized (i.e. pattern recognition receptor-mediated) mycobacteria [198]–[200], [403], [407], [424], although internalization of *M. marinum* by neutrophils was not observed at the site of infection in zebrafish larvae [80]]. While my results indicate kidney neutrophils have the capacity to internalize *M. marinum in vitro*, it is possible that the inflammatory site may alter this ability, through soluble mediators, or preferential internalization by other innate immune cells (e.g. macrophages).

Whether neutrophils can kill internalized mycobacteria is controversial. Studies showed a killing response [200], [402], [425]–[427] and restriction of growth of internalized bacilli [183], [428]. In contrast, others reported a lack of bacterial killing [198], [406], [429]. Earlier findings by our group indicated that exposure of *M. marinum* to goldfish monocytes to resulted in an inhibition of cytokine induced respiratory burst activity [98]. Interestingly, lower concentrations of both live and heat-killed *M. chelonae* and *M. fortuitum* enhanced an ROI response, while only high concentrations of live *M. marinum* was capable of inducing respiratory burst function. Similarly, a killing response was shown in naïve neutrophils exposed to 5:1 MOI *M. chelonae* and *M. fortuitum* but not *M. marinum*. Therefore, it appears that neutrophils are capable of killing *M. chelonae* and *M. fortuitum* by oxidative burst,

but not *M. marinum,* suggesting that neutrophils must generate additional antimicrobial response(s) for control of this pathogen. The differences in neutrophil anti-mycobacterial responses observed for different species of the bacilli, may in part be due to the ability of *M. marinu*m to withstand ROI by elaborating KatG catalase, which has been previously shown for its close relative, *M. tuberculosis* [133].

The generation of ROI has also been shown to induce apoptosis of immune cells via oxidative stress [430]. Mycobacterial induction of neutrophil apoptosis has been observed in mouse and humans models [399], [431], [432], which is consistent with my results. The consequence of mycobacteria-driven neutrophil apoptosis is critical in determining the outcome of infection by influencing a pro-inflammatory or pro-resolving state. Typically, neutrophil apoptosis induces a pro-resolving state in surrounding cells, particularly macrophages [318], although internalization of mycobacteria-induced apoptotic neutrophils by macrophages has been shown to promote an inflammatory response, aiding in destruction of internalized mycobacteria [432]–[436]. It has also been reported that *M. bovis* strain BCG internalization and subsequent neutrophil apoptosis increased pro-resolving mediators TGF β and prostaglandin E2 [184], which may be due to site-specificity, or pathogen-host differences. My results showed that goldfish kidney neutrophils undergo primarily apoptosis, and to a lesser extent necrosis, following exposure to *M. marinum*. Further work is necessary to determine these effects on downstream immune events during infection in teleosts.

This is the first attempt to characterize the functional response of fish neutrophils to natural mycobacterial pathogens *in vitro*. My findings highlight key

differences in activation states, which may well lead to protection or lack of protection during early infection stages. Further analysis into the protective role of neutrophils in mycobacterial infection *in vivo* would further elucidate possible evasion techniques, as well as mechanisms of protective immunity in fish.

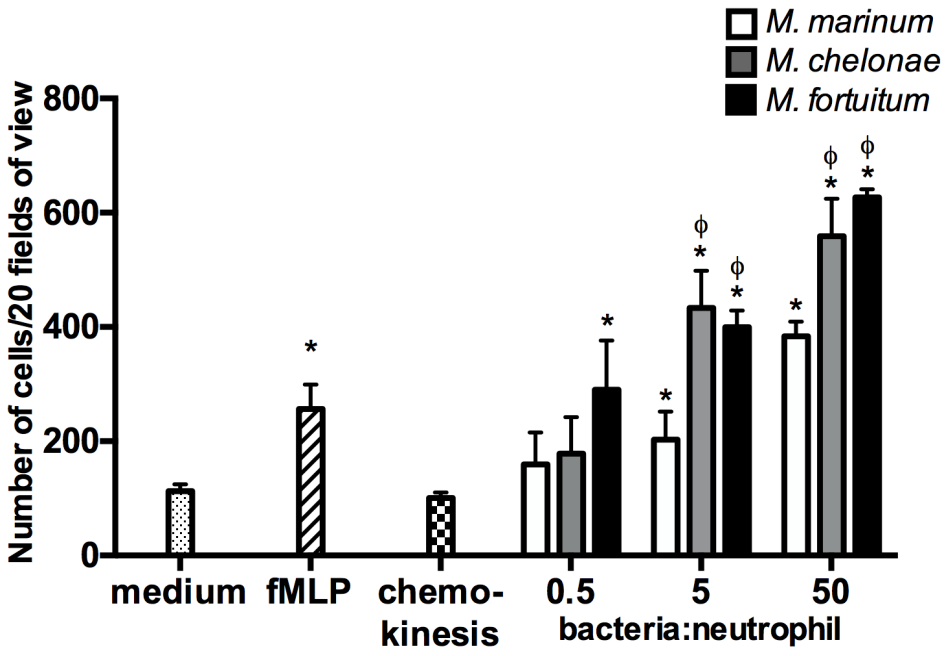

Figure 5.1. Chemotaxis of primary kidney neutrophils towards mycobacteria. Duplicate filters separating kidney neutrophils and *M. marinum*, *M. chelonae* or *M. fortuitum* in bacteria: neutrophil ratios indicated were stained with Gills Solution 3 after 1 h incubation. Total number of cells in 20 random fields of view were counted under oil immersion (1000X). Medium alone was the negative control, and the positive control was 10 ng/mL fMLP. Equal concentrations of fMLP in the upper and lower chemotaxis apparatus chambers served as a chemokinesis control. Kidney neutrophils were obtained from four individual fish (n=4). Data were analyzed by one-was ANOVA with multiple comparisons followed by Tukey post-hoc test. (*) denote significantly different ($p<0.05$) from the medium control group, and (Φ) denotes significant differences within bacteria: neutrophil groups.

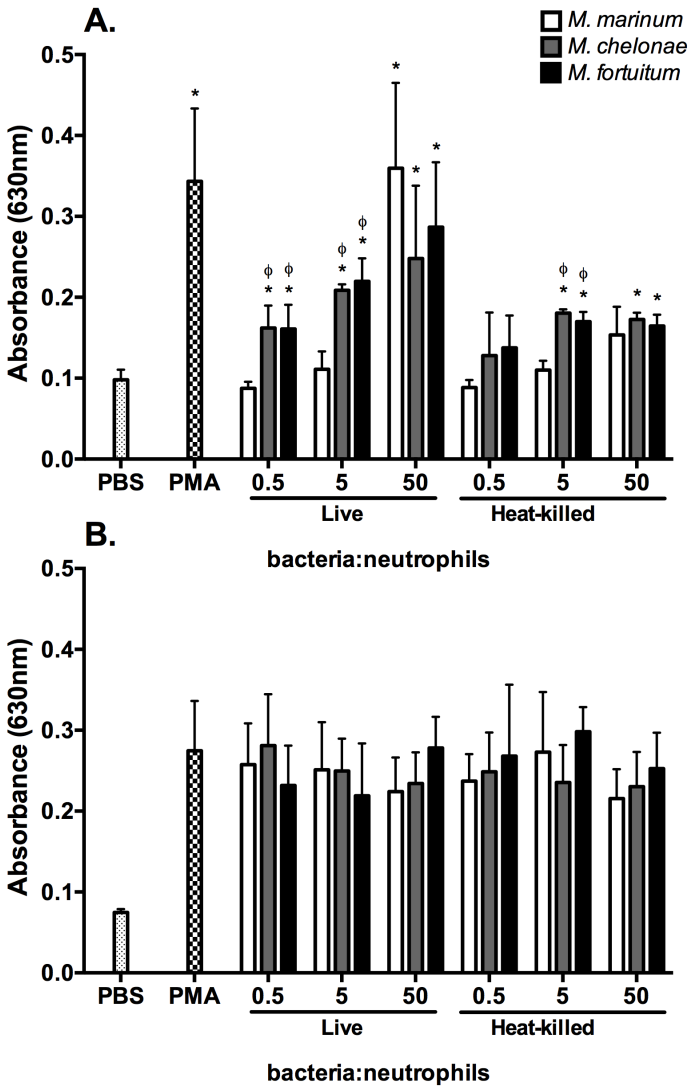

Figure 5.2. Reactive oxygen production of goldfish primary kidney neutrophils after exposure to *M. marinum*, *M. chelonae* and *M. fortuitum*. (A) Kidney neutrophils were incubated for 30 min with either live or heat-killed mycobacteria at the ratios indicated in the presence of 400 µg/mL nitroblue tertrazolium (NBT). PBS treatment indicated background ROI production, and 100 ng/mL phorbol myristate acetate treatment served as a positive control for NBT reduction. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test. (*) denotes significantly different ($p<0.05$) from the negative control group and (Φ) denotes significant differences within bacteria: neutrophil groups. (B) Kidney neutrophils were exposed to either live or heat killed mycobacteria for 2 h in the ratios indicated. Following incubation, cells were activated with 100 ng/mL PMA in the presence of 400 µg/mL NBT for 30 min. Kidney neutrophils were obtained from four individual fish (n=4). Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. (*) denotes significantly different ($p<0.05$) from the PMA-alone treatment group.

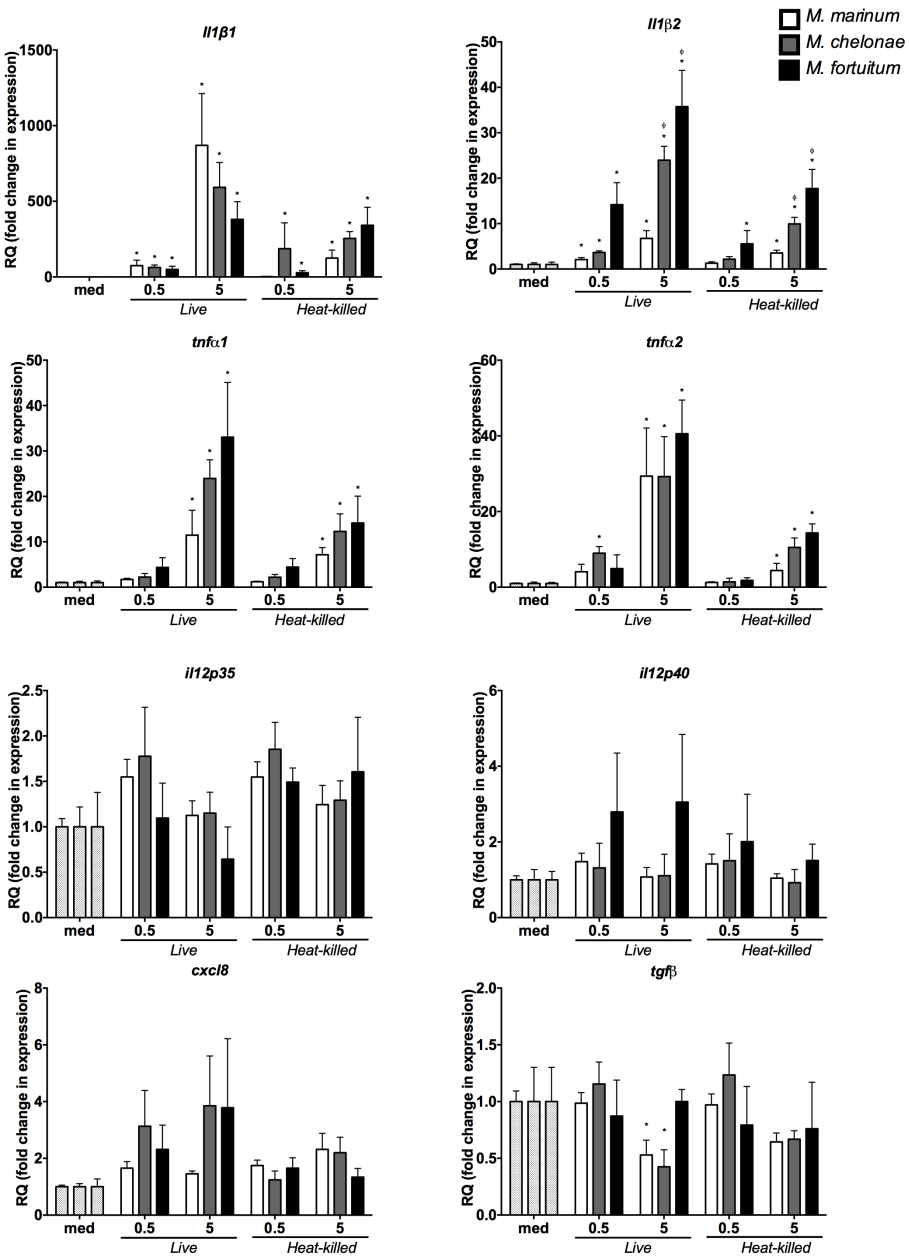

Figure 5.3. Mycobacteria induces changes of immune gene expression in goldfish kidney neutrophils. Neutrophils from 4 individual fish were exposed to live or heat-killed *M. marinum*, *M. chelonae,* or *M. fortuitum* at the ratios indicated on the x-axis (n=4). Cells were incubated for 24 h and expression data were normalized against cells treated with PBS alone. Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. (*) denotes significantly different ($p<0.05$) from the negative control group and (Φ) denotes significant differences within bacteria: neutrophil groups.

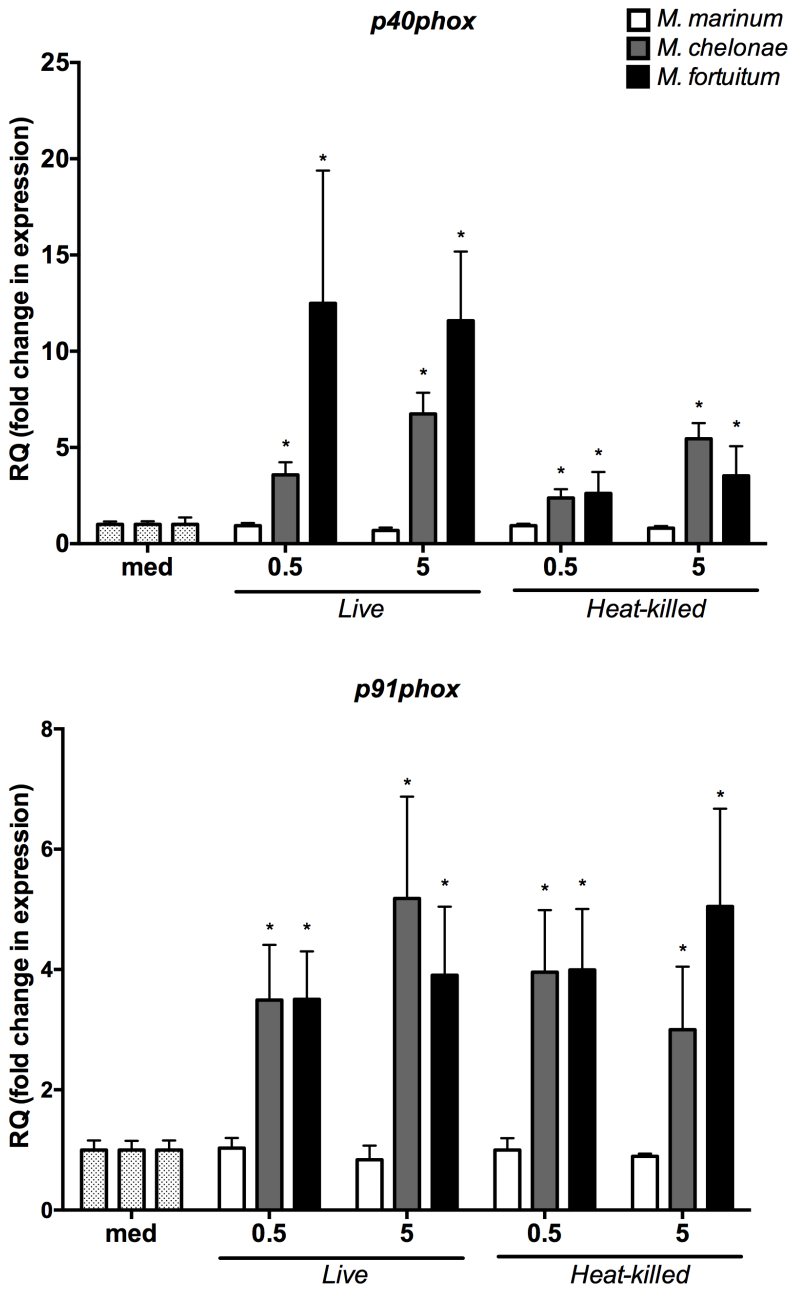

Figure 5.4. Mycobacteria induce changes in primary kidney neutrophil expression of phox genes. Neutrophils from 4 individual fish were exposed to live or heat-killed *M. chelonae* at the ratios indicated (n=4). Cells were incubated for 24 h and expression data were normalized against cells treated with PBS alone. Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. (*) denotes significantly different ($p<0.05$) from the negative control group and (Φ) denotes significant differences within bacteria: neutrophil groups

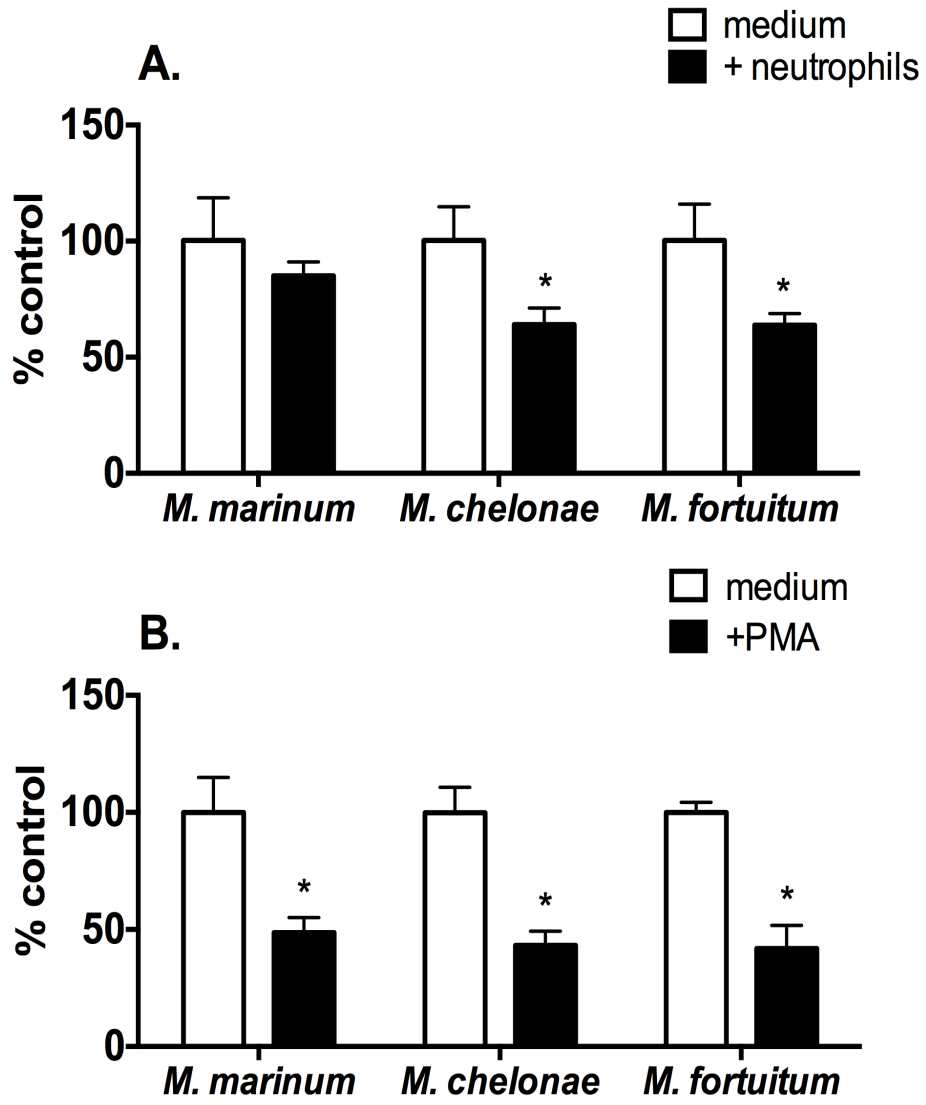

Figure 5.5. Killing of internalized mycobacteria by PMA stimulated goldfish kidney neutrophils. (A) *M. marinum, M. chelonae* and *M. fortuitum* were incubated with and without goldfish neutrophils (5:1) for 6 hours, and the number of CFUs determined. (B) Goldfish neutrophils were incubated for 4 h, washed 3X in PBS to remove extracellular bacteria, and stimulated with PMA for 30 min. Resulting lysate CFUs were enumerated by plating serial dilutions on Middlebrook 7H9 medium growth plates. Data were analyzed by using an unpaired Student's t-test, (*) denotes significance ($p < 0.05$) from the medium control group.

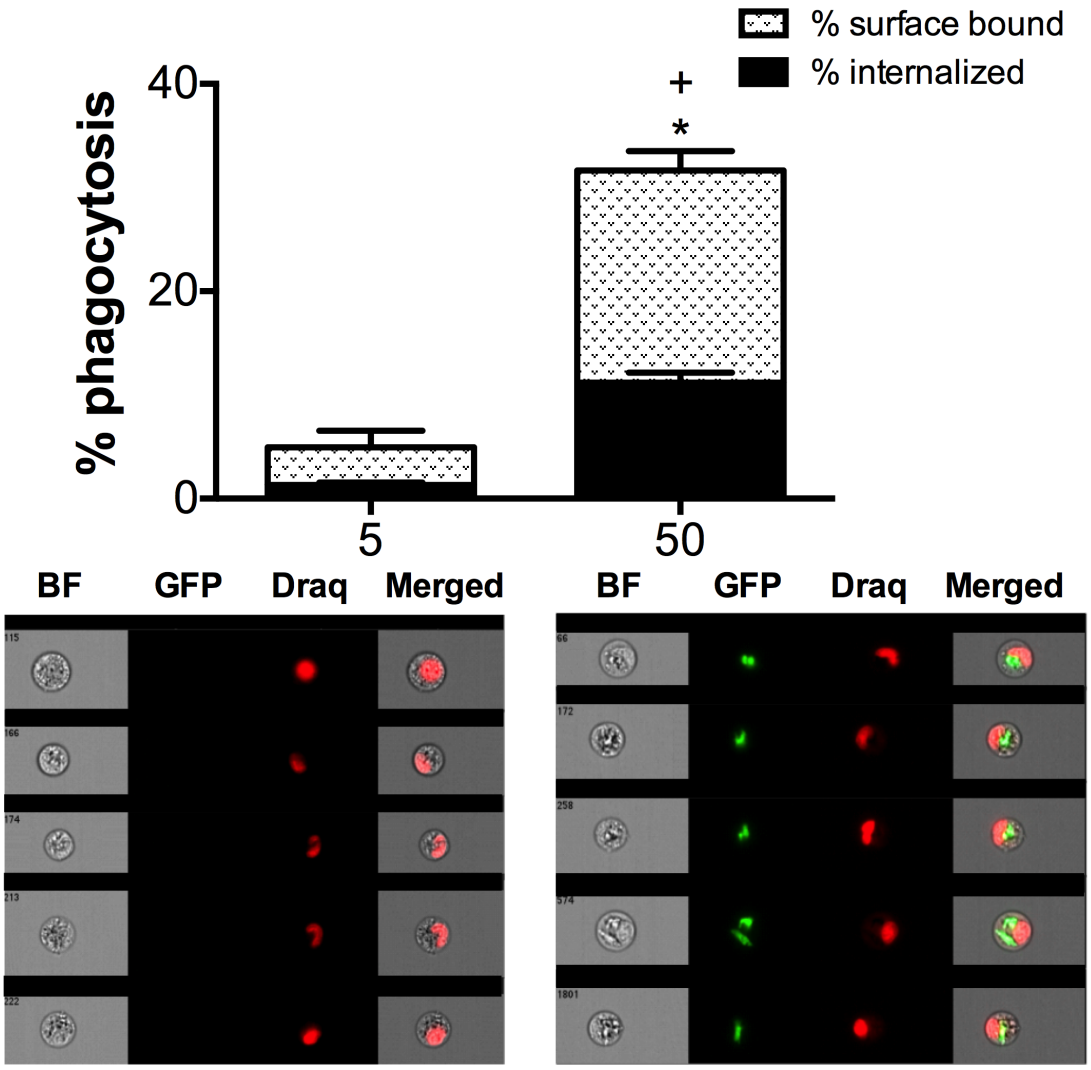

Figure 5.6. Phagocytosis of *M. marinum* by goldfish primary kidney neutrophils. GFP-*M. marinum* were incubated with neutrophils at ratios of 5:1 and 50:1 for 2 h at 25ºC. Cells were then fixed, and phagocytosis was quantified by flow cytometry. Upper panel: Black bars indicate the percent of cells that cells with internalized *M. marinum*, and white patterned bars indicate the percent of surface bound events as a percentage of the entire population. Lower panels: Representative images of neutrophils that have not internalized *M. marinum* (lower left) and neutrophils that have internalized *M. marinum* (lower right). Data were collected using an ImageStream MkII cytometer (Amnis). Two independent experiments were performed (n=5). Data were analyzed by a paired student's t-test. (+/*) denote significantly different ($p < 0.05$) from the 5:1 exposure group.

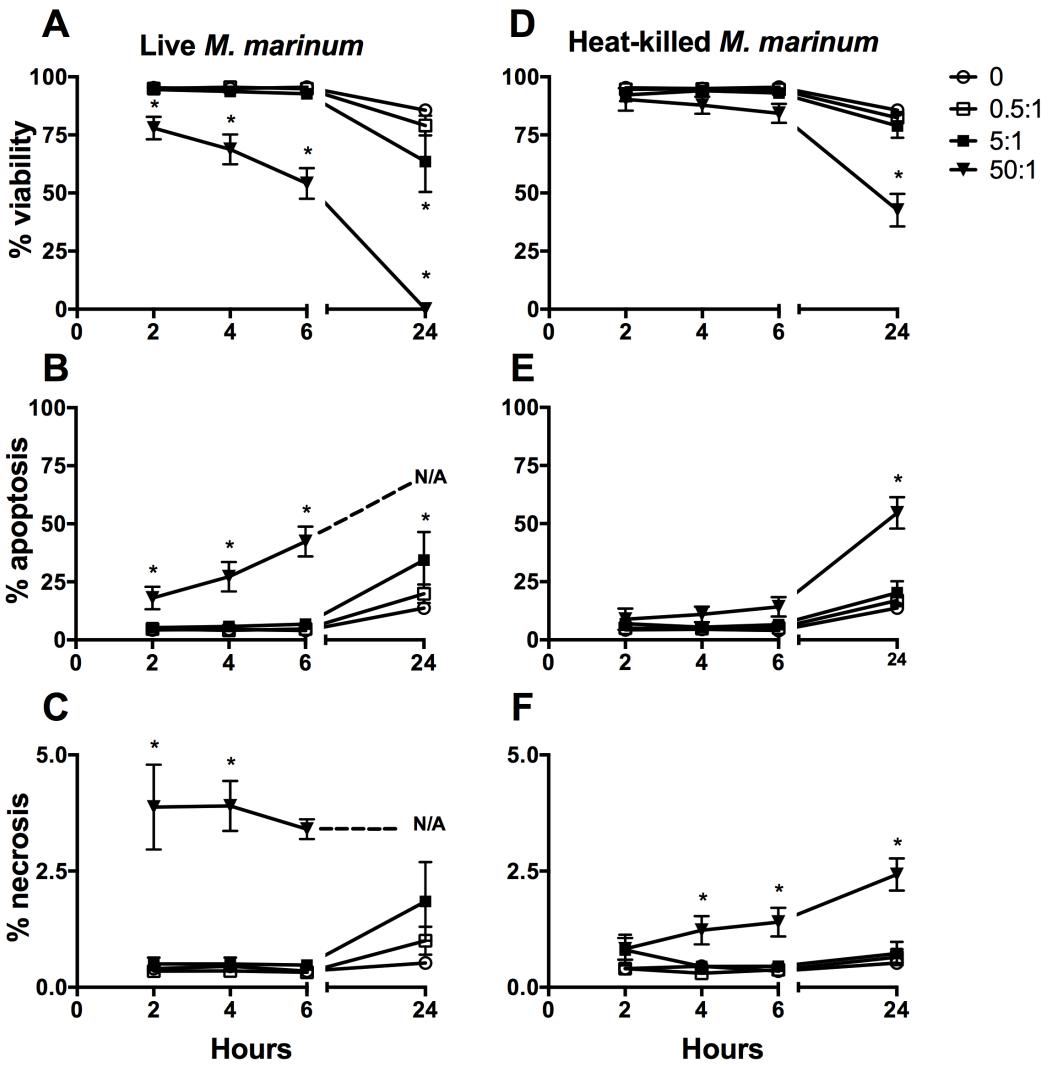

Figure 5.7. Apoptosis of goldfish kidney neutrophils after exposure to *M. marinum*. Neutrophils were incubated with *M. marinum* for 2, 4, 6 or 24 h at the ratios indicated. Apoptosis was measured using Annexin V FITC (eBioscience) and data were acquired using FACS Calibur flow cytometer (BD). Data were analyzed by one-way ANOVA followed by Dunnett's post-hoc test. (*) denote significantly different ($p < 0.05$) from the medium control group ($n = 4$).

Chapter VI: Antimicrobial responses of goldfish primary kidney macrophages to mycobacterial pathogens

6.0 Introduction

Pathogenic *Mycobacterium* spp. are responsible for considerable morbidity and mortality worldwide. Primarily intracellular pathogens, mycobacteria are responsible for serious human diseases including tuberculosis (*M. tuberculosis*) and leprosy (*M. leprae*), as well as considerable disease in a variety of animal species. Despite an ongoing effort to characterize host-pathogen interactions, research has been hampered by slow replication times of *M. tuberculosis* (24 h) and difficulty in *in vitro* model development, especially with regard to suitable primary phagocyte model systems. More recently, efforts have focussed on development of surrogate model systems to study host-pathogen interactions with more attractive biosafety levels, doubling time, and recapitulation of natural interactions. Use of fish, especially zebrafish (*Danio rerio*) models and *M. marinum* have gained attention as a useful surrogate model, as *M. marinum* grows considerably faster than *M. tuberculosis* and shares 99.4% 16S sequence identity [437]. Similarities in pathology, including granuloma formation in body tissues, intracellular residence and immune evasion strategies have been shown in a variety of fish and amphibian species [78], [365], [366], [438], [439].

The ability of phagocytes to control mycobacterial growth is paramount for host protection. Although highly pathogenic mycobacterial pathogens such as *M. marinum* reside and replicate within host phagocytes, it has been demonstrated that phagocyte depletion in zebrafish embryos results in increase bacterial burden [78].

Therefore, permissiveness of infection of different mycobacterial species depends heavily on phagocyte activation states and effector function, especially RNI production, which is paramount to antimycobacterial host protection [440]. Accordingly, a number of studies have attempted to determine the influence on macrophage polarization with regards to alternative activation, which results in increased arginase activity and competition with iNOS for a common substrate, L-arginine [441], [442]. Indeed, pathogenic mycobacteria such as *M. tuberculosis* have been shown to interfere with classical activation with the virulence factor ESAT-6 [443], and increased levels of IL-4/IL-13 has been observed in patients with progressive pulmonary TB [444]–[448]. The critical nature of iNOS is well understood in mycobacterial protection. Less information exists on the regulation of arginase during infection, although *in vitro* studies have shown increases in arginase -1 expression with mycobacterial challenge through PRR stimulation [449], [450].

In this chapter I focused on the *in vitro* analysis of primary kidney macrophages following exposure to *M. marinum*, *M. chelonae* and *M. fortuitum*. The activation states and killing capacity of macrophages was assessed with an emphasis on NO production and arginase activity. The lack of NO production in *M. marinum* exposed macrophages and increased arginase activity may influence the consequence of infection in fish or amphibian hosts and may serve as an insight as a model for human mycobacterial disease.

6.1 Results

6.1.1 Expression analysis of pro inflammatory genes in primary kidney macrophages following exposure to *M. marinum*, *M. chelonae* and *M. fortuitum*

To examine the potential differences in regulation of pro inflammatory cytokines by fish mycobacterial pathogens, primary kidney macrophages were exposed to 0.5 and 5:1 mycobacteria per cell, and gene expression was determined using quantitative PCR (Fig. 6.1). Compared to non-treated controls, macrophages incubated with both *M. marinum* and *M. fortuitum* exhibited significant and dose dependent increased expression of IL-1β1 at each exposure level. IL-1β1 expression was also significantly increased following incubation of macrophages with *M. chelonae* at 5:1, although unchanged at the 0.5:1. Macrophages incubated with either quantity of mycobacteria showed no significant increase of IFNγ expression. TNFα2 expression in macrophages was increased in both *M. chelonae* and *M. fortuitum* treated groups, while *M. marinum* was modest, albeit non-significant increases in expression levels (Fig. 6.1).

6.1.2 Expression analysis of regulatory genes in primary kidney macrophages following exposure to *M. marinum*, *M. chelonae* and *M. fortuitum*

The effects of *M. marinum*, *M. chelonae* and *M fortuitum* on goldfish macrophage regulatory gene expression was also assessed. At both bacterial levels and for all species tested, no increases of IL-10 or TGFβ was observed. Treatments of macrophages with 5:1 of either *M. marinum* or *M. fortuitum* significantly increased the expression of suppressor of cytokine signaling-3 (SOCS3) (Fig. 6.2).

6.1.3 Expression analysis of M2-type genes in primary kidney macrophages following exposure to *M. marinum*, *M. chelonae* and *M. fortuitum*

The expression of macrophage M2-type genes was investigated following exposure of macrophages to *M. marinum*, *M. chelonae* and *M fortuitum*. Exposure of macrophages to 5:1 *M. marinum*:macrophages resulted in significant increases in expression levels of IL-4/13A, IL-4/13B and arginase-2, while only arginase-2 was upregulated following 5:1 exposure to *M. fortuitum*. No significant changes in arginase-1 occurred for any treatment type, although a modest decrease in 0.5 *M. fortuitum* was observed (not significant) (Fig. 6.3).

6.1.4 Nitric oxide production of primary kidney macrophages exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*

It is well established that goldfish kidney macrophages possess the ability to exhibit robust RNI production upon activation with PAMPs or endogenous cytokines. Here, I was interested the innate ability of macrophages to produce RNI with exposure to mycobacteria live and heat-killed mycobacteria at 0.5:1, 5:1 and 50:1 bacteria to macrophages. At any level of exposure, *M. marinum* was incapable of inducing RNI production, while live versions of *M. chelonae* and *M. fortuitum* both induced RNI production at 5:1 and 50:1 exposure levels (Fig. 6.4).

6.1.5 Respiratory burst activity in primary kidney macrophage cultures exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*

In addition to RNI production, the ability of primary kidney macrophages to

produce reactive oxygen intermediates (ROI) was assessed following exposure to mycobacteria. Only *M. chelonae* significantly primed an ROI response, showing significant increase in the production of ROI in 5:1 and 50:1, both live and heat killed. No significant increases in ROI production was observed for any treatment of *M. marinum* or *M. fortuitum*, but rather at the highest levels of live mycobacterial exposure, a relative decrease in ROI compared to control cells was observed (Fig. 6.5).

6.1.6 Arginase activity in primary kidney macrophages exposed to *M. marinum*, *M. chelonae* and *M. fortuitum*

Following the variance in ability to elicit a nitric oxide response, I measured the arginase activity in primary kidney macrophages following exposure to mycobacteria. Macrophages were incubated with either 0.5 or 5:1 mycobacteria and assessed for arginase activity. A decrease in arginase activity was observed in *M. fortuitum* and *M. chelonae* exposed macrophages. Conversely, 0.5:1 *M. marinum* was unchanged from medium alone cell controls, and 5:1 showed a significant increase in arginase activity, both compared with controls and other mycobacterial treatment groups (Fig. 6.6).

6.1.7 Killing response of activated primary kidney macrophages

The ability of naïve and activated macrophages to kill mycobacteria was also measured. For these experiments mycobacteria was added to the PKMs cultures at a ratio of 5 bacilli to 1 host cell, and cells were lysed and plated on Middlebrook agar

following 6 hours exposure. No significant decrease in mycobacteria was observed compared to the bacteria alone control (Fig. 6.7A). However, addition of rgIFNγ significantly reduced *M. chelonae* and *M. fortuitum* compared with bacteria alone, and rgTNFα2 significantly reduced viable bacteria for all 3 species (Fig 6.7A). To determine the ability of cytokine stimulated macrophages to kill internalized bacteria, macrophages were also incubated with mycobacteria and washed to remover extracellular bacteria. Following the addition of cytokines, the number of internalized viable bacteria was assessed. The treatment of macrophages with either rgIFNγ and rgTNFα significantly reduced the number of bacteria for all 3 species of mycobacteria. Neither rgIL-4/13A or rgIL-4/13B altered the number of intracellular bacteria compared to medium control (Fig 6.7B).

6.2 Discussion

In this chapter I described the *in vitro* response of primary kidney macrophages to natural mycobacterial pathogens. The discrepancy in pathogenicity between *M. marinum*, *M. chelonae* and *M. fortuitum* can be accounted for in terms of alterations of an effective immune response resulting in persistence in host animals. Upon mycobacterial infection, the interaction between host and pathogen is key to determining whether infection will lead to (i) apparent acute infection; (ii) latency, where surviving microbes are controlled by the immune system; or (iii) clearance of mycobacteria by the immune system, most common of non-pathogenic mycobacterial species. Investigations focused on the early immune response to mycobacteria are particularly important, as epidemiological and genetic studies suggest the early phase

of infection may represent a therapeutic window prior to establishment of disease [451]. Here, I described the *in vitro* response of primary macrophages as a model of early exposure events to tissue macrophages.

The necessity of Th1 immunity against acute mycobacterial infection has been well established, primarily with regards to IFNγ production and macrophage activation [452]. An inability to generate a strong Th1 response, such as in IL-12$^-$, STAT-4$^-$ and T-bet$^-$ animals, greatly increases host susceptibility [452], [453]. Interference with Th1 activation has been well documented in *M. tuberculosis* infection, and more recently, growing evidence suggests activation of an ineffective Th2 response heightens vulnerability to mycobacterial infection [445], [454]–[456]. It has become apparent that innate activation of M1 macrophages leads to promotion of a Th1 response (and not the other way around), propagating a positive feedback loop for further macrophage activation [457]. Therefore, it is crucial that initial exposure lead to classical activation for innate protection and shaping of adaptive immunity. Existence of classical and alternative activation states have been demonstrated in fish macrophages. Interferon and various microbial stimuli (e.g. LPS) effectively polarize M1 macrophages, resulting in nitric oxide production and respiratory burst activity akin to mammals [384], [458], [459]. Similarly, parasitic infection with *T. carrassi*, and more recently, IL-4/13 orthologues, are capable of inducing a M2 phenotype, resulting in increased arginase expression and activity [230], [356], [385]. Upon classical activation, macrophages express iNOS enzymes, responsible for the production of reactive nitrogen intermediates (RNI). These reactive radicals are highly toxic and damage nucleic acids, proteins, lipids and carbohydrates, and play an

essential role in killing as demonstrated in the murine tuberculosis model [460], [461], although early mycobacteriostatic activity in human models are found to be independent of NO [462], [463]. This controversy may be due to the temporal and relative contribution of innate NO and adaptive NO activation, where innate levels are more easily withstood through microbial mechanisms such as mycobacterial peroxiredoxins, degradation of nitrosylated proteins by mycobacterial proteasomes, and inhibition of NOS accumulation at the level of host phagosomes, which can effectively evade this response [395], [462], [464].

I observed that exposure of primary kidney macrophages to *M. marinum*, *M. chelonae* and *M. fortuitum* showed differential ability in macrophages to produce nitric oxide intermediates. *M. marinum* did not increase nitric oxide beyond background control levels at any level of infection, while both *M. chelonae* and *M. fortuitum* enhanced nitric oxide production. This suggests that *M. marinum* may actively counteract this antimicrobial function of macrophages or fail to induce the response (or both). Previous reports in our laboratory illustrated the ability of *M. marinum* to reduce interferon primed NO, most likely due to active attenuation [63], although co-treatment of *M. marinum* with *M. fortuitum* did not inhibit NO production compared to *M. chelonae* alone. It is possible that *M. marinum* is capable of impairing interferon-stimulated NO production and not NO generated by PRR stimulation. The demonstrated M1 polarization of macrophages my *M. chelonae* and *M. fortuitum* most likely help influence eventual Th1 activation, which manifests in more effective control of infection. This coincides with my observation of a general

decline of viable mycobacteria during *in vivo* infection courses, presumably through an effective Th1 response, as discussed in chapter 7 of this thesis.

Reactive oxygen intermediates (ROS) are produced by NADPH oxidase components in phagocytes including neutrophils, monocytes, macrophages and dendritic cells [465] and are also a hallmark of M1-type activation. Superoxide anion (O_2^-) is the initial ROS produced and subsequently converts into other oxygen derivatives. Like RNS (and in combination with RNS), it is conceived that RNS and ROS exert their bactericidal effect mainly by direct oxidation of mycobacterial structures leading to bacterial damage and death. The role of ROS in mycobacterial protection is controversial, although ROS seems to have a bactericidal effect [460], [466]. This is underlined by the increased incidence of *M. tuberculosis* in patients suffering from chronic granulomatous disease (CGD), that is caused by a deficiency of an NADPH oxidase component [465], although mice with defective NADPH oxidase components show no less resistance to infection [131], [467]. My results show that like NO production, *M. marinum* is not capable of priming ROS in PMA-triggered PKMs and show less overall ROS production than PMA-alone treated macrophages, suggesting down-regulation. Similarly, *M. fortuitum* showed decreased ROS production at the highest levels of infection, while *M. chelonae* enhanced ROS in a dose dependant manner, with both live and heat-killed treatments. Taken together, it is possible that a lack of ROS production may infer lack of classical activation in macrophages treated with *M. marinum*, and potentially also *M. fortuitum*, although *M. fortuitum* was capable of stimulating RNS

production. It is also possible that mycobacterial catalase is responsible for interfering with NBT oxidation, since catalase has been shown to react with superoxide ions [468].

Gene expression studies suggest differential activation states following exposure to each mycobacterial species, in line with the observed functional differences. Interestingly, exposure to each species increased IL-1β1 expression, a potent pro-inflammatory cytokine and critical to mycobacterial defense [278], [282]. Translation of proteolytic processing of IL-1β1 into its active form was not studied here although *M. tuberculosis* inhibits AIM2-inflammasome activation via its ESX-1 secretion system. This may be the case in *M. marinum*, which also possesses ESX-1 and the region of deletion 1 (RD-1), a 9.5kb section of DNA containing several genes involved in pathogenicity and virulence, although it is unclear if inflammasome processing of Il-1β1 is conserved in fish [469]. More work is necessary to determine whether the increase in gene expression corresponds to an increase in mature active IL-1β1 peptide. Not surprisingly, no treatment of macrophages resulted in an increase of IFNγ, although increases in TNFα2 was seen in *M. chelonae* and *M. fortuitum* exposed macrophages, further suggesting innate M1 activation, where these expression increases were not seen in *M. marinum* exposed macrophages. No changes of IL-10 and TGFβ were observed following mycobacterial exposure, however, significant increases in SOCS3 was observed was measured in *M. marinum* and *M. fortuitum* treated macrophages, consistent with what is seen in *M. tuberculosis* infected macrophages [328].

Expression of IL-4/13 and arginase genes was measured following exposure to the pathogens, where both IL-4/13A, IL-4/13B and arginase-2 was significantly increased following *M. marinum* exposure. It has been demonstrated that alternatively activated mammalian macrophages are capable of producing low levels of IL-4 [470], although this has yet to be not been demonstrated in fish macrophages. Here, we show that *M. marinum* increases expression of IL-4/13 genes in primary kidney macrophage cultures. Increases in arginase-1 levels of *in vitro* exposed to mycobacteria has been shown in mouse macrophages through a TLR pathway [449]. Similarly, arginase-2 expression was increased in both *M. marinum* and *M. fortuitum* exposed macrophages, where unlike mammals, arginase-2 is upregulated by cAMP stimulation and thought to be responsible for arginine metabolism and polarization of macrophages [230], [356]. The induction of arginase in early exposure to bacterial pathogens may interfere with initial killing by macrophages and may also alter the subsequent T helper response needed for effective immunity to mycobacteria. Measurement of arginase activity *in vitro* corroborated gene expression studies, where 5:1 *M. marinum* to host cells resulted in increased arginase activity, while *M. chelonae* and *M. fortuitum* decreased activity, in line with the increases of nitric oxide production I observed.

Contribution of cytokines from exogenous sources can further polarize/activate macrophages. Recombinant cytokines IFNγ, TNFα2, IL-4/13A and IL-4/13B were generated to determine whether cultured macrophages were capable of killing *M. marinum*, *M. chelonae* and *M. fortuitum*. In all cases, IFNγ and TNFα2 activated a killing response by goldfish macrophages, while neither IL-4/13A or IL-

413/B affected bacterial CFUs. Accordingly, increases in Th2/IL-4 responses in mycobacterial infection in fish is likely counterproductive to host immunity, as is established in mammalian literature.

In this chapter, I reported for the first time the M1/M2 activity of teleost macrophages following mycobacterial exposure. These findings indicate that initial exposure of macrophages to mycobacterial pathogens may play an important role in determining acute control of infection, as well as possible contribution to adaptive immunity during chronic infection.

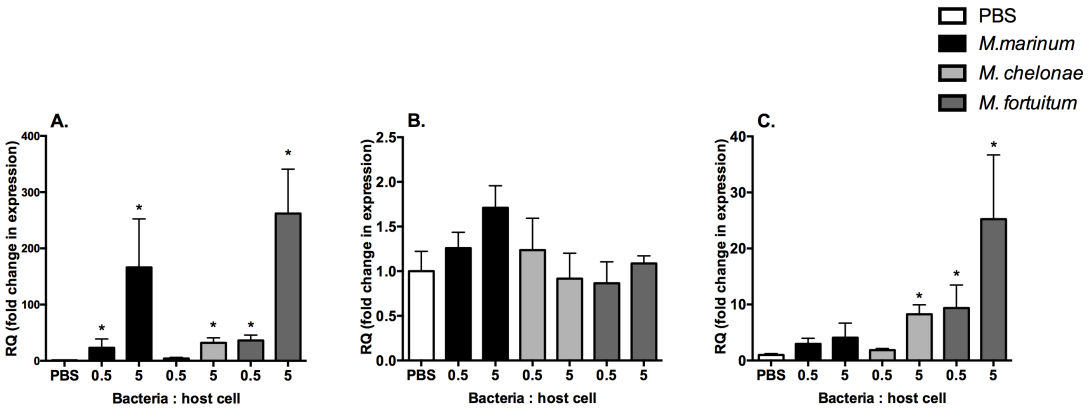

Figure 6.1. Pro-inflammatory gene expression following exposure of primary kidney macrophages to mycobacterial pathogens. (A) IL-1β1, (B) IFNγ and (C) TNFα2 were analyzed for relative expression in PKM cultures following 18 hours exposure to *M. marinum*, *M. chelonae* and *M. fortuitum* at the ratios shown. Expression data were normalized to PBS exposed controls. Results are means ± RQ values from 5 individual fish (n=5). Statistical analysis was performed by one-way ANOVA followed by a Dunnet's post hoc test (*) indicates significant difference from control cells ($P < 0.05$).

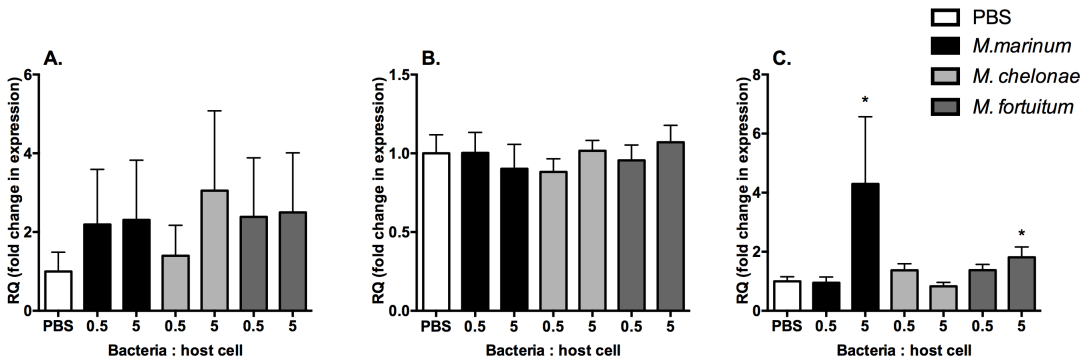

Figure 6.2. Regulatory gene expression following exposure of primary kidney macrophages to mycobacterial pathogens. (A) IL-10, (B) TGF-β and (C) SOCS3 were analyzed for relative expression in PKM cultures following 18 hours exposure to *M. marinum*, *M. chelonae* and *M. fortuitum* at the ratios shown. Expression data were normalized to PBS exposed controls. Results are means ± RQ values from 5 individual fish (n=5). Statistical analysis was performed by one-way ANOVA followed by a Dunnett's test (*) indicates significant difference from control cells ($P < 0.05$).

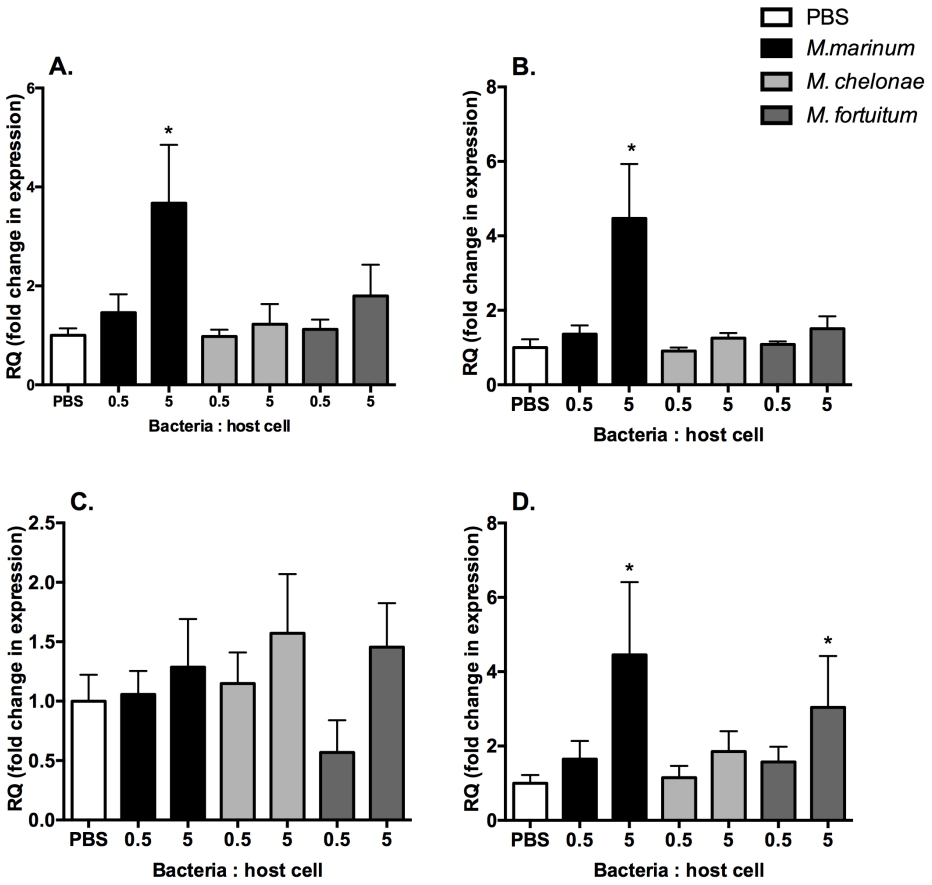

Figure 6.3. M2-type gene expression following exposure of primary kidney macrophages to mycobacterial pathogens. (A) IL-4/13A, (B) IL-4/13B, (C) Arginase-1 and (D) Arginase-2 were analyzed for relative expression in PKM cultures following 18 hours exposure to *M. marinum*, *M. chelonae* and *M. fortuitum* at the ratios shown. Expression data were normalized to PBS exposed controls. Statistical analysis was performed by one-way ANOVA followed by a Dunnett's test. Results are means ± RQ values from 5 individual fish (n=5). (*) indicates significant difference from control cells ($P < 0.05$).

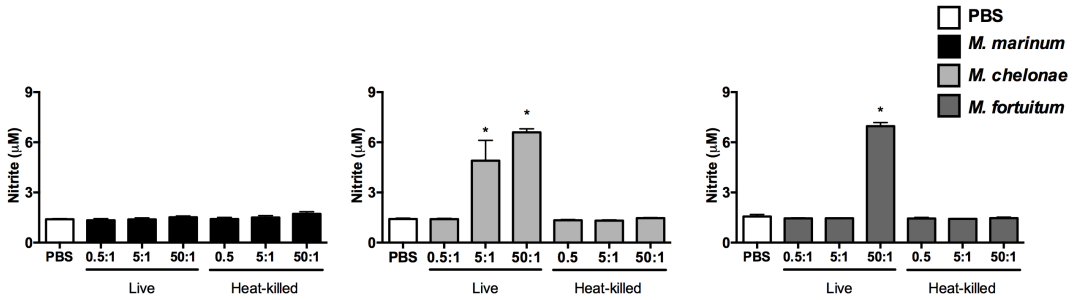

Figure 6.4. Nitric oxide production of primary kidney macrophages following exposure to mycobacterial pathogens. Macrophage cultures were incubated with 0.5:1, 5:1 and 50:1 live or heat-killed bacteria for 48 hours. Nitrite production was determined by Griess reaction and nitrite was determined using a nitrite standard curve. Macrophages were obtained from day 6 primary kidney macrophage cultures from individual fish (n=5). Statistical analysis was performed by one-way ANOVA followed by a Dunnett's test. (*) indicates significant difference from control cells ($P < 0.05$).

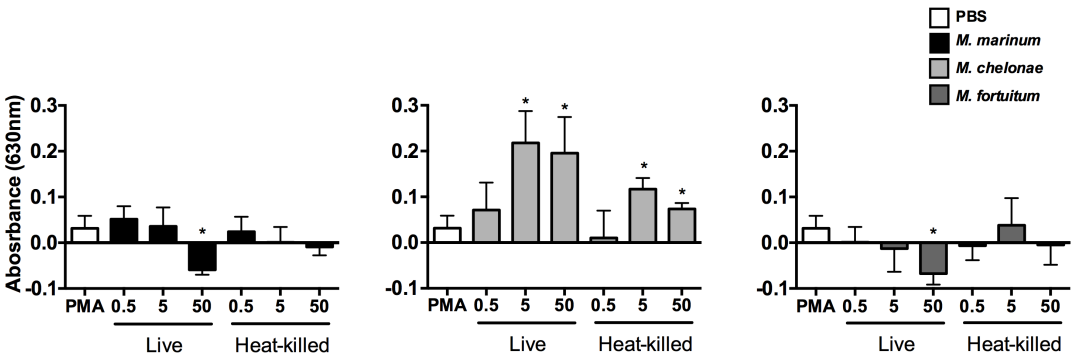

Figure 6.5. Respiratory burst of primary kidney macrophages following exposure to mycobacterial pathogens. Cultures were incubated with 0.5:1 and 5:1 and 50:1 live or heat-killed bacteria for 18 hours and the ROI response was then induced with PMA (100 ng/mL). Cells were obtained from day 4 primary kidney macrophage cultures from five individual fish (n=5). Statistical analysis was performed by one-way ANOVA followed by a Dunnett's test. (*) indicates significant difference from control cells ($P < 0.05$).

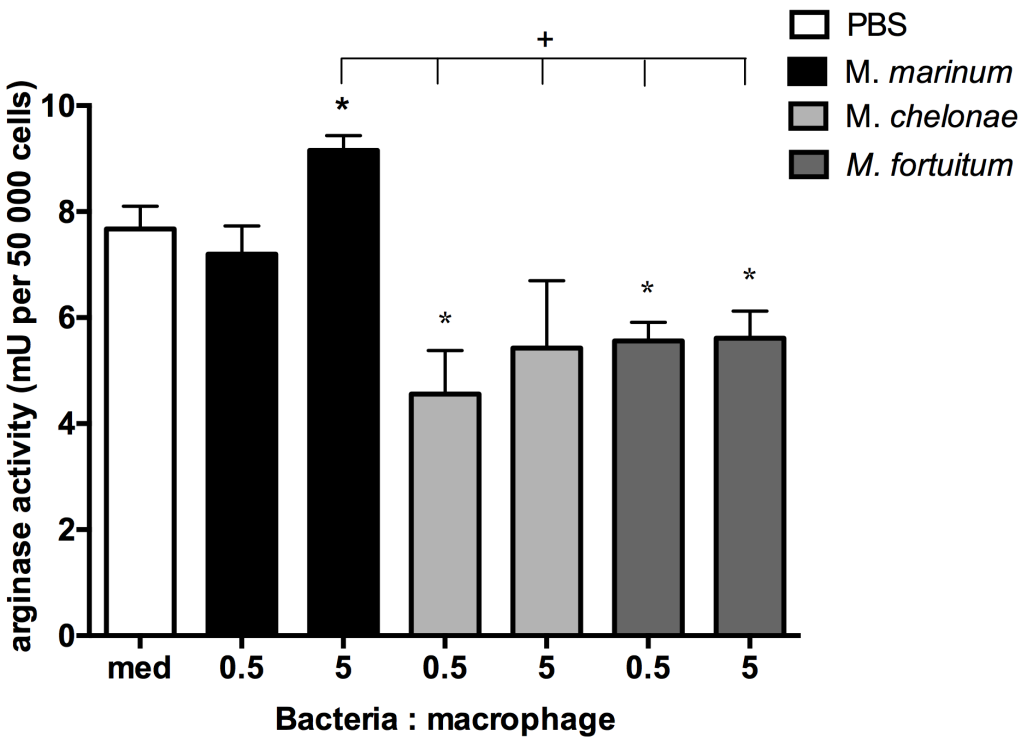

Figure 6.6. Arginase activity in primary kidney macrophages following exposure to mycobacterial pathogens. Day 6 primary kidney macrophages (5×10^5) were untreated or stimulated with 0.5:1 or 5:1 *M.* marinum, *M. chelonae* or *M fortuitum* for 18 h at 20 °C in triplicate. Data are representative of four individual fish, shown as mean ± SEM (n = 4). Statistical analysis was performed using one-way ANOVA followed by a Tukey's post hoc test. Significant differences from control samples are denoted using an asterisk (*) and (+) above lines denotes a significant difference indicated by the lines ($P < 0.05$).

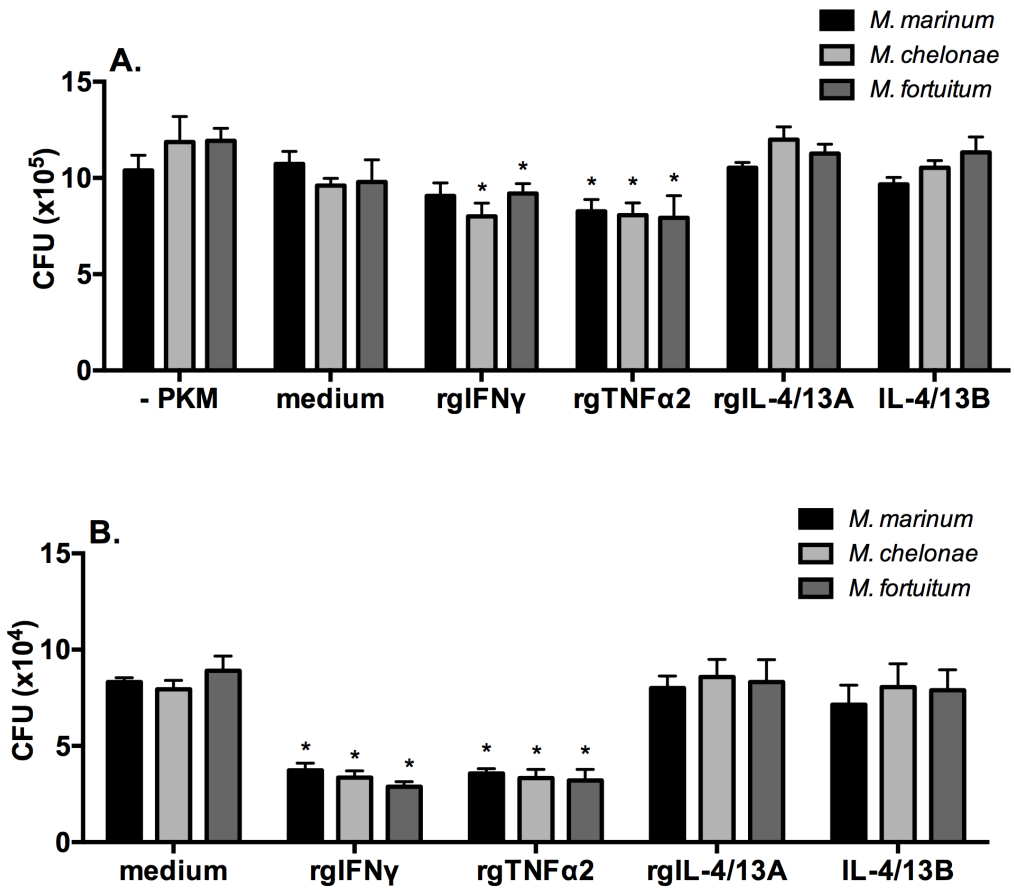

Figure 6.7. Killing of mycobacteria by cytokine stimulated macrophages. (A) Mycobacteria was incubated in media alone or with PKMs for 2 hours at a MOI of 5:1. Cells were then stimulated with the recombinant cytokines shown and incubated for 4 h. Cells were lysed, serially diluted and plated on Middlebrook 7H10 to determine viable CFUs. (B) Intracellular survival of mycobacteria in cytokine stimulated macrophages. Macrophages were incubated with mycobacteria for 3 hours and were washed 3x in complete MGFL to remove cell-free bacteria. Cells were stimulated with recombinant cytokines and incubated for 3 h. Cells were lysed, serially diluted and plated on Middlebrook 7H10 to determine viable CFUs. Macrophages were derived from individual fish (n=5). Statistical analysis was performed by one-way ANOVA followed by a Dunnet's post hoc test, and the results were deemed significant at $P < 0.05$. Significant differences from control samples are denoted using an asterisk (*).

Chapter VII: Characterization and functional analysis of the goldfish (*Carassius auratus* L.) interleukin 4/13[4]

7.0 Introduction

IL-4 is a highly pleiotropic type 2 cytokine with diverse roles in immune regulation and homeostasis. Several cell types produce IL-4 including CD4+ T cells [471], CD8+ T cells [472], mast cells [473], basophils [472], eosinophils [474], NKT cells [475] and macrophages [470] in response to specific receptor-mediated activation. Functionally, IL-4 is well defined as a central cytokine in mediating the Th2 phenotype in lymphocytes, promoting differentiation into cells capable of producing other type 2 cytokines [476], in addition to suppression of IFNγ-producing CD4+ (Th1) cells [477], [478]. Along with altering the Th1/Th2 balance, IL-4 can influence the activation status of macrophages, antagonizing classically activated macrophage and promoting a 'pro-heal' alternative phenotype [442]. Alternatively activated macrophages (M2-macrophages) are characterized by increased intracellular arginase activity, thereby differentially utilizing L-arginine catabolism and polarizing macrophage function [479]–[482]. Similar to IL-4, IL-13 is a type 2 cytokine with overlapping roles in alternative activation and the recognition by the IL-4Rα, although alternate receptors [e.g. IL-13Rα2] have also been identified [483], [484].

It appears likely that a single IL-4/13 gene existed in ancestral Gnathostomes, which has been duplicated in different lineages by whole genome duplications, and/or

[4] A version of this chapter has been published: Hodgkinson J. W., Fibke C, and Belosevic M. 2017 Recombinant IL-4/13A and IL-4/13B Induce Arginase Activity and down-Regulate Nitric Oxide Response of Primary Goldfish (*Carassius auratus* L.) Macrophages." *Developmental and Comparative Immunology* 67:277-384.

tandem duplication events. Functional similarities to mammalian IL-4 and IL-13 have been demonstrated for IL-4/13A and IL-4/13B with regards to their ability to promote Th2-type responses. Teleost IL-4/13A has been reported to promote B cell proliferation and differentiation *in vivo* [485], while IL-4/13B has been shown to induce B cell proliferation *in vitro* [486]. T cell activation has also been demonstrated following injection of the recombinant IL-4/13A in zebrafish [487]. Moreover, IL-4Rα has been shown to bind IL-4/13A *in vitro*, and soluble IL-4Rα interfered with IL-4/13A-mediated B cell proliferation [488]. Together, confirmation of a conserved Th2-inducing role of IL-4/13 paralogues has been relatively well defined. However, the role of IL-4/13A and IL-4/13B in alternative activation of teleost macrophages has yet to be shown, although an increase in arginase activity, a putative marker for M2 macrophages has been reported in cyclic adenosine monophosphate (cAMP) stimulated macrophages [489]. M2 macrophage polarization has also been demonstrated in carp following *Trypanosoma carrassi* challenge [356], [384], [490].

This chapter describes the identification and functional characterization of goldfish IL-4/13A and IL-4/13B. To date, the role of IL-4/13A and IL-4/13B with regards to macrophage polarization has not been examined, and characterization of the effects on macrophage plasticity is required to understand the scope of macrophage activation with regards to mycobacterial challenge in teleosts, and to gain evolutionary perspective. The main objectives of this chapter were to (1) identify IL-4/13A and IL-4/13B transcripts, (2) assess the distribution of IL-4/13A and IL-4/13B mRNA in goldfish tissues and immune cells in response to pathogens, (3) determine the effects of recombinant IL-4/13A and IL-4/13B forms on arginase

mRNA levels and enzyme activity and (4) determine the effects of IL-4/13A and IL-4/13B on the IFNγ-induced nitric oxide production in goldfish macrophages.

7.1 Results

7.1.1 Sequence analysis and characterization of gfIL-4/13A and gfIL-4/13B

The coding sequence for gfIL-4/13A was cloned by PCR amplification and consisted of a 414 bp ORF encoding a polypeptide of 133 amino acids (Fig. 7.1). The predicted molecular mass and isoelectric point were determined to be 15.4 KDa and 6.05, respectively, and a signal peptide was predicted (Signal P 4.1). The predicted amino acid sequences of gfIL-4/13A contained an IL-4/13 domain (SMART). The coding sequence for gfIL-4/13B consisted of a 408 bp ORF encoding a polypeptide of 131 amino acids (Fig. 7.1). IL-4/13B was predicted to be 15.3 KDa with an isoelectric point of 6.50. Similar to gfIL-4/13A, an IL-4/13 domain was predicted based on the secondary structure of the polypeptide (SMART), as well as a signal peptide (SignalP 4.1) (Fig. 7.2). Phylogenetic analysis of IL-4/13A and IL-4/13B indicated the closest relationship of orthologous carp molecules (*Cyprinus carpio*) (Fig. 7.2). IL-4/13A and IL-4/13B showed 19% amino acid similarity towards each other. Coding sequences of goldfish IL-4/13A and Il-4/13B were submitted to GenBank under the accession numbers KX574594 and KX574595, respectively (Table 3.7).

7.1.2 Analysis of gfIL-4/13A and gfIL-4/13B expression in normal goldfish tissues and immune cells

To determine the distribution and mRNA levels of goldfish IL-4/13A and IL-4/13B in healthy goldfish tissues, the gill, liver, muscle, intestine, kidney, brain, spleen, and heart of five individual fish were harvested for expression analysis by quantitative PCR. The levels of relative mRNA expression ranged from ~1.5 and 25-fold differences relative to the reference tissue, gill. Both IL-4/13A and IL-4/13B were ubiquitously expressed through examined tissues and were in relatively similar expression patterns. Highest expression levels were observed in heart, spleen and brain tissue, while lowest levels were in gill, liver and muscle (Fig 7.3A and B).

To examine IL-4/13A and IL-4/13B expression in immune cells, isolation and cultivation of neutrophils, peripheral blood leukocytes, splenocytes, monocytes and macrophages was performed. The mRNA levels of relative mRNA expression ranged from ~5.5 to 400-fold differences as compared to goldfish kidney neutrophils, which was the reference tissue. Highest observed expression in the immune cells samples were in kidney macrophages and monocytes, followed by splenocytes, peripheral blood leukocytes, and kidney neutrophils (Fig. 7.3B).

7.1.3 Recombinant goldfish IL-4/13A and IL-4/13B purification

The open reading frame of IL-4/13A and IL-4/13B without the signal peptide was cloned into pET SUMO vector. Following protein purification both recombinant molecules were run on SDS-PAGE gel and stained with Commassie brilliant blue to assess relative purity (Fig. 7.4 *left*). Additionally, IL-4/13A and IL-4/13B were run on

SDS-PAGE, transferred to nitrocellulose and blotted with an anti-His antibody (Fig. 7.4 *right*). The predicted molecular weight of each recombinant with the attached SUMO and Hisx6 tag was ~28.3 and 28.4 KDa, corresponding to the observed MW. Mass spectrometry analysis of the ~30 KDa band identified each protein as most similar to goldfish IL-4-like proteins when compared against the NCBI database and matching the generated peptides with the goldfish IL-4/13 predicted amino acid sequence.

7.1.4 Goldfish IL-4/13A and IL-4/13B increase expression levels of arginase-2 in primary kidney macrophages

To investigate the effect of IL-4/13A and IL-4/13B on activation state, we measured the expression of arginase mRNA (arginase-1 and arginase-2) following treatment of recombinant IL-4/13A and IL-4/13B. In the presence of 50 ng/mL of either rgfIL-4/13A or rgIL-4/14B, increases in arginase-2, but not arginase-1 expression levels were observed at all time points assayed ($P<0.05$; one-way ANOVA) (Fig. 7.5). Equivalent concentrations of rgIFNγ showed no ability to increase either arginase-1 or arginase-2 at any of the assayed time points.

7.1.5 Recombinant gfIL-4/13A and gfIL-4/13B induced arginase activity in primary kidney macrophages

To investigate the effect of IL-4/13A and IL-4/13B on activation state, we measured the expression of arginase mRNA (arginase-1 and arginase-2) following treatment of recombinant IL-4/13A and IL-4/13B. Duplicate samples were run using

day 7 macrophages cultivated from 2 individual fish, and duplicate experiments were performed (n=4). $50x10^5$ cells were seeded into duplicate wells and the treatments consisted of PBS in media alone, cAMP, shown previously to induce arginase activity in carp macrophages; [356], and a range of either rgIL-4/13A or rgIL-4/13B. Cells were incubated for 18 hours due to preliminary mRNA expression experiments. PKM cultures with rgIL-4/13A exposure showed a dose dependant increase in arginase activity (50 ng/mL to 5 pg/mL), significantly more than PBS samples (Fig. 7.6A). Recombinant IL-4/13B also induced arginase activity at 50 ng/mL and 5ng/mL, but lower concentrations were unchanged compared with control (Fig. 7.6B).

7.1.6 Goldfish recombinant IL-4/13A and IL-4/13B down-regulate the IFNγ-induced macrophage nitric oxide response

Recombinant goldfish IFNγ has been previously shown to potently activate reactive nitrogen species in goldfish macrophages [352]. Due to an observed increase of arginase activity, it was postulated that rgfIL-4/13A and/or rgfIL-4/13B might affect macrophage nitric oxide production. To test this, day 7 primary head kidney macrophages were treated with varying concentrations of rgfIL-4/13A or rgfIL-4/13B. 50ng/mL rgfIFNγ was added to the cultures 18 hours later and incubated subsequently for 48 hrs. Treatment of goldfish macrophages with rgfIFNγ alone (positive control) induced significant production of nitrite. The pre-treatment with either rgfIL-4/13A or rfgIL-4/13B decreased nitrite production of rgfIFNγ-activated macrophages in a dose-dependent manner (Fig. 7.7).

7.2 Discussion:

In this chapter, I described the cloning and characterized goldfish IL-4/13A and IL-4/13B effects on goldfish macrophage activation. Distinct subsets of macrophages have been well characterized in mammalian models, which generate remarkable heterogeneity based largely on the Th1-Th2 cytokines IFNγ and IL-4 [441]. In teleosts, IFNγ-stimulated classically activated macrophages have been well characterized in a wide range of models [491], [492]. By contrast, alternatively activated macrophages have been far less studied, and IL-4/IL-13 induction of an M2 phenotype (sometimes denoted M2a macrophages in mammalian literature) remains to be fully elucidated, although the intrinsic cellular structures for alterative activation of macrophages have been recognized in fish. For example, arginase activity in carp macrophages was induced by cAMP treatment and after exposure to pathogens [356], [490], closely resembling the paradigm described for mammalian macrophages. Recent transcriptome analysis of cAMP stimulated carp macrophages showed that arginase-2 has been upregulated, further confirming the existence of a M1/M2 heterogeneity of macrophages in teleosts [489]. In this study, the functional capacity of IL-4/13 paralogs to alter the activation states of primary goldfish macrophages was explored.

Goldfish IL-4/13A and IL-4/13B share the highest similarity with those of common carp. Both molecules contain predicted IL-4/IL-13 motifs akin to those of the mammalian IL-4 or IL-13 genes, although there appears to be relatively low amino acid sequence conservation of IL-4/13A (~16%) and IL-4/13B (~18%) when compared to murine IL-4. The inherent lack of conservation made it initially difficult

to find IL-4/13 in fish genomes [493], relying on syntenic regions to find IL-4/13 loci. Following the initial reports of IL-4/13 in pufferfish [494] IL-4/13 genes have been identified in medaka [493], zebrafish [488], salmon [495], common carp [486], trout [496], fugu [497], and now in the goldfish. Interestingly, IL-4Rα, IL-13Rα1, IL-13Rα2 and γC receptors are present in lower vertebrates [498], and despite the relative lack of conservation of the IL-4/13 ligands, Zhu and co-workers, demonstrated that IL-4Rα specifically recognized IL-4/13A in zebrafish [488]. However, the comprehensive mechanisms of receptor-ligand interactions remain to be fully elucidated, especially with regards to the identity of potential binding partners of IL-4/13B.

The constitutive expression of IL-4/13A and IL-4/13B in goldfish tissues is similar for IL-4/13A and IL-4/13B, with highest mRNA levels observed in kidney and spleen. Higher expression of IL-4/13A and IL-4/13B in goldfish immune organs support the demonstrated effects of teleost IL-4/13 recombinants on different myeloid and lymphoid cell populations. Indeed, functional similarities to mammalian IL-4 and IL-13 have been reported for teleost IL-4/13A and IL-4/13B with regards to their ability to promote B and T cell differentiation and proliferation [485]–[488].

Heterogeneity of macrophage activation was first demonstrated in murine macrophages, where IL-4 showed potent activation of macrophage mannose receptor (MMR) and MHC class 2 antigen expression, distinct from classical activation by IFNγ marked by nitrogen radicals [499]. Subsequently, it was shown that up-regulation of arginase by IL-4/IL-13-dependant signalling results in differential metabolism of L-arginine leading to polyamine and proline biosynthesis, promoting

collagen production and tissue repair [500], [501]. Fish macrophages have also been shown to possess inducible arginase expression and arginase activity [356], [384], [489]. In this report, we reported that recombinant gfIL-4/13A and gfIL-4/13B induced significant arginase activity in goldfish macrophages in a dose-dependent manner. In addition, an increase in arginase-2 mRNA levels were observed following the treatment with both IL-4/13 recombinants, whereas arginase-1 mRNA levels remained unchanged compared to PBS controls. That IL-4/13A and IL-4/13B induced alternative activation of macrophages is indicative of a conserved function of these cytokines in teleosts.

The inhibitory function of arginase activity on nitric oxide response of macrophages has been documented for mammalian macrophages, where both arginase and iNOS competitively utilize the common substrate L-arginine [502], [503]. Although this axis of metabolism has been reported in carp macrophages [490], the IFNγ-IL-4/13 regulatory paradigm has not been established in teleosts. To test whether the induction of arginase activity affected IFNγ-induced nitric oxide production, we pre-treated macrophage cultures with IL-4/13A or IL-4/13B prior to addition of goldfish recombinant IFNγ. I observed a dose dependent reduction in nitrite production after pre-treatment of macrophages with IL-4/13A and IL-4/13B, indicating that, like in mammals, IL-4/13 regulates IFNγ-induced macrophage antimicrobial functions.

The results of this study demonstrate a conserved role for IL-4/IL-13 in alternatively activating macrophages in teleost fish, the first report of its kind,

confirming the importance functional regulation and plasticity of macrophage activation in higher vertebrates.

gfIL-4/13A

```
1       atgaagactatactactgctcacttttgctgtcttggtctccagcattcctttggaaaag
         M  K  T  I  L  L  L  T  F  A  V  L  V  S  S  I  P  L  E  K
61      agcgataagaaattattgggagagcttattgatgagctaaacagtgaagtgaaaagattg
         S  D  K  K  L  L  G  E  L  I  D  E  L  N  S  E  V  K  R  L
121     tcagagaataatgagacaaatgaaacctacgtgaccgacctgcaaatgcataactgtaag
         S  E  N  N  E  T  N  E  T  Y  V  T  D  L  Q  M  H  N  C  K
181     agtgtgtttttctgccaggccgaacaagaactgttaaaggaggtttctggacggtctggt
         S  V  F  F  C  Q  A  E  Q  E  L  L  K  E  V  S  G  R  S  G
241     gccgaatttgaccatttccgtactgacaagaaactaatgaggaatttacacaagtataac
         A  E  F  D  H  F  R  T  D  K  K  L  M  R  N  L  H  K  Y  N
301     cagcaccatgtggaaacctgcaaacctgctcttaaagacgaagaaatgactctgcttgat
         Q  H  H  V  E  T  C  K  P  A  L  K  D  E  E  M  T  L  L  D
361     ttcttgaaaaatctcttgacatgtgctaggcatgtgaatggccaaaagtcataa
         F  L  K  N  L  L  T  C  A  R  H  V  N  G  Q  K  S  -
```

gfIL-4/13B

```
1       atgaggactttcatgctgttggtgctggcgattgtagccgttactgggttgaaacataaa
         M  R  T  F  M  L  L  V  L  A  I  V  A  V  T  G  L  K  H  K
61      gcgggaccaatcctcctaatggaaatcatagatgatgtgaagaagatcttgaatcagagt
         A  G  P  I  L  L  M  E  I  I  D  D  V  K  K  I  L  N  Q  S
121     tcagcggtgaacctcaaccaatttgtaagagatgtatttcctccagtgggctgctcggag
         S  A  V  N  L  N  Q  F  V  R  D  V  F  P  P  V  G  C  S  E
181     gaacacatttgccaagcagcaatggtcatgatgaacacagagttaagctacagcatggta
         E  H  I  C  Q  A  A  M  V  M  M  N  T  E  L  S  Y  S  M  V
241     cacagaggactgtttgcctatgttaattattcagggcaccttcaatgcaacgttacagct
         H  R  G  L  F  A  Y  V  N  Y  S  G  H  L  Q  C  N  V  T  A
301     tcagaagaacacagaatggatgttttcctggaagaaatcaaaaattgctgcaaagaacaa
         S  E  E  H  R  M  D  V  F  L  E  E  I  K  N  C  C  K  E  Q
361     ttctttaaactcttaaagcaaccagatgagaaaaaacaagtgaattgactctagaattac
         F  F  K  L  L  K  Q  P  D  E  K  K  Q  V  N  -
421     tgcttatgatcccaatactatatttttgtattccatttaatattaaattatttttttatta
481     aatgttttctattgtttatatttattgtatttaggaataaatattttctgaccaaaaaaa
484     aaaaaaaaaaaa
```

Figure 7.1. Nucleotide and amino acid sequence of goldfish IL-4/13A and IL-4/13B. Darker shading on amino acid regions corresponds to signal peptide domains (Signal 4.1) and lighter shading on amino acids corresponds to regions containing IL-4-like domains (SMART).

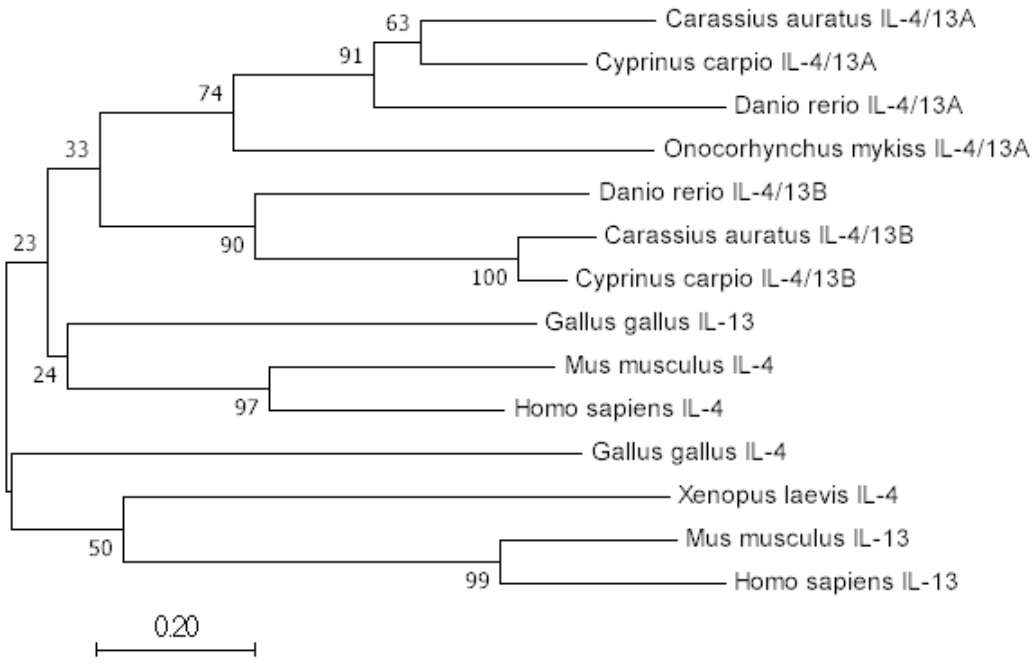

Figure 7.2. Evolutionary relationships of goldfish IL-4/13A and IL-4/13B
The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Bootstrap values are shown for clades (in 10 000 sampling replicates). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7.

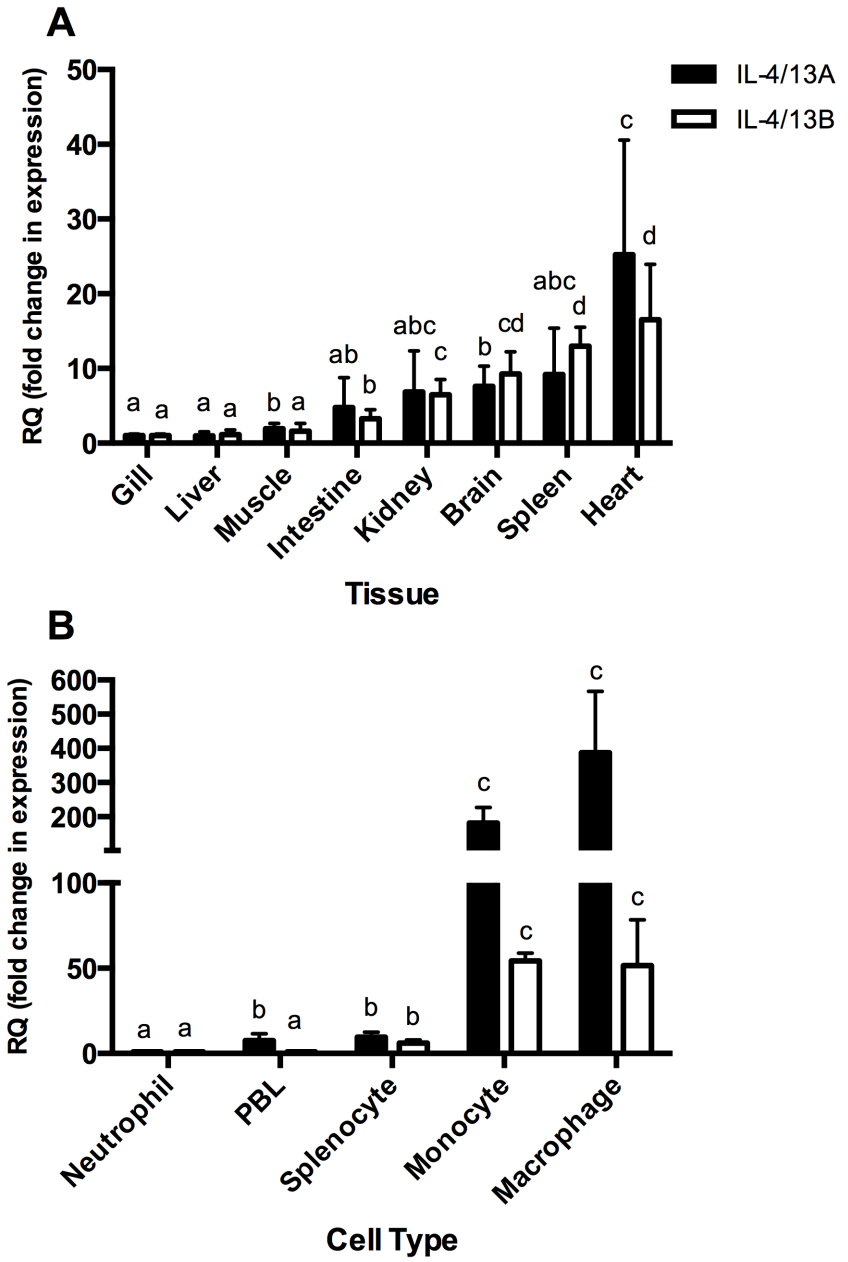

Figure 7.3. Quantitative expression analysis of goldfish IL-4/13A and IL-4/13B in tissues and immune cell populations in healthy fish. A) goldfish IL-4/13A and IL-4/13B tissue expression. IL-4/13A and IL-4/13B expression levels are relative to endogenous control gene, elongation factor-1 alpha (EF-1α) All results were normalized against the gill which had the relative lowest expression level. Tissues were analyzed from five individual fish (n = 5). B) IL-4/13A and IL-4/13B expression levels in immune cell populations from five individual fish (n = 5). Results were normalized against resting kidney neutrophils which had the lowest expression level. Statistical analysis was performed using one-way ANOVA followed by a Tukey post hoc test. Different letters above bars denote statistical difference ($p < 0.05$). The same letter infers no statistical difference.

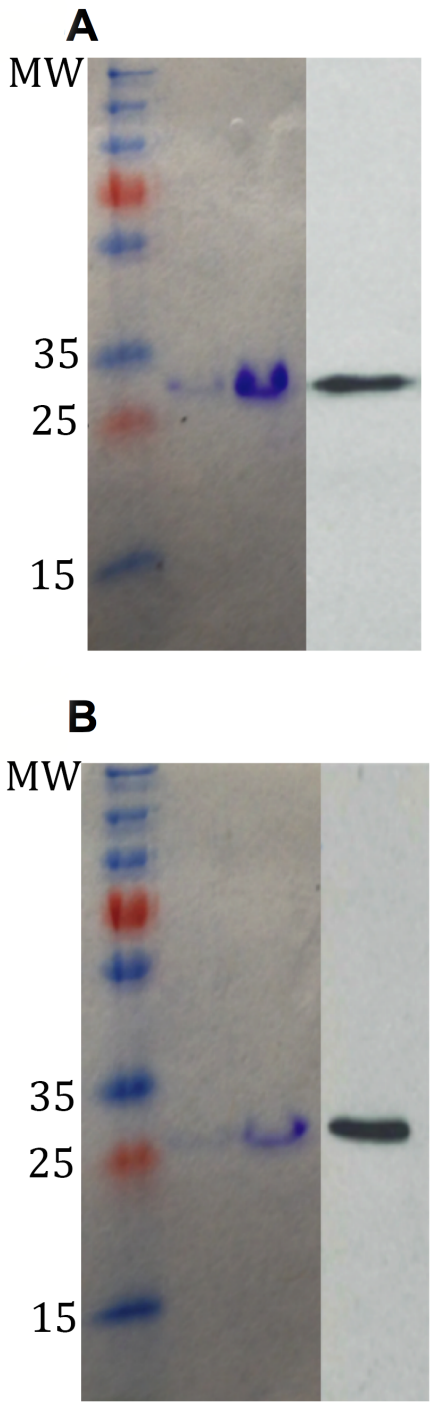

Figure 7.4. Commassie brilliant blue staining and Western blot of purified IL-4/13A and IL-4/13B elutions. Recombinant IL-4/13A (A) and IL-4/13B (B) was purified and dialyzed against 1X PBS. SDS-PAGE gel was run, followed by Commassie (left) and Western blot (right). MW – molecular weight markers.

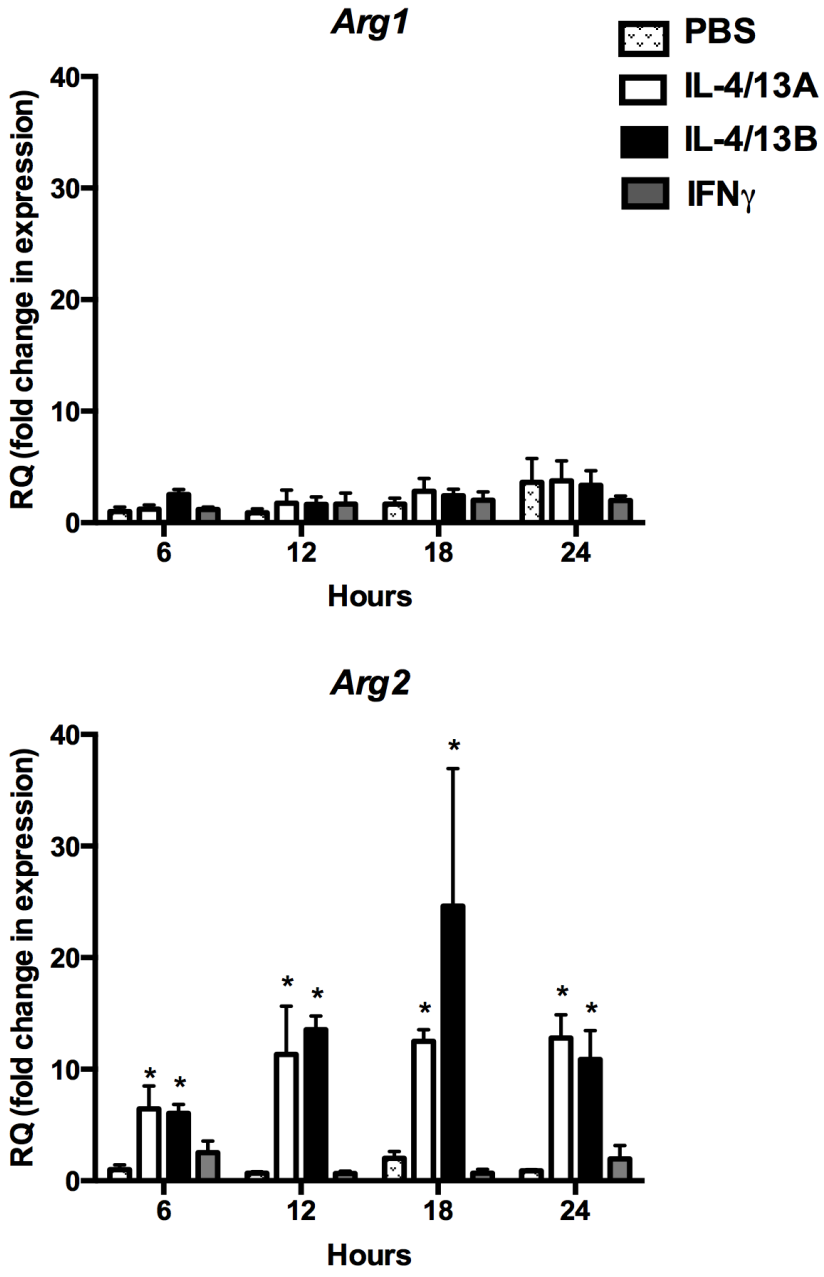

Figure 7.5. Recombinant goldfish IL-4/13A and IL-4/13B increase arginase-2 expression in goldfish macrophages. Day 7 primary kidney macrophages (1×10^6) were untreated or stimulated with either 50 ng/mL IL-4/13A, IL-4/13B or IFNγ. Cells were washed and collected at 6, 12, 18 and 24 h post exposure. Expression levels of arginase-1 and arginase-2 are relative to untreated controls at 3 h. Cells were analyzed from four individual fish (n = 4). Statistical analysis was performed using one-way ANOVA followed by a Dunnett's test. Asterisks (*) above bars denote statistical difference from timed control ($p < 0.05$).

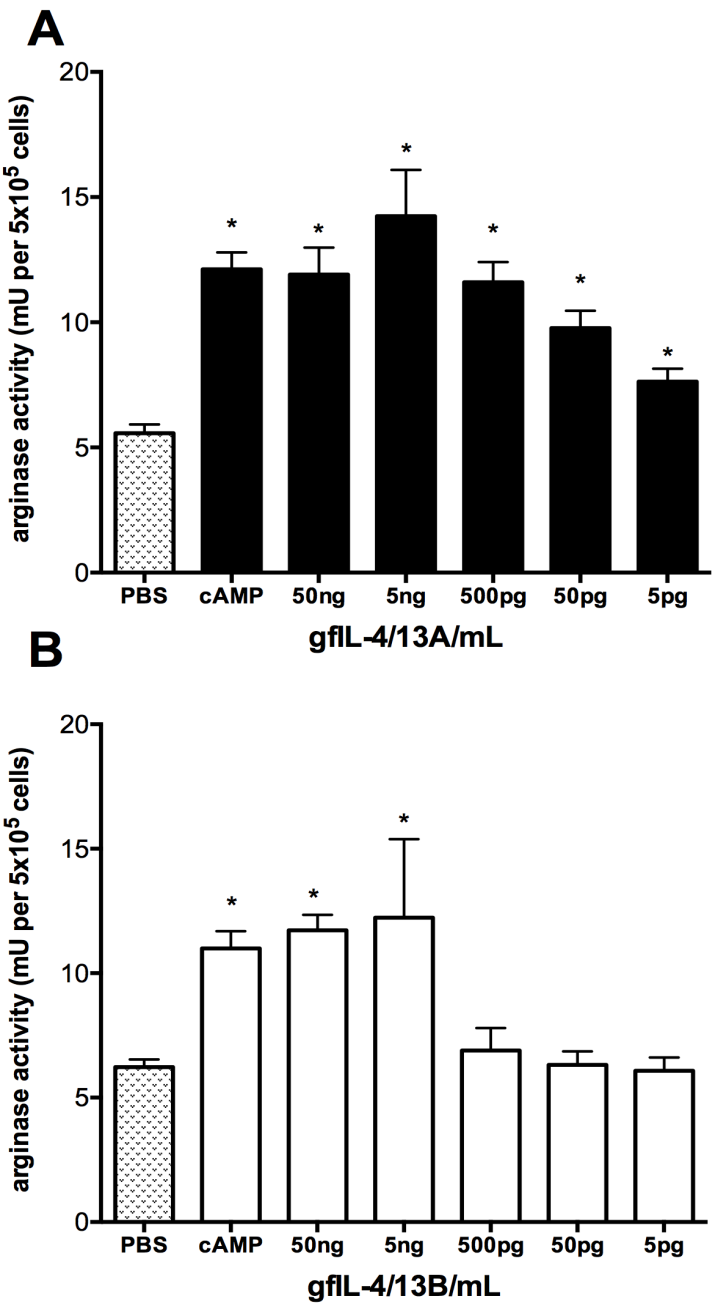

Figure 7.6. Recombinant goldfish IL-4/13A and IL-4/13B enhances arginase activity in goldfish macrophages. Day 7 primary kidney macrophages (5×10^5) were untreated or stimulated with cAMP, rgIL-4/13A and rgIL-4/13B in the concentrations shown for 18 h at 20 °C in triplicate. Data are representative of four individual fish, shown as mean ± SEM (n = 4). Statistical analysis was performed using one-way ANOVA followed by a Dunnett's test. Significant differences from control samples are denoted using an asterisk (*).

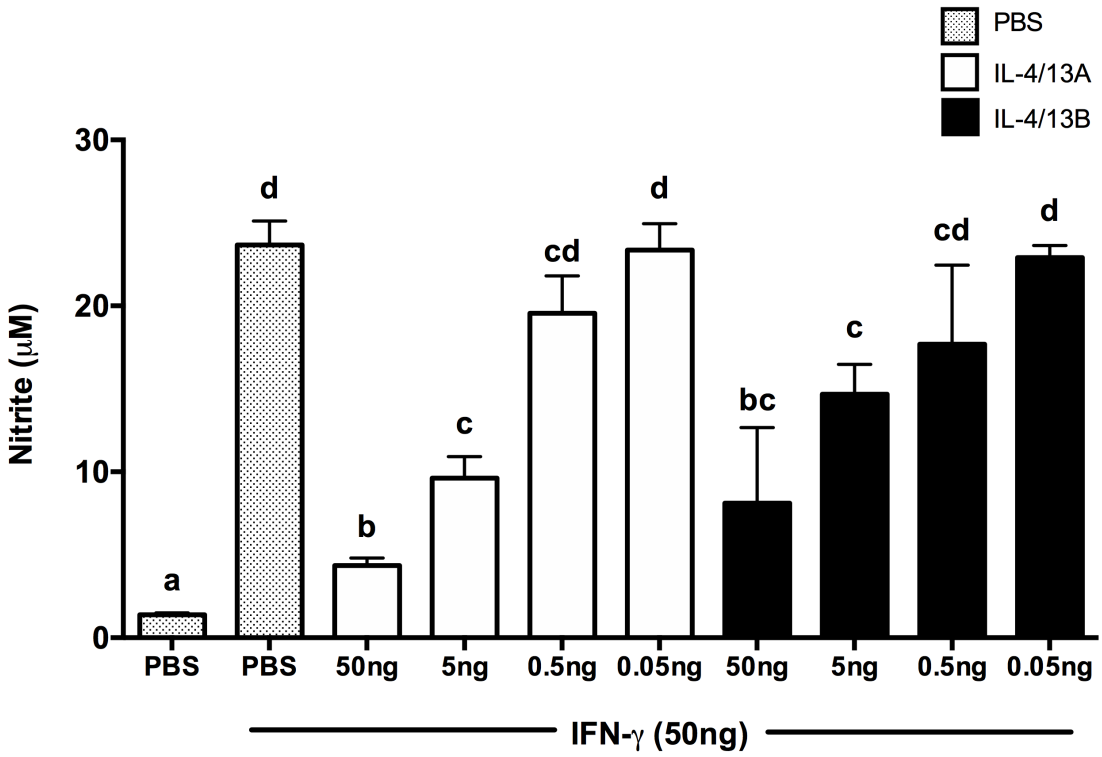

Figure 7.7. Recombinant goldfish IL-4/13A and IL-4/13B counteract the nitric oxide priming of recombinant goldfish IFNγ in primary kidney macrophages. Day 7 primary kidney macrophage cultures were either left untreated or primed with IL-4/13A and IL-4/13B in the concentrations shown and incubated overnight. The following day, PKMs were stimulated with 50 ng/mL IFNγ and incubated for 48 h. Supernatants were tested for nitrite levels with the Greiss reaction. Data are representative of five individual fish, shown as mean ± SEM (n = 5). Statistical analysis was performed using one-way ANOVA followed by a Tukey's post hoc test. Significant differences are denoted by dissimilar character.

Chapter VIII: General discussion

8.0 Introduction

Orchestration of an effective immune response is a highly complex, tightly regulated enterprise, that results in tolerance of innocuous or commensal microbes and selective destruction of infiltrating pathogens, while maintaining the integrity of normal tissue function. Following inflammation and the corresponding associated tissue damage due to the infection, tissue repair and restoration of homeostasis is imperative. Due to the relatively nimble evolutionary tendencies of pathogenic microbes, a number of different host defense mechanisms have been evolved by vertebrates to counter pathogenic insults. Particularly well adapted pathogens, such as those of the genus *Mycobacterium*, have numerous evasion mechanisms to combat host defense, including resisting effector phagocyte function and evolving strategies to reside within the immune cells [330], [504], [505].

While the birth of modern immunology has given rise to enumerable public health victories including eradication of small pox and near eradication of a number of other serious human pathogens, *M. tuberculosis* remains prevalent across the globe. The first isolation of 'tubercle bacillus' was famously demonstrated by Robert Koch in 1882, and since that time, mycobacterial pathogens have been one of the most studied pathogens in history [12], [506]. As of early 2018, research articles with 'tuberculosis' in the article returned over 245,000 PubMed search results, but despite the ongoing efforts, only a partially effective vaccine has been generated almost a century ago by multiple passage of *M. bovis* by Albert Calmette and Camille Guérin:

the bacillus Calmette-Guérin (BCG) vaccine. In children, BCG is quite effective at protecting against miliary (TB), a serious disease resulting in 100% mortality if untreated [507]. However, BCG is ineffective in protecting against adult pulmonary TB, which is the most common form of disease [508]. Thus, intellectual advancement has yet to fully elucidate the factors behind persistence of mycobacterial pathogens in vertebrate hosts, and continues to represent significant public, economic and ecological concern.

This dissertation focuses on the characterizing of the immune response of adult goldfish during infection with natural mycobacterial pathogens *M. marinum*, *M. chelonae* and *M. fortuitum*. For over two decades, *M. marinum* has served as a surrogate pathogen for the study of *M. tuberculosis*, owing to genetic relatedness, less stringent biosafety requirements and highly similar pathology, especially in natural fish models. Despite the lack of lungs, the most common site of infection for *M. tuberculosis*, morphologically similar granuloma formation occurs in body tissues of fish and it has been demonstrated to require homologous immune components, including cytokines, lipid mediators and immune cells [62].

From a pathogen perspective, *M. marinum* possesses highly conserved virulence factors and evasion techniques that are similar to those of *M. tuberculosis*. The overwhelming majority of studies of *M. marinum* in natural fish hosts have been done using the zebrafish embryo model, where optical transparency and genetic tractability allow for visualization of infection events at a cellular level. Research on the formation and function of granulomas (generated rapidly in zebrafish embryos), has contributed significantly to our understanding of the formation and the

mechanisms of granuloma formation in infected hosts. Because this infection model is developmentally immature, the vast amount of research is the study of the innate immune system in isolation, where animals are hyper-susceptible to infection and granuloma formation [342]. Since the majority of natural mycobacterial infections occur and persist by evading fully developed immune systems, the study in adult models both expands and validates findings derived from embryonic models.

In addition to the study of *in vivo* infections, the goldfish provides a unique *in vitro* model for the study of primary macrophages and neutrophils derived from the kidney. The adult teleost kidney serves as the main site of hematopoiesis, akin to the mammalian bone marrow. Soluble molecules released in response to infection mobilize progenitor pools, where they are recruited into the vasculature and commit/polarize in response to local stimuli. The Belosevic group has characterized the development of goldfish kidney derived leukocytes containing pluripotent cell populations. In the absence of exogenous stimulation, isolated kidney leukocytes selectively differentiate into myeloid progenitor cells *in vitro*, generating endogenous growth factors that govern cell differentiation [345], [509], [510]. During *in vitro* development, primary kidney cultures are comprised of at least three distinct populations, including progenitor cells, monocytes, and macrophages, which have been characterized by morphology, immunohistochemistry and function. In earlier phases of development, cultures are composed primarily of monocytes, while macrophages are the predominant cell type in later stage cultures. Characterization of these immune cell populations provides for a potent model of primary cell interactions, which were utilized in the investigations of this thesis. In addition to the

characterization of immune cell populations, the Belosevic group has characterized a number of soluble mediators of inflammation and resolution, including gene duplicates TNF-α/TNF-α2, IFN-γ/IFN-γrel and IL-1β1/IL-1β2, IL-10, M-CSF and their cognate receptors. Cytokine and growth factor function in inflammation and resolution is largely similar to orthologous molecules in mammals with the overlapping roles of gene duplicates [352], [511]–[514].

8.1 Use of a fish model system for the study of host defense to mycobacterial pathogens

Animal models play an integral role in the scientific process, providing similar physiology to advance the understanding of host defence. With regards to modeling mycobacterial infectious disease, a number of organisms have emerged to study immune function due to a relatively imperfect recapitulation of disease in humans, each with particular advantages and disadvantages. *In vitro* models systems for *M. tuberculosis* have largely relied on the murine model system [58], [130], although their natural resistance to mycobacterial infections make them difficult to interpret. Use of human cell lines typically requires differentiation with phorbol esters or LPS prior to infection studies which also complicates findings. Given that *M. tuberculosis* is a serious human pathogen, adding complications for biosafety, and the extremely slow growth rate compounds the difficulty of studying host pathogen interactions in the laboratory.

Similarities between *M. tuberculosis* and *M. marinum* make the later a popular alternative to study host-pathogen interactions because it is a safer,

genetically amenable and faster growing organism. The zebrafish has become the model of choice as discussed in Chapter 2 of the thesis, where embryonic transparency and genetic tractability allow for tweaking and visualization during infection. However, *in vitro* studies in zebrafish are problematic due to their relatively small size and cell numbers. Instead, cultivation and characterization of goldfish primary cells for the study of these interactions *in vitro*, as well as *ex vivo*, I believe, provides a complementary system of immune function analysis. Decades of research in characterizing goldfish growth factors and cytokines, including those generated in this thesis allows for the study of the functional fate of cells infected with mycobacteria. Overall, the breadth of our understanding of teleost macrophage function has been expanded in this thesis, and the ability of *M. marinum* to circumvent host defence provides the framework for future research in this area.

While zebrafish will no doubt continue to lead the way for *in vivo* modelling of mycobacterial infection, the results generated using an embryonic system will likely need to be validated using adult teleost species that possess a fully developed immune system, such as the goldfish or carp. Similar to the inability to generate *in vitro* cell cultures, *ex vivo* analysis of the functional state of immune cells is impractical due to the size of zebrafish. Utilization of goldfish, and the infection models generated in this thesis will aid in complementing established systems of study as we continue to refine or understanding of the complicated relationship between host and mycobacterial pathogen.

8.2 Overview of findings

The main goal of my thesis was to elucidate functional differences in immune outcomes using a comparison of natural mycobacterial pathogens. Specifically, I utilized *in vitro* macrophage and neutrophil interactions to assess effector functions and cytokine expression levels. Additionally, I observed the outcome of infections in adult goldfish, with regards to cytokine expression levels and corresponding bacteremia in tissues.

Although the central mediators of inflammation were previously characterized in goldfish, the role of IL-4/13A and IL-4/13B had not yet been addressed with regards to macrophage effector function. Given that macrophage effector function was a central focus of my thesis, I decided to generate recombinant goldfish (rg) IL-4/13A and IL-4/13B to assess their capacities to modulate macrophage function in order to generate a more comprehensive profile of phenotypic potential (Chapter 4). I

observed that both rgIL-4/13A and rgIL-4/13B were capable of eliciting arginase activity in primary macrophages as well as arginase-2 mRNA expression, which was previously shown to be induced in macrophages isolated from *T. carrassi* infected carp [229]. Additionally, I observed inhibition of IFN-γ-induced NO activity in macrophages that were co-treated with rgIL-4/13A and rgIL-4/13B, demonstrating a conservation of function that is seen in IL-4 and IL-13 in mammals [230]. This suggests that the two IL-4/13 isoforms have largely overlapping roles in influencing the metabolism of L-arginine and promoting an M2 macrophage phenotype. Although it is not fully elucidated which receptor each isoform binds, recombinant zebrafish IL-4/13A was shown to bind the zebrafish IL-4Rα chain [515]. In mammals, IL-4 and IL-13 bind receptor heterodimers comprised of three subunits; IL-4Rα, IL-13Rα1 and a common γ-chain, γC. IL-4 interacts with the a IL-4Rα and γC heterodimer (type I receptor) and a IL-4Rα and IL-13Rα1 heterodimer (type II receptor), whereas IL-13 interacts only with the type II receptor [516]. It is likely that goldfish IL-4/13A and IL-4/13B signal through these subunit combinations, as each has been identified throughout the jawed vertebrates and duplicates of each have been cloned in salmonids [517], [518].

I utilized goldfish kidney neutrophils to assess their *in vitro* functional response to mycobacterial pathogens (Chapter 5). Goldfish neutrophils were highly chemotactic towards each of mycobacterial pathogen, and readily internalized *M. marinum.* Despite controversial reports in the literature [80], [205], [412], I observed internalization of free mycobacteria by the kidney neutrophils. My work suggested that activated neutrophils were capable of killing *M. chelonae* and *M. fortuitum* but

not *M. marinum,* and that lower numbers of *M. chelonae* and *M. fortuitum* induced significant higher respiratory burst response compared to *M. marinum*. Exposure to high numbers of *M. marinum* also induced a significant respiratory burst response, however, exposure of neutrophils to equivalent numbers of *M. marinum in vitro* caused neutrophil death, likely due to excessive activation. I observed a general resistance to effector function generated by neutrophils when exposed to *M. marinum*, when compared to *M. chelonae* and *M. fortuitum*, which may result in greater persistence when bacilli are encountered in initial stages of infection, or once liberated by necrotic cell death at the level of the granuloma. I saw increase in expression levels of IL-1β1/IL-1β2 and TNFα1/TNFα2 following exposure to each pathogen, although the contribution to levels of protein produced has yet to be demonstrated in fish neutrophils.

I also utilized the established primary kidney macrophage culture system to observe functional consequences following exposure to mycobacterial pathogens (Chapter 6). I documented large increases of IL-1β1mRNA induced by all mycobacterial pathogens studied, while large increases in TNFα2 was only after exposure of macrophages to *M. chelonae* and *M. fortuitum*. Importantly, I observed no nitric oxide intermediate production at any level of exposure to *M. marinum*, but the activation of NO was observed after exposure of macrophages to either M. *chelonae* and *M. fortuitum*. IL-4/13A, IL-4/13B and arginase-2 expression was increased in *M. marinum* exposed cultures, although it is unclear which cell type in the mixed culture was responsible for these increases in expression. In mammals, it is disputed whether macrophages are capable of producing IL-4 or IL-13, but it appears

that they are likely an innate source of IL-4 in mouse macrophages [470]. Although increased in expression of these genes was observed, I am not certain whether fish macrophages produce IL-4/13A or IL-4/13B. Only *M. chelonae* exposed monocytes were capable of initiating a respiratory burst response. I observed modest increases in arginase activity in *M. marinum* exposed macrophages, while *M. chelonae* and *M. fortuitum* stimulation resulted in a significant reduction in arginase activity, which paralleled with the NO response. Activation of macrophages with recombinant IFN-γ and TNF-α2 resulted in killing of all internalized bacilli, although I observed that innate activation by bacteria alone that resulted in modest killing of *M chelonae* and *M. fortuitum*. The functional responses of phagocytes exposed to each mycobacterial pathogen is summarized in Table 8.1. A critical overall difference in innate activation of macrophages was observed following exposure to mycobacterial species, especially regarding arginase and iNOS activation levels. The early innate response to mycobacterial pathogens by resident macrophages likely serve as the impetus for activation of downstream adaptive components that may or may not be host beneficial. Due to the fact that *M. chelonae* does not possess critical virulence factors ESAT-6 secretion system-1 (ESX-1), 6 kDa early secretory antigenic target (ESAT-6), 10 kDa culture filtrate antigen (CFP-10), *p*hthiocerol *dim*ycocerosates (PDIM) and Pro-Glu (PE) and Pro-Pro-Glu (PPE) proteins, and *M. fortuitum* lacks PDIM and PE/PPE proteins [519], this may lead to the differences in functional outcomes observed (Figure 8.1)

I assessed the course of infections in adult goldfish (Chapter 7) at time points of 7, 14, 28 and 56 days post infection (dpi). *M. fortuitum* and *M. chelonae* tissue

bacteremia were reduced at every time point past 7 dpi, while *M. marinum* increased in bacterial load for the first 28 dpi, suggesting a relative lack of control of the pathogens during the acute phase of the infection. I documented early increases in pro-inflammatory cytokines (IFN-γ, IL-12p35, IL-12p40 and IL-1β1) at 7 dpi. By contrast, early and sustained increases in proinflammatory cytokines occurred in goldfish tissues infected with *M. fortuitum* and *M. chelonae*, despite this, bacteria persisted in animals throughout the observation period. *M. marinum* infections generated increases in pro-regulatory type molecules (IL-10, TGF-β and SOCS-3) at 14 dpi, which was similar to decreases in the expression of proinflammatory cytokines. Conversely, the expression of SOCS-3, a negative regulator of IFN-γ signalling, was significantly higher at every time point in *M. chelonae* infected fish. Increase in IL-10 at 56 dpi was also observed in infected fish. General decreases in pro-regulatory cytokine expression levels were observed at 7 dpi, while increases in TGF-β and IL-10 were observed at 56 dpi in *M. fortuitum*-infected fish. General increases in M2-type genes (IL-4/13A, IL-4/13-B, arginase-1 and arginase-2) were observed through the course of infection with *M. marinum*. I observed modest increases in IL-4/13A and IL-13/B through the course of infection with *M. chelonae*, and relatively sporadic regulation of these genes were seen in *M. fortuitum*-infected fish at each time point. Interestingly, iNOS expression levels were significantly enhanced in kidney leukocytes at 7 and 56 dpi in all infections.

8.3 The conservation of macrophage plasticity

The majority of our understanding of macrophage biology comes from research using mammalian models, where distinct macrophage subsets of have been characterized, including classically activated cells by IFNγ and TNFα (M1); alternatively activated cells by IL-4 and IL-13 (M2a); macrophages activated by immune complexes or apoptotic cells (M2b); and regulatory macrophages, deactivated by IL-10, TGF-β, or glucocorticoids (M2c), which culminate in the various effector subtypes, broadly described as having a "kill" or "heal" response [520]. Recent transcriptomic and proteomic analyses of macrophage populations derived by these distinct stimuli have underlined the complexities of these mechanisms at the molecular level, that control and influence the various physiological responses of macrophages.

In fish, research of macrophage plasticity has focused primarily on the phenotype comparable to an M1 activation state, which serve a critical role in inflammation and host protection. These cells rapidly kill pathogens by engulfment and production of toxic reactive intermediates [509], phagolysosomal acidification [521], and restriction of nutrient availability [63]. Furthermore, M1 macrophages are robust factories of cytokines, chemokines, and lipid mediators, which act to potentiate and fine-tune the inflammatory and adaptive immune responses. Over the same time period, comparatively little has been accomplished in characterizing alternate macrophage phenotypes, although similar deactivating roles of glucocorticoids (GC), immune complexes, IL-10 and TGF-β have been demonstrated in teleosts, suggesting conserved functions of these ligands in deactivating or aiding in the tissue repair [322], [385], [522]. The work described in this thesis advances our understanding of

the alternative activation states of teleost macrophages through the characterization of IL4/13A and IL4/13B with regards to their ability to alter arginase activity, thereby implying a conserved M2a phenotype [356], [495]. This conservation of phenotypic variants in macrophages is a testimony to the importance of these processes undergirding the orchestration of host immunity, as well as the integral contribution to repairing and restoring a normal physiological state.

8.4 Future Directions

8.4.1 Mechanisms of host defense during co-infections

Relatively little has been studied in the context of parasitic coinfection with *M. tuberculosis*, although it has been observed that pulmonary tuberculosis (PT) and some parasitic diseases can be risk factors for each other. Leishmaniasis, giardiasis, strongyloidiasis and helminthiasis have been associated with higher risk of *M. tuberculosis* infection and prognosis [523]. It has also been documented that coinfection of echinococcosis with PT shifted the immune profile from predominantly Th1 to a Th2 response [524]. The existence of alternatively activated macrophages in teleosts was first reported in *T. carassii* infected carp [229], [385]. Based on the work in this thesis and the influence of macrophage effector functions in goldfish host defense, it would be interesting to utilize the established infection models of *T. carassii* and *M. marinum* to observe the immune outcomes of during co-infections.

To investigate the effects of co-infection, it would be interesting to establish 28 dpi sub-chronic *M. marinum* infections in goldish, where bacteremia begins to

plateau, likely due to the onset of an effective Th1/M1 response. Infection at this point with *T. carassii* may influence the Th1/Th2 and M1/M2 balance in the fish, leading to lower inhibition of bacteremia, which could be measured by serial plating of homogenized tissue, compared to fish infected with *M. marinum* alone. Additionally, *ex vivo* analysis of head kidney leukocytes during *T. carassii* infection has revealed that peak arginase activity occurs 3-4 weeks post infection [385]. Establishing *T. carassii* infections corresponding to peak arginase activity and infecting with *M. marinum* may help elucidate the risk factors associated with contracting mycobacterial disease. Together, utilizing both infection models could be invaluable at modelling the risk associated with polarization of macrophage function.

8.4.2 Identification of potential host defense mechanisms in teleosts exposed to mycobacteria

Although expression studies are a useful step in elucidating and characterizing host defense, it would be useful to capture the entire suite of molecular regulation by utilizing RNA sequencing (RNA-seq) technology both in whole tissues and immune cells isolated from infected animals. This approach would be beneficial to isolate novel genes that may be attenuated in due to mycobacterial pathology, and it would also serve to expand the genetic repertoire of the goldfish model. Observing the tissue and/or isolated leukocyte mRNA levels with each infection at specific time points outlined in this thesis would provide a vast molecular snapshot throughout the course of infection.

In addition to RNA-seq, development of polyclonal antibodies against the goldfish cytokines described in this thesis would be beneficial. This would enable a more complete picture of immune regulation and a higher resolution description of the immune status in a variety of context (i.e. Western Blotting, ELISA, immunohistochemistry) utilizing both *in vitro* and *in vivo* models. Additionally, neutralizing specific cytokines with blocking antibodies would serve to characterize the importance (Th1-type) or perhaps interference (Th2-type) of host cytokines to a productive immune response.

To validate the *in vitro* findings in this thesis with regards to macrophage polarization, utilizing the established time points in these studies for *ex vivo* analysis of goldfish macrophage activation states would provide much more clarity on cellular polarization, rather than tissue expression that can obfuscate cellular phenotypes. To investigate this, *ex vivo* analysis of arginase and iNOS activity, isolation of kidney leukocytes and stimulating with recombinant cytokines (rgIFN-γ/rgIL-4/13) or LPS/cAMP would help determine the functional polarity of macrophages. Interestingly, the recent development of flow cytometric assays to determine iNOS/arginase levels allows for rapid detection of M1/M2 phenotypes in murine macrophages [525]. Validation of these assays in teleost leukocytes would be a useful and expedient means of assessing specific cellular populations and activation states from infected fishes.

Finally, the degree to which M1/M2 macrophage polarization plays a role in the various stages of mycobacterial infection could be understood by generating transgenic zebrafish lines with fluorescent reporters for iNOS and arginase-2

markers, alongside infection with fluorescent *M. marinum* pathogens. Development of reporter lines by insertion into the genome using bacterial artificial chromosome (BAC) recombineering has already allowed for visualization of macrophages and neutrophils using fluorescent *mpeg* and *mpx*, respectively. Using this system, real time visualization of the plasticity and polarization of macrophages should be possible, including the effects on polarization of macrophages exposed to *M. marinum*, in addition to the profile of surrounding macrophages or recruited monocytes, and activation profiles at the granuloma. Establishing such a model would provide greater insight into the necessary conditions to activate the killing phenotype in infected organisms.

8.4.3 Assessing virulence of *M. marinum* using goldfish macrophage cultures *in vitro*

The inability of *M. marinum* to innately activate and polarize kidney-derived goldfish macrophages, which is seen in *M. chelonae* and *M fortuitum* exposed cultures is likely of critical importance to both the direct killing effects, as well as the further activation of adaptive immunity. It would therefore be very beneficial to isolate the bacterial factors responsible for this attenuation. Using a whole-genome comparative analysis between *M. marinum* and *M. chelonae* or *M. fortuitum* to identify prospective mycobacterial genes for disrupting may uncover the gene or genes responsible for disrupting NO, or addition of these genes to the less virulent mycobacteria to endow virulence. Site directed gene mutagenesis, using nitric oxide production as a functional readout of mycobacteria virulence would serve as a

relatively high-throughput system for identifying mutants unable to disrupt NO production. The interference of iNOS by induction of arginase activity has also been demonstrated in mouse macrophages, which is TLR dependant [526], although the bacterial component triggering this response is undefined. Isolation of a bacterial factor that interferes with iNOS activation by arginase induction is paramount for understanding mycobacterial virulence and may eventually serve as a therapeutic target for future vaccine development.

8.5 Summary

The establishment of fish model systems are beneficial for the study of host defense mechanisms against prominent natural pathogens, such as those from the genus *Mycobacterium.* The high degree of conservation in molecular, cellular and tissue immune functions is testimony to the finely tuned underlying structures, and evasion of these structures through common strategies utilized by fish and mammalian pathogens alike. For this reason, surrogate analysis the immune response of *M. marinum* in fish models provides an interesting insight into the immune response in higher vertebrates.

In this thesis, I reported on the molecular identification and functional characterization of key cytokines with regards to macrophage polarization, as well as effector functions of teleost phagocytes when encountering natural mycobacterial pathogens. I believe that the findings in this thesis both contribute to our understanding of immunology in teleost fish and may provide a model of understanding of immune mechanisms in higher vertebrates.

Table 8.1. Summary of phagocyte function with mycobacterial exposure

	M. marinum	*M. chelonae*	*M. fortuitum*
Body tissue bacteremia	↑	↓	↓
Neutrophil ROS (low MOI)	—	↑	↑
Neutrophil ROS (high MOI)	↑	↑	↑
Neutrophil Chemotaxis	↑	↑	↑
Neutrophil killing	—	↑	↑
Macrophage ROS	↓	↑	↓
Macrophage NOS	—	↑	↑
Macrophage Arginase	↑	↓	↓
Macrophage Killing (innate)	—	↑	↑
Macrophage Killing (cytokine)	↑	↑	↑

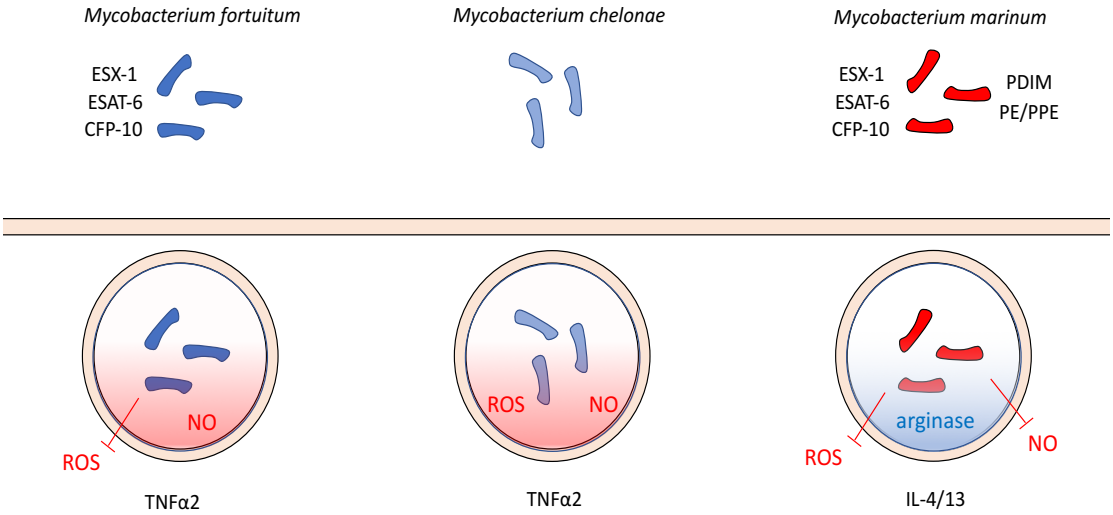

Figure 8.1 Functional consequences of mycobacterial exposure to goldfish macrophages. Defined mycobacterial virulence factors expression by each species. ESAT-6 secretion system-1 (ESX-1), 6 kDa early secretory antigenic target (ESAT-6), 10 kDa culture filtrate antigen (CFP-10), *p*hthiocerol *dim*ycocerosates (PDIM) and Pro-Glu (PE) and Pro-Pro-Glu (PPE) proteins are variably expressed by each pathogen. The functional consequence of mycobacterial exposure, leading to nitric oxide (NO), reactive oxygen species (ROS), or arginase production. Resultant increases in TNFα2 or IL-4/13 expression.

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