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Teleost contributions to the understanding of mycobacterial diseases

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ABSTRACT

Few pathogens have shaped human medicine as the mycobacteria. From understanding biological phenomena driving disease spread, to mechanisms of host-pathogen interactions and antibiotic resistance, the *Mycobacterium* genus continues to challenge and offer insights into the basis of health and disease. Teleost fish models of mycobacterial infections have progressed significantly over the past three decades, now supplying a range of unique tools and new opportunities to define the strategies employed by these Gram-positive bacteria to overcome host defenses, as well as those host antimicrobial pathways that can be used to limit its growth and spread. Herein, we take a comparative perspective and provide an update on the contributions of teleost models to our understanding of mycobacterial diseases.

INTRODUCTION

Mycobacterium spp. are Gram-positive, acid-fast staining, non-motile, non-spore forming bacilli, further defined based on growth rate (rapid or slow growing) and pigmentation (Runyon, 1959). The *Mycobacterium* genus consists of over 190 species that occupy a broad range of ecological niches, and include serious human pathogens like *M. tuberculosis* and *M. leprae*, as well as more innocuous soil-dwelling organisms. *M. tuberculosis* has afflicted humans for approximately 70 000 years, and it is thought to have killed more persons than any other microbial pathogen (Daniel, 2006). In 1865, Jean-Antoine Villemin, a French military surgeon first demonstrated the infectiousness of tuberculosis when he inoculated a rabbit with lung tissue from an individual that had died of tuberculosis (Daniel, 2006). Eighteen years later, Koch identified the bacillus responsible for TB and posited his famous postulates, setting a gold standard for demonstrating etiology of infectious disease (Daniel, 2005; Koch, 1952). The Bacille de Calmette et Guerin (BCG) vaccine was developed by serial passage of *Mycobacterium bovis* in 1921, reducing the risk of acquiring TB by approximately 50% (Colditz et al., 1994), although varying levels of effectiveness have been observed based on region, genetic factors, exposure to other pathogens, and BCG culturing practices (Venkataswamy et al., 2012). In 1944, the first effective antibiotic against tuberculosis was introduced by Selman Waksam. Currently, there are a number of antibiotic therapies with relatively high treatment success (approx. 85%) (Uplekar et al., 2006) though the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) create new and urgent challenges in antibiotic therapy. Despite these advances, it is estimated that one-third of the world's population is currently infected; the majority of these cases remain asymptomatic, while approximately 9 million cases of active tuberculosis (TB) and 1.3 million deaths are reported annually (Daniel, 2006; Herzog, 1998). Latent infections create a unique public health challenge as asymptomatic disease reservoirs, particularly given ongoing issues with antibiotic resistance and prevalence of human immunodeficiency virus (HIV) infections (Comstock et al., 1974; Vynnycky and Fine, 2000).

Beyond 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*) (Hines et al., 1995). These 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 (Pagan and Ramakrishnan, 2015). In the case of *M. marinum*, relative genetic relatedness to *M. tuberculosis* [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 (Stinear et al., 2008). Fish disease models based on *M. marinum*, *M. chelonae* and *M. fortuitum* infection have further helped our group to gain additional insights into natural mycobacterial infections. Distinct bacterial growth patterns and granuloma formation have been identified, although much remains to be learned about what constitutes an effective host immune response for control of these infections.

This review summarizes key parameters in the immune response to mycobacterial pathogens in mammalian and teleostean model systems, which highlight the complexity of these host-pathogen interactions and impact the outcome of infection. Our goal is to build on established insights derived from mammalian systems and offer novel points of discussion based on recent findings using teleost fish models. We focus on two teleost species, *Danio rerio* (zebrafish) and *Carassius auratus* (goldfish). Each provides unique attributes for examination of mycobacterial infections. The zebrafish embryo was developed for use as a model for developmental biology and has been used as such for the last quarter of a century, primarily due to its external fertilisation and development, visual transparency, and fecundity of the adults (Brittijn et al., 2009). This model offers well recognized advantages based on unique molecular tools, genetic manipulation capacity, and *in vivo* observation of immune cell transit and host-microbial interactions early in development. Goldfish, on the other hand, has added opportunities because of the greater number of cells available, well-established cultivation methodologies for primary cells from adult fish, and protocols for *ex vivo* characterization of immune cell function.

***MYCOBACTERIUM* SPP.**

Mycobacteria range in length between 1-10 µm long and 0.2-0.6 µm in diameter (Gauthier and Rhodes, 2009; Jacobs et al., 2009). Mycolic acids, α-alkyl, β-hydroxyl long-chain fatty acids, are the most abundant lipid in mycobacterial cell walls, making up 60% of the dry weight. Composition of mycolic acids are diverse within the *Mycobacterium* genus and are used in conjunction with genomic approaches for identification of species and strains (Portevin et al., 2014). Broadly, fast growers 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 (Saviola et al., 2006). Slow growing species include prevalent pathogens like *M. tuberculosis*, *M. marinum*, *M. leprae* and *M. avium*. Faster growing mycobacterial species are largely saprophytic, although *M. fortuitum* and *M. chelonae* have been shown to be pathogenic, especially in fish and frog species and under nosocomial settings. As mentioned, *M. marinum* and *M. tuberculosis* are very closely related from a morphological and molecular standpoint.

TUBERCULOSIS

Tuberculosis (TB) is predominantly a lung disease, which accounts for 70% of infection cases (Harisinghani et al., 2000). TB can be divided into two major patterns, primary and post-primary TB. In a recent paper, Behr and colleagues (Behr et al., 2018) suggested simplified terms to describe progression of TB in patients. Primary tuberculosis is defined as the initial infection as evidenced by a positive tuberculin skin test or a new positive interferon gamma release assay; active TB with evidence of progressive disease of the lung; and tuberculous reactivity which is indirect evidence of present or past infection with *M.tuberculosis* as inferred by adaptive immune response to *M. tuberculosis* antigens (interferon gamma release assay, positive tuberculin test) in an asymptomatic person (Behr et al, 2018). Primary infection is typically controlled by an immunocompetent individual, although serious disease can manifest in children and immunocompromised individuals (Hunter et al., 2018). The lesions in the lung that develop during primary tuberculosis often heal, but despite the reduced pathology, the infection is rarely sterilized and bacteria can persist for up to several decades as

subclinical infection, where mycobacteria remain within individual granulomas at a variety of growth states (Paige and Bishai, 2010). Post-primary tuberculosis 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 (Hunter, 2011). While the majority of post primary infections also spontaneously recover, 80% of the estimated 9 million yearly clinical cases of TB are the result of post primary infections (Paige and Bishai, 2010). Ironically, immunocompetent individuals between the ages of 15-40 are most likely to die from acute post primary TB (Cheeseman, 1952; Lawn and Acheampong, 1999; Tucker and Dooley, 2018). Further, survivors of post primary TB have an increased risk in contracting the disease again, rendering no immunity to previous bouts of the disease (den Boon et al., 2007; van Rie et al., 1999). Symptoms of active pulmonary TB include chest pain, coughing (with or without blood), difficulty breathing, excessive sweating, fatigue, fever, weight loss, wheezing, cachexia (Campbell and Bah-Sow, 2006). Active TB can also manifest as miliary disease, meningitis, abdominal disease and sepsis (Jacob et al., 2009).

TELEOST MODELS OF MYCOBACTERIAL DISEASES

Although *M. tuberculosis* can infect most warm-blooded animals, traditional laboratory infection models prove difficult in recapitulating human disease. Aerosol transmission is not observed in non-primate models, there is limited capacity to observe post-primary infection and disease, and only a few models (e.g. guinea pigs) generate necrotic (caseous) granulomas (Basaraba and Hunter, 2017; Harper et al., 2012). As a result, a range of animal models continue to be used in an effort to understand mycobacterial disease, including non-human primates, mice, rabbits, guinea pigs, frogs, and fish. The lack of a standardized model restricts easy integration of findings; however, strengths within each of these models continue to yield important insights into the mechanisms mediating immune responses to mycobacterial pathogens.

Teleosts are evolutionarily one of the first group of organisms to have both the innate and classical adaptive arms of the immune response, both of which are key to the pathogenesis of human TB. Moreover, *in vivo* fish models offer unique advantages over traditional mammalian models of TB, as mice are not natural hosts of *M. tuberculosis* and

primate models are expensive and difficult to work with. Three *Mycobacterium* spp. are most often used to elicit disease in fish: *M. marinum*, *M. chelonae* and *M. fortuitum*, although an increasing number of species have been isolated from fish in recent years (Gauthier and Rhodes, 2009). *M. marinum* is the closest related strain to human TB complex, and as such is the most widely used in the laboratory setting. *M. marinum* so closely resembles the human pathogen that it can also infect humans resulting in fish tank granuloma. This disease occurs when *M. marinum* enters the extremities through abrasions on the hand or arms, and produces granulomatous nodes (Petrini, 2006). The pathology of fish tank granuloma is indistinguishable from cutaneous tuberculosis.

Piscine mycobacteriosis typically manifests as chronic disease, which may or may not produce clinical signs both externally and internally. Externally, pathology includes non-specific scale loss and dermal ulceration, pigmentation changes, spinal defects, lethargy, ascites and emaciation (Jacobs et al., 2009; Leibovitz, 1980). Internal pathology includes enlargement of the kidney, spleen and liver, as well as grey or white nodules on internal organs (Gauthier and Rhodes, 2009). Similar to disease in mammals, granulomatous inflammation represents a classical hallmark of infection. In fish, granulomas can be found in multiple organs and tissues, and are highly variable in size and organization (Jacobs et al., 2009). The relative relatedness to *M. tuberculosis*, as well as the remarkably similar pathology in fish infections has rendered *M. marinum* a popular surrogate model for human tubercular diseases.

Mycobacterium marinum

Infection with *M. marinum* was one of the first teleost disease models to be adopted for increased understanding of the biology of mycobacterial infections, and has become one of the most successful. Initial studies of infected zebrafish embryos and adults, and were used to follow disease pathology over time. It was established that, on a histopathological level, *M. marinum* infection in adults is a chronic granulomatous disease that is hard to distinguish from human TB (Meijer et al., 2004; Meijer et al., 2005). Infection of the one-day post fertilization zebrafish embryo led to the formation of granuloma-like structures within just a few days, in the absence of adaptive immune cells (Davis et al., 2002). The *M. marinum*/zebrafish model has also yielded insights into

mycobacterial virulence factors, drug tolerance and potential therapeutic targets. Zebrafish infection experiments, for example, showed that WhiB6 modulates levels of mycobacterial infection, granuloma formation, and dissemination (Chen et al., 2016a). *M. marinum* strains lacking phthiocerol dimycocerosates (PDIMs) and structurally related phenolic glycolipids (PGLs) were also avirulent and hypersensitive to antibiotics (Yu et al., 2012). Multidrug-tolerant organisms have also been identified soon after *M. marinum* infection driven through bacterial-macrophage interactions, but appear susceptible to bacterial efflux pump inhibitors like verapamil (Adams et al., 2011).

The emergence of transgenic lines labelling innate immune cell populations has also paved the way for a tractable zebrafish/*M. marinum* embryo model in which the innate immune response to mycobacterium infection could be investigated (Hall et al., 2007; Walton et al., 2015)Ellett et al., 2011; Gray et al., 2011; Mathias et al., 2006; Renshaw et al., 2006). Early morpholino knockdown oligonucleotide technologies also uncovered important roles for many genetic and enzymatic systems in granuloma formation, including cytokines (e.g., TNFα), cell signalling components (e.g., MyD88) and cell matrix enzymes (e.g., MMP9) (Clay et al., 2008; van der Vaart et al., 2013; Volkman et al., 2010). Furthermore, the ability to follow granuloma formation over time using fluorescence timelapse microscopy has identified multiple rounds of phagocytosis of *M. marinum* and cell death leading to the formation of hallmark granuloma structures, cell behaviours that are likely to occur in and around the human lung upon infection and/or TB reactivation, but require invasive imaging to observe in mammalian systems (Hosseini et al., 2016). Importantly, findings from the zebrafish/*M. marinum* model have been directly related to human genetic variation and TB disease (Tobin et al., 2012). With the now widely adopted CRISPR-Cas9 system making it easier to knockout genes in zebrafish, we expect many additional novel genetic findings in TB pathology from this *M. marinum* model. In addition to the power of the zebrafish model, other fish models of *M. marinum* include the goldfish allowing added flexibility for examination of responding leukocyte populations and their antimicrobial functional responses *ex vivo* as well as *in vivo* using adult fish (Grayfer et al. 2011; Hodgkinson et al. 2012;(Grayfer et al., 2014).

Mycobacterium abscessus

The success of the zebrafish/Mm model has made zebrafish an attractive model for other infectious diseases, and these include non-tuberculous mycobacterial diseases. *Mycobacterium abscessus* is an important emerging human pathogen and is especially associated with cystic fibrosis patients (Jonsson et al., 2007). An embryonic zebrafish model of *M. abscessus* has helped to shed light on the innate immune mechanisms behind the increased severity of a rough variant compared to the less sever smooth variant. These distinct morphotypes have also been shown to contribute to different stages of infection and display differential capacity to activate and respond to phagocyte antimicrobial mechanisms (Malcolm et al., 2018). The rough morphotype also has a propensity to form cords, large areas of extracellular bacteria too large to be phagocytosed by macrophages or neutrophils. This cording leads to abscess formation, an important physiopathology of *M. abscessus* infection, leading to greater larval death (Bernut et al., 2014). Additional features of this important emerging mycobacterial disease including progression and fate of *Mabs* infection, host and pathogen modulators of disease severity, and developing opportunities for therapeutic intervention have recently been reviewed (Bernut et al., 2017).

Mycobacterium leprae

Recently, a *Mycobacterium leprae* zebrafish model has been established to understand the pathophysiology and the interplay between innate and adaptive immune components in leprosy disease (Madigan et al., 2017b). *M. leprae*, is unique amongst mycobacteria in that it causes peripheral neuropathy, leading to the devastating paralysis and deformities associated with the disease. The initial nerve damage is caused by macrophages activated through *M. leprae*-specific phenolic glycolipid 1 (PGL-1), rather than neural interactions with the bacteria itself, highlighting a potential novel future treatment opportunity (Madigan et al., 2017a).

EARLY INFECTION EVENTS

Recognition

Following inhalation of *M. tuberculosis* in humans, a variety of host phagocytes localize to the site of infection, including resident macrophages, dendritic cells and recruited neutrophils (Schlesinger, 1996). Host recognition of mycobacteria is mediated 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) and Nod-like receptors (NLRs) (Berrington and Hawn, 2007; Kleinnijenhuis et al., 2011; Reiling et al., 2008). 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 (Caws et al., 2008), and MyD88 and CARD9, master adaptors for TLRs and NLRs, are essential for host protection to *M. tuberculosis* (Kleinnijenhuis et al., 2011; Schlesinger, 1996). Mice deficient in MyD88 lose resistance to *M. tuberculosis*, marked by reduced IL-12, TNF and Th1 cytokine production and iNOS expression (Scanga et al., 2004). Similarly, *Card9*$^{-/-}$ mice have impaired host resistance to *M. tuberculosis* and higher bacterial burden (Dorhoi et al., 2010), as well as mice deficient in NOD-2 (Divangahi et al., 2008). 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 (Carmona et al., 2013).

In fish, recognition of *M. marinum* PAMPs by PRRs can induce the nitrosative defense mechanism in leukocytes, which is attenuated in *M. marinum* containing the RD-1 virulence locus (Elks et al., 2014), or in *M. marinum* absent cell wall phthiocerol dimycoceroserate (PDIM) lipids (Cambier et al., 2014). 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 in MyD88 of zebrafish can also accelerate granuloma formation (van der Vaart et al., 2012), although this is likely also due to the loss of IL-1 signalling, which is also 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) (Meijer and van der Vaart, 2014), demonstrating the importance of TLR signalling in

300 later stages of infection. The implication of NLR signalling has also been suggested in
301 expression studies of goldfish primary kidney leukocytes following stimulation with *M.*
302 *marinum* (Xie et al., 2013).
303
304 **Early regulation of cytokine production**
305 Early recognition of mycobacterial PAMPs by mammalian PRRs results in the
306 production of a number of cytokines, including tumour necrosis factor (TNF), interferon
307 (IFN)-γ, and IL-1 family cytokines (IL-1α, IL-1β, IL-18 and IL-33), IL-12, IL-17 and IL-
308 23. Orthologous cytokines of the fish have also been studied in acute mycobacterial
309 infections, although not extensively. Evaluation of cytokine gene expression following
310 BCG vaccination in Japanese medaka has shown early increases in IL-1β, IL-6, IFN-γ
311 and TNFα, akin to mammalian observations. Similar responses have been observed in
312 goldfish and adult zebrafish (Berrington and Hawn, 2007; Reiling et al., 2008; Yang et
313 al., 2012a).
314 Among the cytokines participating in these responses, TNF has long been
315 recognized as critical for protection against mycobacterial infection. The importance
316 of TNF signaling was first revealed by blocking with anti-TNF receptor antibodies,
317 which increased bacillary load within the granuloma and expedited host death
318 (Bean et al., 1999; Flynn and Chan, 2001). Moreover, interference with TNF
319 signaling during latent infection resulted in reactivation, suggesting an important
320 role in maintaining a latency programme and fending off active disease (Botha and
321 Ryffel, 2003; Chakravarty et al., 2008; Mohanty et al., 2015). Impaired TNF function
322 in mycobacterial infection reduces iNOS and increases IL-10 production, while IFN-γ
323 and IL-12 remain unchanged (Mohan et al., 2001). It has been suggested that TNF is
324 required for the generation and maintenance of granulomas (Bean et al., 1999;
325 Chakravarty et al., 2008), although this has been shown to not necessarily be the
326 case, where various infection models have demonstrated granuloma formation in
327 the absence of TNF (Flynn and Chan, 2001; Iliopoulos et al., 2006; Lin et al., 2010).
328 Experiments using goldfish and zebrafish have revealed the importance of a
329 balanced TNF response in the control of mycobacterial infection. Similar to
330 mammalian models, TNF induces a mycobacterial killing response by stimulating

effector RNS and ROS in macrophages (Grayfer et al., 2011; Roca and Ramakrishnan, 2013). TNF is induced in early infection events in zebrafish, leading to restriction of the growth of *M. marinum* (Clay et al., 2007). Unsurprisingly, morpholino knockdown of TNF receptor (TNFR) led to increased bacterial growth and decreased containment (Clay et al., 2008), although hyper-expression of TNF is also unfavorable to the host, enhancing inflammation and necrosis (Roca and Ramakrishnan, 2013; Tobin et al., 2012). The temporal duality in outcome of TNF signaling has been clearly described in the zebrafish system (Roca and Ramakrishnan, 2013). Interestingly, chemical inhibition of necrosis in high TNF fish have been shown to uncouple the positive and negative effects and is a possible therapeutic approach for treatment of TB.

PHAGOCYTOSIS ANTIMICROBIAL RESPONSES

Internalization of mycobacterial bacilli by responding phagocytes is mediated by mannose, complement, and scavenger receptors (Kleinnijenhuis et al., 2011). Following internalization, mycobacteria are either neutralized by activated phagocytes (through the respiratory burst, nitric oxide production, or phagolysosomal events), or bacteria may persist within the phagocyte (Castaneda-Delgado et al., 2010; Fabri et al., 2011). Evasion of host effector responses can result in latent infection or acute, active disease (Frieden et al., 2003). Generation of an appropriate immune response during early events play a critical role in determining the fate of infection, either through protective innate immunity or adaptive responses (Frieden et al., 2003).

The cellular interactions between host leukocytes and *M. marinum* have been well documented early during fish infection. In the zebrafish embryo model transparency enables visualization of these interactions. Similar to mammalian models, resident fish macrophages readily internalize *M. marinum* (Davis et al., 2002) and neutrophils rapidly migrate to the site of infection and uptake the pathogen (Abadie et al., 2005; Clay et al., 2007), although neutrophil phagocytosis of *M. marinum* is largely dependent on the site of infection (Yang et al., 2012a).

Phagosome maturation

Pathogenic mycobacteria have evolved a number of evasion strategies to subvert the hostile environment following professional phagocytic internalization. Pathogenic *Mycobacterium* spp. are particularly adept at interfering with the maturation of the phagosome, restricting acquisition of late endosomal or lysosomal characteristics (Clemens and Horwitz, 1996; Sturgill-Koszycki et al., 1994). In mammals, a number of mycobacterial factors have been identified that interfere with phagosomal maturation and lysosomal fusion. The mycobacterial cell wall lipid phtiocerol dimycocerosates (PDIM) contributes to host cell entry through receptor-dependant phagocytosis, and also impairs phagosome maturation and acidification, compared to PDIM mutants (Astarie-Dequeker et al., 2009; Pethe et al., 2004). Trehalose 6,6'-Dimycolate (TDM) and lipoarabinomannan (LAM) have also been shown to play an important role in arresting of normal phagosome processing (Axelrod et al., 2008; Welin et al., 2008). More recent findings suggest pathogenic mycobacteria can escape the phagosome by translocating to the cytosol, although this may only occur at later stages in infection at the level of the granuloma (Bafica et al., 2005).

M. marinum infection of teleost models corroborate interference with phagosome maturation findings described above. Circumvention of the phagosome and lysosome has been demonstrated by *M. marinum* in trout and carp macrophages (Barker et al., 1997; El-Etr et al., 2001), and the pathogen has also been shown to localize in non-acidified phagosomes (El-Etr et al., 2001). Most recently, it has also been shown that *M. marinum* are able to survive and grow within the phagolysosomal compartment, albeit at slower growth rates, through action of the virulence determinant MarP (Levitte et al., 2016).

Respiratory burst

The fate of internalized particles depends heavily on the activation state of the phagocyte. Increased risk of mycobacterial diseases is observed in humans with chronic granulomatous disease, where defective NADPH oxidase is incapable of producing ROS in phagocytes (Deffert et al., 2014). However, controversy over the role of ROS exists, as it has been shown that mice deficient in NOX2 are relatively resistant to mycobacterial infection (Adams et al., 1997; Cooper et al., 2000; Jung et al., 2002). *M. tuberculosis* possesses 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$^{-/-}$) (Ng et al., 2004). 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* (Ehrt and Schnappinger, 2009). 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 (Piddington et al., 2001). *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 (Dussurget et al., 2001), where *Sod*A mutants were attenuated in mouse infection models (Edwards et al., 2001).

Fish possess orthologous NADPH oxidase machinery and are similarly capable in producing toxic reactive oxygen intermediates from both TLR and cytokine activation (Grayfer et al., 2014). *In vitro* and *ex vivo* studies of *M. marinum* in the goldfish have shown attenuation of IFN-γ and TNF-α activated ROS production by kidney macrophages (Grayfer et al., 2011; Hodgkinson et al., 2012). 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 extracellular milieu, which is more growth permissive (Roca and Ramakrishnan, 2013). More recently, *M. marinum* cell wall component phosphoribosyltransferase has been shown to enhance bacterial survival by inhibition of oxidative stress and autophagy pathways (Mohanty et al., 2015). These findings highlight the importance of fine-tuning the regulation of ROS, where both inhibition and excessive activation enhances bacterial growth and disease pathology.

Nitric oxide antimicrobial response

In addition to respiratory burst, activated macrophages express inducible nitric oxide synthase (iNOS/NOS2), enzymes that catalyzes the conversion of L-arginine to L-citruline, resulting in the production of a potent antimicrobial compound, NO

(MacMicking et al., 1997). 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 (Gordon, 2003). 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) (Stuehr and Marletta, 1987). Parallel production of superoxide and NO can also result in the formation of peroxynitrite ($ONOO^-$), a potent antiparasitic/antimicrobial agent (Henard and Vazquez-Torres, 2011). Like ROS, RNS can modify DNA, proteins and lipids, resulting in antimicrobial function, but can also damage host cells (Yang et al., 2009). Activation of iNOS is essential for the destruction of intracellular pathogens, including mycobacteria (Adams et al., 1991; Schon et al., 2004). Indeed, iNOS knockout studies have demonstrated uncontrolled bacterial replication, dissemination, tissue destruction and mortality (MacMicking et al., 1997).

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 (Voskuil et al., 2003; Wayne and Sohaskey, 2001). 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 (Green et al., 2014). Other virulence factors involved in denitrification include TpX, a thiol peroxidase that reduces peroxynitrite (Jaeger et al., 2004), AhpC, a catalase peroxidase (Sherman et al., 1999), PknE, a serine/threonine kinase E, an inhibitor of NO-mediated apoptosis (Jayakumar et al., 2008). Forrellad et al. recently published a comprehensive review on mycobacterial virulence factors that combat oxidative and nitrosative stress (Forrellad et al., 2013).

Infections of *M. marinum* in fish have yielded corroborative insights into the role of iNOS in mycobacterial immunity. In zebrafish, TLR (MyD88 dependent) recognition of *M. marinum* resulted in NO production, although bacteria containing the RD1 locus, were capable of attenuating the response (Elks et al., 2014). Interestingly, *M. marinum* has recently been shown to preferentially recruit permissive macrophage phenotypes during early infection, which do not produce nitric oxide upon internalization (Cambier et al., 2014). This evasive "screening" of macrophages is accomplished using cell-surface-

associated phthiocerol dimycocerosate (PDIM) lipids which mask underlying PAMPs and recruit permissive macrophages through a host chemokine receptor 2 (CCR2)-mediated pathway (Cambier et al., 2014). In PDIM deficient *M. marinum*, TLRs stimulation leads to recruitment of macrophages with microbicidal potential through nitrosative mechanisms. Further reports using the zebrafish model demonstrated that the enhancement of iNOS reduced bacterial burden, while impaired iNOS increases host susceptibility, and the Dos dormancy survival regulon is also seemingly conserved (Chen et al., 2016b). *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 (Grayfer et al., 2011; Hodgkinson et al., 2012).

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-formylkynurenine (Taylor and Feng, 1991). Expression of IDO can be induced by either IFN or TLR pathways, and is generally accepted to deprive supply of tryptophan to pathogens, limiting growth and persistence (Green et al., 2014; Wayne and Sohaskey, 2001); (Wang et al., 2014). The catabolism of L-tryptophan also results in production of metabolites known as kynurenines, which promote a broad spectrum of downstream effects, including immunotolerance and suppression of T cell proliferation (Grohmann and Bronte, 2010).

Despite the presence of the IDO-mediated approach to pathogen control, pathogenic mycobacteria are capable of *de novo* L-tryptophan biosynthesis, rendering a deprivation strategy by IDO ineffective. In fact, increased IDO expression correlates with severity of pathology in *M. tuberculosis* infected individuals (Plain et al., 2011). *M. tuberculosis* actively promotes host IDO production and suppression of IDO reduces bacterial burden, pathology, and clinical signs of TB disease, leading to increased host survival (Gautam et al., 2018). Downstream effects of kynurenine metabolite production are also likely to interfere with T cell activation, a critical component of anti-mycobacterial immunity. Thus, interference of IDO has been proposed as a potential

485 therapeutic target, in addition to the L-tryptophan biosynthesis machinery in
486 mycobacteria, anthranilate synthase (TrpE) (Warner, 2015; Zhang et al., 2013).
487 Fish IDO orthologues (proto-IDOs) appear to have less efficient tryptophan
488 degradative capacities as compared to the mammalian IDOs, suggesting the possible
489 presence of alternative fish IDO substrates that are yet to be identified (Yuasa et al.,
490 2007). To date, only one expression analysis of proto-IDO orthologues has been
491 presented in the context of mycobacterial infection. Goldfish macrophages infected with
492 *M. marinum* up-regulated proto-IDO gene expression and exposure of macrophages to
493 live *M. marinum in vitro* and induced substantially greater proto-IDO mRNA levels than
494 the heat-killed bacteria, suggesting a possibly similar tryptophan metabolism strategy
495 seen in mammalian hosts pathogen infection models (Grayfer et al., 2011). Similarly, our
496 group has observed large increases in IDO expression levels in *M. marinum* infected
497 spleen and kidney tissue.
498
499 **MODULATION OF MACROPHAGE SURVIVAL**
500 **Macrophage apoptosis**
501 Regulated cell death by infected cells is an important mechanism to contain
502 pathogen replication and spread. The importance of apoptosis to an effective immune
503 response is underlined by the various pathogenic virulence factors that inhibit the process
504 (Best, 2008). Apoptosis in *M. tuberculosis* infected macrophages is generally regarded as
505 a host protective response, where intact bacteria encased in plasma membrane are
506 internalized by phagocytes in a process called efferocytosis (Martin et al., 2012).
507 Sequestration of mycobacteria inside of the apoptotic body is thought to disrupt the
508 interference of phagolysosomal fusion following phagocytosis, thereby delivering the
509 bacilli to lysosomal components and facilitating degradation (Kagina et al., 2010).
510 Apoptosis was first recognized as an important line of defense against mycobacteria
511 when attenuated *M. tuberculosis* strains such as *M tuberculosis* H37Ra exhibited reduced
512 viability and increased apoptotic turnover (Keane et al., 2000), whereas virulent *M.*
513 *tuberculosis* strains induced less apoptosis and persisted intracellularly (Kelly et al.,
514 2008). Pro-apoptotic mutants have led to the discovery of virulence factors that impair
515 apoptotic function, including the *sec*A2 gene, a secretion system that secretes

mycobacterial superoxide dismutase, a strong superoxide scavenger (Hinchey et al., 2007). Contradictory reports suggest apoptosis can also promote mycobacterial spread (Early et al., 2011) 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 (Huang et al., 2016). The increased apoptosis in the granuloma leading to the expansion of infected macrophages has previously been demonstrated in zebrafish larval granulomas possessing the RD1 virulence locus (Davis and Ramakrishnan, 2009), reiterating the immunological context of these processes as paramount.

Macrophage necrosis

Recent insights 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 physical conditions results in unregulated cell death. However, a number of controlled states of necrosis have been defined (Feoktistova and Leverkus, 2014). 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 (Roca and Ramakrishnan, 2013; Tobin et al., 2012; Tobin et al., 2010). 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. Mice deficient in PGE2 are more susceptible to mycobacterial infection due to enhanced necrosis, while LXA4 mutations enhance apoptotic response, leading to less susceptibility (Divangahi et al., 2010).

Comparative 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. The discovery of polymorphisms in *ltah* (leukotriene A4 hydrolase) was originally done in the zebrafish, and was later demonstrated in susceptible human populations (Herb et al., 2008; Tobin et al., 2010). Host mechanisms of control influencing the development of a necrotic core in the granuloma have 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 (Pagan and Ramakrishnan, 2015). Virulence products of the ESX-5 secretion system have also been identified as influencers the necrotic/apoptotic balance in fish, where knockouts shift drive a hyper-virulent necrotic response in the center of the granuloma, though the mechanism underpinning this observation is unclear (Weerdenburg Eveline et al., 2012).

NEUTROPHILS

The roles of neutrophils during mycobacterial infection remain controversial, although neutrophils are infected by mycobacteria, and are the predominant infected cell type during active tuberculosis (Eum et al., 2010; Francis et al., 2014). Numerous reports suggest a negative role for neutrophils in tuberculosis, where respiratory failure and death are associated with elevated blood neutrophil levels (Barnes et al., 1988; Lowe et al., 2013). Further reports have shown neutrophils to facilitate delivery of mycobacteria to dendritic cells, thereby aiding in the subsequent activation of $CD4^+$ T cells (Blomgran et al., 2012). 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 (Blomgran et al., 2012). 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, while depletion of neutrophils at later stages of infection has been shown to reduce the bacterial load (Zhang et al., 2009). Therefore, it may be possible that early protective responses and promotion of T cell activation by neutrophils renders later

578 immune contribution unnecessary, and disruption of this tightly controlled process may
579 lead to neutrophil pathology at later disease stages. Indeed, it has been suggested that
580 neutrophilia during active disease is an indication of failed Th1 immunity (Nandi and
581 Behar, 2011).
582 In fish models, recent interest in neutrophils in *M. marinum* infection has helped
583 to underline their importance in protection. Initially, it was thought that macrophages but
584 not neutrophils internalize *M. marinum*, as this was observed following injection of
585 bacilli into the bloodstream or hindbrain of zebrafish embryos (Yang et al., 2012a).
586 Subsequent reports showed chemotaxis and internalization of *M. marinum* by primary
587 goldfish neutrophils, as well as changes to pro-inflammatory status and killing capacity of
588 these adult teleost neutrophil populations (Hodgkinson et al., 2015). The survival of
589 intracellular mycobacteria was significantly reduced in activated neutrophils. Similarly,
590 in zebrafish internalization of *M. marinum* by neutrophils in subcutaneous infection
591 studies suggested that site specificity of infection exists, and is more likely to reflect the
592 natural role for neutrophils in early infection (Hosseini et al., 2016). Further, a recent
593 report in zebrafish using confocal laser scanning methods illustrated that neutrophils may
594 also contribute more to the dissemination of bacteria than macrophages, due to their high
595 mobility post infection (Hosseini et al., 2016). Clarification of the role of neutrophils in
596 early infection has been greatly aided in the zebrafish model, where death by bacteremia
597 is associated with neutropenia, further suggesting a protective response by neutrophils
598 (Belon et al., 2014). This is corroborated in transgenic zebrafish expressing truncated
599 chemokine receptor Cxcr4 leading to retention of neutrophils in hematopoietic
600 compartments and an increase in the bacterial burden (Yang et al., 2012a). Interestingly,
601 enhancing nitric oxide production of neutrophils prior to infection by manipulation of
602 hypoxia-inducible factor (Hif-α) signaling mediated a protective response, an effect
603 dependent on Il-1β production (Elks et al., 2013; Ogrysko et al., 2018). At the granuloma,
604 neutrophils have been shown to internalize apoptotic bodies of macrophages, scavenging
605 and killing internalized bacilli via oxidative mechanisms (Hosseini et al., 2016; Yang et
606 al., 2012a). In this manner, neutrophils play an important protective role at the later
607 stages of infection in fish models.
608

CD4+ T CELLS

Th1

Adaptive immunity is first detectable between 3-8 weeks post mycobacterial infection (Jasenosky Luke et al., 2015), which is widely accepted as being delayed compared to other bacterial infections (Urdahl et al., 2011; Winslow et al., 2008). This adaptive response is highly dependent on T helper cells, although the evidence is highly correlative, as suggested by the increased susceptibility to TB in HIV coinfection (Cooper, 2009). Following initial infection and internalization events, antigen presenting cells, predominantly dendritic cells, traffic to a nearby lymph node and stimulate CD4+ T cell expansion, although inhibition of MHC class II peptide presentation by *M. tuberculosis* has been observed as a proposed evasion mechanism (Yang et al., 2012a). 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 (Blomgran and Ernst, 2011; Divangahi et al., 2010). However, once migrated, infected dendritic cells are capable of releasing intact bacterial antigens that are taken up and presented by the uninfected dendritic cells, optimizing T cell priming (Srivastava and Ernst, 2014). 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 (Gallegos et al., 2008), demonstrating that 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 (Rossouw et al., 2003). These cytokines are critical for activation of the antimycobacterial function of macrophages, including phagosome maturation, reactive nitrogen intermediates and antigen presentation (described above) (Flynn et al., 2011; Weiss and Schaible, 2015). Indeed, cessation of bacterial growth correlates strongly to the arrival of CD4+ T cells to the infection site or granuloma, although the mechanisms have yet to be fully defined. Evidence for the protective capacity of 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 (Darrah et al., 2007). 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 (Castro et al., 2013a; Castro et al., 2013b). 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 (Yoon et al., 2015). 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* (Myllymaki et al., 2018). However, most of the research in the zebrafish has focused on the embryonic system which predates development of lymphocytes. Correlative importance of Th cells in mycobacterial control has been assessed with regards to IFN-γ expression. Increases in IFN-γ mRNA expression has been observed in adult goldfish and zebrafish *in vivo* (Hodgkinson et al., 2012; Oksanen et al., 2013; Yoon et al., 2015) and in goldfish primary cultures (Grayfer et al., 2011). IFN-γ-primed macrophage effector function was also shown to be attenuated by *M. marinum*, suggesting effector evasion even with IFN-γ stimulation (Grayfer et al., 2011).

Th2

CD4+ T cells can functionally polarize to Th2 cells, which generally produce IL-4, IL-5, IL-10, and IL-13, stimulating a strong antibody response and inhibiting antimicrobial macrophage activation (Romagnani, 1999). Because Th1 responses are generally regarded as host protective, Th2 responses, 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 chronic helminth infection appears to decrease immunogenic response of BCG (Elias et al., 2008) and impairs a productive Th1 response in concurrent *M. tuberculosis* infections

(Babu et al., 2009; Resende Co et al., 2007). Interestingly, IL-4Rα$^{-/-}$ deficient mice infected with a helminth, exhibited 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 (Potian et al., 2011).

Fish possess a Th2/M2 functional state in response to parasitic infection, which has been exhibited by increases in arginase activity in macrophages (Joerink et al., 2006). Fish also have Th2-type cytokines capable of a homologous regulatory and anti-inflammatory role, as has been demonstrated in goldfish macrophages exposed to recombinant forms of IL-4 (Hodgkinson et al., 2017). Zebrafish Th2-like cells have been characterized in response to *M. marinum* infections (Yoon et al., 2015), and interestingly, adequate Th2 gene expression levels are necessary for well-controlled latency (Hammaran et al., 2014). 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.

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 (Collison et al., 2007; Gondek et al., 2005; Read et al., 2000). 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 (Scott-Browne et al., 2007). Accumulation of Tregs has also been shown to prevent eradication of *M. tuberculosis* by suppressing the Th1 response in an IL-10 independent manner, and where depletion of Tregs also resulted in reduced bacterial load (Kursar et al., 2007). Despite the likelihood of an impairment of an effective anti-mycobacterial response, it is still relatively unclear the role of Tregs in different stages of infection, where they likely aid 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 (Wen et al., 2011), sea bass (Nunez Ortiz et al., 2014) and zebrafish (Dee et al., 2016; Hui et al., 2017; Kasheta et al., 2017). A few studies of the contribution of Tregs cells to the immune response to *M. marinum* have been undertaken 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 (Hammaran et al., 2014). 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 mycobacterial infection.

Th17 cells

T helper (Th) 17 may play a role in mycobacterial protection, as they are known to have significant pro-inflammatory effects on intracellular pathogens (Cooper, 2009). In TB, 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 was shown to limit the neutrophilic accumulation and survival, which may decrease inflammation and improve the infection outcome (Nandi and Behar, 2011). Contradictory reports have also shown the IL-17 response is dispensable in with sufficient IL-12p70 production (Khader et al., 2005). Still, partial protection has been reported following transfer of antigen-specific Th 17 cells in to naïve hosts (Gallegos et al., 2011). 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. At present, little has been established regarding IL-17 participation in *M. marinum* infection, although in zebrafish this has been inversely correlated with increases in IL-17 expression levels (Ojanen et al., 2015).

CD8+ T CELLS

Although comparatively 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 (Andersson et al., 2007). Decreases in IFN-γ^+ TNF^+ IL-2^+ trifunctional CD8+ T cells has

732 been observed in active disease states as well as cellular dysfunction in individuals with a
733 high bacterial load (Day et al., 2011), marked by a higher proportion of pro-apoptotic
734 markers and diminished proliferative capacity (Day et al., 2014). It has been suggested
735 that during active disease, *M. tuberculosis*-specific CD8+ T cells are arrested in an
736 intermediate point in differentiation with a reduced capacity for cytotoxicity and
737 proliferation (Jasenosky Luke et al., 2015). Inhibition of T cell function is exacerbated in
738 TB patients taking anti-TNF therapy for auto-immune disorders (Bruns et al., 2009). This
739 may mean that impaired upstream activation events, i.e. macrophage of Th1, lead to
740 arrested CD8+ function.
741 In fish, T cells express typical markers, including CD8, and have homologous
742 cytolytic function (Castro et al., 2013). CD8+ activation has been implicated in fish
743 immunity towards *Edwardsiella tarda* (Rowe et al., 2014), although to date, there is no
744 contribution from fish models on the research of CD8+ T cells during mycobacterial
745 infection.
746
747 **B CELLS**
748 B cells and antibody production are vital for a protective response and vaccination
749 to numerous infectious agents, although their contribution to mycobacterial protection is
750 not well understood. It was originally conceived that B cells do not contribute
751 meaningfully toward protection due to the intracellular nature of *M. tuberculosis*
752 (Kumararatne, 1997), although they likely play a role beyond what was previously
753 thought (Maglione Paul and Chan, 2009). B cells have been observed at the granuloma
754 where they may contribute to host defense (Phuah et al., 2012) and they have been shown
755 to influence inflammatory progression and bacterial containment (Maglione et al., 2007).
756 Most recently, antibodies have been implicated in a protective role during mycobacterial
757 infection via changes to their Fc functional properties (Lu et al., 2016). Individuals with
758 latent tuberculosis infection displayed functional enhancements including
759 phagolysosomal maturation, inflammasome activation, and macrophage killing of
760 intracellular *Mycobacterium tuberculosis*, when compared to individuals with active
761 tuberculosis disease (Lu et al., 2016).

762 Fish and mammalian B cells share many similarities, including the generation of
763 hyper-specific antibody repertoires by somatic gene rearrangement, and heavy chain
764 isotypes IgM and IgD. However, fish possess a unique antibody isotype profile, IgT/IgZ,
765 and lack IgG/IgE, although the exact isotype picture continues to evolve (Bengten and
766 Wilson, 2015). Antibody production following infection with *M. marinum* has been
767 observed, although a definitive link to host protection is yet to be established (Cui et al.,
768 2010; Pasnik et al., 2003).
769
770 **IFN-γ**
771 It is well established that IFN-γ is essential for protective function towards
772 intracellular pathogens, including mycobacteria (Bach et al., 1997). CD4+ Th cells are
773 the predominant source of IFN-γ during mycobacterial infection. CD8+ T may also
774 produce IFN-γ, although they cannot compensate for a lack of CD4+ cells (Flynn and
775 Chan, 2001). Transient sources of IFN-γ from NK cells, NK T cells and γδ T cells has
776 also been observed and is thought to tide over the protective response over until adaptive
777 sources take over, and also seem to be more prominent during infection with hyper
778 virulent *M. tuberculosis* infection (Cooper Andrea and Khader Shabaana, 2008). As
779 previously mentioned, antimicrobial mechanisms of the macrophage are critically
780 important in eradicating intracellular mycobacteria. IFN-γ is largely responsible for this
781 activation following expansion of antigen specific T cells. IFN-γ exerts numerous
782 downstream effects through a suite of transcriptional programs (Boehm et al., 1997),
783 activation of reactive oxygen production and iNOS transcription (MacMicking et al.,
784 1997), autophagy, endosome maturation (Russell et al., 2010), and production of
785 antimicrobial peptides (Fabri et al., 2011). Mice deficient in IFN-γ are unable to control
786 low dose infections of *M. tuberculosis*, where they fail to produce reactive intermediates
787 and bacteria replicate unabated (Cooper et al., 1993; Flynn et al., 1993). Similarly,
788 mutations in cognate receptor IFN-γR1 has been implicated in fatal BCG infection
789 (Jouanguy et al., 1997). Despite the role in controlling infection, IFN-γ levels are
790 correlated with the severity of disease, where excessive levels of IFN-γ are seen in

patients with severe TB (Verbon et al., 1999), and these levels are reduced following productive therapy (Tsao et al., 2002).

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 (Hodgkinson et al., 2012) and primary cultures (Grayfer et al., 2011). Interestingly, *M. marinum* impaired the IFN-γ primed respiratory burst and nitric oxide response in cultured leukocytes (Grayfer et al., 2011). In adult zebrafish, IFN-γ levels have corresponded to a partially protective BCG vaccination (Oksanen et al., 2013) as well as iNOS activation (Parikka et al., 2012).

IL-10

IL-10 is produced by a variety of immune cells, including macrophages, neutrophils, B cells, DCs and T cells (Saraiva and O'Garra, 2010) and provides critical regulatory feedback of inflammation to prevent immunopathology (Cooper, 2009). Similar to IFN or TNF, IL-10 can act as a double-edged sword during infection, where precise control of IL-10 is imperative. Overproduction during mycobacterial infection has been shown to contribute to chronic infection, while excessive inflammation and pathology occurs with insufficient production (Saraiva and O'Garra, 2010). Mice deficient in IL-10 exhibited enhanced protection to mycobacterial infection (Redford et al., 2010; Roach et al., 2001) 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 (Beamer et al., 2008). Interference of immune activation by IL-10 is through a variety of mechanisms, including antigen presentation, limited development of a Th1 response (and subsequent IFN-γ production), leading to inhibition of TNF, and prevention of iNOS expression (Fiorentino et al., 1991; Moore et al., 2001). Accordingly, mycobacterial pathogens have been shown to dampen host defence by modulating the IL-10 response. Increased IL-10 production was shown to arrest phagosome maturation in *M. tuberculosis* infected macrophages, while blocking IL-10 antibodies reversed this effect (O'Leary et al., 2011). The relative pathogenicity of mycobacterial strains also 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

822 early IL-10 and arginase-1 expression via CD4+CD25+FoxP3+CD223+ regulatory T-
823 cells. Coinfection with complementary strains demonstrate a virulence mechanism
824 utilized by *M. tuberculosis* HN878 to exploit host immune systems through induction of
825 IL-10, which is presumed to be an immune evasion strategy (Reed et al., 2004). Thus, the
826 data in humans and other mammalian models largely points to IL-10 impairing the ability
827 to eradicate mycobacteria infection when in excess. Studies in fish, however, have not
828 reached this same consensus. *M. marinum* induced expression of IL-10 has been observed
829 in goldfish primary kidney monocytes and macrophages (Grayfer et al., 2011), although
830 no apparent increases of IL-10 were observed in spleen or kidney tissues during
831 infections (Hodgkinson et al., 2012), and introduction of recombinant IL-10 did not alter
832 the viability of *M. marinum* within goldfish phagocytes (Hodgkinson et al., 2012). In
833 zebrafish, IL-10 mutants showed enhanced survival and enhanced interferon gamma
834 response following chronic *M. marinum* infection (Harjula et al., 2018).
835
836 **MMP9**
837 Metalloproteinase 9 (MMP9) has been isolated as a critically important
838 chemotactic factor for recruiting macrophages and monocytes to the granuloma (Taylor et
839 al., 2006), and is increased in mice and humans infected with *M. tuberculosis* (Price et al.,
840 2001; Taylor et al., 2006). Chemical inhibition of MMPs in mice leads to delayed and
841 smaller granuloma formation (Hernandez-Pando et al., 2000), while heightened levels are
842 correlated with deverity of disease (Chang et al., 1996).
843 Important findings on the contribution of MMP9 to pathology has been generated
844 in the zebrafish and been shown to be modulated by an RD-1 factor, ESAT-6. It was
845 observed that infected cells at the granuloma are not responsible for MMP9 production,
846 but rather nearby adjacent epithelial cells (Volkman et al., 2010). The stimulation of
847 MMP9 by epithelial cells is thought to attract naïve macrophages and monocytes while
848 simultaneously allowing for the dampened antimicrobial response in phagocytes in the
849 granuloma (Ramakrishnan, 2013). Moreover, these findings in the zebrafish offers
850 insights as to why granulomas do not typically grow in skeletal muscle or cardiac tissue,
851 due to the a lack of neighboring epithelial cells, responsible for MMP9 production.
852

LIPID MEDIATORS

Balance of the eicosanoids prostaglandin (PGE2) and lipoxin (LXA4) plays a major role in the outcome of mycobacterial infection. The balance of PGE2 and LXA4 govern whether macrophages undergo apoptosis or necrosis, which is an important determinant in host protection during infection (Behar et al., 2010). Less virulent mycobacterial infections, including the BCG vaccine, preferentially increase PGE2, leading to apoptosis in macrophages and ultimately bacterial containment (Divangahi et al., 2010). Deficiencies of PGE2 in mice resulted in increased bacterial loads and host susceptibility (Behar et al., 2011; Chen et al., 2008). In fish, an *ltah4* mutant led to increased LXA4 production resulting in greater host susceptibility to *M. marinum* infection (Tobin et al., 2010). Heterozygous polymorphisms in the human *LTA4H* promoter were found to be associated with TB severity in Vietnamese patients, suggesting this could serve as the basis for effective therapies against tuberculosis (Tobin et al., 2012), although it should be noted that changes in disease outcomes were not observed in a Russian cohort with the same polymorphisms (Curtis et al., 2011).

GRANULOMAS

The granuloma is an organized cellular aggregate that can form in the presence of ineradicable infectious or non-infections stimuli (Kunkel et al., 1989). The most prominent known cause of granulomas are those generated in response to pathogenic mycobacterial infections, giving rise to the name "tuberculosis" due to the hallmark pathology (Ramakrishnan, 2013). Macrophages, the most prominent cell type in granulomas, can undergo a variety of transformations, including interdigitation with other macrophages as epithelioid cells, fuse into multinucleated giant cells, or differentiate into foam cells, which are rich in lipids, thought to be due to the interference of lipid metabolism by internalized mycobacteria. Finally, they can create caseuous centers within the granuloma by necrotic cell death, mediated by hypoxia (Hunter, 2011; Ramakrishnan, 2013). The inner accumulation of macrophages in these heterogeneous forms, as well as infected neutrophils, are surrounded by lymphocytes and fibroblasts that create a fibrotic encapsulation (Peters and Ernst, 2003). A wide range of chemokine,

cytokine and adhesion molecules orchestrate the formation of granulomas, as reviewed in (Peters and Ernst, 2003).

The granuloma was historically viewed as a generally protective feature, whereby 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 infection (Cosma et al., 2003). This is consistent with more recent identification of phagocyte mediated killing of mycobacteria in early tuberculous granulomas (Yang et al., 2012). Further, multiple forms of deficiencies, such as IFN-γ, TNF, IL-12 or MyD88 prevent the development of an organized granuloma, which corresponded to hyper-susceptibility (Khader et al., 2006; Mohan et al., 2001), 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, suggesting that granulomas often fail at controlling bacterial proliferation, resulting in the alternate view of the granuloma as an immune response that is attempting, but failing, to control the infection (Ramakrishnan, 2013).

It was previously thought that generation of granulomas requires adaptive immunity, coinciding with slower growth of mycobacteria (North and Jung, 2004), however, granuloma formation in zebrafish embryos infected with *M. marinum* occurs in the absence of adaptive immune components, which are not present developmentally (Davis et al., 2002). Visualization studies in zebrafish revealed that nascent granulomas continually accept infiltrating macrophages and monocytes, responding to a chemokinesis gradient, into the structure until they are heavily infected (Davis et al., 2002; Davis and Ramakrishnan, 2009). Macrophage reprogramming that parallel E-cadherin-dependent mesenchymal-epithelial transitions contribute to organized granuloma formation (Cronan et al., 2016). Foam-like cells have also been identified using the zebrafish-*M. marinum* granuloma model, where transdifferentiation of macrophages appears driven by the mycobacterial ESX1 pathogenicity locus (Johansen et al., 2018). Vascularization further supports granuloma formation, driven in part through CXCR4 (Torraca et al., 2017), angiopoietin-2 (Oehlers et al., 2017), and VEGF (Walton et al., 2018). 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. Further, the attraction of macrophages and monocytes to the granuloma by virulent mycobacterial strains results in fresh phagocytes internalizing dead cell components containing live bacteria, thereby multiplying the number of host cells (Ramakrishnan, 2013). 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 (Egen et al., 2011), and RD-1 deficient mycobacterial infections in mice also demonstrate poorly formed granuloma structures (Sherman et al., 2004; Swaim et al., 2006).

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 (Davis and Ramakrishnan, 2009). The zebrafish *M. marinum* model has identified that the vascularisation of granulomas both nourishes the bacteria aiding granuloma growth, but also facilitates dissemination of bacteria from primary granulomas (Oehlers et al., 2015). 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 (Volkman et al., 2004). Together, these data suggest an alternate role to the granuloma as host protective structures. Validation of embryonic studies is necessary due to the vast physiological discrepancies of the models, and the embryo model may not always be the best for elucidating host: pathogen interactions. For example, *M. marinum* deficient in ESX-5 was slightly attenuated in zebrafish embryos, but hyper-virulent in adult zebrafish (Weerdenburg Eveline et al., 2012).

CLINICAL IMPLICATIONS OF TELEOST MODELS

Many of the examples of teleost mycobacterial research discussed in this review, provide relevant and novel insights into the cellular and pathophysiology of mycobacterial disease. The advantages of these model coupled to the difficulties of infecting mice with the human pathogen *M. tuberculosis*, may render teleost models increasingly important as we look to answer clinically relevant questions in TB.

One such clinically relevant question is understanding how TB manifests into life threatening conditions. Many of the teleost models described in this review, address the general mechanisms of granuloma formation, but not specifically addressing the location microenvironment of diseased tissue. Tuberculosis meningitis has high morbidity and mortality in the developing world, however little is known about how pulmonary TB spreads from the lung to the brain (Wolzak et al., 2012). A recent zebrafish model of TB meningitis, using *M. marinum* in embryo and adult infection, showed that the bacilli are able to cross the blood brain barrier, either via a Trojan-horse mechanism by carriage in macrophages, or without the need of macrophage help in an ESX-1 dependent manner (van Leeuwen et al., 2018; van Leeuwen et al., 2014). Furthermore, there has been a recent report of using zebrafish as a model for ocular TB, a condition that can cause vision loss in affected patients (Takaki et al., 2018). These new *in vivo* models show great promise in adding to our understanding of the complex nature of TB disease and has the potential to open up completely novel treatment avenues as more is understood.

A key question in the TB clinic is how to target antibacterial drugs to infected macrophages, and more importantly, to the centre of granulomas where latent and potentially drug resistant, bacilli lie. The possibility of using nanoparticle drug delivery vectors that target macrophages has been explored in the zebrafish *M. marinum* model. Liposomes, containing antimycobacterials such as Rifampicin, have been shown to be efficiently phagocytosed by macrophages in the *in vivo* zebrafish embryo and are effective against *M. marinum* infection (Fenaroli et al., 2014). Furthermore, more recently, liposomes have been modified to more easily cross endothelial barriers, allowing larger nanoparticles to be used that remain able to penetrate granulomas but can deliver larger payloads of drugs. These findings were recapitulated in a murine model of TB, identifying that the zebrafish is a useful model to study nanoparticle drug delivery in mammalian systems (Fenaroli et al., 2018).

Finally, the quest to improve the ageing and relatively ineffective Bacillus Calmette-Guérin (BCG) vaccine is a key clinical aim. This has been addressed in the zebrafish by combining the BCG vaccination, (which is modestly protective in an adult zebrafish *M. marinum* model), with a DNA vaccine containing Ag85B, ESAT6 and a resuscitation-related gene RpfE. This combination of antigens boosted the protective effect of the BCG vaccine (Oksanen et al., 2016). The BCG vaccine neither prevents *M. tuberculosis* infection nor inhibits the reactivation of latent TB in humans, and so alternatives are being actively sought. The zebrafish is a useful screening tool for this search and some alternative candidate antigens have recently been identified showing promise for future novel vaccines (Myllymaki et al., 2018; Myllymaki et al., 2017).

The increase in study of teleost mycobacterial models makes it seem only a matter of time before novel treatment strategies discovered in fish enter clinical trials and the clinic itself rather than being used simply to inform the clinical situation.

SUMMARY

Various immune molecules and mechanisms have been established as relevant for host protection against mycobacteria while many others are either controversial or poorly defined. Despite an extensive body of work delineating various facets of immune mechanisms and host evasion mechanisms, *M. tuberculosis* remains a deadly pathogen responsible for millions of deaths annually. Teleost models offer an expanding platform for understanding of mycobacterial infections and those mechanisms that offer the greatest potential to enhance host protection.

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