Animal Models of Tuberculosis: Zebrafish

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Over the past decade the zebrafish (*Danio rerio*) has become an attractive new vertebrate model organism for studying mycobacterial pathogenesis. The combination of medium-throughput screening and real-time in vivo visualization has allowed new ways to dissect host pathogenic interaction in a vertebrate host. Furthermore, genetic screens on the host and bacterial sides have elucidated new mechanisms involved in the initiation of granuloma formation and the importance of a balanced immune response for control of mycobacterial pathogens. This article will highlight the unique features of the zebrafish–*Mycobacterium marinum* infection model and its added value for tuberculosis research.

Why would one use zebrafish (*Danio rerio*) to study tuberculosis (TB)? Although zebrafish are vertebrates, they do not have lungs, an obvious caveat for studying a pulmonary disease. Furthermore, at present it is unclear whether *Mycobacterium tuberculosis* can give rise to successful infections in cold-blooded animals. Robert Koch tried to infect cold-blooded animals, including a turtle, a goldfish, three eels, and five frogs. After two months, none of them showed any sign of disease, whereas most mammals were either clearly ill or showed tubercles upon autopsy (Koch 1884). Despite these drawbacks, zebrafish have emerged as a valuable organism to study infectious diseases and especially TB (Grunwald and Eisen 2002; Meeker and Trede 2008; Ramakrishnan 2013). The power of the model, real-time imaging of biological processes, was first exploited for TB by the group of Ramakrishnan (Davis et al. 2002),

leading the way to study mycobacterial virulence factors and host characteristics in real time in a living vertebrate animal. In recent years, the strength of the zebrafish model has been greatly extended with the increasing availability of transgenic zebrafish lines, improved imaging techniques, and a growing list of genetic tools and large-scale mutant analysis. This article will highlight the unique features of the zebrafish–*Mycobacterium marinum* infection model and its added value for TB research.

WHAT IS THE ZEBRAFISH–*M. marinum* MODEL OF TUBERCULOSIS?

To appreciate the zebrafish–*M. marinum* model of TB, it is important to discuss the basic traits and tools of both the zebrafish and its natural pathogen *M. marinum*.

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General Properties of Zebrafish

Advantageous features of the zebrafish include their small size (adults are 3- to 5-cm long), the possibility of keeping them at high population density (5 fish/L), and their ease of breeding—a single female can lay up to 300 eggs a week (Meijer and Spaink 2011). Zebrafish embryos develop externally and are transparent during embryo and larval stages, making it possible to follow host–pathogen interaction in real time. In contrast to other animal models, the zebrafish can be studied during the first weeks of development. In this period, the embryo solely relies on the innate immune system (Fig. 1) (van der Sar et al. 2004b; Meeker and Trede 2008; Novoa and Figueras 2012; van der Vaart et al. 2012), which provides the opportunity to study the contribution of innate immunity to disease in an isolated fashion. Furthermore, it allows for distinguishing between this arm of immunity and a combined innate and adaptive immune response in the context of infection, like in adult fish, which have a complex adaptive immune system akin to that of mammals (Fig. 1) (Traver et al. 2003; Meeker and Trede 2008; Renshaw and Trede 2012; van der Vaart et al. 2012). The finalized whole-genome sequence of zebrafish (Howe et al. 2013) reveals that ~70% of human genes have at least one obvious zebrafish ortholog.

Because of the genetic possibilities (Amsterdam and Hopkins 2006; Lesley and Rama-

krishnan 2008; Meijer and Spaink 2011; Blackburn et al. 2013) and the specimen availability and size (and therefore screening options), zebrafish are often seen as a bridge between cell culture systems and mammals (Brittijn et al. 2009).

General Properties of *M. marinum*

Because *M. tuberculosis* does not seem to cause disease in cold-blooded animals, an alternative pathogen is used. Zebrafish are susceptible to a number of mycobacterial pathogens, of which *M. marinum* is the most interesting candidate (Watrall and Kent 2007). *M. marinum* naturally inhabits aquatic environments and is the causative agent of a tuberculosis-like disease in cold-blooded animals (Tobin and Ramakrishnan 2008). Furthermore, this species is a close genetic relative of *M. tuberculosis*. At 6.6 Mb, the genome of *M. marinum* is ~1.5 times the size of that of *M. tuberculosis*, which likely reflects its expanded host range and capabilities to survive in the environment. Orthologous coding sequences share an average amino acid identity of 85% (Stinear et al. 2008). Furthermore, the two species share different mechanisms for intracellular growth and host survival. *M. tuberculosis* genes can usually complement mutations in *M. marinum* orthologs and vice versa (Gao et al. 2003; Stinear et al. 2008; Tobin and Ramakrishnan 2008; Stoop et al. 2011). Similar

Figure 1. Development of zebrafish immunity in comparison with the human immune system. Zebrafish possess a complex immune system, similar to that of humans. Development of zebrafish larvae is shown in A and a time line is shown in B. The appearance of components of the immune system is shown in C, and comparison is made with human immune components. Components of the innate immune system are detectable and active in the first days postfertilization (dpf) (e.g., macrophages, neutrophils, eosinophils, and mast cells). Adaptive immunity takes longer to develop and starts with thymus development at 60 hours postfertilization (hpf) and the appearance of the first lymphocytic markers at ~4 dpf. At 21 dpf, the thymus is fully matured, and the first mature T cells and B cells are detected; humoral immunity is functional at 28 dpf. wpf, weeks postfertilization; TCR, T-cell receptor; NK, natural killer; MHC, major histocompatibility complex; DC, dendritic cell; APC, antigen-presenting cell; TLR, Toll-like receptor; NITR, novel immune-type receptor; TNF- α , tumor necrosis factor- α ; IFN, interferon. References cited in figure as follows: a, Herbomel et al. 1999; b, Herbomel et al. 2001; c, Traver et al. 2003; d, Meijer and Spaink 2011; e, Novoa and Figueras 2012; f, Renshaw et al. 2006; g, Le Guyader et al. 2008; h, Renshaw and Trede 2012; i, Bertrand et al. 2007; j, Balla et al. 2010; k, Meeker and Trede 2008; l, Dobson et al. 2008; m, Lam et al. 2002; n, Trede et al. 2004; o, Danilova et al. 2004; p, Schorpp et al. 2006; q, Meeker et al. 2010; r, Laing and Hansen 2011; s, Lam et al. 2004; t, Danilova et al. 2005; u, Page et al. 2013; v, Yoder 2009; w, Yoder et al. 2010; x, van der Sar et al. 2004b; y, van der Vaart et al. 2012; z, Palha et al. 2013; aa, de Jong et al. 2011; bb, de Jong and Zon 2012; cc, Lugo-Villarino et al. 2010.

A		B		C		Ref				
				Zebrafish	Human
24 hpf	48 hpf	3 dpf	4 dpf	5 dpf	6 dpf	7 dpf	2 wpf	3 wpf	4 wpf	

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to *M. tuberculosis*, specific genotypic lineages of *M. marinum* are associated with variability in virulence (van der Sar et al. 2004a; Ostland et al. 2008; Hernandez-Pando et al. 2012). Apart from the free-living stage, another clear difference for *M. marinum* is its restricted growth temperature, which lies between 28°C and 30°C. Growth is normally halted at 37°C, which is considered as one of the main factors that limits *M. marinum* infections to cooler surface of the skin (Kent et al. 2006). *M. marinum* is primarily associated with human skin lesions called fish tank granulomas. Interestingly, these local granulomas are often histopathologically indistinguishable from *M. tuberculosis* dermal granulomas (Travis et al. 1985; MacGregor 1995) (Fig. 2). *M. marinum* has several other advantages over working with *M. tuberculosis*, including fewer biosafety restrictions (BSL2 instead of BSL3) and a relatively short replication time (4 h) (Tobin and Ramakrishnan 2008).

ROUTES OF INFECTION

The natural infection route for *M. marinum* has not been fully elucidated, but the available evidence strongly indicates that the gastrointestinal tract is the port of entry (Harriff et al. 2007). Furthermore, transmission was significantly enhanced when the bacteria were supplied with in free-living unicellular eukaryotes, including amoeba and paramecium (Harriff et al. 2007; Peterson et al. 2013). However, these more natural routes of transmission are not really applicable for infection experiments, as the infection dose and timing cannot be easily controlled. Therefore, to study mycobacterial pathogenesis in vivo, zebrafish are infected with *M. marinum* via different inoculation routes (Fig. 3). Adult zebrafish are usually infected by intraperitoneal or intramuscular injection, whereas the most commonly used infection route in embryos is injection into the caudal vein at 28 hpf (Meijer and Spaik 2011; Benard et al. 2012). Local in-

Figure 2. Pathology in adult fish compared with human granulomas. Zebrafish granulomas caused by *M. marinum* show great similarities with human granulomas formed after infection with *M. tuberculosis*. Panels represent nonnecrotic early granuloma in human (A) and zebrafish (B) and granulomas with a necrotic center in human (C) and zebrafish (D). Human granulomas obtained from a neuropathology study in our Department of Pediatric Infectious Diseases and Immunology (D Zaharie, S Roest, M van der Kulp, AM van Furth, pers. comm.).

Figure 3. Routes of infection. Zebrafish are infected with *M. marinum* at different time points and via different inoculation routes. Systemic infection is achieved by injection into the caudal vein at 28 hpf or inoculation via the duct of Cuvier in embryos at 2–3 dpf (Benard et al. 2012). Local injection routes are the hindbrain ventricle, muscle, notochord (Alibaud et al. 2011), or otic vesicle. In addition to intravenous injection at 24–28 hpf, yolk injection can be applied at the one- to four-cell stage in a high-throughput setting (Benard et al. 2012).

oculation routes (e.g., via the hindbrain ventricle, muscle, notochord, or otic vesicle [Fig. 3]) can be used to study macrophage and neutrophil chemotaxis. Alternatively, yolk injection at the one- to four-cell stage can be applied for early infections in a high-throughput setting (Meijer and Spaink 2011).

KEY FEATURES OF *M. marinum* INFECTION IN ZEBRAFISH

Actually two zebrafish infection models exist, the adult and the embryonic-larval model. Each has its own characteristics and benefits and both will be discussed.

Pathology in Adult Fish

Adult zebrafish develop on intraperitoneal injection with *M. marinum*, a chronic infection with necrotic (caseating) granulomas, a key feature of human TB (Pozos and Ramakrishnan 2004; van der Sar et al. 2004a; Berg and Ramakrishnan 2012). These granulomas are preferentially formed in fatty tissue and are most commonly found in the pancreas, adipose tissue, liver, spleen, and gonads (Swaim et al. 2006; Parikka et al. 2012; Oksanen et al. 2013; Stoop et al. 2013). The first granulomas can already be found in the first weeks postinfection (Swaim et al. 2006; Parikka et al. 2012). Even the first signs of necrosis, consisting of cytoplasmic and

nuclear debris, are present at this time. The induction of a latent, chronic, or active mycobacterial disease depends on the infection dose and the *M. marinum* strain used (van der Sar et al. 2004a; Swaim et al. 2006; Parikka et al. 2012). A low infection dose results in a latent disease with stable numbers of granulomas over time, whereas a high-dose infection leads to a more progressive and active disease (Parikka et al. 2012). During a chronic disease course in zebrafish, bacterial growth seems to mimic growth curves of various other animal models of TB—growth for the first 3–4 wk and reaching a plateau when adaptive immunity develops (North and Jung 2004; van der Sar et al. 2004a; Swaim et al. 2006; Parikka et al. 2012; Ramakrishnan 2012). At 16–20 wk postinfection, most granulomas contain a necrotic center, which is also the location where the bacteria are predominantly present. Most granulomas form a fibrotic and/or cellular cuff, which separates them from the surrounding tissue at this time point (Swaim et al. 2006; Parikka et al. 2012; Ramakrishnan 2012).

As in human TB, maximal control of *M. marinum* infection in zebrafish is dependent on an intact adaptive immune system (Swaim et al. 2006; Parikka et al. 2012; Ramakrishnan 2012). Because of the lack of immune markers, characterization of the immune response of zebrafish during mycobacterial infection is mainly based on transcriptome and deep se-

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quencing studies (Meijer et al. 2005; Hegedus et al. 2009; Meijer and Spaink 2011; van der Vaart et al. 2012). These studies show a modest but complex host response in the early stages of infection (Meijer et al. 2005; Hegedus et al. 2009). Detailed analysis of immune factors involved in mycobacterial disease depends on the generation of more knockout zebrafish and development of specific antibodies directed against immune cells and chemokines/cytokines.

Pathology in Embryos

Pathology in zebrafish embryos is, because of practical/ethical reasons, usually only studied for 5–6 d. Within this short time frame early granuloma formation can be studied by real-time imaging (Fig. 4). This allows visualization of early steps in mycobacterial pathogenesis in the context of innate immunity. On infection, *M. marinum* is readily phagocytosed by macrophages (Lesley and Ramakrishnan 2008; Yang

et al. 2012; Ramakrishnan 2013), which traverse endothelial and epithelial barriers and form infectious clusters in deeper tissue within 4 d (Davis et al. 2002; Lesley and Ramakrishnan 2008; Tobin and Ramakrishnan 2008). Once early granulomas form, macrophages adopt a distinctive epithelioid morphology. Within these clusters mycobacteria activate genes that are known to be specifically activated within mature granulomas in adults, confirming that these infectious clusters actually resemble granulomas (Tobin and Ramakrishnan 2008). This means that innate immune determinants are sufficient to drive *M. marinum* granuloma formation/initiation (Tobin and Ramakrishnan 2008; Meijer and Spaink 2011; Ramakrishnan 2013).

LESSONS LEARNED FROM THE ZEBRAFISH INFECTION MODEL

We will discuss a number of bacterial features and host characteristics important during the early steps of mycobacterial infection that

Figure 4. Pathology in embryos. (A) Merged bright-field and fluorescent image of a zebrafish embryo infected with red fluorescent *M. marinum* and photographed at 5 dpi. (Adapted with permission from Stoop et al. 2011.) Clustering of mycobacteria and early granuloma formation is shown as red spots. (B–D) Higher magnification of an early granuloma at 5 dpi formed after bloodstream infection, derived from analysis using confocal imaging by our research group. (B) *M. marinum* E11 (in red), (C) phagocytes stained with anti-L-Plastin (in green), (D) merge of B and C confirming the colocalization of these cells in early granulomas in zebrafish embryos. Scale bar, 35 μm.

have been discovered using the zebrafish model (Fig. 5).

Dynamic Granulomas

Classically the granuloma is regarded as a static structure “walling off” bacteria from the rest of the body and therefore critical for host protection (Ulrichs and Kaufmann 2006; Rubin 2009; Schaaf and Zumla 2009). This idea was changed upon observation of the early stages of granuloma formation in zebrafish embryos, which revealed the dynamics of this process (Ramakrishnan 2012). Elegant studies with photo-bleaching of distinctive clusters in zebrafish embryos and reinfection experiments showed that infected macrophages can detach from the established granuloma and wander off to new locations to form secondary granulomas, thereby disseminating *M. marinum* (Lesley and Ramakrishnan 2008; Ramakrishnan 2013). Furthermore, macrophages attracted to existing granulomas consume damaged/apoptotic infected cells and their bacterial content in the center of the granuloma, leading to expansion of the early aggregate. These experiments revealed two things: (1) Granuloma formation might actually aid bacterial proliferation, because accelerated bacterial proliferation coincides with granuloma formation (Lesley and Ramakrishnan 2008); and (2) early granulomas are not fixed in size and location. Subsequently, TB studies in mice and nonhuman primates further supported the notion that granulomas are actually highly dynamic structures (Egen et al. 2008; Lin et al. 2013; Ramakrishnan 2013).

Genetic Susceptibility to TB

A broad variation in TB susceptibility and differences between individuals is a long-understood concept.

In the search for candidates for host susceptibility, the zebrafish model has contributed by using forward genetic screens. Tobin et al. (2010) used this method to identify mutant zebrafish with increased susceptibility to *M. marinum*. Genetic analysis of one such mutant showed that the *lta4h* locus was affected. This locus controls the balance between pro- and

anti-inflammatory eicosanoids. Also in humans LTA4H polymorphisms seem to play a role in the control of infection and inflammation during TB (Tobin et al. 2010). This characterization led to the conclusion that inflammation must be balanced, and misbalance can result in either an inadequate inflammatory or tissue-destructive hyperinflammatory state. Additional research (Tobin et al. 2012) showed that therapies directed to a specific profile could favor disease outcome (Berg and Ramakrishnan 2012), highlighting how well the zebrafish model resembles aspects of human TB and how useful this model can be to study features of this disease.

In addition to the *lta4h* locus, other genes seem to be required to maintain the balance of mycobacterial infection. For instance, *ptpn6* morphant embryos, in which the gene is temporarily knocked down, show a hyperinflammation phenotype (Kanwal et al. 2013). The *ptpn6* gene is associated with chronic inflammatory disease in human and plays an important role as a negative regulator of the innate immune system, probably by regulating the induction levels of several kinases in TLR signaling (Kanwal et al. 2013).

Complementary Features of the Embryo and Adult Systems

An illustrative example in which virulence patterns showed large differences in the embryo model compared with the adult model is described by Stoop et al. (2013). In this study they examined the effect of a knockout in the mycobacterial *mptC* gene, which is required for mannan core branching of lipomannan and lipoarabinomannan. This modification has been linked to TLR-2 activation (Nigou et al. 2008). Interestingly, although this mutant is clearly attenuated in embryos, the effect is only minor in the context of the adaptive immune system. The reverse is also possible, as was shown by Weerdenburg et al. (2012). An *M. marinum* mutant disrupted in ESX-5 secretion was slightly attenuated in embryos, but showed increased virulence in adult zebrafish, characterized by highly increased bacterial loads and early onset of granuloma formation. The molecular basis for

Figure 5. Graphical summary. This summary shows bacterial and host characteristics important in mycobacterial pathogenesis derived from and validated in the zebrafish infection model. The different hallmarks of pathogenesis are shown. For each step, red shows identified mycobacterial factors important for the pathogen to survive and cope with the immune system of the host, and green shows host factors required for an appropriate immune response. Three types of granuloma are described and schematically depicted in this summary. The granuloma in the *middle* is the normal granuloma with a balance between inflammation and infection; at the *left* and *right*, granulomas without the right balance are depicted with high infection and high inflammation, respectively. Labels in the figure refer to the following references: a, Stoop et al. 2012; b, van der Vaart et al. 2012; c, Davis et al. 2002; d, van der Woude et al. 2012; e, Cosma et al. 2006; f, Meijer and Spaink 2011; g, Alibaud et al. 2011; h, Gao et al. 2006; i, Clay et al. 2007; j, Kanwal et al. 2013; k, Davis and Ramakrishnan 2009; l, Volkman et al. 2010; m, Tobin et al. 2010; n, Volkman et al. 2004; o, van der Woude et al. 2013; p, Stoop et al. 2013; q, van der Vaart et al. 2013; r, Roca and Ramakrishnan 2013; s, van der Sar et al. 2009; t, Elks et al. 2013.

this difference has not been identified yet, but seems to be independent of the adaptive immune response, as the hypervirulence phenotype was also observed in zebrafish *rag* mutants. These studies highlight the importance of studying both the embryo and adult systems.

Mycobacterial Virulence Factors

The zebrafish embryo is an excellent model to study the importance of different mycobacterial virulence factors in different steps of infection. The *erp* (*pirG*) gene, coding for a cell wall-associated protein with unknown function, was first identified as required for virulence in *M. tuberculosis* (Berthet et al. 1998; Cosma et al. 2006). Using microscopic examination of infected zebrafish embryos it could be shown that *M. marinum* lacking *Erp* failed to grow and survive upon phagocytosis, an event very early in granuloma pathogenesis (Cosma et al. 2006; Meijer and Spaink 2011). Subsequently, macrophages were eliminated in zebrafish embryos by injection of *pu.1* morpholino, thereby knocking down the *pu.1* transcription factor, which is required for myeloid development (Meijer and Spaink 2011). Now, growth of the *erp* mutant was restored, indicating that in vivo attenuation was specifically linked to defective growth inside macrophages (Lesley and Ramakrishnan 2008).

A number of studies have used different setups to identify *M. marinum* virulence factors, most of which seem to underscore the similarities between *M. marinum* and *M. tuberculosis*. The most elaborate screen was performed by Stoop et al. (Stoop et al. 2011, 2013; van der Woude et al. 2013), who screened in total 1000 random transposon mutants for early granuloma formation and virulence. With nearly half of the highly attenuated mutants, the most prominent virulence locus identified in these experiments was *esx-1*. This is not entirely surprising, as the *esx-1* locus is probably the most extensively studied virulence locus in pathogenic mycobacteria. The *esx-1* locus is coding for components of a protein secretion system and its substrates, and although the actual mechanism is still not completely resolved, the most compelling data suggests that ESX-1 effector

proteins are required for phagolysosomal escape (Stamm et al. 2003; Houben et al. 2012). In addition to the phagosome escape phenotype, macrophage recruitment and dissemination of disease (Volkman et al. 2004; Davis and Ramakrishnan 2009; Stoop et al. 2011) have also been attributed to the ESX-1 system, although these effects could be indirect because *esx-1*-deficient *M. marinum* does not reach its normal location within the phagocytosing cell. Importantly, phagosomal escape of pathogenic mycobacteria was first convincingly shown for *M. marinum* (Stamm et al. 2003) and only later for *M. tuberculosis*, underscoring the importance of this model.

In conclusion, the combination of real-time imaging and high-throughput settings seem ideally suited to screen for bacterial factors involved in the establishment of a successful infection.

Using Zebrafish to Identify New Antimycobacterial Compounds

The search for new antimicrobial compounds or therapies can be accelerated using the zebrafish model. Activity and dosage of antimycobacterial compounds in zebrafish closely resemble characteristics in humans (Adams et al. 2011). In addition, the zebrafish model has helped to challenge the model that persistence is linked to arrested growth (Adams et al. 2011; Philips and Ernst 2011). Using the zebrafish model, it was shown, by spatial monitoring of the behavior of fluorescent bacteria after treatment with antibiotics, that both macrophages and granulomas play a role in the induction and dissemination of drug-tolerant bacteria. The intramacrophage-mediated oxidative stress induces the expression of bacterial efflux pumps in actively replicating bacteria. It was also shown that bacterial efflux pump inhibitors (e.g., verapamil) can be added to the standard antibiotic treatment to reduce macrophage-induced drug tolerance and possibly shorten treatment (Adams et al. 2011; Philips and Ernst 2011; Berg and Ramakrishnan 2012; Zumla et al. 2013).

Another example of using zebrafish embryos in the identification of new antimycobac-

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terial drugs is a recent study by Makarov, who produced and analyzed a new generation of benzathiozinones (Makarov et al. 2014). These compounds bind DprE1 and thereby selectively inhibit the biosynthesis of crucial cell wall components. The most effective second-generation compound (i.e., PBTZ169) was compared with the first-generation lead compound in a zebrafish embryo infection model. Although both compounds reduced bacterial load in zebrafish embryos, this model showed an important difference in toxicity, whereas the original compound led to developmental abnormalities, like deposits in the notochord and subsequent shortening of the anteroposterior axes, and PBTZ169 did not. These examples show that effectiveness and toxicity of antimycobacterial compounds can be assessed accurately using zebrafish embryos.

CONCLUDING REMARKS

The use of zebrafish larvae for studying microbial infection has led to important new insights in host defense mechanisms, which often appear to be common for higher vertebrates (Table 1). However, we still need to extend our comparison of zebrafish model with the mammalian systems to show the translational value for biomedical applications. The rapid increase of available high-throughput technologies in the zebrafish toolbox, such as advances in robotic

injection and automated readouts of zebrafish embryos (Spaink et al. 2013), will lead to new approaches for TB research. In addition, new reporter lines of zebrafish that provide readouts for activation of the immune system are highly useful tools for even better in vivo visualization of mycobacterial infections (Kanter and Rawls 2010; Palha et al. 2013). What we still need are specific antibodies for distinguishing immune cell types and technologies for generating cell-specific and conditional knockout mutants.

Zebrafish provide an excellent opportunity to address questions that are difficult to solve in mammalian systems. In return, discoveries in zebrafish must be confirmed in mammalian systems to maximize their translational impact.

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Table 1. Top five advantages of the zebrafish model in mycobacterial research

- 1 Fast model, small animal, ease of breeding, ease of genetic manipulation
- 2 Transparency and availability of transgenic zebrafish lines make real-time imaging possible
- 3 Innate and adaptive immunity can be studied separately
- 4 *Mycobacterium marinum* is strongly related to *Mycobacterium tuberculosis* and causes granulomatous disease in zebrafish with shared characteristics to human granulomatous disease
- 5 Screens possible for (i) mycobacterial virulence factors; (ii) host factors; (iii) therapeutic compounds, like antibiotics

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