



The Zebrafish Breathes New Life into the Study of Tuberculosis

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Tuberculosis (TB) is a global health emergency. Up to one-third of the world's population is infected with *Mycobacterium tuberculosis*, and the pathogen continues to kill 1.5 million people annually. Currently, the means for preventing, diagnosing, and treating TB are unsatisfactory. One of the main reasons for the poor progress in TB research has been a lack of good animal models to study the latency, dormancy, and reactivation of the disease. Although sophisticated *in vitro* and *in silico* methods suitable for TB research are constantly being developed, they cannot reproduce the complete vertebrate immune system and its interplay with pathogens and vaccines. However, the zebrafish has recently emerged as a useful alternative to more traditional models, such as mice, rabbits, guinea pigs, and non-human primates, for studying the complex pathophysiology of a mycobacterial infection. The model is based on the similarity between *Mycobacterium marinum* – a natural fish pathogen – and *M. tuberculosis*. In both zebrafish larvae and adult fish, an infection with *M. marinum* leads to the formation of macrophage aggregates and granulomas, which resemble the *M. tuberculosis* infections in humans. In this review, we will summarize the current status of the zebrafish model in TB research and highlight the advantages of using zebrafish to dissect mycobacterial virulence strategies as well as the host immune responses elicited against them. In addition, we will discuss the possibilities of using the adult zebrafish model for studying latency, dormancy, and reactivation in a mycobacterial infection.

Keywords: tuberculosis, zebrafish model system, vaccination, *Mycobacterium marinum*, *Mycobacterium tuberculosis*, *Mycobacterium* infections, granuloma, latency

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INTRODUCTION

Tuberculosis (TB) is still the world's second deadliest infectious disease killing 1.5 million people and with an estimated 9.6 million new cases reported to the WHO in 2015 (1). An estimated one-third of the world's population has been exposed to TB. 5–10% of these latent carriers will eventually develop the active disease (1).

The causative agent of TB, *Mycobacterium tuberculosis*, spreads through the air (Figure 1A). Alveolar macrophages phagocytose the inhaled mycobacteria and transport them into the lung tissues (2). A cascade of pro- and anti-inflammatory signaling leads to the recruitment and accumulation of additional macrophages and other leukocytes in the pulmonary tissues. Eventually, the formation of granulomas, the hallmark of pathological TB, is initiated. The granuloma is a heterogeneous, but well-organized, and dynamic accumulation of immune cells, including

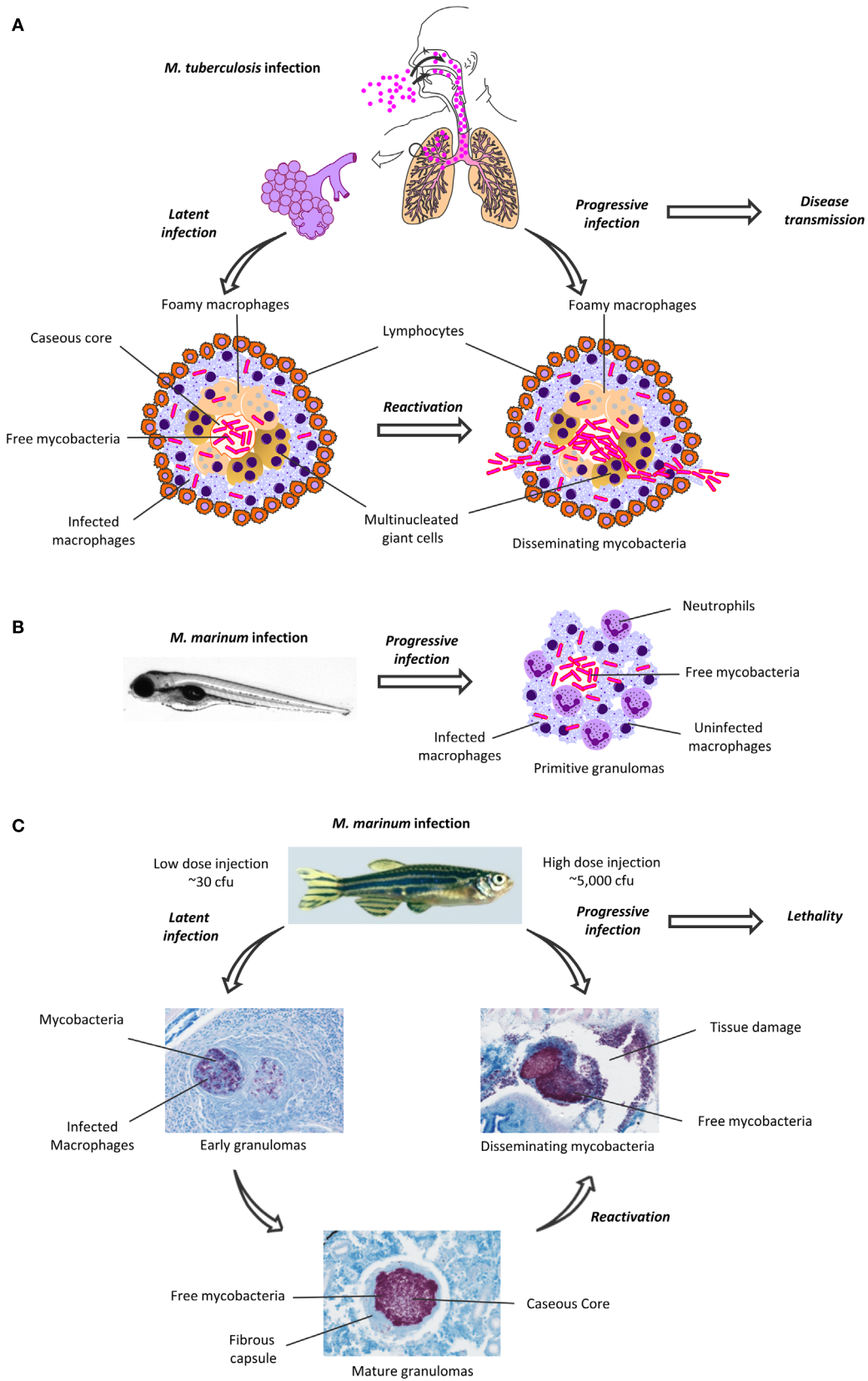


FIGURE 1 | Granuloma formation in human *Mycobacterium tuberculosis* infection and in zebrafish *M. marinum* infection.

(Continued)

FIGURE 1 | Continued

(A) *M. tuberculosis* spreads as an aerosol, and first infects alveolar macrophages. In most individuals, the infection is maintained in a latent, subclinical state, which is characterized by the formation of granulomas (left). The mature granulomas have a caseous, necrotic core, surrounded by infected macrophages and lymphocytes. Upon reactivation, the granulomas are disrupted, causing cavities in the lungs. The mycobacteria escape from the granulomas and are disseminated in cough droplets, which facilitates the transmission of the disease. **(B)** Upon infection with *M. marinum*, the granulomas in the zebrafish embryo develop within a few days and mainly consist of infected and uninfected macrophages and recruited neutrophils. **(C)** Depending on the bacterial dose, the adult zebrafish *M. marinum* infection can lead to a latent or an active, progressive disease. A latent infection is characterized by the formation of granulomas in various organs. As the early granulomas mature, their inner parts become caseous and surrounded by a fibrous wall. The zebrafish with a latent infection remain asymptomatic. A (re) activated, progressive mycobacterial infection is characterized by the disruption of the granulomatous structures, rapid replication and dissemination of mycobacteria and profound tissue damage. Eventually, a progressive mycobacterial disease will lead to death in most fish. The zebrafish granulomas were visualized with Ziehl–Neelsen staining, mycobacteria are seen as purple rods. cfu, colony-forming unit.

TABLE 1 | Characteristics of a mycobacterial infection in humans, adult zebrafish, and zebrafish embryos.

	Human	Adult zebrafish (>3 months)	Zebrafish embryo (<6 days)	Reference
Immune system	Innate and adaptive	Innate and adaptive	Innate only	(6–8)
Mycobacterial pathogen	<i>Mycobacterium tuberculosis</i> and atypical mycobacteria including <i>Mycobacterium marinum</i>	<i>Mycobacterium marinum</i>	<i>Mycobacterium marinum</i>	(9–11)
Natural susceptibility	Yes	Yes	Yes	(9, 10, 12)
Infection route and infectious dose	Airways ID ₅₀ <10 bacilli	Natural infection Digestive tract	?	(9, 10, 12)
	N/A	Experimental infection Multiple injection techniques, intraperitoneal injection is the most commonly used; <30–10,000 cfu	Multiple injection techniques, caudal injection is the most commonly used; <10–>300 cfu	(8, 13–15)
Infection phases	Acute Latent Reactivation	Acute Latent Reactivation	Progressive?	(10, 13–17)
Granuloma types	Early Fibrous Necrotic	Early Fibrous Necrotic	Primitive?	(4, 5, 13–18)
Cell types involved in granuloma formation	Macrophages Neutrophils Dendritic cells Lymphocytes (T cells, B cells, and NK cells) Fibroblasts Epithelial cells	Macrophages Neutrophils Dendritic cells Lymphocytes (T cells and B cells) Epithelial cells	Macrophages Neutrophils Epithelial cells	(4, 5, 13–18)

blood-derived infected and uninfected macrophages, foamy macrophages, and epithelioid cells (3). The inner cell mass is usually surrounded by a ring of leukocytes and fibroblasts (4) (**Figure 1A**; **Table 1**). The localization and control of the bacteria and the restriction of the immune response to a defined area are generally regarded as the main functions of granulomas (5).

Mycobacteria can persist asymptomatic within the granuloma for decades. However, a dysregulation of the immune system can reactivate the mycobacteria, which leads to disease progression (secondary TB) (2) (**Figure 1A**) Primary TB mainly occurs in children, who are at the highest risk for TB meningitis and a disseminated form of the disease (19).

Efforts to eradicate TB are obstructed by the lack of unambiguous diagnostic tools, the lengthy antibiotic treatments required for curation, the growing problem of multi-drug-resistant bacteria, and the poor protection provided by the Bacillus Calmette–Guérin (BCG), the only vaccine available (1). As a

live vaccine, BCG imposes a risk of a disseminated infection in immunocompromised patients (1, 20, 21). Thus, there is a need to develop new effective drugs and vaccines against TB. For this purpose, relevant animal models are essential. The most commonly used animal models in TB research are mice, guinea pigs, and non-human primates (NHP), all of which have their limitations related to either space, costs, ethical aspects, or their ability to replicate the human disease pathology (22, 23). Recently, the zebrafish–*M. marinum* model has gained popularity as a natural pathogen–host system that closely recapitulates the pathology of human TB (**Table 1**) (13, 23). The infection model and its applications are discussed in more detail below.

THE ZEBRAFISH–MYCOBACTERIUM MARINUM INFECTION MODEL

Mycobacterium marinum, the causative agent of fish mycobacteriosis, is a close relative of *M. tuberculosis* (24). *M. marinum*

spreads *via* water, and it also occasionally infects humans, but the infection is usually limited to the skin (fish tank granuloma) (11). Thus, *M. marinum* is safer to work with and has a shorter replication time than *M. tuberculosis* (9, 23). Similar to human TB, fish mycobacteriosis displays an acute and chronic form and the subsequently formed granulomas also resemble the lesions caused by *M. tuberculosis* [Figure 1 (13–15)]. Both bacteria are able to survive and replicate within macrophages (23, 25). In a laboratory setting, the zebrafish is an advantageous choice as a host organism for *M. marinum* for several reasons: multiple infection techniques can be used for both zebrafish embryos and adults (26); for a review, see Ref. (8, 27–29) (Figures 1B,C). The transparency of the embryos allows the use of sophisticated *in vivo* real-time imaging techniques, including multiple leukocyte and macrophage fluorescent reporter lines (30–32), and several techniques for genetic manipulation (6, 33–35). Moreover, zebrafish are small in size, and produce numerous offspring, making them also suitable for large-scale screening studies, including drug screens (6). Despite the anatomical differences between fish and mammals, the zebrafish is a vertebrate model with an innate and adaptive immunity consisting of the same primary components as present in humans (6, 36, 37). As zebrafish lack lymph nodes, immune cells mainly develop and perform their functions in the spleen, the kidney, and the thymus (6, 38, 39). Zebrafish embryos rely solely on innate immunity. In the embryos, functional macrophages and neutrophils emerge 1 and 2 days post-fertilization (dpf), respectively, while lymphocytes start developing after 4 dpf and the adaptive immunity becomes fully functional at 4 weeks post-fertilization (wpf) (7). This facilitates studying the function of the innate and adaptive immune system, as well as different cell types, separately (8, 33).

HOST-MYCOBACTERIUM INTERACTIONS – INSIGHTS INTO EARLY INFECTION EVENTS FROM ZEBRAFISH LARVAE

Zebrafish larvae have been especially useful in elucidating the role of macrophages and the strategies the phagocytosed mycobacteria use to suppress phagosomal maturation, apoptosis, and the antibacterial innate immune response (8, 25, 40). Scavenger receptors of different classes have been studied in the context of the phagocytosis of mycobacteria. For example, Marco binds the glycolipid trehalose 6,6'-dimycolate on the mycobacterial cell wall and affects the regulation of the subsequent proinflammatory response (41). CD36 also appears to be involved in mycobacterial control, though its role and the regulation of its expression seem quite complicated (42). Following phagocytosis, Toll-like receptor (TLR) signaling *via* MyD88 is needed for resistance against an early mycobacterial infection (43, 44). For example, the activation of the TLR signaling leads to the production of antibacterial molecules by macrophages, such as the perforin Mpeg1.2 (45). In the zebrafish, the *myd88*^{-/-} mutants have been a useful tool in elucidating the role of TLR signaling in mycobacterial resistance (46, 47).

Toll-like receptor signaling is involved in the initiation of the production of reactive oxygen and nitrogen species (ROS and RNS, respectively). In the zebrafish, early stabilization of the transcription factor hypoxia-inducible factor 1 α (Hif-1 α) in neutrophils limits bacterial growth by inducing iNOS, which in turn leads to increased protein nitrosylation (48). Later in the course of the infection, this response is decreased in the developing granulomas by a mechanism independent of the early secretory antigenic target 6 system 1/region of difference 1 (ESX-1/RD1) virulence locus (49).

As a mean to counteract the mycobacterial evasion strategies, the host can induce autophagy, a process which enables cells to digest their cytoplasmic contents, including microorganisms and membranous structures, in lysosomes (50). This is dependent on TLR signaling and Myd88, which are linked *via* the DNA damage-regulated autophagy modulator DRAM1. DRAM1 is needed for the formation of autophagosomes and for their fusion with lysosomes, while a DRAM1 deficiency leads to defects in maintaining the mycobacteria inside vesicles in macrophages and in the control of mycobacterial growth (51). Again, zebrafish larvae provide feasible tools for observing the events of autophagy *in vivo* and in real-time utilizing both light and electron microscopy, including the GFP-Lc3-transgenic line, for the visualization of autophagosomal structures (52).

ZEBRAFISH LARVAE MODEL CHALLENGE OLD DOGMAS IN MYCOBACTERIAL RESISTANCE

After a successful infection by mycobacteria, granulomas are seeded. The granulomas in zebrafish embryos mainly consist of aggregated macrophages, intra- and extracellular mycobacteria, together with recruited neutrophils, and form within a few days post-infection (18, 53); for a review, see Ref. (54). Despite their rather primitive structure, the embryonic zebrafish granulomas provide a physiological model for studying cellular processes affecting mycobacterial infections, such as the generation of hypoxia and angiogenesis. Injection of mycobacteria into the caudal vein results in the development of non-hypoxic granulomas in the richly vascularized area of the caudal hematopoietic tissue (48). In contrast, granulomas resulting from a trunk infection reside in a sparsely vascularized area and can become hypoxic and induce vascularization (55).

Importantly, studies on early granulomas in zebrafish larvae have challenged some of the old dogmas. In general, granulomas have long been considered a protection mechanism elicited by the host. However, recent evidence from zebrafish embryos suggests that *M. marinum* uses the macrophages and granulomas for its own expansion and dissemination (16, 17). The bacteria can recruit new, uninfected macrophages to the granuloma site using the RD1 locus (16). The arriving macrophages phagocytize infected, dead cells and thus contribute to the spreading of the bacteria. The recruitment of new macrophages is enhanced by the bacteria by ESAT-6-mediated production of matrix metalloproteinase 9 (17). Mycobacteria also need the RD1 locus for escaping from the phagosomes into the cytoplasm of infected macrophages both in

human cells and in zebrafish (51, 56). Additionally, mycobacteria use cell surface lipids to mask pathogen-associated molecular patterns, hence selectively infect permissive macrophages and avoid microbicidal ones (57). Thus, granulomas present a combat zone for the host immune system and the bacterial response, rather than purely a protection mechanism of the host to prevent the spread of bacteria (4).

In addition to basic research, the zebrafish larvae provide a feasible tool for early-stage drug development and large-scale screens (33, 58, 59). Two major, and related, issues in improving the TB drug development are the lengthiness of the curative treatments and the increasing emergence of drug-resistant bacteria (1). Discoveries made in zebrafish larvae revealed that intracellular mycobacteria use their efflux pumps to acquire a tolerance against the antibiotics commonly used to treat human TB, which allows the bacteria to persist and replicate in the cytosol. *M. tuberculosis* uses the same mechanism (60). However, this tolerance was reversed by an efflux pump inhibitor, such as verapamil, which can therefore reduce the tolerance to antibiotics and thereby shorten treatment times (61). These results prove that the zebrafish larval model can replicate the function of antitubercular compounds (60). In addition, angiogenesis has been shown to be important for granuloma formation. Therefore, targeting vascularization, for example, by inhibiting vascular endothelial growth factor receptor (VEGFR) signaling could provide a means to target mycobacterial infections and inhibit mycobacterial dissemination, resembling the strategy used in cancer therapies (55).

On the host's side, cytokines and their respective receptors play an important role in protection against mycobacteria. For example, the signaling axis mediated by chemokine CXC-motif containing receptor 3 (CXCR3) has been implicated in mycobacterial spreading and could thus provide a therapeutic target (62). In addition, in both humans and zebrafish larvae the leukotriene A4 hydrolase (LTA4H) locus controls pro- and anti-inflammatory mediators that in turn control the expression of TNF (63). While TNF is required for the host response against mycobacteria, its excess renders the host more susceptible to an infection, highlighting the importance of a balanced response (64). Thus, the zebrafish can elucidate the pathways controlling the host immune responses, and this information can be further applied to targeting these pathways with new drugs and developing host-directed therapies (8, 63–66). In addition, further investigation into the evasion strategies that mycobacteria use to interfere with the host defense mechanisms can potentially lead to the discovery of novel drug targets to combat mycobacterial diseases (49, 67).

MYCOBACTERIAL LATENCY VS. ADAPTIVE IMMUNITY – THE ADULT ZEBRAFISH AS A MODEL FOR TB

One of the main advantages of the zebrafish–*M. marinum* model may lie in granuloma formation, which has not been easy to reproduce in the traditional model animals (22). In the adult zebrafish, however, the histology of the mature granulomas resembles those seen in human TB with their caseous, necrotic core surrounded by leukocytes and epithelial cells (4, 17). Granulomas are found

in various organs such as the pancreas, gonads, spleen, and liver several weeks post-infection (**Figure 1C**) (13–15).

The latency of TB is another aspect that has been difficult to replicate experimentally (22). As this is also challenging to study in humans, our knowledge of the required immunological mechanisms for the control of a mycobacterial infection at its different stages remains limited (17). However, this could be improved by studying the zebrafish model, as like humans, adult zebrafish develop a latent, non-progressive disease with dormant bacteria residing within well-structured granulomas, and the fish remain asymptomatic (13, 14). Moreover, reactivation of the bacteria can occur spontaneously, or can be induced experimentally by an immune deficiency, such as exposure to γ -irradiation. In either case, reactivation will lead to the active spreading of the bacteria and the development of symptoms similar to an active infection as well as high lethality, much as in human TB (13).

Although the early cytokine response mediated by the innate immunity plays an important role in determining whether a mycobacterial infection leads to an acute or latent disease, the adaptive immunity is also required to control the bacteria. This is seen in the adult *rag*^{-/-} zebrafish that are devoid of lymphocytes. The mutant fish are unable to generate a latent state of the infection and are, therefore, hypersusceptible to *M. marinum* (13, 14, 68). So far, the significance of different lymphocyte subsets has not been extensively studied in the zebrafish mycobacterial infection. Nevertheless, Th1 as well as Th2 cells seem to be involved in the effective control of mycobacterial infections (69, 70). Interestingly, a novel Th2-like subset of cells capable of inhibiting the growth of *M. tuberculosis* has been found in human TB patients. This observation challenges the old idea that only Th1 cells are important for mycobacterial control (71). Genetic differences in the mycobacterial strains also seem to affect their virulence, for example, strains isolated from infected humans more commonly causing an acute disease, and isolates from poikilothermic species causing a chronic infection in the zebrafish (72).

While the zebrafish larvae provide a feasible tool for screening for drugs against TB, the adult zebrafish appears to be a promising model for early vaccine development. The zebrafish can be partially protected against mycobacteriosis by BCG (68, 73) or attenuated *M. marinum* (74), suggesting the use of adult zebrafish as a model for studying the feasibility of conserved mycobacterial antigens as vaccines. For example, the RD1 virulence locus and the ESX-1 secretion system, which are absent from BCG, have been shown to be important for virulence in *M. tuberculosis*. In *M. marinum*, RD1 is also required for granuloma formation in both larvae and adult fish (14, 49, 75, 76). Indeed, the antigens in this region do show some potential as targets for vaccines in the zebrafish as well as in other models (68, 73, 77–79). The zebrafish can also provide a feasible model for searching for the most effective antigen combinations and for studying different vaccination strategies. For instance, a DNA-based vaccine consisting of three mycobacterial antigens (Ag85, ESAT-6, and CFP10), which has also been studied in other TB models, confers protection against mycobacteria in zebrafish (68, 80–82). Furthermore, the effect of BCG can be boosted by a DNA vaccine (68, 73, 80–82). Adult zebrafish could therefore be used for developing vaccines for

both replacing and boosting BCG, as well as for studying the immunological correlates required for protection.

DISCUSSION

The lack of an animal model that recapitulates the human disease stages and pathology has in part hampered the development of new drugs, vaccines, and diagnostic tools against TB (22). Even though the mammalian animal models mostly used for TB research, namely, mice, rabbits, and guinea pigs, do develop granulomatous structures, only primates are a natural host for *M. tuberculosis* and show true latency and reactivation (83). However, the use of primates as laboratory animals is difficult in terms of ethical and economic issues as well as space limitations. When drug and vaccine development is considered, a natural host–pathogen pair is likely to be a more reliable model. Moreover, choosing to work with (zebra)fish might have an additional practical advantage: as mycobacterial infections are able to cause epidemics in fish farms, aquariums, and zebrafish facilities (9, 84), vaccinating fish against *M. marinum* is of potential economic and ecological relevance. This has been studied to some extent, for example, in the striped bass with the Ag85A DNA vaccine (85) and in the Japanese flounder with BCG (86). Thus, the results obtained in human biomedical research and veterinary studies could potentially augment each other.

The zebrafish–*M. marinum* infection exhibits essentially the same disease phases as those seen in human TB, including latency and reactivation – either spontaneously or following immunosuppression (13, 14). This might have important implications as the different disease phases probably also represent on the one hand different strategies of bacterial adaptation, and on the other hand, different stages of the host immune response (87). Since these aspects can be replicated in the zebrafish infection, the model should facilitate a more detailed dissection of both the effective (and deleterious) immune responses and the bacterial counter strategies in each stage of the infection. The zebrafish model can also be used to complement the human patient data in the identification of reliable biomarkers for the diagnosis of the different stages of TB (88).

Besides biomarkers, new drugs and vaccines are needed to combat TB. For this, a better knowledge concerning the correlates of a protective immune response is essential (89). The HI virus

attacks CD4⁺ T cells, and a co-infection renders the patients highly susceptible to TB. Therefore, it seems that CD4⁺ T cells are important for the host response (90). This does not mean, however, that the study of other cell types should be neglected, as they too can reveal new immunological mechanisms (70, 71). Moreover, deficiencies in the IFN- γ signaling axis lead to hypersusceptibility toward TB, and thus IFN- γ expression has been associated with protection against the disease (90). However, despite inducing high levels of IFN- γ production, a promising vaccine candidate, MVA85A, failed recently to enhance protection in an efficacy trial (91). This suggests that it is unlikely that a single immunological factor could predict the course of a TB infection (90, 92). Thus, there are still gaps in our knowledge of how an effective host defense against TB is elicited, and relevant animal models are needed to fill in the missing information. Furthermore, once the picture of protection mechanisms is more complete, the animal models can aid us in translating this information into the benefit of clinical medicine. We believe the zebrafish will be an important player in fulfilling both of these tasks.

AUTHOR CONTRIBUTIONS

All authors contributed to planning and writing the manuscript and designing of the figures.

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