Insights into tuberculosis from the zebrafish model

Russell D. Berg[1] and Lalita Ramakrishnan[2,3,4]

[1] Department of Molecular and Cell Biology, University of Washington, Seattle, WA 98195, USA
[2] Department of Microbiology, University of Washington, Seattle, WA 98195, USA
[3] Department of Medicine, University of Washington, Seattle, WA 98195, USA
[4] Department of Immunology, University of Washington, Seattle, WA 98195, USA

***Mycobacterium tuberculosis* (MTB) continues to plague humanity because of significant gaps in our understanding of MTB infection, including the nature of a protective versus pathological host response, why antimicrobial cure is so difficult, and the ineffectiveness of vaccination. The development of a zebrafish model, utilizing infection with the natural fish pathogen *Mycobacterium marinum* (Mm), has yielded important insights into tuberculosis with immediate clinical applications.**

Insights into tuberculosis from the zebrafish

Tuberculosis is an ancient disease that still inflicts humanity with a heavy burden of mortality and lost productivity. It is characterized by the formation of hallmark histological lesions called granulomas, comprising differentiated macrophages and other immune cells surrounding a necrotic core. *Mycobacterium tuberculosis* (MTB) bacilli are found in the cellular areas and are especially abundant in the necrotic regions. Not only is the existing vaccine strain, Bacillus Calmette–Guérin (BCG), of extremely limited efficacy, but curative drug treatment takes several months, leading to widespread treatment failure and drug resistance. Moreover, systemically disseminated infections with drug-sensitive MTB are often lethal, even with antimicrobial therapy. Elucidating the pathogenesis of tuberculosis has been difficult: the ubiquitous mouse model does not fully recapitulate human disease, and other models such as rabbits, guinea pigs, and nonhuman primates are expensive and lack genetic and immunological tools. MTB is also dangerous to work with, requiring specialized and expensive facilities. The study of the zebrafish (*Danio rerio*) infected with its natural pathogen *Mycobacterium marinum* (Mm) as a model of tuberculosis has largely overcome these limitations and provided surprising insights into the pathogenesis of human tuberculosis, with immediate clinical implications [1–6].

The zebrafish's amenability to forward and reverse genetics and its optical transparency in the larval stages led to its early adoption as a model for the study of disease and development. More recently, this has been extended to the study of immunity [7]. The increasing availability of transgenic lines with fluorescent labels in phagocyte and lymphocyte lineages has increased its advantages for studying host–pathogen interactions [7]. On the pathogen side, Mm is the closest relative of the *M. tuberculosis*

organism complex [8]. Due to its preferential growth at 35°C, Mm typically causes only a superficial human infection called 'fish-tank granuloma' that is histologically similar to dermal tuberculosis [9]. Zebrafish infection produces the characteristic necrotic (caseating) granulomas seen in human tuberculosis. Importantly, infection of the larval stage recapitulates the steps leading to human tuberculosis, producing a granulomatous infection. Simply being able to directly and non-invasively visualize the steps of pathogenesis in the transparent larval zebrafish under baseline and perturbed conditions has yielded three major insights into tuberculosis (Box 1).

The granuloma is dynamic and promotes bacterial expansion

The hallmark histological structure of tuberculosis is the granuloma, characterized by an infectious focus surrounded by infected and uninfected phagocytes, some of which have assumed an elongated and interdigitated phenotype described as 'epithelioid'. In humans and zebrafish, the center of the granuloma becomes filled with dead phagocytes and extracellular bacteria, comprising a substance called caseum. The prevailing understanding of the granuloma, based on analysis of histological sections from humans, was that the granuloma is a static host-protective structure with epithelioid macrophages serving to 'wall-off' an infectious agent they could not otherwise clear. Findings from the zebrafish have shown that the tuberculous granuloma is dynamic. A mycobacterial virulence determinant, ESAT-6, induces recruitment of macrophages to the site of infection [6]. There, bacteria are phagocytosed directly or through efferocytosis of apoptotic/necrotic macrophages. Subsequently, infected macrophages can then egress from the granuloma and seed additional granulomas [5]. Rather than solely protecting the host, granulomas are now understood to be a mechanism for mycobacterial expansion and dissemination

Tolerance to antimicrobial agents is mediated by bacterial efflux pumps

Tuberculosis cure requires long-term treatment because genetically susceptible mycobacteria become drug-tolerant *in vivo*. The mechanism of phenotypic antibiotic tolerance in mycobacteria has been attributed to latent, non-replicating bacterial populations in the host. The zebrafish model has again shed light on this critical, and frustrating, aspect of treating tuberculosis. It was shown that Mm and MTB within macrophages become drug-tolerant and are then expanded and disseminated by the granuloma, even

Box 1. Key insights into tuberculosis gained from the zebrafish

The granuloma is dynamic
Virulent *Mycobacteria* induce granuloma formation and actively recruit phagocytes to the granuloma [6]. Egress of infected macrophages from a granuloma seeds new infectious foci [5].

Mechanism of antibiotic tolerance
Both *Mycobacterium tuberculosis* (MTB) and *Mycobacterium marinum* (Mm) develop phenotypic antibiotic resistance in response to the intra-macrophage environment. This antibiotic tolerance is mediated by bacterial efflux pumps, which can be inhibited by verapamil [4].

Inflammation must be balanced
The *LTA4H* locus regulates the tumor necrosis factor gene *TNF* through balanced production of pro- and anti-inflammatory eicosanoids. *TNF* restricts intracellular growth but, in excess, leads to cell death [10]. Only patients with increased inflammation benefit from current standard-of-care adjunctive immunosuppressive treatment [3].

during treatment [4]. Following up this observation in human macrophages revealed that intracellular Mm and MTB become antibiotic-tolerant by inducing specific efflux pumps that are primarily required for intra-macrophage growth. It was subsequently shown that verapamil – an old, already approved, and cheap drug – inhibits efflux-mediated tolerance.

Both excess and inadequate inflammation renders the host susceptible to tuberculosis

Tuberculosis has historically been attributed to result from suboptimal inflammation because immunosuppressants such as corticosteroids and TNF antagonists increase tuberculosis susceptibility in humans. A forward genetic screen in the zebrafish for host determinants of Mm susceptibility identified the locus *lta4 h* as critical to the balanced production of pro- and anti-inflammatory eicosanoids required for optimal control of infection [10]. This finding was extended to humans, where a human *LTA4H* polymorphism has been associated with disease severity [3]. Importantly, only high inflammatory state individuals respond to standard-of-care anti-inflammatory adjunctive therapy, whereas low inflammatory state patients may be harmed by it. This work changes our understanding of human resistance to tuberculosis, and has immediate clinical ramifications for the use of personalized pharmacogenomics to guide treatment.

The zebrafish model has many inherent advantages for the study of tuberculosis pathogenesis. Zebrafish naturally develop a tuberculosis-like disease upon infection with Mm, enabling us to examine a multi-layered immune response that has developed over evolutionary timescales. The fish is also cheap, fecund, genetically tractable, and optically transparent, facilitating real-time non-invasive microscopy. Studies in the zebrafish have changed how we think about tuberculosis, teaching us that the granuloma is dynamic, mycobacteria become tolerant to antibiotics by inducing expression of efflux pumps, and that both inadequate and hyperinflammatory states are pathological. The zebrafish model is likely to continue making important inroads into understanding the confusing and often contradictory clinical realities of tuberculosis.

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