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Insights into early mycobacterial pathogenesis from the zebrafish

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Summary

Here we discuss the application of the zebrafish as a relatively new model host for the study of mycobacterial pathogenesis. Recent advances in our understanding of host-mycobacteria interactions from the zebrafish include insights into the role of the innate immune system in both controlling and facilitating infection. Analysis in the zebrafish has revealed that innate macrophages restrict initial bacterial growth, but also convey infecting bacteria into the granuloma, which serves as a place for bacterial growth and spread. Bacterial virulence determinants interact with these processes at different steps in pathogenesis, which can be dissected in these living see-through hosts. As these studies uncover new facets of the bacteria-host interactions in tuberculosis they raise even more questions for future investigation.

Introduction

Tuberculosis (TB) is a chronic disease that requires the infecting mycobacteria survive and replicate in the face of an apparently competent host immune response. The discernible steps of mycobacterial pathogenesis suggest the engagement of a wide range of immune response pathways. In human pulmonary TB, infecting mycobacteria traverse the alveolar epithelium in macrophages and dendritic cells [1,2]. Mycobacteria replicate within these cells by subverting host endocytic trafficking mechanisms [3]. These cells transport mycobacteria to deeper lung tissues where they aggregate with additional macrophages and other immune cells to form organized structures called granulomas. Granulomas become increasingly complex as adaptive immunity and other organizing elements such as collagen and fibrin come into play. The natural history of infection is similarly complex. The infecting bacteria can gain an upper hand from the outset leading to progressive granulomatous disease. In many cases however, infection can be cleared by innate immunity alone, or after adaptive immunity is invoked [4,5]. Still, in about a third of infected individuals, mycobacteria persist long-term within granulomas, leading to asymptomatic infection. A tenth of these asymptomatic cases progress to active disease, often without a discernible waning in host immunity. The molecular and cellular mechanisms regulating the steps of pathogenesis and different infection outcomes have been difficult to approach in any single model of TB pathogenesis [5]. The development of the zebrafish as a genetically tractable and optically

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transparent model to study mycobacterial pathogenesis is helping to fill some of the gaps in our understanding of the early steps of pathogenesis and their consequences. The new findings and areas for future exploration in mycobacterial pathogenesis made possible by the zebrafish model are the focus of this review.

The zebrafish model of mycobacterial pathogenesis

The zebrafish is naturally susceptible to TB caused by *Mycobacterium marinum* (Mm), a close genetic relative of *Mycobacterium tuberculosis* (Mtb) [6,7]. Mm is a natural pathogen of ectotherms and like Mtb, replicates in host macrophages and produces a chronic granulomatous infection using shared virulence determinants [8-12]. The zebrafish has both innate and adaptive immunity and, similar to mammals, both are involved in protection against TB [13-16]. The hallmark cells of human immunity including macrophages, granulocytes, T and B lymphocytes, have all been identified in zebrafish [13]. Homologues of many determinants of innate and adaptive immunity in humans including Toll-Like Receptors (TLRs), complement components, as well as most cytokines and chemokines are present [13,17-19]. A useful feature of the zebrafish is that it can be infected during early developmental stages when it is optically transparent and fully competent macrophages are present and circulating, but when adaptive immunity has not yet developed [14,20]. This allows for the intravital monitoring of host innate immune-mycobacterial interactions and the separation of the roles of innate and adaptive immunity in pathogenesis [9,14,20]. The early events of pathogenesis can be readily visualized in zebrafish embryos infected via injection of fluorescent bacteria into the caudal vein or the hindbrain ventricle at 32-48 hours post fertilization (pf). Macrophages arrive at the infection site within hours, phagocytose the bacteria and then migrate to deeper tissue where they form organized granuloma-like aggregates (see Figure 1, Step 1-6) [14]. In adult zebrafish Mm induces systemic disease with the caseating granulomas typical of human TB [16].

Molecular, genetic and imaging tools are steadily being developed in the zebrafish. Table 1 summarizes the present and potential uses of some of these techniques for the study of mycobacterial pathogenesis. Despite these advances, several limitations continue to beleaguer the zebrafish model including a paucity of cell lines and antibodies, as well as transgenic and inbred congenic fish lines, and thus difficulty in performing cell transfer experiments, all of which contribute to the utility of the mouse as a model host. While mouse models offer abundant immunological tools and incomparable molecular resolution these models have traditionally focused on endpoint analysis for technical reasons. Recent advances in intravital microscopy have enabled the first live images at various stages of mycobacterial infection in the mouse liver [21]. The study of mycobacterial pathogenesis can be powerfully augmented by the unique and accessible real-time observational capabilities of the zebrafish. Long term, serial imaging of live zebrafish to follow infection in a single fish from the earliest stages to granuloma formation [14] can be combined effectively with a variety of techniques (Table 1) for a detailed dissection of the role of individual host or bacterial determinants in mycobacterial pathogenesis. Already several insights and discoveries have emerged from such live imaging studies as discussed below.

One application of the unique advantages of the zebrafish embryo model is in identifying the contribution of bacterial and host immune determinants to specific steps of early pathogenesis. While the manner in which bacterial determinants intercept macrophage endocytic trafficking is well studied in cell culture models, if and how bacteria affect cell migration and granuloma formation *in vivo* remains a mystery [22]. In addition, several key host protective immune determinants, such as TNF, are pleiotropic in their actions, and how and when they exert their protection against mycobacteria is not clear from the combination of *in vitro* studies and endpoint assessments *in vivo* [23]. Even the broader issue of the

relative roles played by the innate and adaptive arms of immunity in TB remains an open question. The finding that Mtb grows exponentially for the first weeks of infection in immune competent hosts suggests that early innate immunity is ineffective in controlling bacterial growth before the onset of adaptive immunity [24]. Yet, there is an increasing appreciation for the role of innate immunity in protection against TB following the identification of a susceptibility gene in mice and humans that modulates macrophage killing of Mtb [25,26]. In addition, the effects of several mycobacterial virulence determinants are apparent in zebrafish embryos, suggesting that these determinants begin to exert their effects in the sole context of innate immunity [9,10,14]. The genetic evidence in humans and mice that a weakened innate immune response can lead to early overgrowth of mycobacteria [27,28] would suggest a corollary hypothesis that a strong innate immune response may be able to eradicate the bacteria and may be an explanation for the low infection rate of Mtb [4,5]. The temporal separation of innate and adaptive immunity in the developing zebrafish embryo in conjunction with the use of defined host and bacterial mutants allows a reductionist study of innate immunity in the absence of adaptive influences. For convenience, we will discuss recent findings from this model as they pertain to the steps of pathogenesis outlined in Figure 1.

Step 1 and 2: Macrophages migrate to and phagocytose mycobacteria

Real time imaging of zebrafish embryos immediately following infection reveals the arrival of phagocytes at the infection site and their uptake of the mycobacteria [14,15]. Using macrophage and granulocyte specific markers on whole infected embryos it has been determined that macrophages are the primary cell type infected with Mm, although infected neutrophils have been observed as well [15,29]. Macrophage migration is specifically induced by bacteria and not by inert latex beads when either is injected into the hindbrain ventricle at a time when this cavity normally lacks macrophages [15]. Using genetic manipulations of both host and bacteria it may be possible to determine the bacterial and host signals responsible for this early detection of bacteria by microenvironmental cells, such as epithelial, endothelial and stromal cells, and how this in turn signals macrophage migration to the site of infection.

Step 3: Migration of infected macrophages to deeper tissue

Cell culture studies using transwells have suggested that mycobacteria traverse epithelial barriers both within macrophages as well as directly [1,30]. In fact, the ESX-1/RD1 virulence determinant has been implicated in the ability of pathogenic mycobacteria to directly cross epithelial barriers [31]. However, a direct examination of mycobacterial transport in the zebrafish embryo has revealed that infecting mycobacteria traverse both endothelial and epithelial barriers mainly within macrophages *in vivo*; very little transit occurs in embryos lacking phagocyte lineages (created by morpholino knockdown of the myeloid transcription factor *pu.1*) [15]. The signals causing these infected macrophages to migrate back into deeper tissue to initiate granuloma formation are poorly understood [1] and this area is ripe for exploration in the zebrafish. For example, the function of chemokines important in migration of myeloid cells to and from the lung in mouse models may be dissected in greater detail in the zebrafish [32]. Significantly, issues of how antigen is trafficked and presented to adaptive immune cells cannot yet be addressed in the zebrafish model due to lack of specific cell markers to identify antigen presenting cells and lack of knowledge of lymphoid organ structure and function in fish.

Step 4: Growth of mycobacteria within individual macrophages

Once the bacteria are within macrophages, they are exposed to bactericidal mechanisms. What are these mechanisms and which are solely innate versus enhanced by adaptive

immunity? The defining feature of pathogenic mycobacteria (with the notable exception of *Mycobacterium ulcerans*) appears to be their ability to grow in cultured epithelial cells or macrophages [33]; this growth can be restricted by the addition of IFN γ to activate the macrophages [34]. Similarly, *in vivo*, Mtb grows logarithmically for the first weeks and plateaus only with the onset of adaptive immunity [24]. Therefore it has been postulated that “innate” macrophages, that is macrophages that have not been primed by Th1 helper T cells, are unable to control mycobacterial growth [5]. However, the zebrafish infection model has revealed that innate macrophages can restrict mycobacterial growth. *pu.1*-deficient embryos lacking macrophages have ten-fold more bacteria after just four days of infection [15]. What host factors mediate these bacteriostatic/bactericidal effects in innate macrophages? Can reactive oxygen or nitrogen intermediates exert any mycobactericidal effects in innate macrophages as they do in IFN γ activated macrophages, or do other mechanisms, such as those mediated by defensins and iron regulation, predominate early in infection? Targeted knockdowns of candidate genes as well as forward genetic screens for zebrafish mutants with alterations in early susceptibility to infection may reveal mechanisms used by innate macrophages to control mycobacterial growth, an area where our understanding is surprisingly lacking. Understanding the mycobactericidal capacity of innate macrophages is important because these mechanisms may contribute to the observed variations in susceptibility of highly exposed individuals to tuberculosis infection.

The other side of this question is how mycobacteria resist the growth restriction imposed by innate macrophages. Mutant analysis over the years has revealed several Mtb determinants that are required for growth in cultured macrophage monolayers and for virulence in whole animals, including Erp and ESX-1/RD1 [35,36]. Erp is a mycobacterium specific cell surface protein [35]. The ESX-1/RD1 locus (hereafter referred to as ESX-1) is absent in all BCG vaccine strains and the locus contains multiple genes encoding secretory machinery and its substrates [31,37-39]. The Mm *erp* and *esx-1* mutants both appear similarly attenuated in macrophages, zebrafish embryos and adult animals by endpoint analyses [10]. The visualization capabilities of the zebrafish embryo has allowed a more detailed dissection of the interaction of these two determinants with host macrophages. Many mechanisms have been proposed for ESX-1 mediated virulence including suppression of macrophage cytokine production and inhibition of phago-lysosomal fusion [39-41]. Other groups have suggested that the *esx-1* locus is not required for intracellular growth per se. Two studies with Mtb and Mm *esx-1* mutants in cultured macrophages suggest that bacteria lacking *esx-1* may be unable to spread from the initially infected macrophages to others in the monolayer, resulting in failure to spread throughout the macrophage culture and achieve maximum growth [11,38]. Similarly in detailed analysis of infected zebrafish embryos the *esx-1* mutant appears to grow in individual macrophages in the fish as evidenced by a normal percentage of macrophages containing numerous bacteria [9,10]. However in this same assay the *erp* mutant bacteria appear to have a macrophage growth defect as the macrophages in the infected fish contain fewer and dimmer fluorescent bacteria [10]. Moreover, the *erp* mutant growth defect is rescued in the *pu.1* morphant embryos that lack phagocytes, further suggesting that its attenuation *in vivo* results from its inability to grow in macrophages [15]. Considering that Erp also plays a role in resisting hydrophobic compounds *in vitro*, it is tempting to speculate that it may be resisting macrophage defensins *in vivo* as the *kasB* locus is thought to do [10,12,42]. In any case, a further dissection of the host molecules whose effects may be intercepted by bacterial Erp should be possible in the embryo model by morpholino knockdowns of candidate molecules, such as defensins, or a forward genetic screen to identify host mutants in which Erp attenuation is rescued.

Steps 5 and 6: Aggregation of infected macrophages and intercellular spread of bacteria

The first surprise that came from the zebrafish embryo infection model was that granuloma-like aggregates formed within only three days of infection. These aggregates represent genuine granulomas in that they are highly organized structures consisting of differentiated macrophages that have undergone epithelioid transformation. Moreover, mycobacteria residing in these structures express the same granuloma activated genes that are expressed in adult granulomas containing adaptive immune cells [14]. The finding that mycobacterial interactions with innate immunity are sufficient to induce granuloma formation challenges the model that granuloma formation requires the participation of adaptive immunity. The ability to monitor and modulate aggregate formation in the zebrafish embryo model has allowed further examination of the function of the granuloma in mycobacterial pathogenesis. Direct visualization of granuloma formation during Mm infection in the embryos revealed an additional ESX-1 mediated phenotype. Mm deficient in *esx-1* cannot efficiently induce the aggregation of infected macrophages into granulomas. This aggregation defect is rapidly rescued by the introduction of a few wild-type bacteria; the macrophages harboring wildtype bacteria seed aggregation of the macrophages containing *esx-1* deficient bacteria [9]. These data suggest that the bacterial ESX-1 locus drives macrophage aggregation and that while macrophages infected with *esx-1* deficient bacteria can receive signals to aggregate, they cannot send such signals. One possible interpretation of this finding is that ESX-1 induces host granuloma formation to promote the arrival of new nutrient rich niches for bacteria to spread to as they replicate. Interestingly, in macrophage cultures where recruitment of new host cells is bypassed by close proximity of macrophages in a monolayer, *esx-1* deficient Mtb and Mm still exhibit a phenotype of failure to grow or spread [11,38] suggesting that failure to aggregate and failure to grow may be separable processes in *esx-1* mutants. Death of infected macrophages has been implicated in the ESX-1 cell spreading phenotype in cultured macrophages [11,31,38] as well as in zebrafish embryos where there is reduced TUNEL-positive death in the few granulomas that form in the absence of ESX-1 [9]. The causal relationships between the many observed ESX-1 mediated phenotypes (macrophage suppression, phagosome maturation arrest, macrophage death, aggregation and intercellular bacterial spread) is not clear. It is possible that the *esx-1* locus mediates these processes independently or in a single pathway. For instance, the induction of cell death could play a role in both aggregation and intercellular spread in the aggregates. Additionally, macrophage subversion could lead to phagosome maturation arrest or vice versa as suggested by a recent study finding that inflammasome activation and phagosome maturation may be linked [43]. Real-time comparisons of *ESX-1* deficient and wildtype infected macrophages during the processes of bacterial growth, intercellular spread and macrophage aggregation may allow dissection of the causative relationship between the various ESX-1 mediated phenotypes.

The role of ESX-1 in granuloma maintenance has been hinted at by the observation that late stage granulomas in adult mice and zebrafish infected with *esx-1* deficient mycobacteria appear loose and unstructured [16,36] but the early phenotype seen in the zebrafish embryo further suggests ESX-1 interacts with host innate immune determinants in granuloma initiation. The zebrafish model can be utilized to determine more precisely how ESX-1 mediates aggregation and virulence. For instance, comparing host factors induced very early after infection with wildtype or ESX-1 deficient bacteria may reveal mechanisms by which mycobacteria promote host cell aggregation and cell death.

Step 7: Granuloma maturation

The finding that macrophage aggregation is promoted by a bacterial virulence determinant suggests that granulomas may not be solely host protective structures, at least early during

the innate immune phase of infection. But it is possible that the granuloma matures to play a more protective role once adaptive immunity comes into play. However, studies on the trafficking of superinfecting mycobacteria into established granulomas reveal a need for reexamination of the model that the established granuloma is a solely host beneficial structure. When adult frogs or zebrafish are infected with green fluorescent Mm and granulomas are allowed to form and mature over several weeks, subsequent superinfection with red fluorescent bacteria results in their rapid trafficking within host macrophages into the established granulomas formed by the original green bacteria. Red bacteria were even found within caseating granulomas, revealing that these are not impermeable structures as previously thought [44]. One explanation for this phenomenon is that it represents the most efficient way for the host to control bacteria, by delivering them to an established site of immune control. However the superinfecting bacteria continue to grow at these sites alongside the initiating bacteria, raising the question of whether mycobacteria derive benefit from granuloma residence even in the adaptive immune stages of infection [22,45].

Conclusions

As exemplified by these early studies, it appears that several complex processes involving cell-cell or cell-bacterial communication can be investigated using the genetic tractability and optical transparency of the zebrafish. Some unexpected discoveries have emerged and given the tools and techniques now available, some mechanistic dissection of these discoveries seems eminently possible. In thinking about approaches, it is important to keep in mind the limitations of the fish system. For instance, cell transfer experiments to determine if a phenotype is cell autonomous or not are not yet easy to perform. However, the capacity for long-term serial visualization provides a significant opportunity to complement traditional disease models with a detailed analysis of the cellular processes in effect early in pathogenesis. Diseases involving a great deal of cell migration and aggregation are most likely to benefit from this new see-through model.

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Figure 1. Infection of zebrafish embryos with fluorescent *Mycobacterium marinum*

A. A zebrafish embryo 30 hours post fertilization (hpf). The arrow indicates injection site in the caudal vein for i.v. infection. The bracket indicates the area of the hindbrain where bacteria can be injected for phagocyte recruitment assays. **B.** Fluorescence image of a zebrafish embryo 5 days post infection (dpi) infected i.v. with ~150 cfu of constitutively green fluorescent Mm. Arrowheads indicate aggregates of infected cells. Scale bar is 300µm. Photos by J.M. Davis.

Figure 2. Schematic of the early stages of mycobacterial pathogenesis. Steps are numbered to correspond to steps referred to in the text. mφ = macrophage. Figure adapted from [10].

Table 1

Technique/tool	Description and utility in the study of mycobacterial pathogenesis
Forward genetics	The fecundity of the zebrafish and the ability to create gynogenetic diploids make forward genetics an efficient means to identify new genes involved in the innate immune response to mycobacteria. <u>Potential application:</u> This approach will identify host genes that modulate TB susceptibility.
Reverse genetics Morpholinos	Transient blockade of gene function, by altering mRNA splicing or inhibiting protein translation [46,47]. <u>Example:</u> This method has been used to determine contribution of host innate immune macrophages e.g. blockade of myeloid transcription factor pu.1 [15].
TILLING	Gene-targeted screening for induced mutations in genomic DNA in a library of sperm generated by random mutagenesis [48]. <u>Example:</u> This tool has been used to identify <i>rag1</i> mutant and the contribution of adaptive immunity in mycobacterial infection in zebrafish model has been tested by using the mutant [16,48].
Dominant negative transgenes	Functional knockdown of host genes by expression of dominant negative protein [49]. <u>Potential application:</u> Using tissue-specific or inducible promoters to drive transgene expression may facilitate the study of spatial and temporal requirements of host gene functions during mycobacterial infection.
Gene expression profiling	Using whole genome arrays available from covering approximately 12,000 Unigene clusters – mostly unannotated [50]. <u>Example:</u> Identify mycobacterium infection regulated genes [50].
Whole mount in situ hybridization	Localization of gene expression in the whole animal [51] <u>Example:</u> Localization of cytokine induction in the context of infection [15]
Fluorescent transgenes	Transgenes integrated into genome by transposition to express fluorescent proteins in selected cell types or under control of other promoters of interest. Transgenic lines with fluorescently tagged myeloid cells [29,52,53] and T lymphocytes are currently available [54]. <u>Example:</u> Mycobacterium-induced cell migration across endothelial barriers [15] <u>Potential application:</u> The development of GFP-macrophage will facilitate the study of the dynamics of granuloma formation.
Photoactivatable fluorescent proteins	The fluorescent protein Kaede can be activated to change color upon UV irradiation [55]. <u>Potential Application:</u> Label individual infected cells and follow over time. Monitor host or bacterial gene expression over time.
Real-time imaging	Live embryos are visualized using differential interference contrast-Nomarski light microscopy, Fluorescence and Confocal microscopy [56]. <u>Examples:</u> Immune cells infected with fluorescent bacteria have been used to monitor the progression of infection in conjunction with bacterial mutants, host MO knockdowns and transgenic lines [9,10,14,15].