New models for the study of *Mycobacterium*–host interactions

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The outcome of *Mycobacterium* infection is determined by a series of complex interactions between the bacteria and host immunity. Traditionally, mammalian models and cultured cells have been used to study these interactions. Recently, ameba (*Dictyostelium*), fruit flies (*Drosophila*) and zebrafish, amenable to forward genetic screens, have been developed as models for mycobacterial pathogenesis. Infection of these hosts with mycobacteria has allowed the dissection of intracellular trafficking pathways (*Dictyostelium*) and the roles of phagocytic versus antimicrobial peptide responses (*Drosophila*). Real-time visualization of the optically transparent zebrafish embryo/larva has elucidated mechanisms by which *Mycobacterium*-infected leukocytes migrate and subsequently aggregate into granulomas, the hallmark pathological structures of tuberculosis.

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Abbreviations
AMP antimicrobial peptide
M. tb *Mycobacterium tuberculosis*
TB tuberculosis
TLR Toll-like receptor

Introduction

Infection with a given pathogen often leads to distinct outcomes in individual hosts; the basis of this variability depends on a complex interplay between pathogen and host. In the case of tuberculous infections, an unusually wide spectrum of outcomes can result: complete clearance of infection, progressive disseminated disease or an initially asymptomatic infection that can progress to overt clinical disease later [1]. This so-called 'latent' infection is presumed to be contained in granulomas, complex structures composed of differentiated macrophages, lymphocytes and other immune cells [2]. In humans and certain other species, the centers of granulomas may undergo necrosis, creating an acellular 'caseous' center containing mycobacteria [2].

The mouse model of tuberculosis

The mouse is the most commonly used experimental animal to model human tuberculosis (TB); the extensive repertoire of immunologic reagents and assays, as well as the facility of generating germline mutations, make it ideal for reverse genetic approaches and *ex vivo* studies [3,4]. Transgenic and knockout mouse lines have advanced our understanding of adaptive immune responses to mycobacteria. However, the mouse model is limited by the difficulty of forward genetic or phenotypic screens; moreover, *Mycobacterium tuberculosis (M. tb)* is not a natural pathogen of mice and the course of TB in mice does not possess certain crucial hallmarks of human disease. For example, mice develop progressively coalescing, multibacillary, noncaseating lesions rather than the organized, discrete, paucibacillary, caseous granulomas of human TB (Figure 1).

New model hosts for tuberculosis

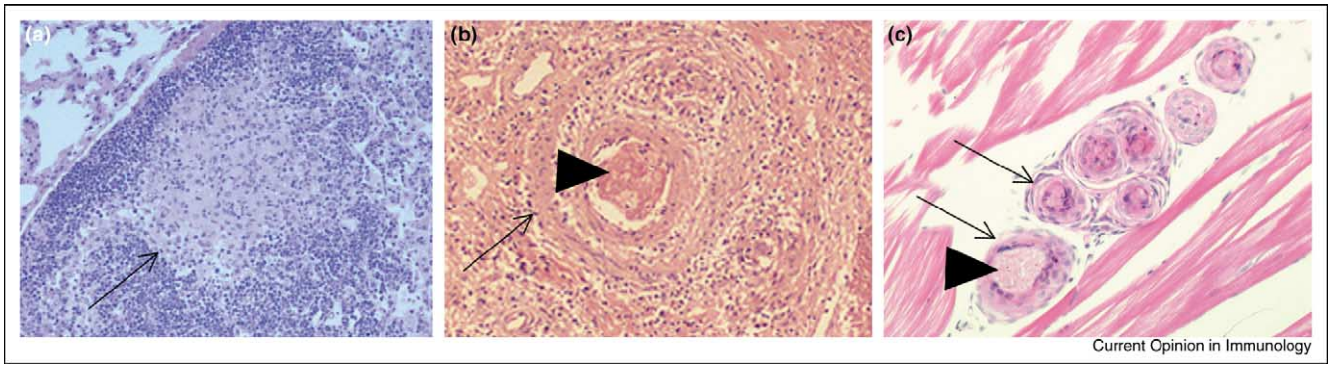
A complete understanding of bacterial pathogenesis requires attention to the still-elusive host factors that determine outcomes of infection (Table 1). Although mice remain commonly used animal models for most infectious diseases, there is a surge of interest in developing alternative hosts to better model selected aspects of bacterial–host interactions. *Dictyostelium discoideum* serves as a surrogate macrophage, *Caenorhabditus elegans* [5] and *Drosophila melanogaster* [6] are useful in the study of conserved innate immune mechanisms, and zebrafish are suited to the study of both innate and adaptive immunity.

To study aspects of TB that are inaccessible in the mouse, researchers have developed *Dictyostelium*, *Drosophila* and zebrafish as model hosts (*C. elegans*, a well-established model host for other bacterial pathogens, is resistant to mycobacterial infection; [5] and C Darby and LR, unpublished results). All of these models take advantage of the low optimal growth temperature of *Mycobacterium marinum*, a pathogen of ectotherms that is closely related to *Mycobacterium tuberculosis* [7] (http://www.sanger.ac.uk/Projects/M_marinum/).

M. marinum as a model pathogen

Owing to its optimal growth range of 25–35°C [8], *M. marinum* is a natural pathogen of ectotherms such as frogs, goldfish and zebrafish. In these hosts, a granulomatous infection develops with key features of human TB [9–11]. Specifically, goldfish and zebrafish granulomas

Figure 1



Examples of mycobacterial granulomas in various hosts. Arrows indicate granulomas, arrowheads indicate caseous, acellular centers. **(a)** Hematoxylin and eosin stain of *M. tuberculosis* granuloma in mouse lung, six weeks post-infection. **(b)** Hematoxylin and eosin stain of caseating *M. tuberculosis* granuloma in human lung. **(c)** Hematoxylin and eosin stain of caseating *M. marinum* granulomas in adult zebrafish muscle tissue, six weeks post-infection.

have the characteristic caseous centers of human TB [11,12]. Consistent with its temperature requirements, *M. marinum* causes a granulomatous infection on the cooler surfaces of warm-blooded hosts, including humans; people who work with fish are susceptible to 'fish tank granulomas' [13] that bear all the same features of *M. tb* granulomas. In addition, reactivation of systemic disease has been reported in fish similar to reactivation TB in humans [14]. *M. marinum* grows more rapidly than *M. tb* and has fewer biosafety restrictions. Given these advantages and

Table 1

Comparison of different model hosts for tuberculosis.

Host–pathogen pair	Pathologic features	Disease course	Relevant immune components present	Potential for genetic study of host-determinants
Human–*M. tb*	Granulomas often caseous. Infection can be asymptomatic (latent) or symptomatic. Disseminated disease can result involving multiple organs.	Wide range: clearance, 'latent' infection (paucibacillary) with potential for later reactivation, disseminated disease can be lethal	Macrophages, dendritic cells, TLR pathway, TNF-α, IFN-γ, chemokines, T cells	Identification of polymorphisms in susceptible populations, identification of spontaneous mutants
Human–*M. marinum*	Caseating superficial granulomas	Skin and soft tissue infection in immunocompetent hosts. Rarely disseminates.	As above	As above
Mouse–*M. tb*	Non-caseous granulomas in majority of strains	Progressive multibacillary disease, eventually lethal	Macrophages, dendritic cells, TLR pathway, TNF-α, IFN-γ, chemokines, T cells	Reverse genetics and transgenic lines established, unparalleled immunologic reagents. Limited forward genetics
Zebrafish adults–*M. marinum*	Caseating granulomas	Adult fish infected as embryos can survive for months with granulomas	Macrophages, TLR pathway, TNF- α, IFN-γ, chemokines, T cells	Forward genetic screens and transgenic lines established. Reverse genetics in adults being developed
Zebrafish embryos–*M. marinum*	Macrophage aggregates with pathological features and molecular features of adult granulomas.	Embryos can survive to adulthood if infected at low levels, or succumb in two weeks if infected with high doses	As above but lacking adaptive immunity. Lymphocytes circulate at 21 days post-fertilization	As above; in addition, reverse genetics in embryos/larvae established using morpholino technology
Drosophila–*M. marinum*	Infected plasmatocytes. Severe tissue damage and bacterial abscesses	Lethal infection with few bacteria	Plasmatocytes (phagocytic cells)	Forward genetic screens and reverse genetics feasible
Dictyostelium–*M. marinum*	N/A	N/A	Macrophage-like	Haploid genome facilitates genetic approaches

Abbreviations: IFN, interferon; TLR, Toll-like receptor; TNF, tumor necrosis factor.

pathogenic similarities, *M. marinum* is used increasingly to model *M. tb* pathogenesis [15•,16,17].

Immune responses to mycobacteria

Whether mycobacteria are inhaled into a lung or breach an epidermal barrier, they first encounter innate immune cells that immediately sense the pathogen and set off a cascade of immune responses [18]. Mammalian Toll-like receptors (TLRs), present on macrophages and dendritic cells, play an important role in the recognition of mycobacteria and subsequent cytokine production and co-stimulatory molecule expression. Nevertheless, in some cases mycobacteria survive and replicate within macrophages, partly through inhibition of phagosome acidification and maturation [19,20]. In mammals, mycobacteria-laden immune cells subsequently migrate into the tissues, including regional lymph nodes [21–23]. Within weeks, an adaptive immune response develops that is critical for granuloma maintenance and disease outcome, as evidenced by the fulminant TB disease in mice lacking T lymphocytes [24,25] and in people with AIDS [26]. The mobilization and aggregation of innate and adaptive immune cells into granulomas is poorly understood, and the use of the newly developed models in conjunction with the traditional mammalian models should facilitate a genetic dissection of the relevant host determinants.

Dictyostelium discoideum

On an evolutionary time-scale, amebae may have served as the training ground for intramacrophage pathogens [27]. *Dictyostelium discoideum*, a free-living ameba, can be considered a genetically tractable macrophage model. This organism has been exploited to investigate the phagocytosis and intracellular survival mechanisms of the pathogens *Legionella pneumophila* and *Cryptococcus neoformans* [28–31]. The haploid genome of *Dictyostelium* facilitates gene mutation making this organism well suited for genetic studies of conserved aspects of macrophage biology.

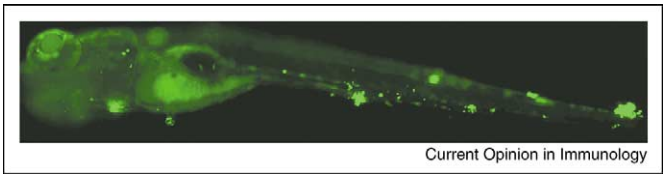
M. avium and *M. marinum* infect *Dictyostelium*, replicate within intracellular vacuoles and show similar growth as in cultured mammalian macrophages [28,32••]. Recent studies with *Dictyostelium* have questioned the role of a vacuolar protein, coronin, in intracellular mycobacterial trafficking. The retention of the phagosomal protein coronin/TACO (Tryptophan aspartate-containing coat protein) in murine macrophages was reported to be required for inhibition of phagolysosome fusion and consequently to promote mycobacterial growth [33]. Human macrophages that are incubated with clumps of BCG also demonstrate retention of coronin on phagolysomes; however, incubation of macrophages with dilute cultures of BCG did not lead to the persistence of coronin on phagolysomes [34], thus differing from these observations by Ferrari and co-workers [33] in murine macrophages. *Dictyostelium* lacking coronin have impaired phagocytosis for inert particles in suspension culture [35]. However, mycobacterial replication was actually enhanced in the coronin mutant [32••], similar to the enhanced growth of *Legionella* in coronin-mutant *Dictyostelium* [30]. Extrapolation from these results to mammalian systems is complicated by the existence of multiple mammalian coronin isoforms; *Dictyostelium* coronin may not be the orthologue of the mammalian coronin relevant to this process. Nevertheless, this report illustrates the feasibility of using easily generated mutant strains of ameba to determine the genetic basis of mycobacterial trafficking in mammalian macrophages.

Drosophila melanogaster

Drosophila is an established model for innate immune function. Indeed, the discovery of mammalian TLRs followed the identification of the *Drosophila* gene Toll, required for certain antimicrobial peptide (AMP) responses [36]. *Drosophila* secrete a wide variety of AMPs in response to infectious threat and possess phagocytic cells called plasmatocytes that engulf and destroy bacterial pathogens. Interestingly, *Drosophila* have a separate hematopoetic cell lineage that differentiates into lamellocytes, which undergo epithelioid cell transformation to surround foreign bodies, such as wasp eggs, too large to be engulfed by a plasmatocyte. This phenomenon is reminiscent of the epithelioid transformation of macrophages in tuberculous and foreign body granulomas of vertebrates. *Drosophila* are susceptible to infection with Gram-positive [37] and Gram-negative bacterial pathogens of humans [38]. In addition to its eminent genetic tractability at the whole organism level, *Drosophila* offers a unique advantage for genetic studies of pathogenesis: the S2 *Drosophila* macrophage cell line has been used in screens for genes important in phagocytosis, and the role of these genes has been subsequently tested *in vivo* [39,40].

As *Drosophila* do not possess adaptive immune responses, the contribution of innate immune responses to mycobacteria can be examined in isolation [6]. Infecting *M. marinum* are phagocytosed by hemocytes with the induction of *Mycobacterium* genes known to be specifically activated in vertebrates following phagocytosis [41••,42]. In *Drosophila*, phagocytosis may be a more relevant host response to *Mycobacterium* than the AMP pathways [41••]. Strikingly, the expression of the five AMP genes examined was induced following *Listeria monocytogenes* (a Gram-positive intracellular pathogen) but not *M. marinum* infection. Moreover, mutants in the *imd(key)* and Toll*(spz)* pathways, important in AMP responses to Gram-negative and Gram-positive/fungal pathogens respectively [6,43] did not have increased susceptibility to *M. marinum*. The role of TLRs in mycobacterial infection is complex [44]; the *Drosophila* results raise the possibility that vertebrate TLR signaling impacts

Figure 2



Entire zebrafish embryo with multiple discrete aggregates, six days after infection, fluorescent image.

infection by crosstalk with adaptive immunity rather than by affecting the microbicidal potential of phagocytic/innate immune responses *per se*. This can be tested in the zebrafish where innate responses can be temporally isolated from adaptive ones (see below).

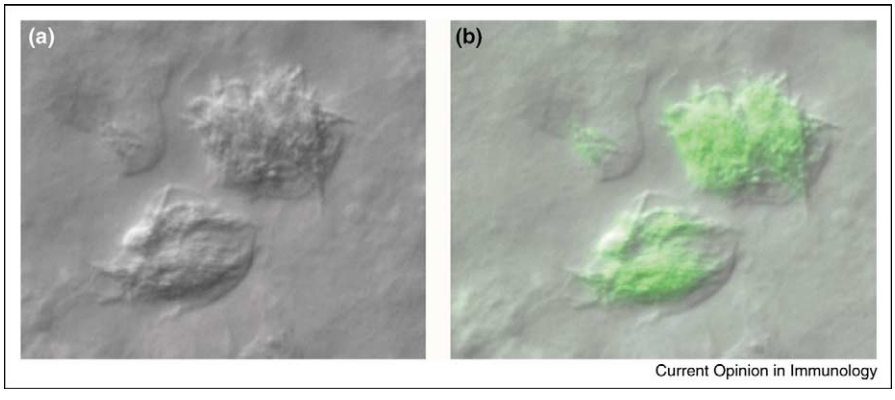
Zebrafish

Zebrafish are particularly relevant model hosts because in addition to innate responses, they have a complex adaptive immune system akin to that of mammals [45,46]. Adult zebrafish have been used to model *Streptococcus* and *Mycobacterium* infections [11,47] while zebrafish embryos and larvae have been exploited to examine *Mycobacterium* and *Salmonella* pathogenesis [48••,49]. Several types of genetic manipulations are possible in the zebrafish: forward genetic screens have identified genes involved in development and hematopoesis [46,50,51] and anti-sense oligonucleotides can be used to functionally inactivate genes in early embryos [52]. The zebrafish system excels for forward genetics but is limited in reverse genetics given the lack of embryonic stem cell lines and homologous recombination. However, *rag1* mutant zebrafish were recently isolated by screening a bank of mutants [53•] opening the way for alternative approaches to reverse genetic techniques developed for the mouse.

Zebrafish embryos provide a particularly enticing system to study pathogenesis because they are transparent for the first three weeks of development, allowing real-time monitoring in live animals of host-pathogen interactions and of fluorescent transgenic immune cells [48••,54] (Figure 2). Germane to the study of mycobacterial pathogenesis, zebrafish embryos have macrophages that are functionally competent for infection [45] (Figure 3). *M. marinum* are phagocytosed by these macrophages which subsequently migrate into deeper tissues and aggregate into granuloma-like structures [48••] (Figure 4). Imaging of the initial *Mycobacterium*–macrophage interactions reveal novel mechanisms of bacterial spread such as the attraction of uninfected macrophages into the aggregates and inter-macrophage transfer of bacteria [48••].

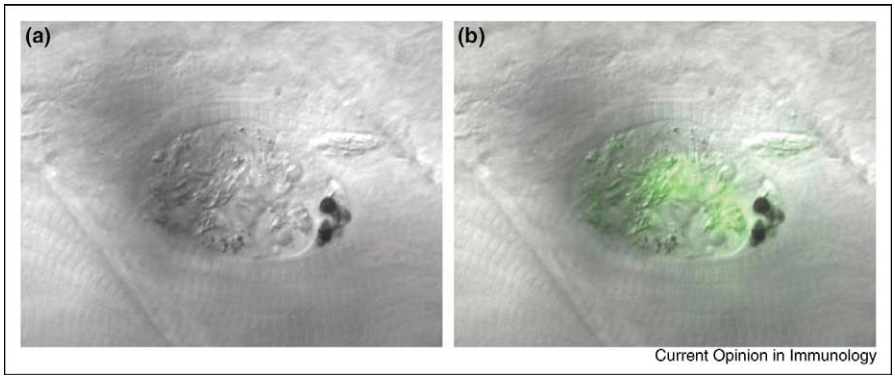
A unique feature of the zebrafish–*M. marinum* system is the possibility of infecting embryos and following macrophage aggregation and granuloma maturation into adulthood. This is done in real time for the first three weeks and by more traditional methods such as tissue histology and immunocytochemistry thereafter. Initially the embryo has only macrophages and neutrophils; thymic development begins at 3 days and circulating lymphocytes do not appear until approximately 21 days post-fertilization [55]. Therefore, it is possible to study the

Figure 3



(a) Individual infected macrophages in tissues, two days post-fertilization, one day post-infection, DIC (differential interference contrast) image. **(b)** Same macrophages, revealing fluorescence of bacteria in DIC/fluorescent overlay image.

Figure 4



(a) Aggregate of infected macrophages in six days post-fertilization zebrafish embryo, DIC image. **(b)** Identical aggregate, revealing fluorescence of bacteria in DIC/fluorescent overlay image.

effects of innate immune interactions in an isolated fashion during embryonic infection and to monitor the impact of adaptive immunity as the infected embryo matures. Conversely, it should also be possible to study the impact of infection on immune development. The zebrafish embryo model has already provided new insight into the contribution of innate immunity to tuberculous granuloma formation. Macrophage aggregates, possessing pathological hallmarks of granulomas and supporting activation of *Mycobacterium* granuloma-specific genes, develop well before the onset of adaptive immunity [48••]. This observation reveals that many specific structural features of granulomas result purely from mycobacteria interacting with innate immunity. As these granulomas mature, they become more paucibacillary confirming that adaptive immunity plays a role in containing the infection, as in mammals [18,25], (D Beery and LR, unpublished observations). Relevant to human granulomas, mature zebrafish granulomas caseate (Figure 1), allowing the exploitation of this novel host to investigate the role of caseation in mycobacterial disease.

Conclusions

Model systems provide insights into specific aspects of complex host-pathogen relationships that are impossible to study in humans. Fundamental aspects of mycobacterial infection, such as bacterial recognition, phagocytosis, and macrophage migration/aggregation are highly conserved throughout evolution. Further understanding of these conserved mechanisms will complement knowledge gained from mammalian studies and yield new insights that will help solve the formidable global challenge of tuberculosis.

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- •• of outstanding interest

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