

Microreview

Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*

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Summary

A thorough understanding of *Mycobacterium tuberculosis* pathogenesis in humans has been elusive in part because of imperfect surrogate laboratory hosts, each with its own idiosyncrasies. *Mycobacterium marinum* is the closest genetic relative of the *M. tuberculosis* complex and is a natural pathogen of ectotherms. In this review, we present evidence that the similar genetic programmes of *M. marinum* and *M. tuberculosis* and the corresponding host immune responses reveal a conserved skeleton of *Mycobacterium* host–pathogen interactions. While both species have made niche-specific refinements, an essential framework has persisted. We highlight genetic comparisons of the two organisms and studies of *M. marinum* in the developing zebrafish. By pairing *M. marinum* with the simplified immune system of zebrafish embryos, many of the defining mechanisms of mycobacterial pathogenesis can be distilled and investigated in a tractable host/pathogen pair.

Introduction

Like *Mycobacterium tuberculosis*, *Mycobacterium marinum* is a macrophage pathogen and causes a chronic, systemic tuberculosis-like disease in ectotherms. It grows optimally at 25–35°C but poorly at 37°C and usually causes only localized disease on the cooler surfaces of humans (Linell and Norden, 1954; Clark and Shepard, 1963). It is easily manipulated on the laboratory bench top under BSL-2 safeguards and has a 4 h generation time during logarithmic growth compared with the > 20 h generation time of *M. tuberculosis*. *M. marinum* infection of fish has long been referred to as fish tubercu-

losis and like human tuberculosis (referred to in the past as consumption) is often recognized by the emaciation it induces (Parisot, 1958; Post, 1987). In contrast to *M. tuberculosis*, which, in humans, is transmitted almost exclusively from lung to lung, *M. marinum* inhabits additional marine and freshwater reservoirs that may include protists (Cirillo *et al.*, 1997; Solomon *et al.*, 2003). *M. marinum*'s expanded niches are reflected in the genomic differences between *M. marinum* and *M. tuberculosis*, which we will detail. However, what is both striking and experimentally useful is the conservation of genetic programmes between the two pathogens and between their respective natural hosts that translates into shared determinants of virulence and protection respectively. These determinants, in turn, form the core of reciprocal host and pathogen evolutionary strategies to contain or expand mycobacterial infection. In this review we compare the pathology, pathogenesis, genomes, genetics and host and pathogen gene expression patterns of *M. marinum* and *M. tuberculosis* infection and disease. Finally, we present insights about mycobacterial pathogenesis made possible by examining *M. marinum* infection in a zebrafish model.

General features of human tuberculosis

Tuberculosis results from a complex choreography between host and bacterium. Infecting bacteria are phagocytosed by host macrophages but can replicate therein by subverting host endocytic trafficking and resisting the immune response (Flynn and Chan, 2001). Infected macrophages migrate into tissues and aggregate into highly organized complex immunological structures called granulomas, the hallmark structures of tuberculosis (Adams, 1976; Dannenberg, 1993). Granulomas are comprised of highly differentiated macrophages that interdigitate and undergo a process known as epithelioid transformation (Adams, 1976). Other immune cells are recruited to tuberculous granulomas and they are adorned by a characteristic lymphocyte cuff (Dannenberg, 1993). Tuberculous granulomas also typically develop an acellular necrotic core called caseum in which mycobacteria can be found to reside extracellularly. While the

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pathogenesis of caseum remains enigmatic, it is responsible for both morbidity and contagion. The rupture of caseating foci into the bronchial tubes has been postulated to be an important prerequisite to mycobacterial transmission in pulmonary tuberculosis (Dannenbergh, 1994). Although tuberculous granulomas have historically been considered to be impermeable, host-protective structures, they are at best only partially efficacious as they can harbour bacteria for long time periods (Adams, 1976; Janeway *et al.*, 2001).

Experimental models of *M. marinum* and *M. tuberculosis* pathogenesis

The ability to grow within host macrophages is an essential virulence attribute of the pathogenic mycobacteria (Cosma *et al.*, 2003). Elegant studies using cultured cells and cell lines have furthered understanding of the intracellular lifestyle of mycobacteria and their interactions with host pathways (Koul *et al.*, 2004); the similarities and differences between *M. marinum* and *M. tuberculosis* in cultured cells will be discussed in later sections. However, animal models are a requisite to fully understand the cross-talk between pathogen and the multitude of host cells involved in granuloma formation and maintenance. Tuberculosis researchers have used mice, guinea pigs, rabbits and macaques to model human tuberculosis. Rabbits, guinea pigs and macaques form caseating granulomas, but mice, the most commonly used model organism, form poorly organized macrophage and lymphocyte aggregates that do not caseate (Flynn, 2006). Therefore, mechanisms of caseation necrosis have remained enigmatic. Of the mammalian models developed to date, experimental infection of macaques most closely resembles human infection in terms of both disease states (latent and active) and granuloma pathology (Capuano *et al.*, 2003). However, due to ethical and cost considerations, use of this model has been limited.

Notably, *M. marinum*-induced granulomas in humans are often pathologically indistinguishable from *M. tuberculosis* dermal granulomas, with epithelioid cells surrounded by a lymphocytic cuff, often with a central necrotic core (Travis *et al.*, 1985; MacGregor, 1995). In ectotherms, *M. marinum* produces a systemic granulomatous tuberculosis-like disease. Many of these naturally susceptible hosts such as fish and frogs can be readily studied in the laboratory (Cosma *et al.*, 2006a). Leopard frogs develop lifelong asymptomatic infection (as do the majority of infected humans) and are found to have highly organized non-caseating granulomas (Ramakrishnan *et al.*, 1997a; Bouley *et al.*, 2001). In contrast, zebrafish, medaka and goldfish develop a symptomatic wasting disease, with zebrafish and goldfish developing caseating granulomas that are reminiscent of active caseating

human tuberculosis (Talaat *et al.*, 1998; van der Sar *et al.*, 2004; Swaim *et al.*, 2006; Broussard and Ennis, 2007) (Fig. 1F and G). Mouse models of *M. marinum* infection have existed for over 40 years and are still in sporadic use (Clark and Shepard, 1963; Robinson *et al.*, 2007). In these infections, bacterial numbers decrease with time, contrary to what is seen in most natural hosts and probably because of temperature incompatibilities. Nonetheless, limited non-caseating epithelioid granulomas do form in organs such as liver and spleen after intravenous infection (Robinson *et al.*, 2007), and it is notable that *M. marinum* immunization results in protection from *M. tuberculosis* challenge (Collins *et al.*, 1975).

In contrast to *M. tuberculosis*- and *M. marinum*-induced granulomas in mammals, *M. marinum*-induced fish and frog granulomas have very few lymphocytes (Bouley *et al.*, 2001; Swaim *et al.*, 2006). Nevertheless, fish lymphocytes are functionally important in restricting bacterial growth; adult *rag1*-deficient zebrafish, which lack B and T cells, are hypersusceptible to *M. marinum* infection similar to *M. tuberculosis*-infected *rag1*-deficient mice (Saunders *et al.*, 2004; Swaim *et al.*, 2006). In summary, the patterns of caseation necrosis and lymphocytic infiltration suggest that the histopathology of mature granulomas differs more substantially across surrogate hosts than between *M. marinum* and *M. tuberculosis*.

Other model hosts have emerged for the study of the early events of *M. marinum* pathogenesis prior to granuloma formation: *Drosophila*, which employ bactericidal phagocytes in host defence but do not form granulomas, and *Dictyostelium*, which has been used to dissect the host and bacterial components of intracellular bacterial replication and localization (Dionne *et al.*, 2003; 2006; Solomon *et al.*, 2003; Hagedorn and Soldati, 2007).

Intracellular localization of *Mycobacterium* in cultured cells

Like most pathogenic *Mycobacterium* species, *M. marinum* survives within host macrophages at least in part by arresting phagosome maturation prior to phagolysosome fusion (Barker *et al.*, 1997; Rohde *et al.*, 2007). In both mammalian macrophages and fish monocytes, live but not heat-killed *M. marinum* localize to non-acidified phagosomes that exclude the vacuolar proton ATPase (Barker *et al.*, 1997; El-Etr *et al.*, 2001). This seclusion has been suggested to protect pathogenic mycobacteria from phagolysosome-mediated killing and possibly to modulate the host adaptive immune response by altering antigen presentation (Russell *et al.*, 2002). The majority of *M. tuberculosis* mutants defective for phagosome maturation arrest are compromised for intracellular growth, but an interesting class of mutants grows as well or better in cultured macrophages despite trafficking to

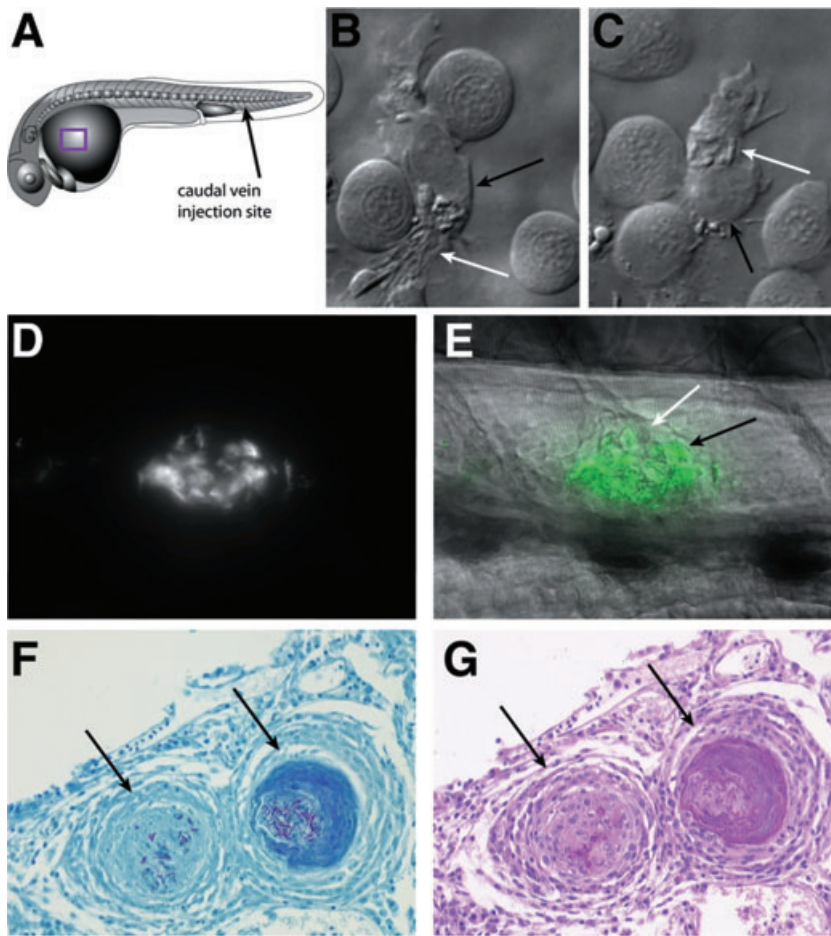


Fig. 1. *Mycobacterium marinum* pathogenesis in the zebrafish.

A. Approximately one hundred bacteria are injected into the caudal vein (arrow) of zebrafish embryos at 30 h post fertilization. Within 1 h, macrophages can be imaged phagocytosing the bacteria in the yolk circulation valley (purple box). B. Macrophage (black arrow) ingesting *M. marinum* (white arrow) approximately 1 h post infection. Round neighbouring cells are erythrocytes, which are nucleated in teleosts. C. Same macrophage as in (B) (black arrow), now with intracellular *M. marinum* (white arrow). By 4 days post infection, recognizable primitive granulomas have formed. D. Green fluorescent bacteria induce the formation of aggregates by infected macrophages in the trunk. E. DIC image with fluorescent overlay of the granuloma shown in (D) that has formed in zebrafish muscle tissue in the trunk. Note the presence of both infected (black arrow) and uninfected (white arrow) macrophages. F and G. (F) Acid fast and (G) haematoxylin and eosin staining of mature zebrafish granulomas (arrows), 6 weeks post infection. The leftmost granuloma is non-caseating, while the rightmost granuloma lacks nuclei at its core and is caseating. This heterogeneous mixture of caseating and non-caseating granulomas is also apparent at early time points in infections of macaques with *M. tuberculosis* (Lin *et al.*, 2006).

acidified phagolysosomes, suggesting that phagosome maturation arrest is not an absolute requirement for *M. tuberculosis* survival, at least in naïve cultured macrophages (Petthe *et al.*, 2004; Stewart *et al.*, 2005; MacGurn and Cox, 2007). Similarly, *M. tuberculosis* that enters cultured macrophages through Fc receptors reaches phagolysosomes but is not impaired for survival or growth (Armstrong and Hart, 1975).

A range of recent observations in cultured cells has suggested that the cell biology of mycobacterial infection may include unexplored complexities. In cultured macrophages a minor population of *M. marinum* can escape from the phagosome into the cytosol and develop actin-based motility (Stamm *et al.*, 2003). These findings were especially thought-provoking because older electron microscopy studies had suggested some level of phagosome escape of *M. tuberculosis* (Myrvik *et al.*, 1984; McDonough *et al.*, 1993). A subsequent study reported that *M. tuberculosis* was capable of escape to the cytosol in cultured dendritic cells as assessed by electron microscopy of cryosections (van der Wel *et al.*, 2007). However, the *M. tuberculosis* data remain controversial and the view that intracellular *M. tuberculosis* remains ensconced

in the phagosome is still predominant (Rohde *et al.*, 2007; Jordao *et al.*, 2008).

A defining feature of pathogenesis for several intracellular pathogens (e.g. *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia rickettsii*) that are localized to the cytoplasm is their acquisition of actin-based motility (Gouin *et al.*, 2005). Such actin-based motility has not been reported even in the few studies that have observed phagosomal escape of *M. tuberculosis*. In contrast, elegant work from the Brown laboratory has shown that about 20% of the *M. marinum* bacteria become motile through the WASP family-dependent induction of actin polymerization, in a mechanism most akin to that employed by *Shigella* (Stamm *et al.*, 2003; 2005). Mutations in the *M. marinum* ESX-1 secretion system (discussed below) abrogate phagosomal escape, the resulting cytosolic actin polymerization and cell–cell spread (Stamm and Brown, 2004; and L.Y. Gao, pers. comm.). Intriguingly, the laboratories that do observe *M. tuberculosis* phagosomal escape also note dependence on ESX-1 (McDonough *et al.*, 1993; van der Wel *et al.*, 2007). How do these observations extend to *in vivo* biology? In extensive electron microscopy studies of

M. marinum in chronic granulomas of frogs, cytoplasmic *M. marinum* has not been observed (Bouley *et al.*, 2001). Consistent with all these data, Stamm and Brown have proposed that actin-based motility may be used by *M. marinum* not during the process of granuloma formation, but in the initial stages of infection, to cross epithelial surfaces after host ingestion or for transmission from an environmental protist to a vertebrate host – both modes of transmission that diverge from those of *M. tuberculosis* (Stamm and Brown, 2004). Perhaps actin-based motility is deployed by *M. marinum* under certain circumstances and represents the expanded repertoire of *M. marinum* transmission strategies within and between niches that have no *M. tuberculosis* counterpart. It will be interesting to address the prevalence and consequences of phagosome escape and actin-based motility in *in vivo* models.

Development of a zebrafish embryo model for mycobacterial pathogenesis

Embryonic and larval zebrafish are optically transparent, presenting the opportunity to witness and manipulate the steps of mycobacterial pathogenesis in real time. The zebrafish has long been a choice vertebrate genetic model, and forward genetic screening and positional cloning approaches are well established. Reverse genetics are possible in embryos and larvae by the introduction of morpholinos, modified antisense oligonucleotides that can be designed to inhibit mRNA translation or splicing (Nasevicius and Ekker, 2000; Draper *et al.*, 2001). Alternatively, retroviral insertion and TILLING approaches can also be used to identify germline mutations in specific genes (Golling *et al.*, 2002; Wienholds *et al.*, 2003; Draper *et al.*, 2004).

Zebrafish contain both innate and adaptive branches of the immune system with conserved orthologues of key human and mouse immune molecules (Traver *et al.*, 2003). As in mammalian models, zebrafish adaptive immunity develops later than innate immunity. T cells can first be detected in the thymus at 3 days post fertilization. However, functional T cells are not thought to exit the thymus until at least 3 weeks post fertilization (Langenau *et al.*, 2004). By infecting zebrafish embryos in the first few days of life, analysis can be limited to the simplified context of the innate immune system, in an optically transparent organism. Thus, whole animal real-time visualization of mycobacterial pathogenesis in a functioning innate immune system is possible.

The ability to dissect innate and adaptive protective mechanisms is useful because although an adaptive immune response is required for ultimate control of infection in both fish and mammals, innate mechanisms of resistance are being increasingly recognized to modulate the inflammatory and bactericidal response to mycobac-

terial infection (van Crevel *et al.*, 2003). In mice, the *sst1/lpr1* susceptibility locus modulates innate resistance to intracellular pathogens including *M. tuberculosis* and *L. monocytogenes* (Pan *et al.*, 2005). In human macrophages, the induction of a vitamin D-mediated innate antimicrobial response appears to be a critical defence (Liu *et al.*, 2006). Hints that innate mechanisms may play important roles in mycobacterial immunity have been present since populations of inbred rabbits with altered susceptibilities to *M. tuberculosis* infection displayed early differences in bacterial loads before the development of an adaptive immune response (Dannenbergh, 1994).

The zebrafish innate immune system

Macrophages that are competent to phagocytose and kill non-pathogenic bacteria populate zebrafish embryos and larvae by 24 h post fertilization (Herbomel *et al.*, 1999) with successive waves of haematopoiesis occurring thereafter (Murayama *et al.*, 2006; Bertrand *et al.*, 2007). Early macrophages and neutrophils respond to a variety of inflammatory stimuli, including wounding (Mathias *et al.*, 2006; Redd *et al.*, 2006; Renshaw *et al.*, 2006). A broad range of TLRs is expressed in zebrafish larvae, some of which are induced during adult *M. marinum* infection, and MyD88 inhibition by morpholino results in increased susceptibility to *Salmonella* infection (Jault *et al.*, 2004; Meijer *et al.*, 2004; van der Sar *et al.*, 2006). Zebrafish also express two classes of genes that are structurally related to Ig-type and C-type lectin mammalian NK receptors, which may contribute to innate immune defences (Yoder, 2004).

As the zebrafish genome project nears completion, a comprehensive *in silico* analysis of innate immune genes in the zebrafish and two related pufferfish species revealed recognizable orthologues for most of the canonical innate immune signalling molecules seen in mammals (Stein *et al.*, 2007). However, some divergence and significant species-specific expansions are present, particularly within the class II cytokines and their receptors and the NOD-like receptors (NLRs). The functional significance of these similarities and differences remains to be determined, but an innate immune framework conserved with that of mammals is present.

M. marinum interactions with the innate immune system

Transparent zebrafish embryos provide a unique platform to examine microbial pathogenesis *in vivo*. Zebrafish embryo infection models have been developed for *M. marinum* and *Salmonella arizonae* as we discuss here (Davis *et al.*, 2002), as well as for *Vibrio anguillarum* (O'Toole *et al.*, 2004), snakehead rhabdovirus (Phelan

et al., 2005), *Edwardsiella tarda* (Pressley *et al.*, 2005), *Salmonella enterica* (Li *et al.*, 2007) and *Salmonella typhimurium* (van der Sar *et al.*, 2003). By intravenous microinjection of fluorescently labelled bacteria into 30-h-old zebrafish embryos, the key early steps in mycobacterial pathogenesis are evident. Within 1 h of injection of *M. marinum* into the caudal vein, zebrafish embryonic macrophages phagocytose the bacteria, but are unable to eradicate them (Fig. 1A–D). Infection results in induction of the pro-inflammatory cytokines TNF and IL-1 β within the first 24 h (Clay *et al.*, 2007). Infected macrophages also express inducible nitric oxide synthase (iNOS), which is known to be important for control of *M. tuberculosis* infection in the mouse (MacMicking *et al.*, 1997; H. Clay and L. Ramakrishnan, submitted).

After extravasation of macrophages into tissue, infected macrophages aggregate within 4 days to form structures that bear pathological hallmarks of granulomas (Fig. 1E and F). These early granulomas have interdigitated macrophages indicative of epithelioid transformation and induce the activation of bacterial genes that are associated with granuloma formation in adult frogs (Ramakrishnan *et al.*, 2000; Davis *et al.*, 2002). The ability of granulomas to form prior to the development of adaptive immunity revealed that innate immune determinants are sufficient to drive *M. marinum* granuloma formation. Similarly, this model has served to dissect the role of macrophages in the sole context of innate immunity, where they play a role both in host protection and in pathogen dissemination (Clay *et al.*, 2007).

Probing initial pathogen/host interactions through host genetics

Various genetically tractable hosts are being used to probe initial host factors that determine infection outcomes. In *Dictyostelium*, mutation of a flotillin homologue results in resistance to infection, as bacteria are unable to prevent accumulation of the vacuolar proton ATPase (Hagedorn and Soldati, 2007). Mutation of the small GTPase RacH results in increased bacterial proliferation due to reduced acidification of the phagolysosome, but reduced cell-to-cell spread (Hagedorn and Soldati, 2007). Forward genetic screens in *Drosophila* have yielded novel host determinants of susceptibility to *M. marinum* infection. *M. marinum* infection in flies induces changes in insulin signalling and a wasting phenotype, and mutations in *Drosophila foxO* result in less wasting and increased survival without altering bacterial replication (Dionne *et al.*, 2006). A large-scale RNAi screen in *Drosophila* S2 cells identified a *Drosophila* orthologue of a β -hexosaminidase subunit as important in the control of *M. marinum* infection (Koo *et al.*, 2008). This finding was validated in mouse macrophages deficient for the orthologous *hexB* gene, and a mechanism was pro-

posed in which β -hexosaminidase is secreted in response to *M. marinum* infection and kills bacteria extracellularly (Koo *et al.*, 2008). It will be interesting to investigate whether these findings also extend to *M. tuberculosis*. Finally, in an ongoing forward genetic screen in our laboratory, we have isolated zebrafish host mutants that show increased susceptibility or resistance to mycobacterial infection prior to the onset of adaptive immunity (D.M. Tobin, R. Lesley, C.B. Moens and L. Ramakrishnan, unpubl. results).

Comparison of the *M. marinum* and *M. tuberculosis* genomes

Sequencing and assembly of the *M. marinum* genome are complete. Its 6.6 Mb genome is about 1.5 times the size of the *M. tuberculosis* genome, reflecting its expanded host and environmental range relative to *M. tuberculosis*. The *M. marinum* genome is 85% identical to orthologous regions of the *M. tuberculosis* genome, and coding sequence amino acid identity averages 85% between orthologues (Stinear *et al.*, 2008). An analysis of 16S rRNA among 80 mycobacterial species both confirmed the intimate evolutionary relationship between *M. marinum* and *M. tuberculosis* and suggested that the two species derived from a common ancestor with the capacity for both host and environmental niches (Tonjum *et al.*, 1998; Gey van Pittius *et al.*, 2006). Based on the shared genetic programmes for intracellular growth and host survival detailed below, we believe that colonization of vertebrates with at least a primitive adaptive immune system preceded *M. marinum*/*M. tuberculosis* divergence.

In terms of sheer bulk, the greatest difference in genome composition is the extra 2.2 Mb that *M. marinum* carries relative to *M. tuberculosis*. It has been hypothesized that much of this sequence resulted from *M. tuberculosis* genome loss as its ancestral species relinquished an environmental niche and specialized to survive exclusively within a host. For example, the light-induced production of beta-carotene protects *M. marinum* from photooxidation damage (Matthews, 1963). Thus, genes specifically required for pigment production like *crtB* – a gene encoding a phytoene synthase – have disappeared from *M. tuberculosis* (Ramakrishnan *et al.*, 1997b). Other genes with dual roles in pigment synthesis and protection from host singlet oxygen species are present in both species (Gao *et al.*, 2003a). Finally, *M. marinum* has continued to acquire new loci; there is evidence of lateral gene transfer and gene duplication to expand the *M. marinum* genome after the divergence from *M. tuberculosis* (Stinear *et al.*, 2008).

The evolutionary history of *M. tuberculosis*, though predominantly marked by genome reduction, also includes 600 kb that is not shared with *M. marinum*. Many of these

M. tuberculosis-specific regions were acquired by lateral gene transfer and preserved, perhaps as part of the evolutionary exigencies of niche specialization. Altogether, 14% of the *M. tuberculosis* genome has no counterpart in *M. marinum*, and 8% of the *M. tuberculosis* genome is thought to have arisen by lateral gene transfer (Stinear *et al.*, 2008). However, mutational analyses of these *M. tuberculosis*-specific genes have yielded very few *in vivo* virulence phenotypes (see below for details). Thus, we speculate that loci associated with the *M. tuberculosis*-specific components of the genome are most relevant to differences in host transmission and organ (e.g. lung) specificity rather than to central mechanisms of pathogenesis.

Macrophage and granuloma-activated genes in *M. marinum*

For both *M. tuberculosis* and *M. marinum*, entry into macrophages and granulomas induces specific patterns of gene expression. In an early analysis based on promoter traps of the *M. marinum* genome, subsets of macrophage-activated and granuloma-activated bacterial promoters (*maps* and *gaps*) were identified (Ramakrishnan *et al.*, 2000). Most of these genes share orthologues with *M. tuberculosis*, but perhaps surprisingly, most of the *M. tuberculosis* orthologues analysed were constitutively expressed rather than induced *in vivo* (Banaiee *et al.*, 2006). Although this may reflect differences in pathogenesis between the two organisms, this result is also consistent with the divergent niches of *M. marinum* and *M. tuberculosis*. *M. marinum*'s transitions between host and environmental residence likely require switching between gene expression programmes specific to a marine reservoir and those that are important to survival within vertebrate hosts. As *M. tuberculosis* lives exclusively within the host, there is no pressure to regulate many of these genes. Thus, the better comparison of the roles of specific genes during pathogenesis is likely to be mutant analysis during *in vivo* infection.

Mycobacterium virulence determinants

Transposon-based mutagenesis screens in *M. marinum* have included an *in vivo* goldfish infection model; here signature tagged mutagenesis isolated 40 mutants, 85% of which had orthologues in *M. tuberculosis* (Ruley *et al.*, 2004). A transposon screen for *M. marinum* mutants affecting macrophage interactions identified 19 *M. marinum* mutants that affected macrophage interactions, 70% of which were in genes orthologous to *M. tuberculosis* (Mehta *et al.*, 2006). The minority of divergent genes could be due either to the cell culture assay system used, to additional evolutionary constraints on

M. marinum not present in *M. tuberculosis* (differences in the biology of infection), or to the presence of a functional equivalent if not a true orthologue. More detailed analyses of individual mutants, described below, reveal that *M. marinum* and *M. tuberculosis* share orthologous virulence determinants, to the extent tested. Moreover *M. tuberculosis* orthologues generally complement virulence determinants discovered in *M. marinum*, demonstrating conserved function (Table 1).

What of the fairly sizeable *M. tuberculosis*-specific genome? Thus far, few fundamental virulence determinants in tuberculosis models have mapped to these regions. A multigene locus in *M. tuberculosis* that is not present in *M. marinum* is responsible for the production of cell surface sulfated lipids such as Sulfolipid-1 that have been proposed to modify virulence (Bhatt *et al.*, 2007; Kumar *et al.*, 2007). However, these particular lipids are not integral to mycobacterial pathogenesis. Accumulation of the sulfolipid intermediate SL₁₂₇₈ in *mmpL8* *M. tuberculosis* mutants results in attenuation of virulence in mice, but bacteria with upstream blocks of sulfolipid synthesis are indistinguishable from wild-type bacteria in mouse or guinea pig infections (Converse *et al.*, 2003; Rousseau *et al.*, 2003; Kumar *et al.*, 2007). Accumulation of the SL₁₂₇₈ intermediate may primarily function through modulation of the host immune system (Domenech *et al.*, 2004). Rv0987-9, another putative virulence locus specific to *M. tuberculosis* and acquired through horizontal gene transfer, was shown to affect macrophage interactions *in vitro*, but *in vivo* infection was unaffected (Rosas-Magallanes *et al.*, 2007). To date the one divergent *M. tuberculosis*-specific gene with an *in vivo* phenotype is the *virS* locus; mutation reduces bacterial loads in the spleen by 800-fold in a guinea pig infection model, although the number of bacteria in the lung is unaffected at the same time point of 20 weeks (Singh *et al.*, 2005). In a more comprehensive analysis using transposon site hybridization (TRASH), the Rubin laboratory identified 126 genes in *M. tuberculosis* that were required for the survival of bacteria in macrophages (Rengarajan *et al.*, 2005) and 194 genes that were required for *in vivo* growth (Sasseti and Rubin, 2003). Of these, only 3% were in *M. tuberculosis*-specific regions acquired by lateral gene transfer. While there has been clear divergent evolution of both pathogens and their respective hosts, mutant analysis suggests that the host-pathogen interactions mediated by *M. tuberculosis*-specific genes are most likely to define refinements within a common programme of pathogenesis rather than fundamental differences.

Cording and cell wall mutants

Two major classes of virulence determinants serve to illustrate these commonalities in *M. marinum* and

Table 1. Comparison of selected virulence determinants of *M. marinum* and *M. tuberculosis*.

Molecule	<i>M. marinum</i>		<i>M. tuberculosis</i>		Cross-species complementation
	Presence	Mutant phenotype	Presence	Mutant phenotype	
Cell wall lipids					
<i>kasB</i> (mycolic acid structure)	+	Attenuated, Rifampicin, defensin hypersusceptibility	+	Attenuated, Rifampicin hypersusceptibility	+
PDIM	Altered stereochemistry, Biosynthetic pathway, transporters present	ND	+	Attenuated	ND
TDM	Biosynthetic pathway present	ND	+	Attenuated	ND
PGL	Biosynthetic pathway present	ND	Some strains (e.g. W-Beijing)	Presence of PGL correlates with hypervirulence	ND
SL1	–	NA	+	None reported	NA
Carotenoids					
CrtB	+	Attenuated	–	–	NA
CrtP	+	Attenuated	+	ND	+
Erp	+	Attenuated, Rifampicin hypersusceptibility	+	Attenuated, detergent sensitivity	ND
ESX secretion					
ESX-1	+	Attenuated, phagolysosome fusion, granuloma formation, haemolysis	+	Attenuated, altered cytokine profile, phagolysosome fusion and others	+
ESAT-6	+	Attenuated	+	Attenuated	ND
CFP-10	+	Attenuated	+	Attenuated	ND
EspA	Probable, but orthologue unclear	ND	+	Attenuated	ND
EspB	+	Attenuated	+	ND	+
ESX-5	+	Attenuated	+	ND	ND

M. tuberculosis virulence mechanisms and the mechanistic insights that have been made possible by the use of *M. marinum*. The cording morphology of virulent mycobacteria during extracellular growth was originally noted by Koch more than a century ago (Koch, 1882). Non-pathogenic mycobacteria species lack this cording, as do a number of *M. tuberculosis* mutants compromised for *in vivo* growth because of changes to cell wall composition. The generation of mutants in *M. marinum* revealed that the correlation between cording and virulence holds true for *M. marinum* as well. Identified in a transposon screen for mutants with defects in macrophage growth, *M. marinum kasB* mutants produce mycolic acids that are two to four carbons shorter than those produced by wild-type *M. marinum*, have reduced levels of keto-mycolates and display cording defects (Gao *et al.*, 2003b). *kasB* mutants are attenuated for *in vivo* growth and virulence in zebrafish and likely have altered permeability that makes them more susceptible to a variety of lipophilic compounds and host defence molecules (Gao *et al.*, 2003b; Cosma *et al.*, 2006b). The corresponding *M. tuberculosis* deletion results in a loss of acid-fast staining due to similar alterations in cell wall composition and dramatic attenuation in a mouse model (Rao *et al.*, 2005).

The *erp* locus, the first mycobacterial virulence determinant to be characterized by targeted deletion, encodes a secreted cell surface protein (Berthet *et al.*, 1998). Mutation of this gene in *M. tuberculosis* and *M. marinum* results in attenuation in both cultured macrophages and animal models of infection as well as hypersusceptibility to lipophilic antibiotics (Berthet *et al.*, 1998; Cosma *et al.*, 2006b). Studies in the zebrafish embryo model revealed that the *erp* mutant is phagocytosed by macrophages normally, but fails to survive within (Cosma *et al.*, 2006b). Moreover, depletion of macrophages rescues the attenuation phenotype of *erp*-deficient bacteria, showing that this virulence determinant exerts its effects specifically with macrophage defences (Clay *et al.*, 2007).

The *M. marinum* locus *iipA* also affects susceptibility to antibiotics and cell permeability by altering cell wall structure (Gao *et al.*, 2006). *M. marinum iipA* and *iipB* are orthologues of *M. tuberculosis* Rv1477 and Rv1478, respectively, and both contain highly conserved NLPC_p60 domains that are thought to mediate peptidoglycanase activity. Mutants in *iipA* fail to cord and have defects in invasion and intracellular survival. Infection of adult zebrafish with high numbers of *iipA* mutant bacteria revealed an attenuated phenotype. Complementation by

the *M. tuberculosis* orthologue suggests that the genes may have conserved functions across the two species, although data from a TRASH screen suggests that the *M. tuberculosis* orthologue is essential (Sasseti *et al.*, 2001; Gao *et al.*, 2006).

In *M. tuberculosis*, specific cell wall lipids such as phthiocerol dimycocerosate (PDIM), trehalose dimycolate (TDM) and a polyketide synthase-derived phenolic glycolipid (PGL) are important determinants of virulence (Cox *et al.*, 1999; Glickman *et al.*, 2000; Reed *et al.*, 2004). Injection of purified cyclopropane-modified TDM (also known as cord factor) is sufficient to induce pulmonary granulomas and to confer a hypoinflammatory phenotype in cultured macrophages, while purified PGL applied to macrophages likewise inhibited release of pro-inflammatory cytokines (Reed *et al.*, 2004; Rao *et al.*, 2005). These results suggest that at least a subset of the mycobacterial cell wall lipids that are altered in cording mutants are capable of direct interactions with the host immune response. The canonical *M. tuberculosis* cell wall virulence lipids are all predicted to be present in *M. marinum* – though the *M. marinum* PDIM equivalent has altered stereochemistry (Onwueme *et al.*, 2005) – based on genomic analysis of the biosynthetic pathways and transporters required for their production and export. There has not yet been a functional analysis of these genes or a comprehensive biochemical analysis of the *M. marinum* cell wall.

ESX secretion systems and their substrates

The ESX secretion systems are specialized secretion systems present across a wide variety of Gram-positive bacteria (Abdallah *et al.*, 2007). They have been the subject of intensive investigation in both *M. tuberculosis* and *M. marinum* due to the central role one of them, ESX-1, plays in mycobacterial pathogenesis. *M. tuberculosis*, *M. bovis* and *M. marinum* mutants lacking the ESX-1 secretion system or its substrates are attenuated in both cultured macrophages and animal models of infection. Both *M. marinum* and *M. tuberculosis* containing a large deletion (named RD1) spanning much of the ESX-1 locus exhibit defects in cell-to-cell spread, altered cytokine profiles and failure to inhibit phagolysosome fusion in cultured cells (Hsu *et al.*, 2003; Stanley *et al.*, 2003; 2007; Gao *et al.*, 2004; Guinn *et al.*, 2004; Volkman *et al.*, 2004; Tan *et al.*, 2006). As noted earlier, *esx-1* may play a role in phagosome escape for *M. marinum* and *M. bovis* in cell culture (Gao *et al.*, 2004; van der Wel *et al.*, 2007).

The ESX-1 locus contains two canonical secreted proteins ESAT-6 and CFP-10 that are co-dependent for secretion. In addition, in *M. tuberculosis*, a third ESX-1-secreted protein EspA lies outside of the ESX-1 locus, but

exhibits co-dependent secretion with ESAT-6 and CFP-10 (Fortune *et al.*, 2005; MacGurn *et al.*, 2005). In *M. marinum*, the role of EspA has not been addressed, but an additional ESX-1 substrate EspB accounts for part of the attenuation of deleting the entire *esx-1* locus and may contribute to defective phagosome maturation arrest (McLaughlin *et al.*, 2007; Xu *et al.*, 2007). It is interesting to note that in both *M. tuberculosis* and *M. marinum*, components of the ESX-1 secretion system are required for phagosome maturation arrest, yet the *M. bovis* BCG strain which lacks the ESX-1 locus is capable of arresting phagosome maturation (MacGurn and Cox, 2007). In *M. marinum*, EspB, ESAT-6 and CFP-10 are all co-dependent for secretion (Xu *et al.*, 2007), while in *M. tuberculosis* EspB is secreted in the absence of CFP-10 (McLaughlin *et al.*, 2007). The *M. tuberculosis* orthologue of *espB*, Rv3381c, restores growth in macrophages to the *M. marinum* mutant, suggesting conserved function of this gene in *M. tuberculosis* (McLaughlin *et al.*, 2007).

Examination of infection with an *esx-1*-deficient strain of *M. marinum* (Δ RD1) in the zebrafish embryo allowed an *in vivo* analysis of the mechanisms of attenuation (Volkman *et al.*, 2004). When injected intravenously, *esx-1* mutant bacteria recruited and were phagocytosed normally by macrophages and were able to replicate intracellularly therein. Although *esx-1*-infected macrophages are unable to seed granuloma formation, they are capable of responding to signals from macrophages infected with wild-type bacteria. If a wild-type superinfection is superimposed on an established *esx-1* infection, *esx-1*-infected macrophages rapidly (within hours) join wild type-containing aggregates, suggesting that the granuloma formation defect results from an inability to transmit rather than to receive cues for granuloma formation.

Other ESX secretion systems are non-redundant, and their substrates may also be important to mycobacterial pathogenesis. The *M. marinum* *esx-5* locus, which has a close orthologue in *M. tuberculosis*, was implicated in secretion of PPE41, one of a large gene family of mycobacteria-specific genes that include the PE and PPE gene families (Abdallah *et al.*, 2006). These gene families comprise up to 10% of the genome of pathogenic mycobacteria, and their gene products are cell wall associated, but their function is not well understood. Mutants in the *M. marinum* *esx-5* locus are compromised in cell-to-cell spread, although it is unclear if PPE-41 is relevant to this phenotype (Abdallah *et al.*, 2006). Nevertheless, the idea that these specialized secretion systems are responsible for export of at least some PE and PPE genes is an important one. One PE-PGRS gene, *mag-24*, identified as macrophage-activated in the screen for *in vivo* activated *M. marinum* promoters, also displayed an *in vivo* defect in both frog and zebrafish models, although it is unclear

whether or not the protein is exported through an ESX secretion system (Ramakrishnan *et al.*, 2000). Intriguingly, expansion of PE and PPE gene clusters in mycobacteria appears to be linked to duplication of nearby *esx* loci (Gey van Pittius *et al.*, 2006). It will be interesting to determine the export requirements and involvement in pathogenesis of other members of these large protein families in both *M. marinum* and *M. tuberculosis*.

Role of granulomas in promoting and restricting infection: insight from the zebrafish

esx-1-deficient bacteria are attenuated for virulence, yet these mutants are defective in the generation of what has traditionally been viewed as a host-beneficial response – granuloma formation. The view of granulomas as host protective structures that are impermeable fortresses of mycobacterial containment has long been a tenet of medical and immunology textbooks (Janeway *et al.*, 2001). However, studies using the *M. marinum*/zebrafish model provide evidence that the granuloma may play a dual role in first promoting and later restricting mycobacterial growth (Volkman *et al.*, 2004; Swaim *et al.*, 2006). *M. marinum* appears initially to employ the *esx-1*-dependent early macrophage aggregates to promote spread and growth (Volkman *et al.*, 2004). Real-time imaging of early granulomas and uninfected macrophages in the zebrafish embryo has revealed spread from infected to uninfected macrophages occurring predominantly in nascent innate granulomas (J.M. Davis and L.R., unpubl. results). Additionally, during real-time monitoring of bacterial growth during infection, the beginning of granuloma formation correlates tightly with an expansion of bacterial numbers (Volkman *et al.*, 2004).

Moreover, even mature, established granulomas are surprisingly porous. Adult frogs and fish with established *M. marinum* infections were superinfected with differentially fluorescent mycobacteria. While pre-infection conferred some immunity to the subsequent infection, superinfecting mycobacteria trafficked to established granulomas in both fish and frogs within 3 days, and mixed granulomas were still found months after the superinfection (Cosma *et al.*, 2004). Thus, even at the epicentre of a mature, established adaptive immune response, newly infecting mycobacteria can infiltrate and persist within. Mature granulomas clearly limit the extent of infection, but there is emerging consensus that pathogenic mycobacteria themselves have evolved to orchestrate granuloma formation in a way that promotes their survival and transmission (Russell, 2007).

Conclusion

In the study of infectious diseases, surrogate pathogens have proved an important tool. The use of *Salmonella*

typhimurium in its mouse host has contributed greatly to the understanding of human typhoid fever caused by its genetic relative *Salmonella typhi*. In the case of tuberculosis, *M. tuberculosis* has been studied in cell culture systems and in a variety of mammalian hosts. The widely used mouse model of tuberculosis has provided remarkable insight into the genetics and immunology of *M. tuberculosis* infection. The last 15 years have seen the use of *M. marinum* as a surrogate organism to understand the many aspects of pathogenesis that it shares with its genetic cousin *M. tuberculosis*. Owing to its lower temperature growth requirement, *M. marinum* can be studied in the genetically tractable model hosts *Dictyostelium*, *Drosophila* and zebrafish. *Dictyostelium* can provide rapid insight into the cell biology of mycobacterial infection and *Drosophila* provides powerful forward genetics and cell culture-based RNAi screens. Likewise, zebrafish have provided a genetically tractable natural host in which the complex interactions leading to tuberculous granuloma formation can be easily visualized. An eclectic approach that draws on the strengths of each surrogate host/pathogen pair should provide the opportunity to understand human tuberculosis more fully.

Acknowledgements

We thank Laura Swaim for adult zebrafish sections and images, Muse Davis for Fig. 1B and C, Eric Brown, Hilary Clay, Lynn Connolly, Christine Cosma and Robin Lesley for valuable discussions and comments on the manuscript. This work was supported by National Institutes of Health Grants RO1 AI036396 and RO1 AI54503 and a Burroughs Wellcome award to L.R. D.M.T. was supported by Postdoctoral Fellowship Grant #PF-04-107-01-DDC from the American Cancer Society and a National Institutes of Health Bacterial Pathogenesis Training Grant AI055396.

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