Using *Mycobacterium marinum* and its hosts to study tuberculosis

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***Mycobacterium marinum*, a close relative of *Mycobacterium tuberculosis* is being used to study mycobacterial pathogenesis. *M. marinum* causes a systemic tuberculosis-like infection and disease in ectotherms such as frogs and fish. This review describes the development of *M. marinum* as a model pathogen and the more recent development of genetically tractable model hosts, namely the zebrafish, Drosophila and Dictyostellium to dissect the complex host-pathogen interactions that lead to tuberculosis.**

THE practice of using surrogate bacterial models to study host–pathogen interactions has a long history and has significantly advanced the field of bacterial pathogenesis. Perhaps the most widely used model is *Salmonella enterica* subsp. Typhimurium, a genetic relative of *Salmonella typhi* which causes human typhoid fever[1]. *S. enterica* causes gastroenteritis in humans, but causes a systemic typhoid-like disease in mice. In contrast, *S. typhi* is human-specific, and does not cause disease in small laboratory animals. Therefore, most researchers who are ultimately interested in understanding the molecular pathogenesis of typhoid fever study the interactions of *S. enterica* with laboratory mice, often using genetically engineered strains with altered expression of specific genes.

The most commonly used animal model for pathogenesis is the mouse, and studies on host aspects of pathogenesis have been aided by advances in mouse genetics and immunology, particularly the ability to create mice with a mutation in a specific gene[2]. More recently, there has been a surge of interest in developing simple and genetically tractable host models to identify specific host determinants that influence outcomes in infection and disease. Bakers yeast *Saccharomyces cerevisiae*, the slime mold *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the zebrafish *Danio rerio* are being used as surrogate host models for various aspects of host–pathogen interactions[3–8].

The study of *M. tuberculosis*, the agent of human tuberculosis, is inherently difficult due to its slow growth and the need for BL3 containment. Tremendous strides have been made in developing genetic tools that have facilitated the identification and characterization of virulence determinants in recent years[3,9,10]. However, a key problem that remains is that *M. tuberculosis* is a human-specific pathogen, and while some laboratory animals can be experimentally infected, these models do not mimic certain important aspects of human infection and disease[3]. *M. tuberculosis* infection of humans is complex and results in a range of outcomes in disease and pathology. Aerosolized bacteria are initially phagocytosed by macrophages and dendritic cells that patrol the lung, and are then carried to deeper tissues. The hallmark of all tuberculous infections is the formation of granulomas, organized structures composed of differentiated macrophages and other immune cells. In humans, granulomas evolve morphologically during infection and disease with the formation of areas of caseous necrosis, and the deposition of fibrin and calcium. At least some of the infecting bacteria can survive for extended time periods within macrophages and in granulomas. The mechanism of their survival in the face of a highly orchestrated innate and adaptive host immune response is a central issue in understanding the fundamental aspects of tuberculosis.

Various animal models are used to study *M. tuberculosis* pathogenesis of humans. Mice are the most commonly used species because they are inexpensive and convenient, and because of the existence of genetically altered animals and inbred strains[11]. However, tuberculosis in the mouse model differs from human infection and disease in several important aspects. Mouse granulomas do not caseate or calcify. In susceptible strains, bacterial burdens can be very high, reaching 10^6 per lung, and are never cleared, while human infections are often paucibacillary. In contrast, many humans appear to clear even an established granulomatous infection within the first several months to years following exposure. Guinea pigs exhibit many pathological features similar to those seen in humans, but unlike humans they are exquisitely sensitive to a progressive pulmonary infection. Most rabbits are resistant to tuberculosis and like guinea pigs demonstrate pathology similar to humans[12]. Rabbits that had been bred for their susceptibility to tuberculosis have been lost, although efforts are in progress to redevelop such strains. Unfortunately, both the rabbit and guinea pig models are beleaguered by the relative paucity of immunological reagents and genetically-defined mutations. Finally, non-human primates are also used to model *M. tuberculosis* infection[13,14], and intratracheal inoculation of cynomologous macaques leads to a spectrum of outcomes similar to that seen in humans[13]. However, the primate models

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are associated with ethical and cost considerations. In summary, despite significant advances in the experimental tractability of *M. tuberculosis*, certain inherent problems remain in the study of its pathogenesis.

M. marinum, one of the closest relatives of the *M. tuberculosis* complex organisms[15], offers the advantage that a number of its natural hosts, such as fish and frogs, are also convenient laboratory animals[16]. Like *M. bovis*, *M. marinum* appears to have a broad host range, causing a systemic tuberculosis-like granulomatous infection in a variety of poikilothermic animals, including fish, amphibians and reptiles[16]. The predilection of *M. marinum* for poikilothermic hosts likely stems from its growth optimum of 33–35°C (generation time ~ 4 h), as it grows about threefold more slowly at 37°C (ref. 16). *M. marinum* also causes superficial lesions called fish tank, acquarium tank or swimmer's granulomas in humans, which are clinically and pathologically indistinguishable from dermal *M. tuberculosis* lesions[17,18]. Consistent with its lower growth temperature optimum, *M. marinum* infection of humans is usually restricted to the cooler body surface areas like the extremities. The disease can penetrate into deeper tissues such as tendons, bones and joints, particularly in the presence of local or systemic immune suppression. However, it rarely disseminates to organs even in severely immunocompromised patients, making it a less formidable pathogen than *M. tuberculosis*. In addition, there are no reports of person-to-person *M. marinum* transmission by aerosol infection or otherwise. The differences in disease tropism between *M. marinum* and *M. tuberculosis* may be dictated by factors other than growth temperature. As alluded to earlier, *M. tuberculosis* infection and disease in humans can also have a dermal localization without systemic spread if transmitted directly via abraded skin[17], suggesting that disease localization may be partly related to the initial point of contact with the organism.

It is noteworthy that *M. marinum* is most closely related to *M. ulcerans*, the causative agent of Buruli ulcers and indeed the two may have arisen fairly recently from a common ancestor. In turn, the two organisms are the closest evolutionary relatives of the *M. tuberculosis* complex organisms[15]. Despite its genetic relationship with *M. ulcerans*, *M. marinum* infection and disease are more similar to those caused by *M. tuberculosis* than *M. ulcerans*. This is likely because *M. ulcerans* secretes a cytotoxic polyketide toxin which produces extensive ulcers in which bacteria grow extracellularly[19]. The complex genetic and pathogenetic relationships among *M. tuberculosis*, *M. marinum* and *M. ulcerans* have been summarized recently[3].

M. marinum: A historical perspective

A brief historical review serves to explain our initial interest in exploring *M. marinum* as a model for *M. tuberculosis*. *M. marinum* was first discovered in 1926 as the agent of a tuberculosis-like disease in fish[20] and rediscovered almost 30 years later as the agent of superficial granulomas in a swimming-pool outbreak[21]. The human isolate was initially considered a new species and named *M. balnei* (*balnei* being a Roman word for baths) and only later was its identity to the previously discovered *M. marinum* determined. It appears that *M. marinum* was investigated as a possible model for *M. lepra*e which is far less tractable than *M. tuberculosis*, since it cannot be grown in culture[22–24]. *M. marinum* and *M. leprae* have superficial similarities in that they both have lower growth temperature optima and cause skin disease in humans. However, this line of research appears to have been abandoned, perhaps as a consequence of finding few pathogenetic similarities. The seminal *M. marinum* pathogenesis study was published by Clark and Shepard in 1963 (ref. 16) who showed that 50 different wild-caught poikilothermic species, including fish, amphibians and reptiles are susceptible to systemic *M. marinum* infection and disease. They also showed that mice injected intravenously with *M. marinum* develop only peripheral disease. However, if the mice were kept at 4°C so that their body temperature was lowered, they developed a systemic disease involving the lungs and spleen that resembled *M. tuberculosis* infection. Subsequently, it was shown that a 37°C-adapted *M. marinum* strain caused systemic disease in mice maintained at ambient temperature and that this infection afforded some protection against a subsequent challenge with *M. tuberculosis*[25]. Similarly, Fenner[26] showed that chick embryos infected with *M. marinum* succumbed to infection at 33°C but not at 37°C. Thus, all of these experiments suggested that *M. marinum* host specificity and disease localization is strongly linked to its growth temperature optimum. The fact that it causes a granulomatous disease in humans made it all the more attractive as a model. There are now several laboratories working in various areas of *M. marinum* pathogenesis and a number of genetic tools as well as host and tissue-culture models have been developed.

M. marinum: Genetics and genomics

An array of genetic tools has been developed for the study of *M. marinum* pathogenesis. Plasmid and cosmid shuttle vectors as well as integrating vectors that use the L5 phage attachment site that were developed for *M. tuberculosis* also work successfully in *M. marinum*[27]. Homologous recombination is relatively facile[27] and multiple transposon mutagenesis transposon mutagenesis systems have been developed. A *Drosophila mariner* transposon family member has been used to generate a library of transposon mutants[28], and a Tn 5367-based transposon system has been developed in the context of a conditionally replicating mycobacteriophage to enhance the transposition frequency[29].

The near completion (theoretical coverage of 99.99% at the time of this writing) of the *M. marinum* genome sequence (http://www.sanger.ac.uk/Projects/M_marinum/) is facilitating molecular manipulations and allowing comparative genomic analyses of *M. tuberculosis* and *M. marinum* as well as whole genome microarray analyses of *M. marinum* gene expression. These analyses should be interesting both with regard to their similarities and their differences. While the organisms have pathogenetic similarities, *M. marinum* appears to have an environmental niche, whereas the *M. tuberculosis* complex organisms do not. Consistent with this difference, the *M. marinum* genome size is currently estimated at 6.6 Mb in contrast to the 4.4 Mb for *M. tuberculosis*. With the caveat that the current *M. marinum* genome size is an overestimate, it is tempting to speculate that the ~ 2 Mb 'excess' genome of *M. marinum* encodes determinants for environmental survival. For instance, at least some genes encoding certain enzymes of the carotenoid biosynthetic pathway present in *M. marinum* (one of the few mycobacteria that turn from white to bright yellow upon exposure to light) are absent in *M. tuberculosis*[27]. It was thought initially that light-induced pigmentation protected *M. marinum* against singlet oxygen arising from light-induced photo oxidative damage[30], suggesting that the genes encoding this pathway might represent genes important for environmental survival. However, a recent study showed that sensitivity to singlet oxygen is encoded by one or more genes (in an operon) that are common to both organisms and thus appears to be at least partially independent of pigment production[28]. Mutation in one of these genes also resulted in decreased macrophage survival of *M. marinum.* This defect was complemented by the introduction of the *M. tuberculosis* homologues on the corresponding operon, suggesting that they play a similar role in *M. tuberculosis*.

The remainder of this review will focus on the biology of experimental *M. marinum* infection and disease in various models, how it compares to experimental *M. tuberculosis* disease, and the new insights that contemporary *M. marinum* studies have provided.

M. marinum and macrophages

A central role for the macrophage in tuberculosis has been long established based on studies of infections of experimental animals and humans[31]. Not surprisingly, macrophages play a key role in *M. marinum* infection as well[32–35]. Macrophages play contradictory roles in infection and disease as they are likely the first host cells to respond to invading mycobacteria, and yet aid in their subsequent dissemination. For instance, in the rabbit and mouse models of pulmonary tuberculosis, alveolar macrophages are thought to transport aerosolized bacteria from the lung to deeper tissues[31,36,37]. Recent real-time imaging studies of live *M. marinum*-infected zebrafish embryos have allowed the direct observation of initial *Mycobacterium*–macrophage interactions *in vivo* in unprecedented detail and have detected several modes of bacterial dissemination by macrophages[6]. Newly infected macrophages were observed migrating from the blood stream or hindbrain ventricle into deeper tissues so as to establish a systemic infection. Other potential modes of dissemination observed were the transfer of bacteria from one macrophage to another via membrane tethers, and uninfected macrophages migrating from distant sites to engulf dead infected macrophages in the tissue, thus creating new infected macrophages. Thus the zebrafish studies have provided direct evidence of the role of macrophages in the dissemination of tuberculosis and have revealed new mechanisms for this process.

While both pathogenic and nonpathogenic mycobacteria can enter cultured eukaryotic cells, only the pathogenic species, including *M. marinum* can survive and replicate therein[38]. This requirement of replication in cultured cells for pathogenicity *in vivo* has been corroborated by the finding that mutants of both *M. tuberculosis* and *M. marinum* deficient for growth in tissue-culture macrophages are also attenuated *in vivo*[35,39,40]. Furthermore, the pathogens *M. marinum*, *M. fortuitum* and *M. avium* but not the nonpathogen *M. smegmatis* can grow in environmental amoebae, which probably served as host cells for intracellular pathogens before complex eukaryotes evolved[41].

The Mycobacterium phagosome: Insights and questions arising from M. marinum studies

Studies of infected, cultured macrophages have provided a detailed understanding of the biology and biogenesis of the *Mycobacterium* phagosome and experiments using *M. tuberculosis*, *M. avium*, and *M. marinum* have yielded similar results[42,43]. Briefly, the phagosome communicates with early endosomes of the host endocytic machinery and acquires specific components from both cell-surface plasma membrane and early endosomes, but fails to fuse to lysosomes and become acidified[42]. Studies with *M. marinum* have further contributed to our understanding of this process as described below.

Shedding light on host proteins associated with the Mycobacterium phagosome: The M. marinum Dictyostelium model

Central to *Mycobacterium* pathogenesis is the generation and maintenance of the *Mycobacterium* phagosome and the molecules that regulate its trafficking[42]. Biochemical analysis of isolated, live *Mycobacterium* phagosomes yielded a candidate host protein that specifically associ-

ates with *Mycobacterium* phagosomes, TACO or coronin, an actin-binding protein whose *Dictylostelium* homologue is required for phagocytosis[44]. These authors speculated that the active retention of TACO on phagosomes that is modulated by live but not dead mycobacteria, prevents phagolysosomal fusion and facilitates bacterial survival. However, this requirement for coronin has been called into question by a recent study using the slime mold *Dictyostelium discoideum* to model *M. marinum*–macrophage interactions[8]. The *Dictyostelium* model appears to be highly relevant for the study of macrophage–*Mycobacterium* interactions in that the intracellular growth properties of *M. marinum* were the same as in cultured macrophages and a mutant attenuated for growth in macrophages also failed to grow in *Dictyostelium*. These researchers used a defined *Dictyostelium* coronin mutant to define the role of coronin in bacterial replication. Surprisingly, although coronin was found to associate with *M. marinum*-infected phagosomes in wild-type *Dictyostelium*, bacterial replication was actually higher in the coronin mutant. The caveat of this study is that there are several mammalian coronin isoforms (with potentially different functions) and it is not clear if the *Dictyostelium* coronin studied corresponds to coronin 1/TACO. However, the coronin studies highlight the importance of genetic approaches to determine the role of various host components in the context of *in vivo* infection. This study also reveals the power of using a genetically tractable host to understand the host's contribution to pathogenesis. Future studies with this model should elucidate host determinants involved in *Mycobacterium*–macrophage interactions.

The complexities of Mycobacterium phagolysosome fusion

As discussed earlier, the avoidance of phagolysosome fusion by *Mycobacterium* is thought to be an important bacterial survival mechanism. These studies have been done studying *Mycobacterium* interactions in cultured macrophages[42]. This nonfusion pattern has been observed in all pathogenic species studied (*M. tuberculosis*, *M. avium* and *M. marinum*), except the mouse pathogen *M. lepraemurium*, which resides predominantly in phagolysosomes during replicative infection[45]. Further, evidence for the importance of nonfusion in bacterial survival comes from experiments in macrophages activated by gamma interferon, where mycobacteria were found predominantly in phagolysosomes and were killed[46,47]. However, other experiments suggest that *Mycobacterium* can survive in acidified compartments. In experiments co-infecting mycobacteria and *Coxiella burnetii* into cultured macrophages, the majority of the mycobacteria co-localized with *Coxiella* organisms in acidified compartments, without significant loss of viability[48]. Also, *M. tuberculosis* infection of freshly isolated human alveolar macrophages revealed that the majority of phagosomes containing intact bacteria were in intimate contact with lysosomes[49]. Furthermore, when opsonized *M. tuberculosis* enters macrophages via Fc-receptors, most bacteria are localized in phagolysosomes and appear to replicate over several days[50]. Fc-receptor-mediated entry may operate in later phases of infection after specific antibodies have been generated[2], and it is possible that the bacteria become adapted to survive in the presence of phagolysosomal fusion in the later stages of *in vivo* infection. In summary, it appears that mycobacteria may have devised mechanisms to resist phagolysosomal fusion under certain circumstances.

M. marinum also appears to survive in macrophage phagolysosomes *in vivo* as revealed by transmission electron microscopy analysis of phagosomes in granulomas of infected frogs over an extended time course[32]. Approximately 60% of the intact bacteria resided in phagolysosomes and the level of phagolysosomal fusion correlated with the level of macrophage activation. It is possible that bacteria within phagolysosomes are dying or destined to die; but this seems unlikely, as the overwhelming majority of bacteria appears intact. It seems more likely that the bacteria have adapted to be able to live within a phagolysosome in the setting of a granuloma. Consistent with this hypothesis, there are *M. marinum* genes specifically activated, not by residence in single macrophages, but only upon aggregation of the infected macrophages[6,35,51]. These data, indicative of bacterial survival in phagolysosomes in granulomas, provide an *in vivo* correlation for the studies of Fc-mediated entry, where antibody-mediated entry into macrophages may lead to phagolysosomal fusion once adaptive immunity has developed[50]. Therefore, it is possible that mycobacteria have at least two sets of adaptive mechanisms: restriction of phagolysosomal fusion early in infection and subsequent adaptation to phagolysosomal fusion within the activated macrophages of granulomas.

***M. marinum* and granulomas**

Similarities and differences between M. marinum and M. tuberculosis granulomas: Virtually all pathogenic mycobacteria produce granulomas, the hallmark structures of tuberculous infection in immunocompetent hosts. Like the macrophage, granulomas may play a dual role in the infection, both containing it and providing an environment where the bacteria can persist. Granulomas likely begin as aggregates of mononuclear phagocytes that surround individual infected macrophages[6,52]. These macrophages become activated, a transformation reflected by an increase in their size and subcellular organelles, ruffled cell membranes, and enhanced phagocytic and microbicidal capabilities[31,52]. A common feature of all *Mycobacterium* granulomas is the further differentiation of the

macrophages into epithelioid cells that have tightly interdigitated cell membranes in zipper-like arrays linking adjacent cells[52]. Several macrophages can fuse to form giant cells.

While the pathological definition of a granuloma requires only an organized collection of differentiated macrophages with a characteristic morphology[52], tuberculous granulomas in humans and mice contain some combination of lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components[2]. While the roles of all the accessory cells in the granuloma have yet to be elucidated, certain T-lymphocyte subsets are critical for maintaining granuloma integrity and restricting bacterial numbers in *M. tuberculosis*-infected humans and mice[2].

A striking feature of certain tuberculous granulomas is the presence of areas of caseous necrosis, regions of acellular debris that have a distinct histology[31,52]. Thus the 'textbook picture' of a human granuloma is that of a central core of necrosis surrounded by epithelioid cells surrounded by a cuff of lymphocytes[31,53]. Bacteria are found both within macrophages of granulomas and, in larger numbers, within the central caseous region[31,32,54]. Human *M. tuberculosis* granulomas can also be fibrotic and calcified and such lesions often do not contain viable organisms, so that fibrosis is often a hallmark of healed or sterile lesions.

In its natural host, humans, *M. tuberculosis* produces highly complex granulomas that can have some or all of the features described above at various points in their evolution. Even within a single infected host, granulomas can be at different stages of evolution[55]. While epithelioid cells are a feature of all tuberculous granulomas, the presence of caseous necrosis, presumably formed by the lysis of infected cells, is highly host species-specific[2,31,32]. Human granulomas can caseate, and rupture of the liquefied caseum into the bronchial tree is the primary source of aerosol tuberculosis transmission[55]. While caseation is thought to be important in human tuberculosis, the actual mechanism of its formation and its impact on pathogenesis other than transmission, remain enigmatic. As mentioned earlier, its formation appears to be governed by host determinants. For instance, mice experimentally infected with *M. tuberculosis* form noncaseating granulomas, whereas rabbits and guinea pigs form caseating ones[11,12,56].

M. marinum granulomas in all species examined have the basic core of epithelioid cells (Figure 1) with the characteristic electron-microscopic features and often with the zippered appearance of cell membranes[18,32,54]. As is the case with *M. tuberculosis*, *M. marinum* also produces caseating or noncaseating granulomas depending on the infected host. Only noncaseating granulomas are produced in the leopard frog even after a year-long infection[32], but both caseating and noncaseating granulomas develop in certain toads, goldfish, zebrafish, and humans[18,32,54] (D. Beery, O. Humbert and L.R., unpublished result). Both caseating and noncaseating granulomas can be found within the same zebrafish (D. Beery, O. Humbert and L.R., unpublished result). Leopard frogs are quite resistant to *M. marinum* disease, appearing healthy and maintaining relatively constant bacterial loads in well-organized granulomas, more than a year post-infection with as many as 10^7 bacteria[57]. In contrast, both goldfish and zebrafish seem to be more susceptible to higher infecting doses, dying within several weeks with large bacterial loads[54,58] (O. Humbert, C. Cosma and L.R., unpublished result). This possible difference in susceptibility could be owing to the larger bacterial numbers in caseating (fish) than in noncaseating (frog) granulomas. Alternately, other host factors could be affecting this potential susceptibility difference.

As discussed earlier, all mammalian *M. tuberculosis* granulomas contain numerous lymphocytes. *M. marinum* human granulomas also contain a high density of lymphocytes[18] (L.R., unpublished result), whereas most frog and fish granulomas have only a few scattered lymphocytes with few lesions having the cuff of lymphocytes common to mammalian tuberculous granulomas[32,54] (D. Beery and L.R., unpublished result). *M. marinum* infection of frogs affords some protection against reinfection similar to the case with *M. tuberculosis* infection of mice (C. Cosma and L.R., unpublished result), suggesting that adaptive immunity plays a similar role in frogs and mammals. Since frogs and fish appear to be able to contain mycobacterioses as well as mammals with the recruitment of fewer lymphocytes, it is possible that the greater number recruited into mammalian lesions represents an excessive and unnecessary immune response. Alternatively, it is possible that the immune systems of ectotherms have evolved to control infection with fewer but more effective lymphocytes. The recent

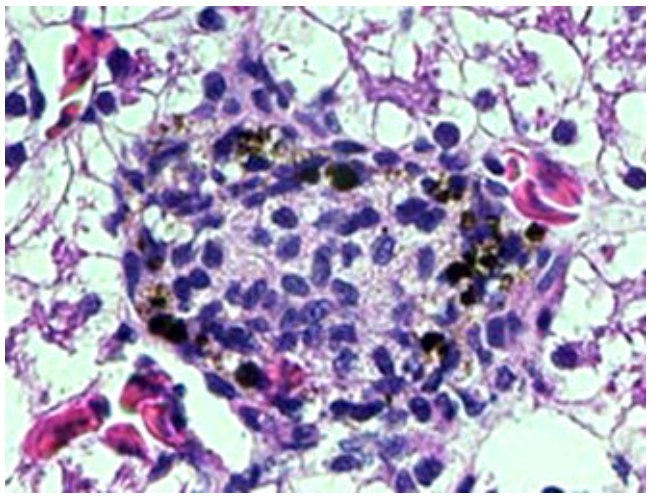

Figure 1. Hematoxylin and eosin-stained *M. marinum* granuloma in adult frog liver. Note the tightly clustered cells without clear cell boundaries (epithelioid cells). Note the black pigment that is commonly found in frog granulomas. Bright pink cells are the nucleated red blood cells. (Image taken by Christine Cosma.)

isolation of a targetted *rag*1 zebrafish mutant should allow the elucidation of the role played by lymphocytes in fish tuberculosis[59].

In summary, *M. marinum* infection of frogs and fish bears some resemblance to human *M. tuberculosis* infection. Fish granulomas caseate and afford the opportunity to study this enigmatic process, whereas frog granulomas maintain a chronic infection with a stable number of organisms in a manner similar to some humans and may be useful for the examination of the bacteria–host interface during a long-term smouldering infection.

Use of M. marinum to probe granuloma-specific gene expression and the granuloma environment

The idea that bacteria adapt to environmental stimuli by changing their gene expression patterns is long established. For the study of pathogenesis, genes expressed solely in the context of the host can provide clues about the conditions bacteria experience as a consequence of host immunity as well as the mechanisms they use to circumvent it. Therefore, an expression screen was undertaken to identify *M. marinum* genes activated specifically during long-term granulomatous infection of frogs[35,51]. The prediction that mycobacteria express specific genes to aid their survival within granulomas was tested using a Green Fluorescent Protein (GFP) reporter-based Differential Fluorescence Induction (DFI) screen. The screen was designed to identify *M. marinum* genes expressed in frog granulomas, but not in bacteriological medium[35,51]. This screen identified two classes of genes: *mags* (macrophage activated genes), induced both in granulomas and in cultured macrophages, and *gags* (granuloma activated genes), induced solely in granulomas. Both classes encode proteins with a wide range of metabolic activities as well as homologues of *M. tuberculosis* genes encoding proteins of unknown function. Interestingly, the *gag*s are not activated *in vitro* under a variety of conditions predicted to mimic the granuloma environment, including hypoxia, stationary phase growth, and *g*-interferon-stimulated macrophages[51]. However, several *gag*s are activated specifically upon aggregation of infected macrophages in the zebrafish embryo[6]. These findings show that the complexities of the granuloma environment uniquely influence mycobacterial gene expression in ways that are not recapitulated *in vitro*. Thus, these studies highlight the importance of performing *in vivo* analyses as a starting point for pathogenesis studies, rather than trying to second guess *in vivo* conditions.

Physiological state of Mycobacterium during long-term granulomatous infection: Insights from chronic M. marinum infection of frogs

Summary of M. tuberculosis studies: All pathogenic mycobacteria, most notably *M. tuberculosis*, *M. leprae* and *M. marinum*, are capable of establishing long-term infections and it is highly likely that all employ similar persistence strategies. Exposure of humans to *M. tuberculosis* via the aerosol route can lead to several potential outcomes, including early clearance of the infection, disease immediately following infection (primary or post-primary tuberculosis), or development of subclinical or asymptomatic infection (latent tuberculosis)[60,61]. Latent tuberculosis has the transition potential to active disease, subsequently making the mechanism of how the bacteria persist not only of scientific interest, but also of public health consequence. Latent tuberculosis has been the subject of several recent reviews[3,60,62,63] and only a brief summary is provided here.

It is not clear whether clinical latency results from bacteria attaining a metabolically dampened, nonreplicative state or from a dynamic equilibrium between bacterial death and replication that maintains low, constant bacterial numbers[60]. Yet, the idea that latent tuberculosis requires physiologically dormant bacilli is pervasive[9,64,65], initially stemming from the observations that clinical latency in humans involves few bacilli and limited pathology in the absence of symptoms. However, direct evidence for such dormancy is limited[3]. Attempts to clarify the state of *M. tuberculosis* in long-term animal infections have also provided results that are both controversial and ambiguous. The idea that the bacteria are not replicating during latency was bolstered by experiments performed in *M. tuberculosis*-infected mice[66], where viable bacterial counts and the numbers of acid-fast bacilli were compared over several months. Bacterial counts enumerated by both techniques remained constant leading to the interpretation that the bacilli must be in a non-replicating state, because the co-existence of bacterial death and replication would have resulted in an increasing number of microscopic bacterial counts over time owing to the accumulation of dead bacilli. However, the caveat of these experiments is that the rate of bacterial death may be slow enough that the discrepancy between visualized and viable bacteria is not within the limits of detection. Modern studies using gene-expression patterns to make conclusions about the bacteria in mouse infection models have also yielded seemingly disparate conclusions. Some suggest a level of bacterial metabolism during latency. *M. tuberculosis* mRNA was detected in the lungs in a drug-induced latency mouse model[67,68]. Another study found that certain genes which had been previously found to be induced in an *in vitro* nonreplicating persistence model were also induced in infected mice, but only after the first week of infection[65,69]. However, this study did not conduct a survey of global gene expression *in vivo* and multiple stimuli can induce the expression of specific genes[70]. Therefore, the interpretation that the induced expression of these genes in the mouse is indicative of nonreplicating persistence *in vivo*, is potentially flawed. Another recent study has

shown that a *M. tuberculosis* mutant in the gene encoding the enzyme Rel_{Mtb} that controls the stringent response and starvation-induced salvage pathways, is attenuated in mice but only late in the course of infection[71]. These results suggest, much like the *M. marinum* granuloma expression studies, that the metabolic state of the bacteria is altered *in vivo*. Again, it is difficult to determine what the precise role of the stringent response is *in vivo*, as our knowledge of this system is based on *in vitro* studies.

M. marinum studies: The DFI study done in *M. marinum*-infected frogs showed that numerous bacterial genes involved in a variety of active cellular processes are highly expressed in seventeen-month-old granulomas of chronically infected frogs, even while the viable bacterial counts remain constant[51]. Indeed, the gene expression profile of these bacteria was remarkably similar to those in logarithmic phase growth in anaemic media. However, this study also has its caveats. Bacterial gene expression is not proof of replication, and the relative stability of GFP as a reporter may preclude detection of a temporal reduction of bacterial gene expression within granulomas. Furthermore, in a latent infection, the bacteria may not all be in the same metabolic state. The *M. marinum*-frog study was done on bacterial populations from granulomas; so it is possible that a subset of these bacteria were in an inactive state and masked by gene expression from an active population. However, the study suggests that at least a subset of the population during chronic asymptomatic infection expresses genes associated with active growth. An electron microscopy series performed on frogs infected between eight weeks and one year also provides indirect evidence for the dynamic nature of host–pathogen interactions in long-term granulomas[32]. Macrophages of a range of activation levels, recent phagocytic events, and bacteria in phagosomes as well as phagolysosomes were seen over a prolonged infection course. Taken together, these two studies suggest a dynamic host–pathogen interplay that continues late into infection.

Insights into granuloma formation: M. marinum–host interactions in zebrafish embryos

In *M. tuberculosis*-infected mice, T-lymphocytes and other immune cells are recruited early during the process of granuloma formation[72] and it is not clear if these accessory cells influence the early stages of granuloma formation. As described in an earlier section, *M. marinum* infection of zebrafish embryos 30 h post-fertilization has allowed the visualization of *Mycobacterium*–macrophage interactions in real time, starting when the bacteria first enter the host[6]. In this model, even few bacteria incited profound macrophage migration and phagocytosis (Figure 2). The infected macrophages in turn recruited additional macrophages and within three or four days post-infection, the macrophages form tight aggregates that have pathological similarities to adult granulomas (Figure 3). The participating macrophages develop tight intercellular boundaries and, in some cases, loss of intercellular membranes to form fused cells with the sequestration of the bacteria. Furthermore, several of the previously described *M. marinum gag*s are specifically activated upon aggregate formations but not upon infection of individual macrophages, demonstrating that the aggregate microenvironment provides at least some of the specific stimuli present in adult granulomas. Notably, these granuloma-like aggregates develop solely in the context of innate immunity, as the developing embryos have not yet developed T-lymphocytes. Thus, the use of this zebrafish

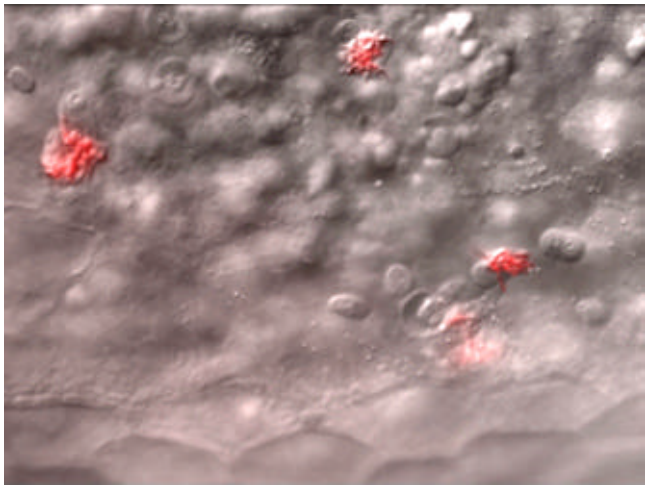

Figure 2. Phagocytosis of *M. marinum* by macrophages of zebrafish embryo. Three macrophages on the yolk ball of a 32 h post-fertilization zebrafish embryo with intracellular *M. marinum* expressing red fluorescent protein. The image is an overlay of fluorescent and differential interference contrast (DIC) images. (Image taken by Olivier Humbert.)

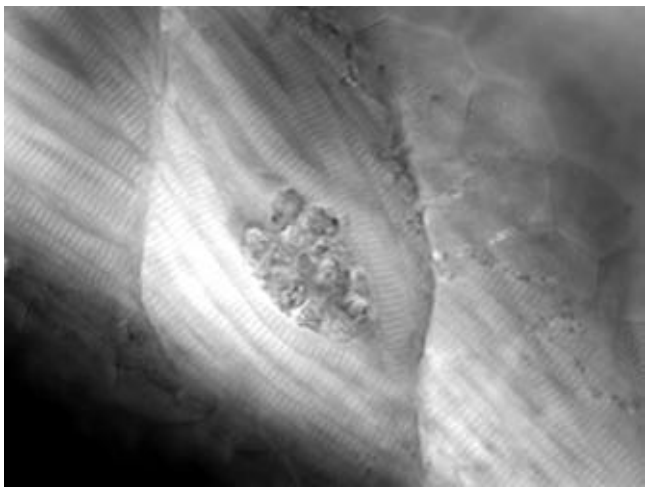

Figure 3. Granuloma-like aggregate in zebrafish embryo. DIC image of a tight aggregate of macrophages that has formed in the muscle tissue of the tail of an embryo that is five days post-fertilization and four days post infection. (Image taken by Olivier Humbert.)

embryo model has revealed that lymphocytes are not required for granuloma formation in its most fundamental aspects. Adaptive immunity undoubtedly plays an important role in further maturation of these granulomas and in curtailing the infection in zebrafish, as it does in mammals. The use of this model should help dissect the relative contributions of innate and adaptive immunity in tuberculosis. The potential to inactivate specific genes in zebrafish embryos as well as to derive mutant strains, such as the already available *rag*1 mutant[59], should further facilitate the dissection of host components involved in tuberculosis. Real-time analysis of these transparent embryos should elucidate the exact mode of action of identified host factors.

Early host–pathogen interactions: The Drosophila model

The *Dictyostelium* model allows for the genetic dissection of *Mycobacterium*–macrophage interactions at the subcellular level, while the zebrafish embryo model offers the potential to dissect the mechanism and impact of cell–cell crosstalk and the interface between innate and adaptive immunity. A *Drosophila* model has also been developed for *M. marinum*, which may be most effective at examining the very early phases of *Mycobacterium* infection[7]. These investigators showed that as few as 5 viable *M. marinum* are lethal to *Drosophila*. This effect was specific in that the attenuated *mag*24-1 PE-PGRS mutant[35] killed the embryos much more slowly. Although bacteria ultimately become extracellular in *Drosophila* infection, experiments utilizing bacteria expressing *mag* : : *gfp* fusions suggested that the majority of the bacteria are located within hemocytes (*Drosophila* blood cells) early in infection. Only later, after killing the host cells and producing extensive tissue damage, are bacteria released into the extracellular milieu. Indeed, intracellular *M. marinum* blocked phagosomal acidification, similar to the effect on cultured mammalian macrophages[43]. Surprisingly, unlike all the other pathogens tested, *M. marinum* failed to elicit the production of anti-microbial peptides in *Drosophila* (see *kasB* section later in this review for implications of this finding). Furthermore, *Drosophila* mutants in the *imd* and *toll* pathways did not exhibit enhanced susceptibility to *M. marinum*. The toll-like-receptor-mediated pathways appear to be important for the control of mammalian tuberculous infections[73]. Because *Drosophila* has components of innate immunity and no adaptive immunity, these findings may be consistent with the idea that these pathways play a role in facilitating effective crosstalk between innate and adaptive immunity in vertebrates with complex immune systems. This hypothesis can be tested in the zebrafish embryo system. Alternately, the baseline sensitivity of the flies may be so high that subtle differences may not be uncovered. If that is the case, testing various attenuated *M. marinum* mutants in this model may uncover more subtle phenotypes.

***M. marinum* virulence genes**

DFI and transposon mutagenesis screens are identifying virulence genes in *M. marinum*[28,35,51,74] (M. Trucksis, pers. commun.). Discussed below are three mutants that have revealed new information about *Mycobacterium* virulence.

PE/PE–PGRS family genes

The highly unique PE protein family constitutes ~ 5% of the *M. tuberculosis* H37Rv genome and is defined by the presence of a conserved 110 amino acid N-terminal domain[75]. Several members have an additional PGRS (polymorphic GC-rich repetitive sequence) domain of variable length, while others also have a unique C-terminus (PE–PGRS–U). Yet other members lack the PGRS, and just have a unique domain linked to the N-terminal PE. PE family members are present in all sequenced *Mycobacterium* genomes, although their number is highly variable. *M. tuberculosis*, *M. bovis* BCG, and *M. marinum* have ~ 80–140 PE/PE–PGRS genes, whereas *M. avium*, *M. leprae* and *M. smegmatis* each have fewer than ten genes[75–77] (www.tigr.org; www.sanger.ac.uk). In *M. leprae*, which has a highly contracted genome, the few remaining PE genes appear to be pseudogenes, a trend shared by other *M. leprae* homologues of *M. tuberculosis* genes[76]. Some PE/PE–PGRS family members have been shown to be surface-localized[78]. The hypothesis that these proteins as well as members of the distinct PPE family, serve as reservoirs of antigenic diversity[75] is bolstered by the finding that 50% of the sequence polymorphisms found between the two sequenced *M. tuberculosis* genomes involve *PE* or *PPE* genes[77].

Direct evidence for a role for some of these PE/PGRS proteins in virulence was first revealed in *M. marinum*[35] and there is also evidence for a similar role in *M. tuberculosis*. Two *M. marinum* PE/PGRS genes are *mag*s, being induced both in cultured macrophages and granulomas[6,35,51]. A *M. marinum* strain mutant in the *mag*24-1 PE–PGRS gene is attenuated in the adult frog, zebrafish embryo, *Drosophila* and *Dictyostelium* infection models[6–8,35]. Interestingly, infection of frogs with this mutant resulted in many loose aggregates consisting predominantly of immature macrophages rather than mature interdigitated macrophages[32]. Certain attenuated mutants of *M. tuberculosis* have also been found to alter granuloma composition[79,80]. A signature-tagged mutagenesis screen of *M. marinum* has also identified members of the PE–PGRS family that are attenuated in goldfish infection (M. Trucksis, pers. commun.).

Evidence is accumulating for the role of PE/PE–PGRS proteins in *M. tuberculosis* virulence. A *M. tuberculosis* PE–PGRS gene is induced selectively in macrophages[81],

while another from *M. bovis* BCG is deficient in adherence to eukaryotic cells[82]. The Rel_{Mtb} mutant shows differential expression of several genes, including six PE–PGRS genes compared to the wild-type strain. Since this mutant is defective for survival *in vivo*, it is possible that some of these PE/PE–PGRS genes that are downstream of Rel_{Mtb} may also play a role in virulence. More direct evidence for PE proteins in virulence comes from a whole genome microarray-based transposon mutagenesis (TraSH) screen for virulence mutants in a mouse infection model that has identified two PE family members[83]. However, the role of PE/PE–PGRS proteins in virulence remains enigmatic. The finding that attenuation results from deletion of individual members of this large family, suggests they have a non-redundant function. In contrast, pathogens like *M. leprae* and *M. avium* persist within granulomas despite having few or no functional copies of these genes. It is possible that they have developed alternate persistence strategies.

WhiB3

Another *M. marinum* gene that is induced in cultured macrophages, *whiB3*, is even more highly activated *in vivo*, both in individual macrophages and granulomas[6,35,51]. The *M. marinum* and *M. tuberculosis* WhiB3 proteins have > 90% identity and both are highly related to a transcriptional activator of early sporulation in *Streptomyces*. There is no evidence that *M. tuberculosis* encodes any structural sporulation proteins, suggesting that if *whiB*3 is indeed a transcriptional regulator in *Mycobacterium*, it must be regulating other processes.

Recently, *whiB*3 has been uncovered as a *M. tuberculosis* virulence factor[84]. Previously, Collins *et al.*[85] identified a missense mutation in the principal sigma factor, SigA (RpoV) of *M. bovis* that had no effect on growth *in vitro*, but resulted in attenuation. Subsequently, Steyn *et al.*[84] used a yeast two-hybrid approach to identify WhiB3 as a protein that interacts with wild type but not mutant SigA. *whiB3* mutations were generated in *M. tuberculosis* H37Rv and in a virulent *M. bovis* strain. Both mutants grew well *in vitro*, but had defects *in vivo*. *M. tuberculosis whiB3* mutants achieved normal bacterial loads in mice, but showed decreased lung pathology, and a slower time to death than wild-type bacteria. In a guinea pig model, *M. tuberculosis whiB3* infection was comparable to that of the parent strain, while in contrast, *M. bovis whiB3* was unable to grow, with a bacterial load far lower than the wild type. Virulence assays on a *M. marinum whiB*3 mutant are ongoing in different models; however at a gross level, this mutant appears to grow as well as wild type in a high-dose frog infection. Given the observed phenotypic differences between the *M. tuberculosis* and *M. bovis whiB*3 mutants, it will be especially interesting to determine the role of the *M. marinum* WhiB3 in the various infection models.

KasB

M. marinum kasB mutants were identified by a transposon mutagenesis screen and found to exhibit poor growth in macrophages, about ten fold less than wild-type bacteria[74]. *kasB* is predicted to encode a *b*-ketoacyl-acyl carrier protein synthase and accordingly, various biochemical and biophysical analyses demonstrated that the *M. marinum kasB* mutants synthesize mycolic acids that are 2–4 carbons shorter than wild-type *M. marinum*, and have a 30% reduction in the abundance of keto-mycolates. All of the phenotypes of the mutant were completely reversed by complementation with the *M. tuberculosis kasB* gene, but not by the highly related *kasA* gene. These data suggest that KasB plays similar roles in these two organisms, not only in mycolic acid biosynthesis, but also in macrophage growth. The *kasB* mutant also displayed diminished cording *in vitro* (a phenotype classically associated with *M. tuberculosis* virulence) and increased membrane permeability, resulting in increased sensitivity to SDS and lipophilic antibiotics like rifampin, but not to the hydrophilic antibiotics isoniazid and streptomycin. Similarly, the mutants were also more sensitive to host antimicrobial peptides found in macrophages, a finding that explains the macrophage growth defect. It will be interesting to determine the phenotype of this mutant in the various *in vivo* models, studies that are undoubtedly in progress. The *kasB* studies are exciting in that they provide a detailed understanding of KasB function and demonstrate functional similarities of the *M. marinum* and *M. tuberculosis* homologues. Furthermore, the increased sensitivity of the *kasB* mutant to rifampin suggests a new, and much needed route of pharmacological intervention in conjunction with rifampin that could apply to both organisms.

Summary

It becomes clear why *M. marinum* is an attractive model for the study of host–pathogen interactions in human tuberculosis. Its relatively rapid growth and the lack of a need for BL3 containment offer obvious experimental advantages. Indeed, the classification of *M. marinum* as a BL2 organism has facilitated studies using fluorescence activated cell sorting and microscopy of live bacteria, both of which would be logistically difficult to perform for a BL3 organism. The lower optimal growth temperature of *M. marinum* has made it possible to study its interactions with model organisms such as *Dictyostelium* and *Drosophila* that require lower temperatures. The *Drosophila* model offers the potential to employ genetics to study the contribution of innate immunity, while the *Dictyostelium* model, a genetically tractable surrogate for macrophages, allows for the identification of host determinants of *Mycobacterium*–macrophage interactions. Zebrafish have both innate and adaptive immunity, and transparent embryos allowing for real-time imaging of infection not afforded by other systems. Finally, its close

genetic relationship with *M. tuberculosis* allows for cross-over of genetic tools and translation of gene function.

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