The Zebrafish Guide to Tuberculosis Immunity and Treatment

LALITA RAMAKRISHNAN
Departments of Microbiology, Medicine, and Immunology, University of Washington, Seattle, Washington 98195
Correspondence: lalitar@uw.edu

During the past 12 years, we have developed the zebrafish as a model for the study of tuberculosis pathogenesis and immunology. We have taken advantage of the optical transparency and the genetic and pharmacological tractability of the developing zebrafish to monitor infection in real time. Detailed information about the sequential interactions among the host and the pathogen, the cell types, and the molecules involved has yielded surprising insights into this ancient disease. We have identified a number of host evasion strategies deployed by pathogenic mycobacteria as well as host responses that provide broad insights into host immunity. Many of these discoveries have relevance to human tuberculosis and suggest new therapeutic avenues for tuberculosis as well as other inflammatory diseases.

Tuberculosis, historically known as the “White Death” and “consumption,” has beguiled doctors and scientists for centuries. In 1882, Robert Koch, one of the all-time greats among medical researchers, firmly established tuberculosis as an infectious disease caused by Mycobacterium tuberculosis. Koch’s discovery fueled a flurry of research that ultimately led to the testing of the BCG vaccine in 1921. This live attenuated vaccine, derived by repeated laboratory passage of a virulent strain of Mycobacterium bovis, is widely used in endemic areas because it offers some protection against the deadliest forms of disseminated tuberculosis in children—miliary tuberculosis and tuberculous meningitis (Rodrigues et al. 1993). In contrast, it offers little protection against the common pulmonary form of the disease. Because tuberculosis can occur in many organs but is mainly transmissible through aerosols from infected lungs, it is this pulmonary disease that sustains the global disease burden. Multiple efforts to make a more protective vaccine have failed (Tameris et al. 2013), which is not surprising given that even natural infection fails to protect against reinfection, both in humans and in animal studies (Cosma et al. 2004).

The first antibiotic therapy against tuberculosis, streptomycin, was introduced in 1948 (Mitchison 2005). Drug resistance quickly rendered streptomycin ineffective, and a 3–4 drug cocktail containing the cornerstone antitubercular drugs isoniazid and rifampicin came into use by the 1970s (Mitchison 2005). However, even this regimen of treatment requires 6–9 mo to achieve cures reliably. Shorter treatment periods result in a temporary clinical improvement, often followed by relapse with transmissible, drug susceptible disease (Connolly et al. 2007). This transient antibiotic resistance is often referred to as phenotypic (as opposed to genotypic) drug resistance or drug tolerance, a phenomenon that is well known in infectious diseases, but perhaps most dramatically manifested in tuberculosis. Because long-term treatment inevitably brings problems with patient compliance, incomplete and inconsistent treatment can additionally lead to selection of genetically drug-resistant bacteria. Today we find that the increase of extensively drug-resistant M. tuberculosis strains forebodes a global epidemic of tuberculosis as incurable as it was in the preantibiotic era (Pietersen et al. 2014).

Thus, against tuberculosis, we remain reliant on a largely ineffective 90-yr-old vaccine and a lengthy and difficult 50-yr-old treatment regimen. The limited success of these empirically derived interventions is reflected in the rise of tuberculosis in much of the world. In 2012, the World Health Organization reported 8.6 million cases of active tuberculosis and 1.3 million deaths. These include nearly a half million cases of multiple-drug-resistant tuberculosis. These alarming statistics place an imperative on searching for more effective interventions through a better understanding of the fundamental mechanisms of disease pathogenesis.

TUBERCULOSIS PATHOGENESIS: CONVENTIONAL WISDOM

During the 133 years since Koch’s discovery, the pathogenesis of human tuberculosis has been deduced from clinical, radiological, and pathological studies of infected or diseased humans coupled with studies using animal models: guinea pigs, rabbits, mice, and recently, nonhuman primates. These studies have led to a framework, presented in medical, immunology, and pathology text-
books, against which new molecular discoveries are interpreted. In this framework, aerosolized bacteria are inhaled and reach the lower lungs, where they are engulfed by phagocytic cells that transport them across the alveolar epithelium and into the lymphatic and circulatory systems so as to render the infection systemic. Although primarily a disease of the lung, tuberculosis can occur in most tissues, with the notable exception of muscle. Within the lung and other organs, highly organized structures called granulomas develop. Consisting of macrophages and a variety of other immune cells, granulomas are the hallmark histological structures of tuberculosis and have long been regarded as critical host-protective structures that wall off bacteria. Within the granuloma, mycobacteria are thought to undergo replicative arrest and become dormant, in response to stresses such as hypoxia. It is these so-called nonreplicating persisters that are thought to become tolerant to antituberculous drugs, which, like most antibiotics, are most effective against rapidly replicating bacteria. Individuals with the walled-off bacteria are considered to be in a state of clinical latency, neither symptomatic nor contagious. As old age or other immune stresses arise, the protective effect of the granuloma is diminished, allowing the bacteria to replicate rapidly and break free of their intracellular niche. The resulting necrotic cores can erode into the airways, releasing the bacteria into the environment in contagious aerosols. Thus, the conventional wisdom is that granuloma benefits the host by rendering the bacteria dormant even if it fails to eradicate them, with the fallout being that the bacteria become antibiotic tolerant.

INSIGHTS FROM A FISH: LUNGS NOT REQUIRED!

During the last decade, my laboratory has developed the zebrafish as a model for tuberculosis by infecting it with its natural pathogen, Mycobacterium marinum, a close genetic relative of M. tuberculosis (Tobin and Ramakrishnan 2008). The idea to use a natural mycobacterial pathogen of a facile laboratory animal, as a surrogate for M. tuberculosis infection, came from my postdoctoral advisor Stanley Falkow. The idea presented advantages, both biological and logistical. Because infectious diseases are often the result of coevolution between pathogen and host, using a surrogate host–pathogen pair can provide understanding that may be elusive when studying a pathogen in a nonnative host, for example, M. tuberculosis infection in a mouse. In addition, although M. marinum is closely related to M. tuberculosis, it has an optimal growth temperature of 33°C–35°C so that it produces a systemic tuberculosis-like disease in ectotherms, such as zebrafish. In humans, M. marinum is an accidental pathogen that produces peripheral granulomatous disease (so-called swimmer’s or fish tank granulomas) restricted to the cooler parts of the body. These lesions can be pathologically indistinguishable from dermal M. tuberculosis infection, demonstrating that M. marinum is possessed of all of the essential functions required to elicit a granulomatous disease in humans. Like tuberculosis, these lesions are often accompanied by lymphatic involvement, can be self-healing, but may also require long antibiotic treatment for reliable cures (Aubry et al. 2002; Decostere et al. 2004). M. marinum infections in humans remain local unless the host is severely immunocompromised (Ramakrishnan 1997). Thus, it can be handled safely using BSL2 precautions, a feature that has been tremendously useful in our studies using sophisticated vital microscopy (Cosma et al. 2006). Fortunately, M. marinum proved to be readily amenable to genetic manipulations, allowing the generation of mutant and transgenic strains.

Although M. marinum is a natural pathogen of a variety of ectothermic species, we chose to develop the zebrafish as a model so as to take advantage of its genetic tractability. Adult zebrafish develop organized necrotic granulomas, structurally similar to human ones, and similarly reliant on adaptive immunity for control of infection, thus validating the model (Pozos and Ramakrishnan 2004; Swaim et al. 2006). Perhaps the most important feature of the zebrafish is its prolonged larval stage during which it is optically transparent. Infection at this stage allows serial, detailed, real-time observations of Mycobacterium interactions with its natural host. Macrophages and neutrophils are present and are functionally competent and specific in their interactions with known pathogens (Davis et al. 2002; Herbomel and Levraud 2005; Yang et al. 2012). However, as in mammals, adaptive immunity takes several weeks to develop (Davis et al. 2002). We have used this developmental isolation of innate immunity to our advantage and have parsed the roles of a variety of innate immune cells and molecules in mycobacterial pathogenesis (Fig. 1).

In this review, I discuss the various mechanistic insights provided by the M. marinum–zebrafish model into a variety of old observations, for example, why tuberculosis initiates in the lower lung rather than the upper airway. I also suggest revisions to long-held dogmas about the tuberculosis based on findings in the zebrafish, for example, that the granuloma also has an important role in the early expansion of infection rather than serving as a solely host-protective structure. We have also shown that actively growing bacteria within macrophages are rendered antibiotic tolerant by the induction of bacterial efflux pumps that are induced to counter macrophage defenses. Finally, the zebrafish has revealed that tuberculosis is not only a disease of failed immunity; increased inflammatory responses also promote susceptibility. These findings have already provided insights into human tuberculosis that have important therapeutic implications.

A ZEBRAFISH OPERATING MANUAL

We have developed methods to rapidly detect and measure bacterial infection serially in hundreds of larvae at a time as well as make detailed serial observations using high-resolution microscopy (Takaki et al. 2012, 2013). We have used M. marinum expressing different fluorescence reporters to infect zebrafish transgenic lines with
fluorescent macrophages and neutrophils to expand the complexity of experiments that can be performed (Fig. 2A,B). We have developed an image-based method of fluorescence quantification that reliably measures relative infection burdens in live larvae. When combined with larval husbandry in 96-well plates and an automated method for high-throughput (inverted) microscopy and image processing, we are able to make serial quantitative and qualitative assessments of infection burdens and larval survival on thousands of larvae (Fig. 2C). The 96-well plate husbandry facilitates screening of small-molecule activities during infection. In vivo assessment of fluorogenic physiological probes (e.g., redox and Ca\(^{2+}\) sensors) and long-term time-lapse fluorescence microscopy are readily performed. Zebrafish genes of interest can be transiently inactivated by injecting antisense oligonucleotides (morpholinos) or may be overexpressed by injecting RNAs at the 1–4-cell stage; such modifications typically last for several days, allowing enough time for infection and a variety of assessments to be performed. More recently, techniques have been developed for facile gene knockouts using TALENs and CRISPR-Cas technologies (Bedell et al. 2012; Hwang et al. 2013). In terms of the actual assays, larvae can be infected by injection of bacteria into the hindbrain ventricle (HBV) (Fig. 3A), a neuroepithelium-lined cavity devoid of phagocytes under baseline conditions. This allows the first encounters between mycobacteria and phagocytes to be studied in great detail. Alternatively, the recruitment step can be bypassed by infecting bacteria into the caudal vein (Fig. 3B), which still allows the monitoring of the progression of infection (Fig. 2C). Bacterial growth within individual macrophages, their aggregation into granulomas, and cellular integrity can all be monitored.

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**Figure 1.** Steps of tuberculosis pathogenesis modeled in the zebrafish. The image shows the host–phagocyte interactions starting from the peripheral site of infection that we have dissected in the zebrafish, with the host and bacterial partners on the top and bottom, respectively. An asterisk marks host factors that have a pathogenic role. Note that TNF has both a beneficial and pathogenic role.

**Figure 2.** Fluorescence microscopy images of fluorescent zebrafish and *M. marinum*. (A) Dual transgenic zebrafish larva with red fluorescent macrophages and green fluorescent neutrophils. (B) A dual transgenic larva with green fluorescent macrophages and red fluorescent neutrophils infected with blue fluorescent *M. marinum* that has developed a granuloma. (C) Low-magnification serial images of the same larva at 1, 3, 5, and 7 d postinfection (dpi), showing the progression from a single infected macrophage at days 1 and 3 to the formation of a granuloma by 5 d. At 7 d, additional infection foci have formed near the primary granuloma. There is some autofluorescence from the yolk in the background.
Neutrophil avoidance is only one part of the two-pronged mycobacterial strategy to avoid being killed effectively by first-responder phagocytes. The second involves avoiding microbicidal macrophages while selectively recruiting permissive ones to better disseminate the infection (Fig. 4) (Cambier et al. 2014). This understanding began with a curious finding: Whereas macrophage recruitment to all other bacteria tested (Escherichia coli, S. aureus, and P. aeruginosa) is reliant on the toll-like receptor (TLR)-MyD88 signaling axis, macrophage recruitment to M. marinum is MyD88 independent. This observation was puzzling because mycobacteria possess abundant pathogen-associated molecular patterns (PAMPs) that stimulate TLR signaling upstream of MyD88 (Philips and Ernst 2012). Indeed, it was the recognition that effective adaptive immune responses in other experimental systems could not be elicited by antigen alone but were dependent on coadministration of complete Freund’s adjuvant (an oil emulsion containing dead M. tuberculosis) that Charles Janeway called “the immunologist’s dirty little secret,” and it led him to first propose the existence of pattern recognition molecules for infectious agents in an influential article in these same proceedings (Janeway 1989). If mycobacteria led Janeway to TLRs, how could macrophage recruitment to mycobacteria be TLR independent, and what alternative mechanism(s) are in play? Pathogenic mycobacteria such as M. tuberculosis and M. marinum produce a series of complex outer-membrane lipids called phthiocerol dimycocerosates (PDIMs) that are absolutely essential for virulence and are made by all clinical isolates (Camacho et al. 1999; Cox et al. 1999; Onwueme et al. 2005). We found that PDIMs function to mask the multitude of underlying TLR-agonizing PAMPs, allowing pathogenic mycobacteria to evade macrophages signaled through TLRs. In the zebrafish and the mouse, we find that TLR/MyD88-sig- naled macrophages are microbicidal owing to expression of the inducible nitric oxide synthase (iNOS); however, it is possible that other microbicidal effectors downstream from MyD88 (e.g., cathelicidins) may predominate in humans (Liu et al. 2006).

These findings explain why microbicidal macrophages fail to arrive in response to mycobacterial infection, but they do not explain how permissive macrophages are recruited. We find that the surface-associated phenolic glycolipid (PGL) of M. marinum (variants of which are produced by many pathogenic mycobacteria) induces expression of a host macrophage chemokine CCL2. Importantly, CCL2, in conjunction with its receptor CCR2, serves to recruit Mycobacterium-permissive, iNOS-negative macrophages that transport mycobacteria deep into the host. Accordingly, a role for PGL (in concert with host CCR2/CCL2) in establishing infection was seen when inocula of one to three bacteria (the inoculum size thought to transmit human tuberculosis) were introduced into the HBV. This finding is consistent both with human studies, showing an association between the high expression of CCL2 and tuberculosis susceptibility (Flores-Villanueva et al. 2005). Also consistent with our findings, the association appears to be stronger in East Asian
populations, where clinical isolates are enriched for the predominantly PGL-expressing W-Beijing strains (Flores-Villanueva et al. 2005; Feng et al. 2012). Why then is PGL, which is only variably produced by clinical M. tuberculosis isolates, dispensable for virulence? It is present in the ancestral Mycobacterium canettii strains as well as in M. marinum, suggesting its integral role in the evolution of mycobacterial pathogenicity. Tuberculosis is an ancient disease, affecting humans more than 70,000 yr ago, and it is possible that the increased infectivity conferred by PGL was essential for most of its history before human crowding with increased opportunities for transmission made it dispensable.

Finally, as depicted in Figure 4, our data suggest an explanation for why M. tuberculosis must reach the alveolar surfaces of the distal lung to initiate infection. Transmission occurs through inhalation of small aerosol droplets of one–three bacteria. Paradoxically, large droplets carrying $\approx 10^4$ bacteria are trapped in the upper bronchial passages and fail to establish infection (Wells et al. 1948; Bates et al. 1965). These observations have been attributed to the alveolar surfaces of the distal lung being more favorable for mycobacterial proliferation, and indeed our work provides a mechanistic explanation. We suggest that commensal microbes from the oropharyngeal surfaces, as well as inhaled environmental organisms, lead to continual TLR signaling in the upper respiratory tract that would override mycobacterial PDIM-dependent immune evasion strategies. In contrast, the relatively sterile and immunologically quiescent lower respiratory tract (Charlson

Figure 4. Model of initial interactions of pathogenic mycobacteria with permissive macrophages, showing how resident bacteria of the upper respiratory tract can affect infection. (Adapted from Cambier et al. 2014.)
et al. 2011) would favor recruitment of *Mycobacterium*-permissive macrophages. Consistent with this hypothesis, we found that coincfetcting *M. marinum* with organisms that recruit macrophages through TLR signaling allowed *M. marinum* killing in a MyD88/iNOS-dependent fashion. Thus, we propose that the commensal flora have a central role in modulating mycobacterial infectivity. Because the dual lipid—masking and recruiting—TLR evasion strategy cannot work in the presence of TLR/MyD88-signaling bacteria, the requisite third component of this survival strategy is the small deeply penetrating droplet size of the infectious *M. tuberculosis* particle. A low-inoculum particle reduces its intrinsic infectivity, making tuberculosis a much less contagious disease than many other respiratory pathogens that are transmitted through the upper airways. Conversely, the persistence of human tuberculosis for over 70,000 yr (Comas et al. 2013) suggests that this strategy of working around the host’s resident flora has been quite effective.

**WHAT DOES NOT KILL THEM MAKES THEM STRONGER: MACROPHAGES MAKE MYCOBACTERIA DRUG TOLERANT**

In work that began with the zebrafish but moved quickly into a human macrophage cell line, we realized the importance of prior findings that *M. tuberculosis* expresses efflux pumps that are induced upon macrophage entry, several of which enable bacterial growth in macrophages and as such comprise host-induced virulence factors (Schnappinger et al. 2003; Rengarajan et al. 2005; Adams et al. 2011; Szumowski et al. 2013). Importantly, we find that these same pumps also mediate tolerance to antitubercular drugs, presumably by efflux mechanisms (Fig. 5) (Adams et al. 2011; Szumowski et al. 2013). As predicted by this finding, the most actively growing bacteria within macrophages are also the most drug tolerant. This finding has implications for tuberculosis because it challenges a widely held belief that only the most slowly or nonreplicating bacteria are drug tolerant because of the scarcity of drug targets (e.g., cell wall determinants, DNA replication forks, ribosomes). Rather, we suggest that drug tolerance may arise by multiple mechanisms, all of which should inform drug development and treatment strategies. A recent study reveals additional complexities in how mycobacteria are killed by antimicrobial agents. Persistent *Mycobacterium smegmatis* were found to grow and divide in the presence of isoniazid, a cell-wall-inhibiting drug, owing to transient expression of the enzyme responsible for prodrug conversion to the active form (Wakamoto et al. 2013).

From a therapeutic viewpoint, these findings offer new avenues that we have pursued. By testing drugs already in use for other diseases that are known to have bacterial efflux pump activity, we found that verapamil, a calcium channel blocking drug that has been used for more than 30 yr to treat hypertension and other conditions, inhibits macrophage-induced tolerance against multiple tuberculosis drugs (Adams et al. 2011, 2014). Because of the dual action of the efflux pumps in mediating bacterial growth and antibiotic efflux, we predicted that verapamil would also inhibit intracellular bacterial growth in the absence of antibiotic coadministration. Indeed, for both *M. tuberculosis* and *M. marinum*, it had a two-pronged attack on intracellular bacteria—by inhibiting their growth per se and by allowing antibiotics to be more efficacious. A recent study testing the efficacy of verapamil in the mouse model of tuberculosis supports the idea that efflux pumps have a central role in drug tolerance; this study found that verapamil shortened the period of treatment required to prevent relapses (Gupta et al. 2013). What was particularly interesting was that verapamil did not have an effect on C3H/HeJ mice, which have been extensively used to study tuberculosis yet fail to reproduce several aspects of human disease. Rather, it was effective in C3HeB/FeJ mice (the so-called Kramnik mice) that display the organized granulomas with bacterium-laden necrotic cores characteristic of active human tuberculosis and are therefore being used for tuberculosis drug testing (Pichugin et al. 2009; Driver et al. 2012; Gupta et al. 2013). In Kramnik mice, verapamil added to standard tuberculosis chemotherapy accelerates bacterial clearance with near sterilization and, importantly, lowers relapse rates when compared with mice receiving standard therapy alone (Gupta et al. 2013).

Based on both of these studies, a multicenter clinical trial is starting in India to determine whether the addition of verapamil to standard drug regimens can shorten the course of treatment. However, although verapamil is a relatively safe drug, its many side effects may still pre-

**Figure 5.** Model of macrophage-induced bacterial efflux pump-mediated tolerance.
clude its widespread use, and as such this trial is only a proof-of-concept study. Ideally, better efflux pumps inhibitors can be designed. Indeed, because the efflux inhibitory activity of verapamil was found to be independent of its calcium channel blockade, we tested norverapamil, the major metabolite of verapamil in humans, which is known to have much less calcium channel activity than verapamil. Norverapamil is as potent as verapamil in blocking both drug tolerance and intracellular growth and should be better tolerated (Adams et al. 2014). Another advantage of using norverapamil is that it has already been effectively tested for safety for all of the years that verapamil has been in use.

Whether and how much efflux pump expression contributes to drug tolerance in human tuberculosis remains to be tested (and the same can be said for the classic nonreplicating models). Are these same efflux pumps expressed in the macrophages of active tuberculosis granulomas? What about the many extracellular bacteria found in necrotic debris in the core of active granulomas? Do they still express these pumps, and do they require them to survive? From a basic immunological point of view, our drug efflux work poses two major questions regarding the identity of the macrophage signal that induces these mycobacterial efflux pumps and the macrophage defenses against which they are being used—questions that bring us back to the core problem of macrophage defenses against mycobacteria.

BUILD IT AND THEY WILL COME: MYCOBACTERIA RECRUIT MACROPHAGES FOR EXPANSION IN THE GRANULOMA

Although we have gained some understanding of how macrophages are recruited to peripheral sites of infection, we remain largely ignorant of how (or why) they return to deeper tissues upon infection. Does this inward movement represent a homeostatic migration? Or is it simply because of the loss of the CCL2 chemokine gradient, once the bacteria are intracellular? We do know that in zebrafish, infected macrophages have a propensity to cross the epithelium (from the HBV infection site) or the vascular endothelium (from the caudal vein infection site) to reach deeper tissues. This is consistent with observations in mammals that mycobacteria migrate into the lung parenchyma (after aerosol infection) or into a variety of organs (after intravenous infection). Soon thereafter, a second wave of migration commences, with new macrophages being recruited to these nascent foci of infection to initiate granuloma formation. At later stages of infection, the tuberculosis granuloma is a complex immune structure, consisting of both innate and adaptive immune cells. However, at this innate stage (the only stage possible in the larvae), phagocytes predominate and by using transgenic zebrafish lines, we have identified these phagocytes to be primarily macrophages with a few neutrophils.

Why do we call this structure a granuloma rather than simply a macrophage aggregate? First, larval granuloma macrophages undergo epithelioid transformation, the hallmark morphological changes that characterize granuloma macrophages in adaptive mammalian granulomas, wherein they form tight interdigitated projections so as to be closely juxtaposed to one another (Adams 1976; Bouley et al. 2001; Davis et al. 2002). Second, we find that bacterial genes that are specifically activated in mature adult granulomas are also induced in these structures but not in solitary infected macrophages (Ramakrishnan et al. 2000; Davis et al. 2002). It has long been thought that tuberculosis granulomas only form after the onset of adaptive immunity at 2–3 wk after infection, depending on the inoculum. This coincides with the time when bacterial numbers plateau, leading to the assumption that granulomas restrict infection. The occurrence of the plateau is highly consistent across models: It is seen in M. tuberculosis–infected mice, guinea pigs, and rabbits, as well as in M. marinum–infected leopard frogs and zebrafish (Davis and Ramakrishnan 2009). On this backdrop, our finding that granulomas in the larvae form much earlier, within 3–5 d of infection and in the sole context of innate immunity (Davis et al. 2002), was unexpected.

Even more surprising, and inconsistent with the protective granuloma model was the observation that granuloma formation is associated with an increased, rather than decreased, bacterial growth (Volkman et al. 2004). A tool that allowed us to address this further came from our concurrent studies using a M. marinum mutant harboring the RD1 deletion. This deletion, which removes a large portion of the ESX-1 secretion system, was originally identified in the BCG vaccine strain as a spontaneous mutation that arose during passage of the parent virulent M. bovis strain and has since been confirmed to be the primary cause of BCG attenuation (Hsu et al. 2003; Guinn et al. 2004). An open-ended investigation of the corresponding M. marinum RD1/ESX-1 mutant in the zebrafish confirmed its attenuation and revealed an important clue both about this virulence locus and the granuloma. The ESX-1 mutant displays no growth attenuation in individual macrophages, yet these bacteria-laden cells fail to attract new macrophages compared with those infected with wild-type M. marinum; as a result, granuloma formation is impaired (Volkman et al. 2004; Davis and Ramakrishnan 2009). The underlying mechanism was revealed by a convergence of descriptive observational studies paired with a screen for differential host responses to wild-type versus RD1/ESX-1 mutant M. marinum (Davis and Ramakrishnan 2009; Volkman et al. 2010). Mycobacteria, through the ESX-1 locus, induce both apoptotic death of infected macrophages and migration of uninformed macrophages to the nascent granuloma. Time-lapse studies revealed that these new recruits migrate within the growing granuloma and phagocytose dead infected macrophages. Typically, multiple new recruits ingest a given dead macrophage, thus expanding the number of infected cells, and thereby the bacterial intracellular niche.

Just as bacterial PGL induces and uses a host determinant (CCL2) for initial macrophage recruitment, this next wave of recruitment is dependent on a bacterial locus (ESX-1) in conjunction with a host factor, matrix metal-
loproteinase 9 (MMP9) (Fig. 6) (Volkman et al. 2010). Like CCR2, MMP9 also functions as a host susceptibility factor in the zebrafish, a finding that is corroborated in mice and in humans (Price et al. 2003; Taylor et al. 2006; Sheen et al. 2009; Volkman et al. 2010). Intriguingly, MMP9 is most highly induced in epithelial cells surrounding the growing granuloma rather than the macrophages themselves, a pattern that has also been seen in human lung tuberculosis granulomas (Elkington et al. 2007; Volkman et al. 2010). It is possible that this “transactivation” affords mycobacteria some benefits. For instance, a single infected macrophage may be sufficient to activate MMP9 in multiple epithelial cells, thus allowing signal amplification. Overall, mycobacteria may have evolved a dual strategy to inhibit inflammation within the macrophage (so as to prevent microbicidal activities), while increasing MMP9 (a protease associated with inflammation) in neighboring cells so as to recruit more macrophages for its own niche expansion. We are interested in finding out the details of this interplay. Although we do not really understand how MMP9 is induced in epithelial cells, this finding may have some bearing on the clinical observation that tuberculosis seldom affects muscle (Rich 1946). In the larval fish also, muscle granulomas generally dwindle, whereas those in other organs may thrive even in the same animal (Adams et al. 2011). In the zebrafish, if a granuloma begins in muscle, the closest epithelium capable of expressing MMP9 is often at a distance from the infected macrophages (Volkman et al. 2010; Ramakrishnan 2012). It is possible that this imposed distance does not permit the granuloma to grow.

The *M. marinum* ESX-1 mutant is attenuated in adult zebrafish and frogs and is characterized by loose granulomas (Volkman et al. 2004; Swaim et al. 2006), similar to its *M. tuberculosis* counterpart in mice (Sherman et al. 2004), further suggesting that the bacterium-expanding role of the granuloma may extend through adaptive immunity. Bacterial growth may slow down with adaptive immunity, simply because the macrophages are rendered more growth restrictive (by T-cell-derived interferon-γ), rather than any type of physical containment wrought by the granuloma. Very recent work using an in vitro human lung tissue model also finds a requirement for ESX-1 in early granuloma formation (Parasa et al. 2013). This understanding of the granuloma as a bacterium-promoting rather than bacterium-restricting entity has therapeutic implications (Ramakrishnan 2012). In most diseases, granulomas are pathological, and it now appears that tuberculosis is no exception. There may be common pathways involved and their interception may lead to host-targeting treatments for tuberculosis and other granulomatous diseases. This is a subject of active investigation in our laboratory. How do the products of the bacterial ESX-1 system, located inside an infected macrophage, act at a distance to induce MMP9 in epithelial cells? What might be the benefit of activating the protease in neigh-

![Figure 6. Model of bacterial ESX-1-mediated granuloma formation through induction of host MMP9 in surrounding epithelial cells.](#)
boring cells rather than in the macrophage itself? Other questions relate to how MMP9 causes macrophage recruitment. Does it activate a specific chemokine? And why are there two distinct mechanisms to recruit macrophages at the two stages of infection? We are also interested in how newly recruited macrophages recognize dead and dying macrophages. Are common efferocytosis pathways replete with “find-me” and “eat-me” signals in play (Hochreiter-Hufford and Ravichandran 2013)? If so, inhibiting efferocytosis by incoming macrophages should attenuate infection, a testable prediction of the model.

Our studies of macrophage behavior in the granuloma have recently also brought us back to neutrophil function in tuberculosis and may provide an argument for why mycobacteria have evolved strategies to evade direct recognition by neutrophils (Yang et al. 2012). It appears that the bacterial strategy to spread into new macrophages through death and rephagocytosis (efferocytosis) is without cost; macrophage apoptosis signals the recruitment of neutrophils (Yang et al. 2012). As discussed earlier, neutrophils do not recognize mycobacteria themselves, yet we find that they are able to ingest mycobacteria indirectly through phagocytosis of infected macrophages. In this way, neutrophils are then able to kill mycobacteria through NADPH oxidase–dependent mechanisms. These results provide a mechanistic link to the observed patterns of neutrophils in human tuberculosis granulomas and the susceptibility of humans with chronic granulomatous disease to mycobacterial infection (Movahedi et al. 2003; Lee et al. 2008; Dogru et al. 2010). It is possible that neutrophils have additional roles in killing extracellular bacteria within the necrotic cores of casedate granulomas—a topic of active investigation in our laboratory.

Finally, our detailed studies of granuloma ontogeny have revealed that the granuloma is a surprisingly dynamic entity (Davis et al. 2002; Cosma et al. 2004, 2008; Davis and Ramakrishnan 2009). Our first clue came from reinfection studies in M. marinum–infected frogs, where we found rapid trafficking of infected macrophages into established granulomas, suggesting their structural and immunological permeability (Cosma et al. 2004). In later studies, tracking individual macrophages in zebrafish larvae revealed that infected macrophages can exit the primary granuloma and travel through tissues or the bloodstream to initiate new foci, thus disseminating infection within the host. We do not yet understand the mechanisms behind this egress. However, these observations are consistent with classic radiological studies showing that individual lesions can expand and regress, all in one patient (Bobrowitz 1980). Moreover, this dynamism is observed even in the context of overall effective antibiotic therapy. In the larval model, we find that during the course of overall effective antibiotic treatment, tolerant bacteria can still expand by spreading to new macrophages within the granuloma as well as disseminate to new areas through the egress of infected macrophages (Adams et al. 2011). Very recent work in cymonomulogus macaques monitored the progression of disease using individually marked isolates of M. tuberculosis and confirmed both the human radiological and our zebrafish findings on two counts (Lin et al. 2014). First, it showed that individual lung granulomas arise from a single founder, and second, it found that individual granulomas within a single host can have variable fates, from sterilization to expansion. The authors attribute a part of this variation to adaptive immunity. However, as discussed earlier in this section, our work suggests that granulomas can suffer different fates based on innate immune mechanisms alone (Adams et al. 2011). Overall, it appears as if much of the granuloma’s formation and fate are dictated by innate immunity, with further influence brought by adaptive immune mechanisms.

**ALL THINGS IN MODERATION: TNF DEFICIT AND EXCESS BOTH CAUSE HYPERSUSCEPTIBILITY THROUGH GRANULOMA NECROSIS**

The proinflammatory cytokine TNF is a well-known host-protective factor in tuberculosis: TNF-deficient mice and humans undergoing TNF-neutralizing therapies are more susceptible to mycobacterial infection (Flynn et al. 1995; Keane 2005). The mouse studies led to the assignment of TNF as a driving factor in granuloma formation and thereby mycobacterial containment (Flynn et al. 1995). This assignment was based on the susceptibility of TNF-deficient mice being concomitant with disorganized necrotic lesions. Our ability to directly monitor granuloma formation at its very earliest stages led us to examine the role of TNF in the larval model and has resulted in a revised view of TNF in mycobacterial infection (Clay et al. 2008). Rather than the notion that TNF is required for granuloma development, we found that TNF signaling deficiency (due to TNFR1 knockdown) actually resulted in accelerated granuloma formation. This acceleration appeared to be the consequence of increased bacterial growth in the infected macrophages, likely resulting in an increase in the ESX-1 products that stimulate granuloma formation. In TNFR1-deficient animals, accelerated granuloma development was soon followed by necrotic death of the overladen macrophages, with the ultimate effect of causing granuloma breakdown. Given the rapid progression of granuloma development and demise, it is likely that the mouse studies missed this distinction because of an inability to monitor early events. Thus, TNF is not required for tuberculosis granuloma formation per se, but rather maintains granuloma integrity indirectly by restricting mycobacterial growth and preventing macrophage necrosis. Elegant live-imaging studies in the mouse showed that TNF blockade in the context of established granulomas led to their disintegration (Egen et al. 2008). Moreover, our zebrafish findings have again been confirmed in the cymonomolus macaque, where TNF neutralization produced susceptibility with preservation of normal granuloma structure (Lin et al. 2010). These authors argue that the disparity in the monkey and zebrafish findings from those in the mouse may be attributable to inherent differences in mouse versus zebrafish and pri mate granulomas, because mouse granulomas are generally less organized. In either case, the zebrafish has again
uncovered a mechanism that has been verified in a primate model and perhaps most important is consistent with observations in humans that patients given TNF antagonists can develop tuberculosis with organized granulomas and areas of caseum (Garcia Vidal et al. 2005; Iliopoulos et al. 2006).

Our initial foray into TNF biology confirmed the idea that TNF is required for mycobacterial resistance, but we then made a surprising discovery: TNF excess, just like TNF deficiency, also results in mycobacterial susceptibility. This insight began with a forward genetic zebrafish screen for hypersusceptible mutants. One mutant mapped to the gene encoding leukotriene A4 hydrolase (LTA4H), an enzyme in the arachidonic acid metabolism pathway that converts the unstable epoxide LTA4 to its highly proinflammatory derivative LTB4 (Tobin et al. 2010). Further exploration revealed that LTA4H deficiency causes the redirection of LTA4 to anti-inflammatory lipoxins, which are immunosuppressive by virtue of inhibiting TNF induction in response to infection (Tobin et al. 2010, 2012). We have discussed the details of the eicosanoid pathway in recent papers and a review article (Tobin et al. 2010, 2012; Tobin and Ramakrishnan 2013); here, I focus on how we related these findings to TNF dysregulation and their relevance to human infection.

We first identified a variant in the human LTA4H promoter with a C–T transition that altered expression of the enzyme in normal controls: The homozygous CC variant had the lowest expression, the TT variant had the highest expression, and the CT heterozygotes had intermediate expression. We interrogated two human cohorts—a tuberculous meningitis cohort in Vietnam and a leprosy cohort in Nepal—for associations between susceptibility and LTA4H genotype and made an unusual observation. Both homozygous genotypes were associated with more severe disease, whereas heterozygotes were protected in comparison (Tobin et al. 2010). Heterozygous advantage is unusual in human genetics, and this finding implicated both insufficient and excessive inflammation in tuberculosis pathogenesis. Follow-up work in the zebrafish revealed the mechanism of this heterozygous advantage. First, we confirmed that LTA4H overexpression rendered fish as susceptible to M. marinum, as does LTA4H deficiency. Next, we found that TNF was the principal driver of the susceptibility of both poles: LTA4H deficiency produced TNF deficiency and could be corrected by administration of recombinant TNF, whereas LTA4H excess resulted in TNF excess, which was remedied by TNF knockdown.

As expected, LTA4H deficiency mimics TNF deficiency in that mycobacteria are poorly controlled, leading to macrophage lysis and release of bacteria into the extracellular milieu. How then does TNF excess promote susceptibility? As might be expected, TNF excess enhances bacterial growth restriction in macrophages. Yet surprisingly, these scantily infected macrophages still lyse, delivering the bacteria to the extracellular milieu. In this way, eventual macrophage depletion allows exuberant extracellular growth of the mycobacteria, so that within days, bacterial burdens have caught up to the low-TNF state (Tobin et al. 2012). Indeed, multiple different genetic routes to TNF excess can result in the identical phenotype (Tobin et al. 2013). This then raises the question of how excess TNF lyases infected macrophages. Through a combination of gene knockdowns and chemical genetics, we find that TNF excess triggers a recently discovered pathway of programmed necrosis (necroptosis) using the RIP1 and RIP3 kinases (Fig. 7). The RIP1/3 pathway triggers mitochondrial reactive oxygen species (ROS) production that imbues the macrophage with greater microbicidal capacity, explaining the observation that TNF excess initially leads to greater mycobacterial control. However, activation of this pathway subsequently kills the cell through at least two mechanisms: (1) translocation of cyclophilin D, a redox-sensitive mitochondrial matrix protein, to the mitochondrial membrane, where it participates in the formation of the transmembrane permeability transition pore complex (mPTCP) and (2) activation of lysosomal acid sphingomyelinase, which causes overproduction of ceramide. Whether ceramide causes necrosis through the mPTCP or independently, we do not know. Ceramide has been primarily studied in apoptosis, and our findings suggest that it additionally has a role in necrosis in vivo, a finding that is gaining ground in other systems as well (Hetz et al. 2002). The identification of these two pathways in RIP1/3-mediated necrosis has allowed us to test an important prediction. Knockdown of both death pathways downstream from ROS production would be expected to allow macrophages to retain their enhanced microbicidal capacity while avoiding lysis; such a combination should produce enhanced resistance. This is indeed the case; treating fish with drugs blocking both pathways (alisporivir acts against cyclophilin D, and the tricyclic antidepressants are known to promote degra-
dation of acid sphingomyelinase), which renders them hyperresistant to infection. Alisporivir has been in Phase 3 clinical trials for the treatment of hepatitis C infection, and tricyclic antidepressants have been in use since the 1950s.

These findings may have substantial therapeutic implications. To elaborate, I return to the Vietnam tuberculous meningitis cohort discussed earlier. Tuberculous meningitis carries a high mortality, even with proper antibiotic treatment, partly because of increased inflammation in the brain. Glucocorticoids have been empirically used as adjunctive treatment along with antibiotics to reduce this inflammation. The Vietnam cohort that we examined was the same one used in the most definitive study on the effect of glucocorticoid that confirmed a small but significant decrease in mortality with glucocorticoid use. Because we had found that mortality in this cohort was associated with both the (hyperinflammatory) LTA4H-high and the (hypoinflammatory) LTA4H-low genotypes, the next obvious question was whether glucocorticoids were selectively effective in the LTA4H-high group. Indeed, this was the case—adjunctive glucocorticoid treatment increased survival from 50% to 100% in this small cohort. In contrast, the LTA4H-low-genotype individuals had a small (but nonsignificant) increase in mortality. Thus, we suggest that host-genotype-guided use of glucocorticoids may allow them to be used only in those individuals that would benefit and not in those for whom reducing inflammation might be harmful. Of course, this study used a relatively small cohort and, like all human genetic studies, requires verification. Replication cohort studies are underway in Vietnam and will soon start in India. It will also be interesting to see whether glucocorticoids could be used to benefit high-LTA4H-genotype individuals with pulmonary tuberculosis, where again there is some empirical evidence of their benefit at the population level (Crichtley et al. 2013). Another potentially interesting avenue to pursue would be to determine whether drugs such as alisporivir and tricyclic antidepressants, which we find confer hyperresistance in high-LTA4H larvae, might also be useful in treating humans with TNF excess, either from the high-LTA4H genotype or from other variations in the pathway. This approach has several advantages over the use of steroids. First, these drugs are likely to be less broadly active than the glucocorticoids, which have a myriad of adverse effects, including global immunosuppression. The second possible advantage is that treatments that block macrophage necrosis downstream from ROS might be used without the need for prior patient genotyping because, at least in the zebrafish, they do not adversely affect the wild-type and low-TNF genotypes, as steroids do (Tobin et al. 2012; Roca and Ramakrishnan 2013).

Macrophage apoptosis and its effects on mycobacterial survival in vivo are current points of contention in the field. Elegant in vitro studies have shown that bacterially induced apoptosis leads to decreased mycobacterial proliferation in cell culture infection models (Behar et al. 2011). However, overlaid on the cell-autonomous effects of apoptotic death on mycobacteria are our in vivo findings that apoptosis can provide a means of spread and expansion within the granuloma. In contrast, we find that necrotic cell death (as also shown in cell culture models) is even more permissive of bacterial growth. These in vivo observations lead us to a more nuanced view of the consequence of apoptosis versus necrosis. As we have detailed previously (Davis and Ramakrishnan 2009; Ramakrishnan 2012), we find that apoptosis favors the bacteria by allowing them to expand within the granuloma and that necrosis is even more permissive of bacterial growth. The latter point is supported by clinical findings that individuals with large necrotic granulomas are more contagious and may thus increase transmission to new hosts, which is the ultimate goal of a pathogen.

The discovery of hyperinflammatory high-TNF-mediated necrosis and its role in disease raises a plethora of new questions. For one, excess TNF predominantly kills infected macrophages (Roca and Ramakrishnan 2013), suggesting that one or more bacterial factors are required to activate the necroptosis pathway. What are these? Another puzzle is why extracellular bacteria are not engulfed by new macrophages the way initially infecting ones are. Does macrophage residence change them in a way that prevents their direct uptake by phagocytes? Or are macrophages simply being depleted? Finally, why do extracellular bacteria flourish? How do they overcome immune defenses such as complement and defensins? These are questions that can be readily addressed in the zebrafish.

CONCLUSIONS

The zebrafish has enabled us to piece together a cohesive picture of the innate aspects of the innate interactions between mycobacteria and host, taking us through all of the steps of pathogenesis, from gaining entry into the host through phagocytes, working around phagocytes to grow intracellularly, and finally lysing out of them to grow extracellularly so as to facilitate transmission. These innate interactions appear to extend to later stages of pathogenesis in adult animals and other animal models, where adaptive immunity is also engaged. This highlights the importance of innate immunity in shaping the final host–pathogen interface and reinforces the idea that alterations of the host response to these early, well-defined interactions may provide new therapeutic and preventive approaches. In the next phase of our work, we hope to elaborate on the mechanisms by which mycobacteria interact with host inflammatory pathways to obtain a deeper understanding of not only tuberculosis but also the many inflammatory diseases with which it shares commonalities.

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The Zebrafish Guide to Tuberculosis Immunity and Treatment

Lalita Ramakrishnan

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