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Host Evasion and Exploitation Schemes of *Mycobacterium tuberculosis*

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Tuberculosis, an ancient disease of mankind, remains one of the major infectious causes of human death. We examine newly discovered facets of tuberculosis pathogenesis and explore the evolution of its causative organism *Mycobacterium tuberculosis* from soil dweller to human pathogen. *M. tuberculosis* has coevolved with the human host to evade and exploit host macrophages and other immune cells in multiple ways. Though the host can often clear infection, the organism can cause transmissible disease in enough individuals to sustain itself. Tuberculosis is a near-perfect paradigm of a host-pathogen relationship, and that may be the challenge to the development of new therapies for its eradication.

Introduction

Tuberculosis (TB) has afflicted humans for about 70,000 years and continues to take a huge toll on human life and health, with 8.6 and 1.3 million cases and deaths, respectively, in 2013 (Zumla et al., 2013; Comas et al., 2013). TB's timelessness in the face of significant human lifestyle changes over the millennia and the advances of modern medicine over the last century bespeak the agility and toughness of its causative pathogen *Mycobacterium tuberculosis*. *M. tuberculosis* may be the paradigm for human host-pathogen adaptation.

TB's notoriety as one of the great bacterial terrors of humanity alongside plague, typhus, cholera, typhoid, and diphtheria has led to descriptors such as the "great white plague" and "the captain of all those men of death." When compared to other major bacterial diseases, there are some interesting and potentially informative aspects of TB's pathogenesis. Human infection and disease is essential for the transmission and therefore the evolutionary survival of *M. tuberculosis*. This is in contrast to plague, which, despite its enormous impact on human history, is a zoonosis in which human disease is essentially an accident with no bearing on the pathogen's subsequent survival. The same could be said for many commensal pathogens, e.g., the pneumococcus, meningococcus, or the flesh-eating streptococci, in which human disease, though terrifying, is of marginal benefit to the long-term survival of the pathogen. Despite the inextricable connection between disease and transmission and thereby its survival, *M. tuberculosis* appears to lack the classical virulence factors that are the badges of honor of many of these pathogens. These include capsules to avoid phagocytosis, pili, or other adhesins for adherence to host tissues; flagella for motility; and enzymes and toxins to poison host cells. How does *M. tuberculosis* produce the disease so devastating to humans and so vital to the pathogen? The classical virulence factors of the mucosal commensal pathogens, many of which reside in the nasopharynx, are really colonization factors that, in the right host, run amok to cause a disease that is of questionable benefit to the pathogens' evolutionary survival. These factors probably give the microbe a selective colonization advantage on mucosal surfaces, where bacterial competition is rife. Not surprisingly, vaccines against individual virulence factors—be it a capsule of the pneumococcus or a toxin—sometimes eradicate colonization along with disease. As an example, the diphtheria toxin that has been responsible for countless million deaths in the past is likely a colonization factor that allows *Corynebacterium diphtheria* to compete effectively with other mucosal bacteria to establish a privileged niche in the tonsils. The diphtheria vaccine that is directed against this single toxin has wiped out colonization together with disease. These represent the more recently evolved "crowd" diseases that emerged in the neolithic age associated with the development of agriculture and the domestication of animals. In contrast, *M. tuberculosis* is an ancient companion of man since before the neolithic age and its associated crowding (Comas et al., 2013), making TB a "heritage" disease for much of its history. We argue that *M. tuberculosis* and many of the other host-adapted mycobacteria have evolved a different strategy for insuring persistence in the host—they have honed their lifestyle to obviate the need for virulence (née colonization) factors like toxins and enzymes that break down anatomic barriers to outcompete other pathogens. Instead they use host macrophages to traverse host mucosal barriers to sterile sites deep in the body. As we see it, *M. tuberculosis*' dirty little secret is to be hydrophobic and to "fly" more efficiently in a tiny droplet to bypass the innate immune system. Rather than jostling with other pesky microbes, *M. tuberculosis* can deal just with its host, and we suggest that host immune evasion, modulation, and exploitation are the trump cards of the pathogenic mycobacteria. This recognition of *M. tuberculosis*' tactics brings a new understanding of host and pathogen biology that can potentially be parlayed into new therapies and interventions.

This Review will examine key new discoveries about TB pathogenesis against a backdrop of the natural history of infection and disease and its difficult treatment. We note that TB pathogenesis was last reviewed in *Cell* in 2001 (Glickman and Jacobs, 2001), a time that marked the derivation of a basic molecular genetic toolkit for *M. tuberculosis* and the postgenomic era of TB research being ushered in following the elucidation of its genome sequence (Cole et al., 1998). The earlier *Cell* Review highlighted the problem of lengthy drug treatment as a factor that made global eradication of TB difficult, described new insights into the strategies used by *M. tuberculosis* to persist in macrophages, and discussed newly identified lipid effectors in virulence. Since then, many additional mycobacterial genomes have been sequenced, enhancing our understanding of mycobacterial evolution. More sophisticated genetic approaches and new animal models have provided new and often surprising insights into how the pathogenic mycobacteria survive and replicate in macrophages and indeed orchestrate the formation of granulomas, macrophage aggregates, and exploit them for their expansion. We will discuss how the mechanisms used by mycobacteria to resist macrophages also render them drug tolerant, a finding that has potential therapeutic implications. Finally, we will discuss how mycobacteria paradoxically can benefit from an over-exuberant host immune response to increase their numbers further and be transmitted to a new, susceptible host. The significance of these discoveries may be most fully appreciated in the context of both mycobacterial evolution and host adaptation. Where appropriate or interesting, we will compare or contrast mycobacterial pathogenic strategies to those of other pathogens. Many of our insights and ideas have come using *Mycobacterium marinum*, a close genetic relative of *M. tuberculosis* (Stinear et al., 2008), which we have developed as a valid and tractable model for *M. tuberculosis* pathogenesis (Ramakrishnan, 2013; Tobin and Ramakrishnan, 2008). We will therefore use *M. marinum* as a stand-in for *M. tuberculosis*, as well as a comparator. We will organize our thoughts around the "pathogenic personality" of *M. tuberculosis* and its many facets as it goes through its pathogenic lifecycle—entry into the host, attainment of a unique niche, multiplication within, and exit from the host—all by avoiding, circumventing, or manipulating host defenses with a unique "pathogenic signature" (Falkow, 2008) (Figure 1).

Stealth Entry Affords Mycobacteria a Privileged Host Niche

The optimal niche for a host-adapted pathogen within a host is the environment in which the pathogen is readily able to replicate. To arrive at and replicate in this niche, pathogens must circumvent host defenses, (Falkow, 2006), which are in turn substantially influenced by the commensal microflora that abundantly populate our skin and mucosal surfaces. The host-commensal alliance that forms the barrier to pathogens has recently been reviewed in *Cell*, as part of this 40th anniversary series (Belkaid and Hand, 2014).

M. tuberculosis is known to initiate infection most efficiently in the lower lung through small aerosol droplets that contain only one to three bacteria, a constraint that makes it less contagious than respiratory pathogens, such as the group A streptococcus and *Corynebacterium diphtheriae* that initiate infection in the nasopharynx to cause, respectively (1) strep throat and scarlet fever and (2) diphtheria, which are spread though large, wet droplets. The insight that small droplets are most likely responsible for transmitting TB comes from human epidemiological studies examining transmission from index cases in confined spaces (Bates et al., 1965; Houk, 1980). Corroborating the human studies are studies that track infection serially in rabbits, demonstrating that aerosol droplet size negatively correlates with infection burdens (Wells et al., 1948). When large aerosolized particles containing 10,000 bacteria were administered, they got stuck in the trachea and the rabbits got no or very little infection. In contrast, upon receiving small aerosols containing one to three bacteria that reached the alveolar spaces of the lung, the rabbits all got progressive infection.

A teleological explanation for why TB initiates in the lower lung at the cost of infectivity comes from the zebrafish larval model of TB (Cambier et al., 2014). The zebrafish larva is optically transparent so that infection with fluorescently labeled bacteria can be monitored in exquisite detail (Takaki et al., 2013). To examine the earliest interactions with the host, bacteria can be injected into the hindbrain ventricle, a microbiologically sterile neuroepithelium-lined cavity in which macrophages and neutrophils are not present normally but migrate with the expected specificity in response to the microinjection of specific chemokines or bacteria (Takaki et al., 2013; Yang et al., 2012). The zebrafish work suggests that pathogenic mycobacteria have developed strategies to avoid the microbicidal macrophages that are the default recruits to keep mucosal commensal pathogens at bay. These macrophages already primed to be microbicidal are recruited through Toll-like receptor (TLR)-mediated signaling that is activated by the so-called pathogen-activated molecular patterns (PAMPs) present on bacterial surfaces. In mouse and zebrafish macrophages, the TLR-induced microbicidal activity is from reactive nitrogen species produced by the action of inducible nitric oxide synthase (iNOS) (Cambier et al., 2014); different microbicidal effectors may be induced in human macrophages (Liu et al., 2006). Mycobacteria, including *M. tuberculosis* and *M. marinum*, are replete with PAMPs. Indeed, complete Freund's adjuvant that is used to prime immune responses is nothing but an oil emulsion containing dead *M. tuberculosis*. However, these mycobacteria express a surface lipid phthiocerol dimycocerosserate (PDIM) that masks the PAMPs so that they are not "seen" by the host innate immune system. Concomitantly, they use a related surface lipid, phenolic glycolipid (PGL), to induce the macrophage chemokine CCL2 to recruit and infect macrophages that are growth-permissive for them. However, this strategy of using a masking lipid to avoid the microbicidal macrophages and a recruiting lipid to infect the permissive ones would be ineffective in the upper airway, an environment replete with an endless supply of TLR-stimulating commensal bacteria. On this battlefield, mycobacteria would be collateral damage caught in the crossfire; they would be killed by the microbicidal macrophages that are continually being recruited. Hence the need for the third component of their tripartite immune evasion strategy: small infection droplets that deliver them directly into the alveolar spaces of the lower lung, which harbors few, if any, commensals (Charlson et al., 2011) (Figure 2).

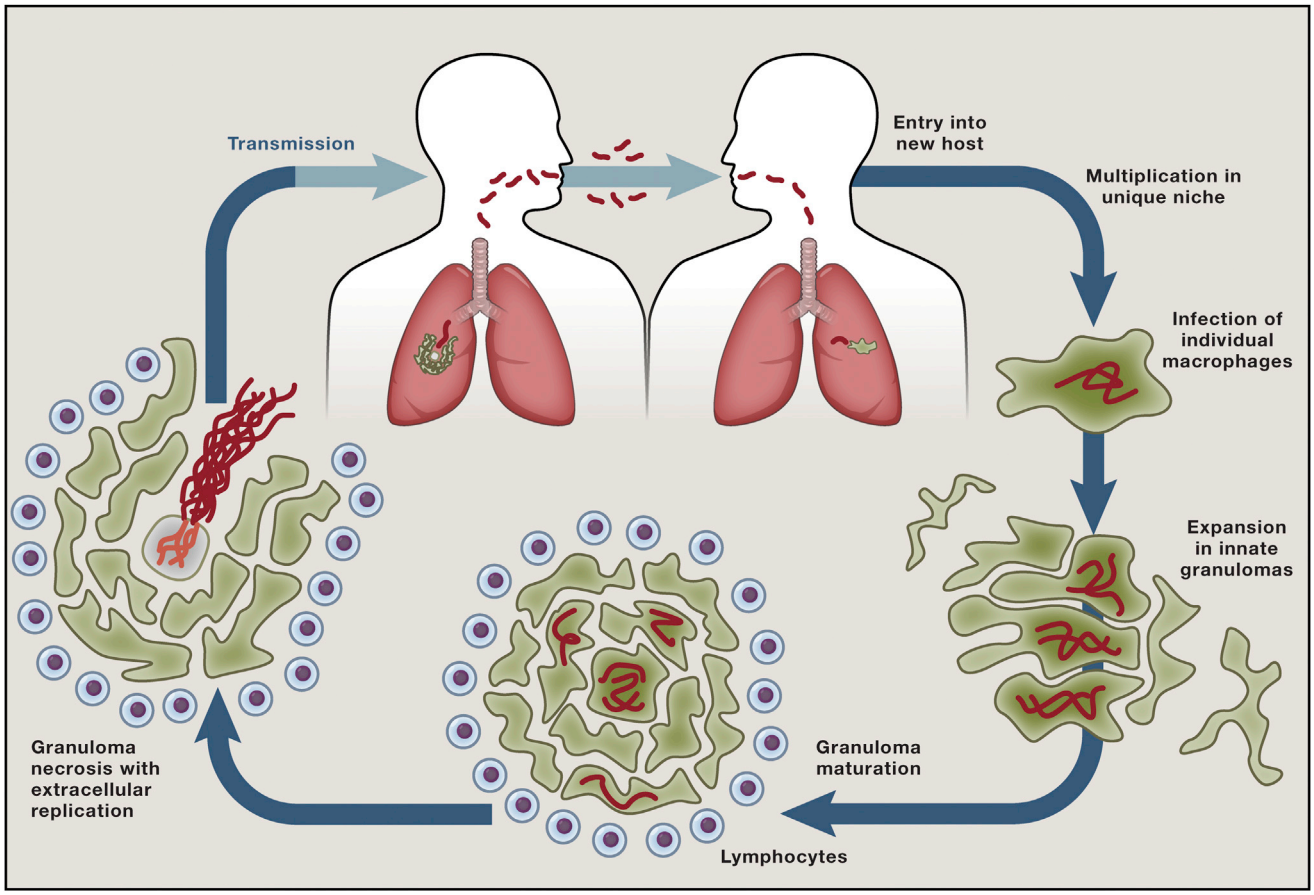

Figure 1. Pathogenic Life Cycle of *M. tuberculosis*

M. tuberculosis infection initiates when fine aerosol particles containing the bacteria coughed up by an individual with active disease are deposited in the lower lungs of a new host. The bacteria recruit macrophages to the surface of the lung, which become infected, and serve to transport the bacteria across the lung epithelium to deeper tissues. A new round of macrophage recruitment to the original infected macrophage is initiated, forming the granuloma, an organized aggregate of differentiated macrophages and other immune cells. The granuloma in its early stages expands infection by allowing bacteria to spread to the newly arriving macrophages. As adaptive immunity develops, the granuloma can restrict bacterial growth. However, under many circumstances, the infected granuloma macrophages can undergo necrosis, forming a necrotic core that supports bacterial growth and transmission to the next host.

There is a growing appreciation for a commensal-primed barrier immunity that pathogens must evade, tolerate, or interrupt. *Helicobacter pylori*, a commensal pathogen that famously causes gastric ulcers, is also a heritage pathogen and has adapted to survive in the stomach, where competition from commensals is minimal (Monack, 2013). *H. pylori* too has evolved to avoid detection via TLRs: its flagellin is not recognized by TLR5 (Gewirtz et al., 2004), and its lipopolysaccharide (LPS) has a lower affinity for TLR4 than that of other bacteria (Moran, 2007). Therefore, like *M. tuberculosis*, *H. pylori* has evolved to avoid proinflammatory host detection by initiating infection in anatomical locations in which commensal competition is minimal. Although *M. marinum* and *M. tuberculosis* have developed tactics to evade reactive nitrogen species, *Mycobacterium avium* that causes TB-like disease in birds appears to have evolved a strategy to tolerate and even benefit from them and, accordingly, does not express PDIM (Dhama et al., 2011; Dumarey et al., 1994; Gomes et al., 1999; Onwueme et al., 2005) (Figure 3). The case of host-adapted Salmonella, another macrophage-dwelling class of pathogens, may be illustrative as well. Salmonella infects via the terminal ileum that is replete with colonizing bacteria. To facilitate its transit through the commensal-laden gut, Salmonella appears to first drive an inflammatory response that generates the reactive nitrogen species to which the commensals are sensitive, but it, like *M. avium*, is tolerant at least early during infection (Fang, 2004; Henard and Vázquez-Torres, 2011). Upon reaching the terminal ileum, the invading Salmonella enters into M cells, specialized cells of the follicle-associated epithelium, a region that is again relatively free from commensal competition (Jones et al., 1994), and invades underlying macrophages. This multipronged strategy to interrupt the commensal barrier so as to reach the M cells affords Salmonella access to the systemic phagocytes of the host (called the reticuloendothelial system). The common theme emerging from these scenarios is that host-adapted pathogens must develop strategies to circumvent the host-beneficial commensal-primed immune barrier in order to reach their replicative niche. We emphasize that, in turn,

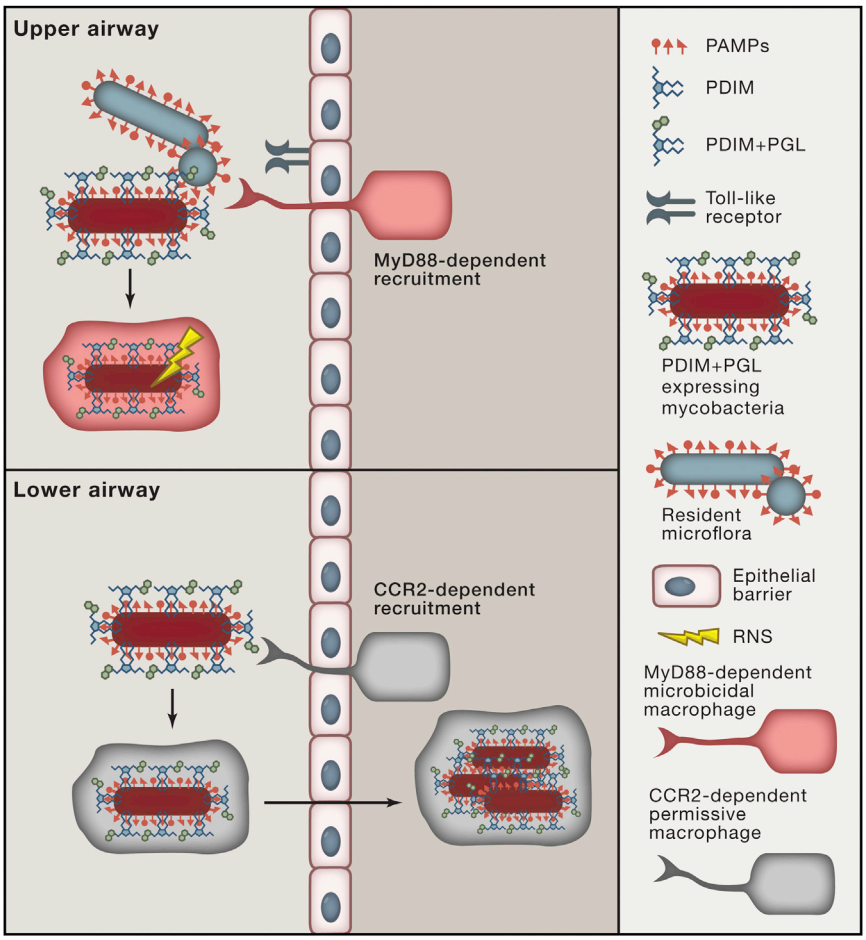

Figure 2. *M. tuberculosis* Evades Commensal Bacteria to Infect Its Host

M. tuberculosis avoids the recruitment of microbicidal macrophages to the site of infection by masking its PAMPs with the PDIM lipid. A related surface lipid PGL recruits permissive macrophages that can transport the bacteria into deeper tissues. However, the upper airways are colonized by resident microorganisms whose PAMPs recruit microbicidal macrophages. Therefore, this mycobacterial strategy to evade microbicidal macrophages is only effective if infection is initiated in the relatively sterile lower lung.

commensals exert a selective pressure that has shaped pathogen evolution.

Mycobacteria have to engage with the host to become phagocytosed by permissive macrophages by simultaneously using the surface lipid, PDIM, to dampen TLR signaling and by using the related surface lipid, PGL, to induce CCL2 signaling. This finding has additionally provided an understanding of the role of these lipids in virulence (Siegrist and Bertozzi, 2014). PDIM is expressed only by pathogenic mycobacteria, is absolutely required for virulence, and is present in all *M. tuberculosis* clinical isolates (Onwueme et al., 2005). Yet PDIM synthesis is metabolically costly so that it is readily lost in axenic culture (Kirksey et al., 2011). PGL, in contrast, is not absolutely required for virulence; it is not present in all clinical *M. tuberculosis* isolates (Reed et al., 2004). However, it is present in many of the W-Beijing strains that have predominated in outbreaks in North America, where TB is not prevalent. In the zebrafish, wherein low-dose infections can be examined longitudinally from the first instances of infection, PGL specifically increases infectivity of inocula of one to three bacteria (that mimic those of human infection) by enhancing the recruitment of mycobacterium-permissive macrophages. It is only in the context of examining the ability of the pathogen to establish infection, rather than sustain it, that the role of PGL is revealed. The presence of PGL on the bacterial surface substantially increases the organism's chances of reaching its preferred replicative niche. These findings may also serve to explain human studies showing an association between TB susceptibility and the high expression of CCL2, PGL's host partner in recruiting permissive macrophages (Flores-Villanueva et al., 2005). Further, the finding that PGL increases virulence through enhanced infectivity provides an understanding of why PGL is present in *M. cannetti* strains, ancestral to *M. tuberculosis*, as well as in *M. marinum*, the closest genetic relative of the *M. tuberculosis* complex (Onwueme et al., 2005), suggesting its integral role in the evolution of mycobacterial pathogenicity. As noted before, TB is generally thought to have infected humans for ~70,000 years, thus predating by ~60,000 years the neolithic demographic transition and its resultant crowding (Bos et al., 2014; Comas et al., 2013). Thus, PGL may have been an essential virulence determinant for most of its history. Perhaps, the greatly increased transmission opportunities arising from human crowding made it dispensable.

Multiplication within the Host—The Macrophage Niche

The strategy elaborated by *M. tuberculosis* to traverse host epithelial barriers within permissive macrophages is, of course, predicated upon its ability to survive within these highly evolved phagocytic host cells. Indeed, macrophages comprise the replicative niche for most of the lifecycle, not only of *M. tuberculosis* but of most other pathogenic mycobacteria (Figure 4). Accordingly, the ability to replicate in host cells is a defining feature of the pathogenic mycobacteria—be they human or animal pathogens—and reliably distinguishes them from their nonpathogenic soil-dwelling cousins like *Mycobacterium smegmatis* (Shepard, 1957) (Figure 3). A clue for how this ability to grow in host macrophages might have evolved comes from the remarkable finding that the ability of mycobacteria to replicate in macrophages tracks completely with their ability to grow in unicellular free-living amebae. Pathogenic mycobacterial species can replicate in amoebae, whereas *M. smegmatis* cannot (Cirillo et al., 1997). Moreover, to the extent tested, the same mycobacterial

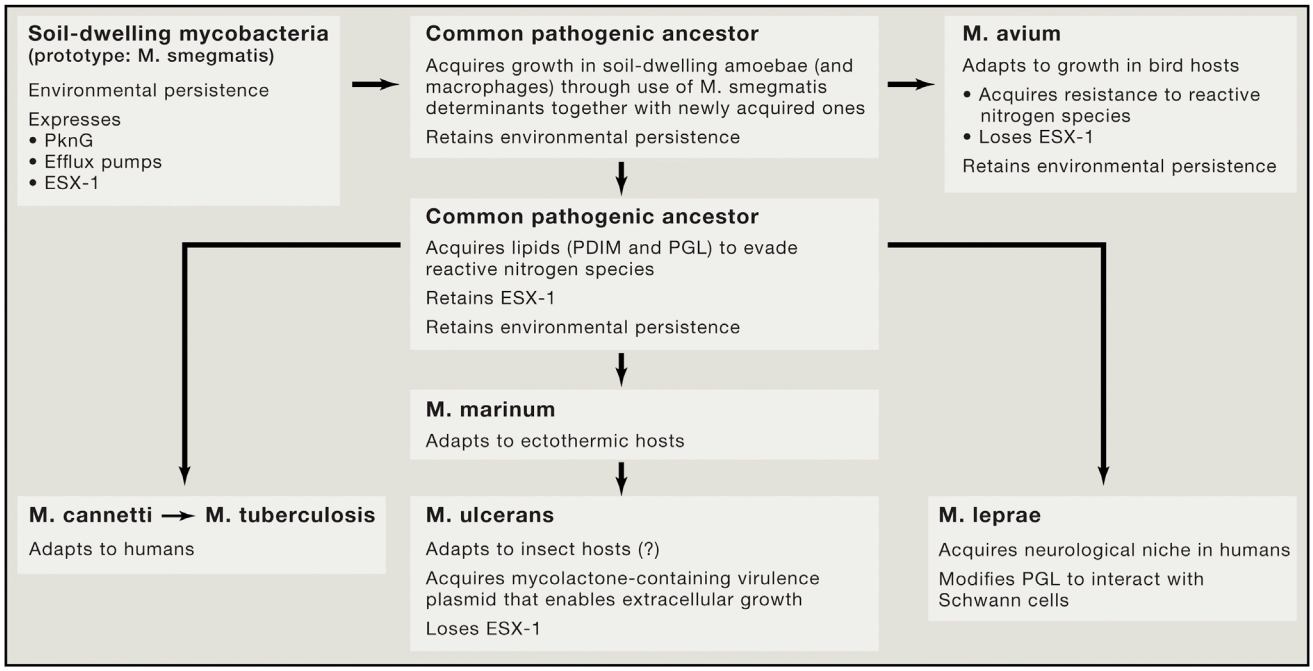

Figure 3. An Evolutionary Perspective of Mycobacterial Pathogenicity
An analysis of the pathogenic traits and preferred hosts of diverse mycobacterial species in relation to their genomes.

determinants are required for growth in macrophages and amoebae (Alibaud et al., 2011; Hagedorn et al., 2009; Hagedorn and Soldati, 2007; Solomon et al., 2003). Thus, predatory environmental amoebae may have served as the ancient evolutionary training ground for mycobacterial pathogens to survive in the macrophages of their multicellular hosts. This has been postulated for *Legionella*, an accidental human pathogen that can cause serious pneumonia after being aerosolized from potable water sources where it is thought to be sustained through replication in environmental amoebae (Fields et al., 2002).

Any foreign particulate that is phagocytosed by macrophages is destined to be processed through the endocytic pathways. Thus, intracellular pathogens have evolved diverse ingenious signature strategies to thwart, modulate, exploit, or avoid host endocytic pathways. Broadly speaking, these pathogens can resist lysosomal fusion to reside in non-acidified endosome-like compartments, survive (or even require) acidification so as to be able to reside in acidified lysosome-like compartments, or break out of the phagosome altogether to reside in the cytosol (Alix et al., 2011) (Figure 4). Most experimental studies on the virulence of intracellular mycobacteria have been conducted using either mouse or human cultured macrophages. For *M. tuberculosis*, observations in cultured macrophages have produced disparate results probably because the cell lines, culture conditions, and kinetics of infection differ considerably between different laboratories. *M. tuberculosis* is reportedly found localized to non-acidified early endosomes or found in acidified lysosomes with a small proportion of the bacteria eventually breaking out of the phagosome to reside in the cytosol (Cosma et al., 2003; van der Wel et al., 2007). Indeed, mycobacteria have specific virulence determinants that promote, at least in cultured cells, both the avoidance of acidification as well as acid resistance, suggesting that, despite their best attempts, they might find themselves in acidified compartments (Rohde et al., 2007). In addition, the ability to break out into the cytosol is dependent on a specialized bacterial secretion system, ESX-1 (van der Wel et al., 2007), whose role we will elaborate upon in the context of the tuberculous granuloma in the following section.

The multiple subcellular compartments that *M. tuberculosis* can occupy within macrophages speak to the plethora of defenses with which they must contend even in the most permissive of macrophages. It is hardly surprising that diverse mycobacterial determinants are required for macrophage survival (Forrellad et al., 2013). What is surprising is that the obvious prediction that these determinants were acquired during mycobacterium's jump to becoming an amoeba dweller does not stand scrutiny. Although our search was not exhaustive, virtually all of the important *M. tuberculosis* virulence determinants that specifically promote intracellular growth are present in *M. smegmatis* (Forrellad et al., 2013)! What is more, in the cases tested, the *M. smegmatis* gene can substitute for its *M. tuberculosis* gene in mediating macrophage growth and virulence, suggesting that no or few further modifications were needed to confer this function (Houben et al., 2009). For instance, the eukaryotic-like serine-threonine protein kinase PknG is secreted into the phagosomal lumen and promotes macrophage growth by inhibiting lysosomal fusion and thereby acidification of the mycobacterial phagosome (Walburger et al., 2004). Although the *M. smegmatis* PknG homolog is able to restore macrophage growth of the *M. tuberculosis pknG*

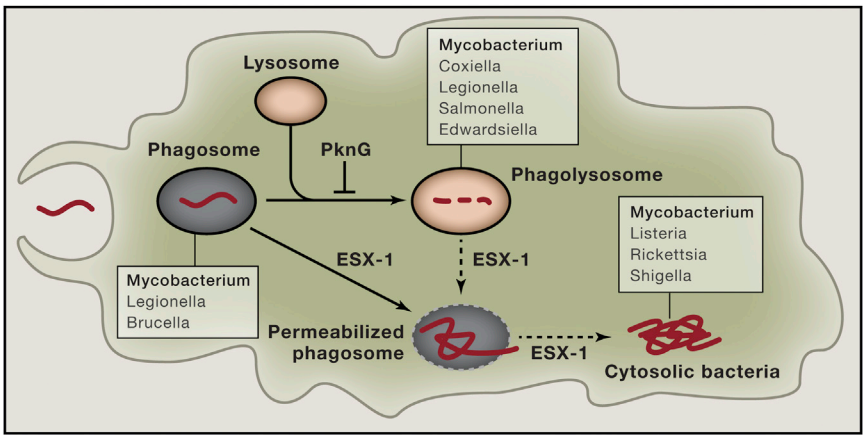

Figure 4. Intracellular Niches of *M. tuberculosis*

The observed intracellular niches of *M. tuberculosis* within macrophages are shown with other pathogens occupying those niches also listed. Confirmed trafficking pathways are indicated with continuous arrows and putative ones with dashed arrows. Pathways dependent on the mycobacterial ESX1 secretion system are indicated.

mutant, its function in the context of this saprophytic organism remains unknown. PknG is translationally repressed in *M. smegmatis* at least under axenic growth, suggesting that there is some specific "real-life" situation during life in the soil when it is induced, presumably to perform some specific function (Houben et al., 2009).

Perhaps the most fascinating example of conservation across mycobacteria is that of the mycobacterial energy-dependent efflux pumps, which we recently discovered to be *M. tuberculosis* macrophage growth factors by a circuitous route when looking for the basis of antibiotic tolerance (Adams et al., 2011; Rengarajan et al., 2005; Szumowski et al., 2013). In addition to developing genetic drug resistance through fixed mutations, *M. tuberculosis* famously develops what is called "phenotypic drug resistance" or "drug tolerance," wherein it becomes transiently resistant to antibiotics (in the absence of fixed genetic mutations) in the host. This necessitates long treatment periods to achieve clinical cures (Connolly et al., 2007). Mycobacterial drug tolerance has long been attributed to the bacteria being in a non-replicating or dormant state in the host (Chao and Rubin, 2010; Rittershaus et al., 2013). However, our recent work shows that, when *M. tuberculosis* enters macrophages, it is, in fact, the actively replicating bacteria within the macrophages that develop antibiotic tolerance through the induction of specific macrophage-induced efflux pumps (Adams et al., 2011; Schnappinger et al., 2003). These same efflux pumps that mediate macrophage-induced drug tolerance also promote intracellular mycobacterial growth (Adams et al., 2011; Schnappinger et al., 2003). This suggests that these pumps may have evolved in the soil dwellers to defend against environmental toxins and inhibitors (including naturally occurring antibiotics) but came to be useful for the contemporary lifestyle of the pathogenic mycobacteria, facilitating intracellular survival, perhaps by protecting them against the antimicrobial peptides in macrophages. With the advent of chemotherapy, their ancestral function to defend against antibiotics or other growth inhibitors affords added benefit in surviving within the host; the same pumps may efflux the natural macrophage defenses as well as the administered antibiotics (Adams et al., 2011; Schnappinger et al., 2003). These findings additionally have therapeutic implications because efflux pump inhibitors should prevent the replication of intracellular *M. tuberculosis*. Moreover, combining efflux pump inhibitors with standard antibiotic therapy should be a "double whammy" to intracellular *M. tuberculosis* because they should inhibit intracellular growth in their own right and additionally allow the antibiotics to kill these bacteria better by preventing their efflux. Indeed, we have found this to be the case using inexpensive, well-tolerated approved human drugs that are currently used for other purposes (e.g., verapamil) (Adams et al., 2011, 2014). Moreover, the addition of verapamil to standard antituberculous chemotherapy reduces relapse rates in *M. tuberculosis*-infected mice (Gupta et al., 2013). On the basis of all these findings, clinical trials of verapamil as a TB treatment-shortening agent are imminent.

In summary, returning to our comparative theme, it would appear that the environmental mycobacteria, e.g., *M. smegmatis*, had determinants that allowed them to survive in the soil even though they could not survive within unicellular predators. The selection for the ability to survive within unicellular amoebae and eons later within the macrophages of multicellular creatures required using these determinants together with acquiring new as yet unknown ones, possibly by horizontal gene transfer (Figure 4). One could argue that mycobacteria come "pre-loaded" with the means to survive within a professional phagocytic cell. It is perhaps a general strategy for other soil organisms adapting to animal hosts as illustrated as well for *Rhodococcus equi*, the horse and occasional human pathogen that diversified from soil-dwelling *Rhodococci* (Letek et al., 2010). In the case of mycobacteria, the pathogenic forbearer selected for growth in amoeba appears to have then followed different evolutionary branches to dwell in different hosts, sometimes involving the acquisition of plasmids and, as is so often the case, gene loss, so as to fine-tune adaptation to specific hosts (Boritsch et al., 2014; Wang and Behr, 2014) (Figure 3).

In the following section, we will discuss the elaborate macrophage manipulation strategies used by *M. tuberculosis* to form the granuloma—the hallmark pathological structure of TB. We argue that it is as much a mycobacterial strategy for survival as it is a host defense response.

Multiplication in the Host—Exploiting the Granuloma

A granuloma is fundamentally an organized aggregate of macrophages whose membranes become tightly interdigitated like those of epithelial cells, leading them to be called epithelioid cells

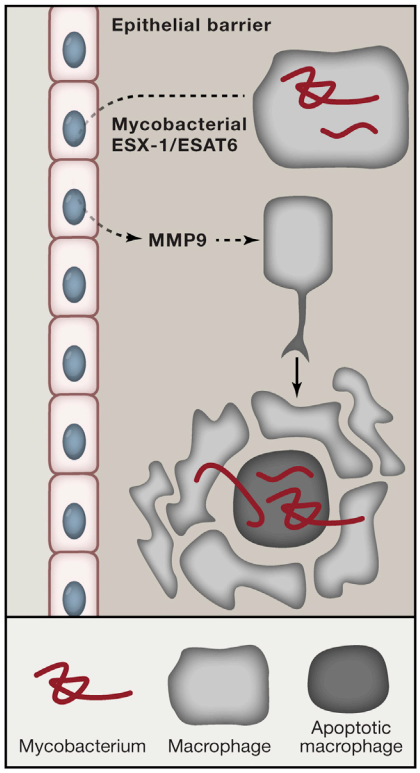

Figure 5. Mycobacteria Exploit the Granuloma to Expand Their Numbers in Early Infection

Mycobacteria within infected macrophages induce in an ESX1-dependent fashion MMP9 expression in epithelial cells surrounding the nascent granuloma. MMP9 stimulates the recruitment of new macrophages to the granuloma. Multiple new arrivals phagocytose the bacterial contents of a given dying infected macrophage, thus spreading the bacteria to new macrophages and providing them new expansion niches.

(Adams, 1976; Bouley et al., 2001). Granulomas can form in response to any number of persistent stimuli, both infectious and noninfectious, so that they are associated with myriad diseases (Ramakrishnan, 2012). They were first recognized as distinct structural entities in the context of human TB in the 17th century, preceding by some 200 years the discovery of *M. tuberculosis* as its cause, and even today, TB remains the most common cause of granulomas worldwide (Ramakrishnan, 2012). For a very long time, the tuberculous granuloma has been held to be an essential host protective structure—a fortress containing a complex mixture of diverse host cells that walls off bacteria (Saunders and Cooper, 2000) (Chao and Rubin, 2010; Rittershaus et al., 2013). Indeed, clinical and epidemiological studies clearly support the idea that the granuloma can sterilize infection in many cases (Cosma et al., 2004; Feldman and Baggenstoss, 1938). Yet, we argue that there is an inextricable link between highly organized granulomas and heavy bacterial burdens in TB, suggesting that, in many cases, the granuloma can be at least conducive to high bacterial burdens if not downright supportive of them (Connolly et al., 2007). Indeed, our findings over the last decade suggest that mycobacteria actually enhance the formation of granulomas and have adapted to exploit these structures for their expansion and dissemination (Davis and Ramakrishnan, 2009). This modified view of the granuloma was made possible by studies in the zebrafish in which we could visualize the earliest events of granuloma formation around the first infected macrophage that had arrived in the deep tissues. Bacterial expansion in granulomas is accomplished by the spread of bacteria from dying macrophages to newly arriving ones (Davis and Ramakrishnan, 2009). When mycobacterial numbers increase to a certain threshold in individual macrophages, they undergo an apoptotic death that leaves viable bacteria still encased within the dead cells (Figure 5). Concomitantly, multiple new uninfected macrophages are recruited to the nascent granuloma that engulf the bacterial contents of a given dead or dying macrophage, thus enabling them to fill up the new cells. This process of macrophage death and re-phagocytosis enables a tremendous expansion of the bacterial niche, all within macrophages (Davis and Ramakrishnan, 2009).

At a molecular level, this coordinated macrophage death and re-phagocytosis is mediated through a specialized mycobacterial secretion system called ESX-1, most likely through its secreted effector ESAT6 (Davis and Ramakrishnan, 2009; Volkman et al., 2004; Volkman et al., 2010) (Figure 5). ESAT6 has been shown to induce apoptosis of infected cells in culture through multiple pathways, one or more of which may be operant in the granuloma (Choi et al., 2010; Derrick and Morris, 2007; Keane et al., 1997; Mishra et al., 2010; Swaim et al., 2006). ESX-1/ESAT-6 also recruits macrophages by inducing host matrix metalloproteinase 9 (MMP9) in epithelial cells surrounding the nascent granuloma (Volkman et al., 2010). If host MMP9 function is decreased, infection is attenuated, with reduced granuloma formation (Volkman et al., 2010). These discoveries, initially made in the zebrafish, are corroborated by findings in human TB showing that MMP9 is induced in epithelial cells surrounding lung granulomas, and increased MMP9 secretion is associated with increased severity and mortality in tuberculous meningitis (Elkington et al., 2007; Price et al., 2001).

In summary, *M. tuberculosis* appears to use at least two distinct pathways to recruit macrophages. When *M. tuberculosis* first enters the host animal, it uses its PGL surface lipid to recruit macrophages through host CCL2, which then bring the mycobacteria across host epithelium to deeper tissues (Cambier et al., 2014) (Figure 2). The intracellular bacteria then orchestrate the recruitment of additional macrophages to form the granuloma through their ESX-1 locus (Figure 5). Why the bacteria transition from using PGL to using ESAT6 to drive macrophage recruitment is unclear. What is clear is that, in both phases, macrophage recruitment benefits the mycobacteria as much as the host! Thus, CCL2 and MMP9 may both represent host determinants that have been co-opted by mycobacteria for their benefit, and increased CCL2 and MMP9 expression are both linked to human susceptibility to TB (Elkington et al., 2007; Price et al., 2001; Flores-Villanueva et al., 2005).

It is curious that, although the growing granuloma supports *M. tuberculosis* expansion, the macrophages within this structure generally become more microbicidal suggesting that the bacterium should be put at a further disadvantage (Adams, 1976; Bouley et al., 2001). But it appears that the mycobacteria,

in turn, rapidly adapt to the more hostile environment in the granuloma by transcriptionally inducing new genes (e.g., efflux pumps) upon entering a macrophage, which aid in intracellular survival. Moreover, when an infected macrophage forms or joins a granuloma, its intracellular bacteria rapidly induce additional genes that help it to counter the additional stresses of living within the cellular environment of the granuloma (Cosma et al., 2004; Davis et al., 2002; Ramakrishnan et al., 2000). If an infected animal with mature granulomas (frogs or zebrafish with *M. marinum*, or mice with *M. tuberculosis*) is superinfected with new mycobacteria, these bacteria enter new macrophages that preferentially migrate to existing granulomas rather than avoiding them as hostile sites (Cosma et al., 2004, 2008). It is as if mycobacteria "know" that, although the granuloma may not be a place where "the living is easy," it still does afford them a preferred and, paradoxically, a protected multiplication niche and, as we shall see in the following section, a transmission niche as well.

Our proposed mycobacterial-centric view of granuloma biology and function returns us yet again to questions about how mycobacteria evolved to pathogenicity. We focus now on the ESX-1 secretion system and its effector, ESAT6. ESX-1/ESAT6, like most other *M. tuberculosis* virulence factors, is also present in *M. smegmatis.* However, in *M. smegmatis*, this secretion system regulates bacterial conjugation (Coros et al., 2008; Parsons et al., 1998) (Figure 3). The *M. tuberculosis* and *M. smegmatis* homologs are functionally conserved (Converse and Cox, 2005; Flint et al., 2004), but the co-option of bacterial gene-exchange systems for virulence is not special to mycobacteria. In diverse bacterial pathogens like *H. pylori*, *Legionella*, and *Agrobacterium*, the type IV secretion system that is required for virulence also mediates DNA transfer or uptake. Equally interesting is the finding that ESX is not even mycobacterium specific; an ESX homolog mediates virulence in *R. equi* while also being present in the soil *Rhodococci* and, indeed, being widely distributed in GC-rich soil bacteria (Letek et al., 2010). The mechanistic details of how ESX-1/ESAT6 mediates granuloma formation and virulence remain to be understood. ESAT6 is reported to be associated with the ability of *M. tuberculosis* subpopulations to break out of the phagosome (De Leon et al., 2012; Hsu et al., 2003; van der Wel et al., 2007) (Figure 4). In fact, prior to full-fledged phagosomal rupture, ESAT6 may permeabilize the phagosomal membrane enough to expose mycobacterial DNA to the host cytosolic DNA-sensing pathway (Manzanillo et al., 2012). This results in the induction of type I interferon, a cytokine that is best known for its antiviral activity; however, mycobacteria appear to drive this cytosolic DNA-sensing response in their favor. Components of the cytosolic pathway and type I interferon mediate host susceptibility in animal models. Type I interferon receptor knockout mice are more protected against TB, similar to the protection against TB afforded by knocking out MMP9 (Manzanillo et al., 2012; Mayer-Barber et al., 2014; Taylor et al., 2006; Watson et al., 2012). In humans too, a type I IFN transcriptional signature has been associated with active versus subclinical TB, suggesting it is a host susceptibility factor for disease progression (Berry et al., 2010). Whether ESAT6 modulates host MMP9 activity and granuloma formation through its membrane permeabilization activity and/or by triggering the cytosolic DNA detection system still remains unclear. What is clear is that it represents another fascinating case in which a ubiquitous bacterial determinant has been co-opted and fine-tuned through genetic selection—not just to modulate bacterial growth in macrophages, but also to become integrated with other bacterial genes for a choreographed manipulation of macrophage migration and death to build the granuloma.

Of course, this initial phase of bacterial expansion in the granuloma is followed by the advent of an adaptive immune response that can often eradicate the tubercle bacilli, presumably by increasing the microbicidal capacity of the granuloma macrophages (Ramakrishnan, 2012). Epidemiological evidence would suggest that, with a little help from adaptive immunity, the granuloma can sterilize infection in most cases (Cosma et al., 2004; Feldman and Baggenstoss, 1938). Conversely, the very large number of active TB cases with full-fledged mature granulomas that are replete with lymphocytes suggests that mycobacteria have evolved additional strategies to evade adaptive immunity. As we have detailed in prior reviews, many of these strategies have been elucidated recently and include delaying both T cell priming in the lymph nodes and their arrival and activity in the granuloma (Pagan and Ramakrishnan, 2014; Ramakrishnan, 2012). Thus, *M. tuberculosis* reduces macrophage responsiveness to signaling by γ interferon, the main T cell cytokine (Banaiee et al., 2006). Finally, *M. tuberculosis* can synthesize its own tryptophan so that, unlike some other intracellular pathogens (e.g., Chlamydia), it is able to survive the intracellular tryptophan starvation brought on by γ interferon (Zhang et al., 2013). Thus, bacterial interactions with the host adaptive immune system add layers of complexity to the host-pathogen interface. The tubercle bacillus first induces an innate inflammatory response to accelerate macrophage responses (recruitment, phagocytosis, apoptosis) that are normally protective to turn the granuloma response into a bacterial production factory. By then delaying T cell priming, arrival, and activation together with macrophage responsiveness by what appears to be a highly orchestrated strategy, the bacteria buy themselves yet more time to establish a strong replicative niche in the granuloma.

Many diverse microorganisms induce granulomas, and it will be interesting to compare the pathways and consequences of granuloma formation for each of them. Within the mycobacteria, a tantalizing difference in granuloma formation pathways is already apparent by comparing *M. marinum* and *M. tuberculosis* to *M. avium* (Figure 3). ESAT6/ESX1 is absent from *M. avium*, which is not a particularly successful pathogen in human hosts (Dhama et al., 2011; Dumarey et al., 1994; Gomes et al., 1999; Onwueme et al., 2005; Sørensen et al., 1995) (Figure 3). However, in birds, *M. avium* causes a full-blown granulomatous disease that is transmissible, usually by ingestion. So *M. avium* granulomas must form through a different pathway. Meanwhile, *M. tuberculosis* ESAT6 has been recently revealed to have yet another function in supporting bacterial expansion in the granuloma—it induces immunosuppressive regulatory T cell populations that delay the migration of effector T cells into the granuloma (Shafiani et al., 2013; Shafiani et al., 2010). Thus, ESAT6 may prolong the phase of bacterial expansion in the innate granuloma that it orchestrates in the first place (Davis and Ramakrishnan, 2009). In this context, it is intriguing

that birds, in contrast to mammals and zebrafish, appear to have lost the transcription factor FoxP3 that is required for regulatory T cell development (Andersen et al., 2012). Therefore, a plausible explanation for *M. avium*'s loss of ESX-1/ESAT6 is that it is superfluous for granuloma expansion in its bird hosts. In contrast, its retention in *M. tuberculosis* and *M. marinum* may reflect its central role in granuloma expansion in mammalian and fish hosts. The selective forces of the host's immune system are reflected in the array of expressed virulence genes seen in each host-adapted mycobacterial species. Although our conjecture on the evolutionary connections may well be incorrect, our revised view of the role of the granuloma in the pathogenesis of tuberculosis can be tested further experimentally and may have potential clinical relevance. We predict that the reduction of granuloma formation by pharmacological inhibition of the relevant host pathways (e.g., MMP9) should ameliorate infection.

Exit from the Host—Escaping the Granuloma

A host-adapted pathogen's final step to ensure its evolutionary success is to exit the host and enter a new host for the infection cycle to start anew. In the case of *M. tuberculosis*, it must exit the granuloma of its infected host to enter and establish infection in a new host. Epidemiological evidence suggests that transmission occurs most efficiently from individuals with organized granulomas that have undergone central necrosis (Sharma et al., 2005; Reichler et al., 2002; Bekker and Wood, 2010; Huang et al., 2014). The necrotic areas rupture into the bronchial tree, thus exposing the mycobacteria to the airway whence they can be aerosolized in cough droplets. Recent work from both our laboratory and others takes a slightly modified view of the dynamics of cell death in a granuloma (Ramakrishnan, 2012). Broadly speaking, infected granuloma macrophages can die in two ways: by apoptosis or by necrosis. Apoptosis leaves the host cell membranes intact so that the bacteria remain encased within the macrophage corpse and are readily phagocytosed by new entering cells. In contrast, macrophage necrosis, or lysis, releases the intact bacteria into the extracellular milieu. This necrotic debris, or caseum, seems to be an ideal bacterial growth medium as the multiplying bacteria reach much higher numbers extracellularly and grow in characteristic serpentine cords. Thus, in our view, apoptotic death favors bacterial expansion or maintenance of the granuloma by providing new cells to grow in, albeit still restricted by macrophage defenses. Bacterial lysis from the macrophages allows more exuberant growth, reflecting the ineffectiveness of extracellular host defenses against mycobacteria. Moreover, recent work suggests that these corded extracellular mycobacteria are not readily engulfed by new macrophages the way that bacteria within apoptotic cells are (Bernut et al., 2014). It is not fully understood how the dynamics of the granuloma shift to favor necrosis, but some interesting insights are emerging.

Our studies in *M. marinum*-infected zebrafish have uncovered two pathways that lead to macrophage necrosis, each through opposite dysregulation of TNF, resulting in too little or too much TNF. TNF is required for macrophage microbicidal activity, although we do not understand the specific mechanisms and effectors (Clay et al., 2008). Whereas host TNF deficiency causes mycobacteria to grow exuberantly within macrophages that then die and release them to the extracellular milieu (Clay et al., 2008), we have found that an excess of host TNF causes infected macrophages to undergo necrosis, through a programmed pathway called necroptosis (Roca and Ramakrishnan, 2013; Tobin et al., 2012). Host TNF excess causes the activation of the RIP1 and RIP3 kinases that then, through a series of steps, induce reactive oxygen species in the macrophage mitochondria (Roca and Ramakrishnan, 2013; Tobin et al., 2012). Reactive oxygen has dual effects: it kills both mycobacteria and macrophage. The net result is that, just as the macrophage is on its way to killing its infecting mycobacteria, it dies. The few surviving mycobacteria that are released extracellularly can expand their numbers rapidly.

Perturbation of several pathways could lead to TNF dysregulation and, in turn, granuloma necrosis. One that we identified in a zebrafish mutant screen for susceptibility to *M. marinum* involves dysregulation of the leukotriene A4 hydrolase (LTA4H), a synthetic enzyme in the eicosanoid pathway that catalyzes the synthesis of the highly proinflammatory lipid leukotriene B_4 (Tobin et al., 2010, 2012). LTA4H deficiency prevents the synthesis of this leukotriene and instead causes the accumulation of the anti-inflammatory lipid lipoxin A_4, which represses the TNF response. In contrast, LTA4H excess causes an overproduction of leukotriene B_4 and an excess of TNF. In humans, a common *LTA4H* promoter variant regulates gene expression and homozygotes for both the low- and high-expression variants that are associated with low and high inflammation, respectively. Individuals with low- and high-expression variants of LTA4H get severe tuberculous meningitis, with a high mortality. In contrast, the heterozygotes, with an intermediate (presumably optimal) level of LTA4H expression, are protected. These findings, in turn, have therapeutic implications—in a Vietnamese tuberculous meningitis cohort, adjunctive treatment with the broadly immunosuppressive glucocorticoids, which are now routinely administered along with antitubercular chemotherapy, only prevented mortality of the high LTA4H group while possibly increasing mortality of the low LTA4H individuals (Tobin et al., 2012; Tobin et al., 2010). These results suggest that patient genotype-directed glucocorticoid treatment may optimize TB treatment.

Our detailed understanding of the two pathways through which excess TNF-induced mitochondrial reactive oxygen causes macrophage necrosis also suggests new approaches to TB therapy (Roca and Ramakrishnan, 2013; Tobin et al., 2012). Reactive oxygen causes the translocation of the matrix mitochondrial protein cyclophilin D to participate in the formation of a pore on the mitochondrial membrane, thus causing leakage of mitochondrial contents. Additionally, the reactive oxygen also causes overproduction of a cellular lipid called ceramide that induces necrosis through mechanisms that are not yet clear. We have identified currently available oral drugs that can block each of these pathways. Alisporivir, a drug in phase 3 clinical trials for another disease, blocks cyclophilin D, and desipramine, a tricyclic antidepressant, inhibits ceramide production. In the zebrafish, the combined use of these drugs allows the reactive oxygen to kill the bacteria without killing the macrophages and thereby converts the hypersusceptible state of TNF excess to hyperresistant. It is possible that these drugs will have a similar effect in humans who induce excess TNF during infection.

Necrosis from excessive TNF may be further exacerbated by the participation of adaptive immunity, as much of the TNF induced in TB is produced by T cells (Roach et al., 2002; Saunders et al., 2004). Perhaps *M. tuberculosis* has co-opted T cells into producing the TNF that causes macrophage necrosis and thus encourages transmission. Indeed, T cell epitopes of all known mycobacterial T cell antigens are reported to be hyperconserved across strains globally, even more so than those of essential genes (Comas et al., 2010). This finding suggests that T cell recognition favors the survival and transmission of mycobacteria, arguably by inducing TNF-mediated necrosis as described above. The hyperconservation of T cell epitope regions of expressed mycobacterial antigens highlights the close evolutionary relationship between T cell recognition and bacterial fitness. Epidemiological evidence suggests that individuals with diminished adaptive immunity (e.g., HIV-infected individuals or children) tend to have smaller and less necrotic granulomas than immunocompetent adults, and these former individuals—though more susceptible to TB—do not transmit it as well as the latter (Huang et al., 2014). Globally, >80% of active TB occurs in individuals who are not HIV infected (Zumla et al., 2013). This link between disease and an intact host adaptive immune system may suggest that *M. tuberculosis* takes advantage of adaptive immunity for its transmission.

In summary, our view is that *M. tuberculosis* and many other pathogenic mycobacteria are not innocent bystanders during the formation of granuloma. They can modulate the host response to infection to build and modify this complex immunological entity into a niche that can sustain infection, first through intracellular growth and then through extracellular growth that also favors transmission.

Concluding Thoughts

The case of *M. tuberculosis* exemplifies how a series of genetic adaptations can convert a soil-dwelling microbe into one of the most successful and enduring pathogens of humanity. More people are thought to have died of TB than of any other infectious disease throughout history, and more people are afflicted with active TB disease today than at any other time in history (Lawn and Zumla, 2011). While marveling at the exquisite bacterial adaptations that have honed this microbe's success in its human niche, it is important to remember that most infected individuals (classically reported to be 90%) can successfully contain or clear the infection (Zumla et al., 2013). This occurs either through an initial mobilization of innate immune mechanisms or, failing that, through adaptive immunity. In this Review, we have tried to point out how the outcome of each step of the host-pathogen interaction can represent "success" for the host—infection can be suppressed or cleared at the first site of infection, in the innate granuloma, or later, when the granuloma is further re-enforced by adaptive immunity (Cambier et al., 2014; Lin et al., 2014; Adams et al., 2011; Rengarajan et al., 2005; Szumowski et al., 2013).

Suppression of infection can result in a clinical latency during which the bacteria persist indefinitely in the host and can produce active disease even decades later—a scenario that is emphasized in the literature (Chao and Rubin, 2010; Cosma et al., 2003; Rittershaus et al., 2013). However, careful longitudinal studies from the pre-antibiotic era suggest that most contemporary human disease manifests within a few months of infection or is cleared (Cosma et al., 2003). In today's world, it is these relatively recently infected individuals who transmit the bulk of the disease, rather than those in whom disease has recrudesced after many decades. From a medical perspective, this places the impetus on understanding how the majority of infected individuals progress to disease relatively rapidly. However, it is likely that clinical latency played an important role in sustaining the organism through the ~60,000 years before TB became a "crowd" disease. The organism early on in its evolution did not have the advantage of large susceptible populations that resulted from the Neolithic revolution of domestication and the development of agriculture. It is hard to imagine how the early hunter-gathers living in small groups could have sustained *M. tuberculosis* without the benefit of activation of transmissible disease in previously healthy infected individuals who were able to travel long distances. We speculate that, in the latter part of its history, TB has shifted from being a heritage disease to a crowd disease, and the opportunities afforded by a growing susceptible host population may have led not only to increased transmission, but also to a more aggressive stance against innate immune defenses, leading to epidemic spread rather than persistence.

It is interesting in this context that we may be witnessing a shift in transmission of another major mycobacterial disease leprosy. Leprosy is caused by *Mycobacterium leprae*, which appears to have also evolved from the common *M. tuberculosis-M. marinum* ancestor (Figure 3). *M. leprae* is particularly intriguing because it has undergone substantial gene reduction to the point where it has lost its capacity for axenic growth (Cole et al., 2001). At the same time, it has become specialized in its pathogenic niche, infecting Schwann cells of the peripheral nervous system through complex mechanisms (Masaki et al., 2013). For most of its pathogenic human history, *M. leprae* has been a strict human pathogen, with transmission occurring only through prolonged contact with infected humans. Yet, in very recent times, the nine banded armadillo in the Southeastern United States became infected from humans and has now become a full-fledged reservoir for disease, so that leprosy is now mainly a zoonotic disease in this area (Truman et al., 2011). Albeit in a less dramatic way, *M. tuberculosis* must clearly have made adaptations to the very great changes in human lifestyles to retain its success. Understanding these changes may have more than academic value; it may help us better understand the disease itself and hence its treatment. The parallel evolution of pathogens to keep up with changing environments is hardly unique to mycobacteria but is shared with many, many other pathogens.

Finally, our recent work has repeatedly confronted us with the fact that TB is not as much a disease of failed immunity as it is of coevolution. At every step of infection, the bacterium appears to be inducing and benefitting from an over-exuberant response using the very inflammatory pathways that are thought to have evolved to thwart bacteria—CCL2 induction to enter the host, MMP9 induction to expand in the granuloma, and finally TNF and T lymphocytes to exit the granuloma for transmission. Despite all of this, both host and pathogen have prevailed. That is why we consider *M. tuberculosis* to be a paradigm of a host-adapted microorganism. It has coevolved with the human immune system, discarding and gaining genes to be in tune

with it. As an ancient disease agent adapting to humans, the microbe could not have anticipated that humans would be so successful. Yet its stealth and subtlety have allowed it to thrive even in the face of modern medicine. While new therapeutic avenues will most likely be found, we must not undervalue the power of genetic selection for the survival of any microorganism—and, based on past performance, particularly for *M. tuberculosis.*

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