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The evolutionary pressures that have molded *Mycobacterium tuberculosis* into an infectious adjuvant

David G. Russell
Microbiology and Immunology College of Veterinary Medicine Cornell University Ithaca, NY 14853

Abstract

Mycobacterium tuberculosis is highly immunogenic yet appears to have evolved to preserve its antigenicity. The retention of antigenicity is important to the maintenance of a robust immune response that contributes greatly to the late-stage tissue damage required for transmission and completion of the pathogen's life cycle. Bacterial persistence is achieved through the remodeling of the tissue at site of infection and maintaining the lymphocytes distant to the infected macrophages in the granuloma core. The tissue metabolism within the granuloma leads to lipid sequestration that supports bacterial growth. However, growth on host lipids places metabolic stresses on Mtb, which has evolved to incorporate potentially harmful metabolic intermediates into the very cell wall lipids that induce the remodeling of the host tissue response.

Introduction

Mycobacterium tuberculosis (Mtb) is a human-specific pathogen with an impressive penetrance of its host population. In the modern era, infection with Mtb leads to active disease in approximately 5-10% of those individuals during the course of their lifetime. This ability to infect many yet cause active disease in only a few at any given instance has likely contributed to the pathogen's success through co-evolution with its host. The life cycle of the pathogen is shown in Figure 1, which highlights the key points discussed in this article.

Co-evolution of Mtb and mankind

Many publications, even recent ones, describe Mtb as a zoonosis evolving from the bovine pathogen *Mycobacterium bovis* (Mb) during the development of agriculture around 12,000 years ago [1-3]. However sequencing of both genomes over 10 years ago revealed that the Mtb chromosome was clearly larger than the Mb chromosome, and the size difference was due predominantly to deletions [4-6]. More recent whole genome analysis of Mtb strains representing the sequence diversity in the Mtb complex across the globe generates a fascinating picture of the co-evolution of this pathogen with its human host [7]. The proto-Mtb strain likely existed across Central Africa and infected mankind as they emerged from Africa around 50,000 years ago. Mankind carried the pathogen as they radiated out across the planet and the bacteria evolved independently in different geographic locations until the waves of colonialism lead to the neo-colonization of South American and Southern Africa

Tel 607 253 4272 FAX 607 253 4058 dgr8@cornell.edu.

from the 1500's onwards. This period of human migration introduced European and Asian strains back into Africa so that the current strain collection on that continent is a genetic mosaic that documents the human migration from its emergence from Africa to its recent re-introduction.

Had Mtb been derived from Mb in the Fertile Crescent 12,000 years ago then much of mankind would have evolved absent selection pressure from Mtb. Were this to be true, one would expect considerable heterogeneity in human susceptibility to this pathogen. But while differential susceptibility to tuberculosis is present, it is comparatively subtle [8-11], except for those catastrophic phenotypes that would be non-sustainable genetic traits absent modern medicine [12]. Such extreme deficiencies have questionable evolutionary significance. The marginal susceptibilities observed are easier to rationalize if, as is now accepted, all of mankind ran the gauntlet of Mtb infection during their emergence from Africa.

Mtb proteins exhibit minimal evidence for antigenic diversity

But what of the evolutionary pressures that shape the Mtb genome? The bacterium requires the human host in order to replicate so clearly must have evolved under consistent selective pressure. However, this selective pressure lacks the environmental diversity experienced by free-living organisms and, like many pathogenic organisms, Mtb has experienced genomic down-sizing [13]. As with any pathogenic organism immune avoidance would constitute a major evolutionary pressure. However, when Hershberg and colleagues extended their analyses to examine the ratio of the rates of non-synonymous and synonymous changes (dN/dS) in 89 genes representing housekeeping genes, virulence-associated genes, and genes encoding proteins that were surface-exposed or secreted they found, surprisingly, that all 3 categories of genes demonstrated comparable ratios of dN/dS mutations [7]. The authors concluded that these genes were selection-neutral with respect to the immune interface with the host.

Mtb contains 2 large gene families that encode the PE and PPE proteins, which have been postulated to represent antigenic variation in Mtb [14]. The genes are thought to encode secreted or cell wall-associated proteins that would interface closely with the host. Both these families of genes show a high degree of genetic variation between family members from different isolates, and the mechanisms of variation, SNPs, intels and deletions also vary considerably. However, analysis of SNPs across an extensive subset of *pe* and *ppe* genes revealed a dN/dS ratio close to 1 suggesting that there was no selective advantage for productive mutations [15]. These data indicate that, absent the highly conserved PE region of the protein thought to be involved in association with the cell wall, there is little selective or purifying pressure fixing structural variants. This result adds context to an earlier study where Comas and colleagues found that antigenic epitopes in other genes were highly conserved, which actually implies strong selective pressure against sequence diversity in immunogenic regions [16]. The suggestion that there is no negative selection against Mtb being immunogenic is contrary to most accepted notions of how a pathogen should evolve. Moreover, Mtb is a rich source of agonists for both the TLR and NOD pattern receptors, the activation of which is known to augment the strength of any developing adaptive immune response [17-21].

Why would Mtb not care if it is immunogenic?

At the level of the infected macrophage this seems a bad idea. The macrophage is an antigen-presenting cell capable of informing both $CD4^+$ and $CD8^+$ cells of its infection status, and if activated by interferon-γ (IFN-γ) capable of either killing Mtb or rendering it non-replicative [22]. In the majority of animal models infection by Mtb is marked by a period of rapid bacterial replication preceding the development of the adaptive immune

response. Several investigators have noted that this immune response is delayed in comparison to other infections. Wolf and colleagues reported that in murine infections the immune response is driven by the antigen burden in lymph nodes and not in the lung, the major site of bacterial expansion [23]. More recently, temporal analysis of the progression of infection in the rabbit model, which generates a more human-like granuloma, confirmed the delayed nature of the immune response [24]. Transcriptional profiling revealed a delay in peak expression of genes involved in macrophage activation and anti-microbial responses in the lung to 8-12 weeks post-infection. Both these observations suggest either that the lung is a privileged site for Mtb infection, or that the bacterium is able to modulate the immune response at site of infection. This delay is likely critical to the success of the infection allowing bacterial replication and preliminary remodeling of the infection site prior to the development of an adaptive immune response.

Once an adaptive immune response develops, the bacterial load in most immune-competent animal models plateaus. Whether this sub-clinical, or “latent” infection is the product of non-replicating or slowly-replicating bacteria is unclear. Ford and colleagues studied the rate of mutation or SNP acquisition in Mtb throughout the course of disease in Cynomolgous macaques and found that the rates were constant during latency and reactivation, and were the same as those in a logarithmically-growing culture [25]. These data are broadly consistent with those of Gill and coworkers who used Mtb transformed with a replication or “clock” plasmid to measure bacterial replication rates in a murine infection model [26]. They observed sustained loss of the plasmid throughout the course of the infection, although the rate of loss was enhanced by immune suppression of the mouse with dexamethasone indicating that rates did vary as a consequence of immune pressure. These data indicate sustained replication, and therefore a sustained capacity for mutation.

Tempering the impact of immunity through the granuloma

The key to appreciating how Mtb manages host immune pressure comes with the realization that Mtb actually requires the adaptive immune response to complete its life cycle. The late stage tissue damage that culminates in transmission is driven by the host immune response therefore the bacterium has to find other means of modulating host immune function without impairing the robustness of the systemic immune response.

A human TB granuloma in possesses several conserved characteristics, however, within an infected individual you can find granulomas in many different forms, Figure 1B. These are usually discussed as different stages in a continuum [27] but, while this is a useful vehicle, we have little evidence which characteristics are indicative of progression to active infection [28]. The classic granuloma has a caseous center surrounded by a macrophage-rich zone in which you can observe multinucleated giant cells, and foamy macrophages. The bulk of the bacilli are in this region. Outside this are epithelioid macrophages and the collagen-rich fibrotic capsule. Lymphocytes are present in low number within this capsule but both are abundant around its periphery. Transmission is postulated to occur when the necrotic center of the granuloma collapses into the airways release viable, infectious bacteria, however recent reports from both human infections and primate models indicate that neutrophils may carry bacteria to the airways during late stage inflammation without the necessity for cavitation of the granuloma [28,29], Figure 1C.

Although host-derived, the granuloma likely represents the efforts by Mtb to shape the immune response and blunten its edge locally. Mycobacterial lipids released by intracellular Mtb traffic from infected to uninfected cells. When inoculated into mice these lipids can induce many of the characteristics of the Mtb granuloma [30-32]. Trehalose dimycolate, TDM, appears the most bioactive of the bacterial effectors [19]. Although the environment

within the granuloma does not represent the extremes experienced by free-living bacteria, it does present a range of conditions that are potentially limiting to bacterial growth [27,28]. The manner in which the bacterium senses and responds to these pressures is key to its success. Although extracellular bacteria can be observed in granulomas, the ability of Mtb to sustain its infection in the macrophage is crucial. Clearly the bacterium's ability to assess its environment and respond accordingly would be an important pressure shaping its genome.

The intracellular environment shapes bacterial metabolism

Mtb is able to arrest the normal maturation of its phagosome and resides in a vacuole that retains many of the characteristics of a sorting endosome [33]. It has a pH of 6.4, and it remains accessible to early endosomal contents. However, upon activation of the macrophage this blockage is overcome and the bacterium is exposed to a lower pH, more hydrolytically-competent environment. The physiological gradients in the endosome-lysosome continuum provide useful cues to Mtb. pH is well studied and Mtb is known to respond robustly to the acidification of its environment [34,35]. Interestingly, the response to pH can be divided into two types; the physiological response, linked to the maintenance of cytosolic homeostasis [36,37], and an adaptive response that is not obviously linked to pH homeostasis but appears connected to metabolic shifts required for growth in the endosomal continuum [38].

Mtb senses pH through the two-component sensor PhoPR and mutants deficient in *phoPR* are avirulent [39,40]. We have studied the transcriptional response on Mtb during invasion of the macrophage and the reduction in pH appears responsible for approximately 50% of the genes up-regulated early in infection [34]. Amongst the genes up-regulated is a 3 gene operon, *aprABC*, which appears to have been acquired by horizontal gene transfer and is unique to the Mtb complex [38]. Deletion of the operon leads to impaired survival in macrophages and a loss of production of polyketide-containing cell wall lipids, such as the virulence-associated phthiocerol dimycocerosates, PDIMs [41].

Recent literature indicates that intracellular Mtb relies of host cholesterol and fatty acids as major carbon sources [42-44], however the utilization of cholesterol can come at a cost to Mtb. Degradation of the side chain of cholesterol gives rise to propionyl-CoA [45-47]. Mtb is exquisitely sensitive to increases in the propionyl-CoA pool and the bacterium has three different means of metabolizing this precursor of potentially-toxic metabolite(s) [48-50]. ICL1 catalyzes the last reaction of the methylcitrate cycle (MCC) that converts methylisocitrate to succinate and pyruvate that feeds into the TCA cycle [43,48]. Alternatively, propionyl-CoA carboxylase can generate methylmalonyl-CoA that will enter the methylmalonyl pathway (MMP) leading to the production of succinyl-CoA [49]. Finally, and of considerable significance to infection, these 3-carbon intermediates in the form of methylmalonyl-CoA may be used as building blocks for the bacterium's cell wall lipids [51-53]. These bioactive lipids includes phthiocerol dimycocerosates (PDIM), sulfolipid-1 (SL-1), diacyltrehalose (DAT), triacyltrehalose (TAT), and polyacyltrehalose (PAT) [54], which provide an effective "sink" for excess propionyl-CoA as well as a means of manipulation of the host tissue response [19,30-32,55,56].

Subversion of host tissue metabolism to support infection

Transcriptional profiling of caseous human TB granulomas [56,57] revealed dysregulation of lipid metabolism as a pathogen-induced pathology that may drive tissue breakdown. Host proteins key to lipid overload and sequestration localized to cells subtending the caseum by immuno-histology. Furthermore, the caseous core of the TB granuloma contained triacylglycerides, cholesterol and cholesterol ester, together with sphingomyelin and lactosylceramide. The presence of cholesterol ester indicated that foamy macrophages were

the most likely source of the lipids in the caseum. Foamy macrophage formation is induced by Mtb infection of macrophages in culture, and most significantly, by trehalose dimycolate (TDM)-coated beads inoculated sub-cutaneously into mice [56,58]. Co-incidentally, TDM is an excellent sink for propionyl-CoA. The data imply that the dysregulation of lipid metabolism is a pathogen-induced phenomenon that plays a key role in the pathology associated with progression to disease [56,57], and may also expand the nutrient pool accessible to the bacilli [59].

Concluding Remarks

Mycobacterium spp. are highly immunogenic and were used previously as constituents in Freund's Complete Adjuvant. While it is not unusual for pathogens to induce an inflammatory response to promote transmission, such as *Vibrio cholera* and *Salmonella*, such a trait is atypical of a chronic infection. Instead of diminishing its immunogenicity Mtb has evolved to model its site of infection to support its persistence despite a strong immune response. This remodeling of the granuloma leads to the exclusion of lymphocytes and the dysregulation of host lipid homeostasis, both of which appear to favor bacterial survival. In a pathogen that relies so heavily on lipid metabolism both for nutrition and for the synthesis of effectors to regulate host behavior it is unsurprising that it has evolved to devote a significant portion of its genome [4] and the majority of its core intracellular transcriptome [60] to these activities. The fact that Mtb has evolved to sustain an infection in the face of a robust systemic immune response remains a considerable problem that has yet to be addressed effectively by any vaccine development program.

Acknowledgments

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Highlights

1. Mtb has evolved with mankind for 50,000 years after human migration from Africa.
2. Mtb shows minimal genetic mutations linked to the avoidance of antigenicity.
3. Mtb is immunogenic, which is critical to the tissue damage required for transmission.
4. Mtb avoids the systemic immune response by local modulation of the infection site.
5. The strategies employed by Mtb have serious consequences for vaccine development.

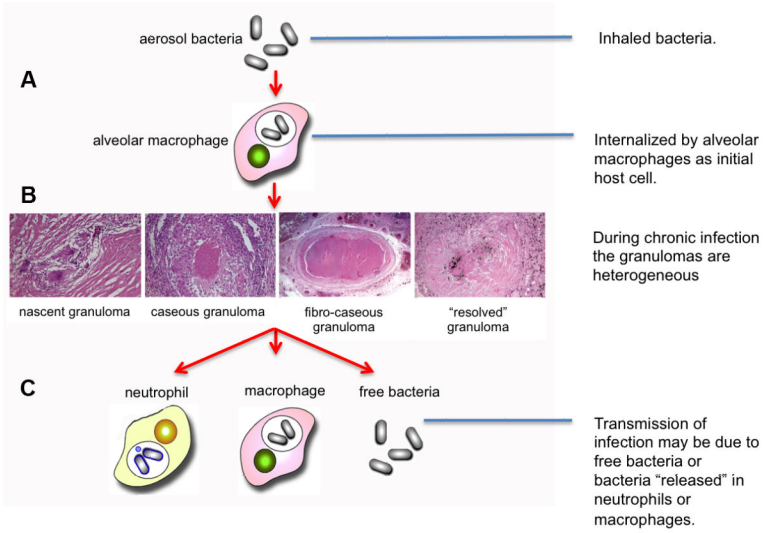

Figure 1. The infection cycle of *Mycobacterium tuberculosis*

(A) Infection is initiated by inhalation of infectious bacilli that are likely internalized by alveolar macrophages that patrol the airway surfaces of the lung. Mtb induces a proinflammatory reaction through the activation of TLR and NOD pathways and initiates the formation of a macrophage-centric granuloma. (B) During the chronic or latent phase of infection in humans one can observe a wide variety of granulomas in lung tissue that vary from the productive to the controlled. The phenotype(s) of the productive, infectous granulomas have yet to be formally determined. (C) While transmission is conventionally regarded as cavitation of an infected granuloma(s), there is an increasing body of data arguing that migration of infected neutrophis or macrophages to the airways plays a critical role in the release of bacilli and induction of transmission .