Mycobacterium tuberculosis and macrophages: who is the boss?

Varshneya Singh and Kanury V. S. Rao*

Immunology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India

***M. tuberculosis*, the causative agent of tuberculosis, has persisted in the human population despite decades of intervention strategies. While our knowledge about the pathogen and the disease process it causes have grown substantially over the years, we are still grappling with devising strategies for its control. This article attempts to understand the infection biology of *M. tuberculosis* and the importance of foamy macrophages in the entire disease process. It also highlights the diverse virulence properties displayed by the *M. tuberculosis* secretory protein, ESAT-6 and touches upon the problem of drug resistance in tuberculosis and the various approaches being employed to deal with it.**

Keywords: Drug resistance, ESAT-6, foamy macrophages, GPR109A, *Mycobacterium.*

Introduction

IT was in the year 1882 when Robert Koch isolated and described the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis* (*Mtb*). More than a century later, *M. tuberculosis* continues to persist as a leading cause of morbidity and mortality worldwide. The World Health Organization (WHO) estimates that one-third of the world's population is infected with *M. tuberculosis* and that more than 8 million new cases of active TB occur annually. TB is transmitted by aerosols containing *Mtb*, released from the lungs of an infected individual through coughing and subsequently infecting the alveolar macrophages in a new host[1]. Although macrophages are designed to kill pathogens, mycobacteria have evolved effective mechanisms that neutralize the anti-microbial responses of the macrophage. Such mechanisms include inhibition of phagosome–lysosome fusion and suppression of other processes such as ROI/RNI production, antigen presentation and host cell apoptosis[2].

The remarkable success of *M. tuberculosis* as a pathogen is also closely associated with its ability to persist in humans for extended periods without causing disease. The resulting reservoir of latent infection greatly complicates efforts aimed at TB control as it requires prolonged drug therapy presumably due to persistence of the dormant tubercle bacilli that are refractory to current treatment regimens. TB is treated with several drugs used in combination. These include isoniazid, rifampin, pyrazinamide and ethambutol. Presently the only vaccine for *M. tuberculosis* is an attenuated strain of *M. bovis* called *M. bovis* BCG (Bacillus Calmette Gúerin). However, the efficacy of this vaccine appears to be restricted to providing protection against extrapulmonary TB in children only. Although management of TB has always been difficult, the problem is further exacerbated today because of two major issues: the HIV epidemic and the increasing incidence of drug resistance. The two most populous countries of the world, China and India account for 40% of all TB cases worldwide and also have significantly high rates of multi-drug resistant (MDR)-TB[3]. In recent years MDR-TB and extensively drug resistant TB (XDR-TB) have gained recognition as a potentially catastrophic challenge to global public health. A recent report from India described four TB patients infected with an *Mtb* strain showing phenotypic resistance to twelve drugs examined and the term totally drug-resistant TB (TDR TB) was coined[4]. These findings have reinvigorated interest in the development of new drugs that can address this looming problem.

This article discusses some of the salient aspects related to understanding the infection biology of *M. tuberculosis* and the relevance of these studies for TB drug development. In this context it specifically emphasizes the central role of foamy macrophages in disease and underscores the diverse virulence properties displayed by the *M. tuberculosis* secretory protein, ESAT-6. Finally, we discuss the problem of drug resistance in TB and approaches being considered to combat it. Here, recent data from our laboratory pertaining to these issues is also described.

Mycobacterial strains: from the laboratory to the clinic

The *M. tuberculosis* H37-strains, *M. tuberculosis* Erdman, *M. bovis* BCG and *M. smegmatis* are most commonly used to study the pathogenesis and virulence mechanisms of mycobacteria in different *in vivo* and *in vitro* systems. H37 is a laboratory strain of *M. tuberculosis* that was isolated from a 19-year-old pulmonary TB patient in 1905 and based on virulence in guinea pigs later dissociated into a virulent

*For correspondence. (e-mail: kanury@icgeb.res.in)

strain (H37Rv) and an avirulent strain (H37Ra)[5]. Although both strains can be cultured in the laboratory on suitable nutrient rich medium, only the virulent H37Rv strain is capable of growth and replication inside human macrophages[6]. H37Rv and H37Ra differ genetically and phenotypically and the major difference lies in a mutation in the *phoP* gene which leads to a defect in the secretion of ESAT-6, from the avirulent strain H37Ra[7]. Of late, *M. marinum* has also found favour, as it shares several features of *Mtb*, is easy to handle as it is less prone to cause disease in humans and can be used to infect zebrafish embryos and the amoeba Dictyostelium[8].

However, naturally occurring *M. tuberculosis* infections in humans are caused by strains that vary widely in their genotype and phenotype. Initially it was believed that little sequence variation existed in *M. tuberculosis*, but a whole-genome comparison of the *M. tuberculosis* clinical (CDC 1551) and the laboratory strain (H37Rv) demonstrated that polymorphisms among *M. tuberculosis* strains are more extensive than initially anticipated[9]. Since then, 6 main lineages and 15 sublineages of the pathogen have been described exhibiting a phylogeographic population structure[10]. Several lines of study have focused on the *M. tuberculosis* Beijing genotype, in understanding how genetic background of *Mtb* strains influences the disease pathology and progression. Strains of the *M. tuberculosis* Beijing genotype were first identified in 1995 from TB patients in China and Mongolia[11] but today it has a global representation. The Beijing genotype is believed to be hyper-virulent compared to most other strain genotypes and this may be due to differences in its interaction with the host immune system[12]. The Beijing strains also have an increased ability to become drug resistant[13].

Although there have been now, several successful attempts to characterize these clinical isolates genotypically, we still know very little about most of these clinical isolates in terms of their basic biology, virulence, *in vivo* pathogenicity, interactions with the host and susceptibility to new vaccines and chemotherapeutics being developed against TB. Independent studies carried out in our laboratory show that the clinical isolates show diverse growth properties in THP-1 macrophages and in human peripheral blood derived monocytes with apparent growth rates that are either significantly higher or lower than that of the laboratory strain, H37Rv[14,15]. It was also observed that *Mtb* exhibits strain-specific variations in the dependence on the host cellular machinery for intracellular survival[14] and an aerosol infection of guinea pigs with different clinical strains of *Mtb* resulted in a wide range of virulence[16]. All these observations point towards the importance of including such clinical isolates in studies aimed at understanding the virulence and pathogenicity of *M. tuberculosis* and for testing new vaccines and experimental TB drugs, as results obtained with the laboratory strains alone might not represent the true picture of what happens in naturally occurring TB.

Foamy macrophages, a secure haven for *M. tuberculosis*

Accumulation of lipid bodies (LBs) within different cell types is induced following infection with pathogens such as bacteria[17], parasites[18] and viruses[19]. The LBs have a distinctive architecture – a core containing neutral lipids mainly triacylglycerols (TAG) and sterol esters (SE) surrounded by a phospholipid hemimembrane with associated proteins such as perilipin[20]. Foamy macrophage (FM) is a cell population, characterized by its high lipid content compartmentalized in LBs that impart the foamy phenotype. The association of FMs with TB was made as early as in 1925 by Pagel, when it was shown that postprimary TB begins as lipid pneumonia, with the accumulation of large amounts of lipid-rich macrophages. These FMs populate the center of granulomas in the lung and contribute to the first appearance of the disease as lipid pneumonia[21]. Foamy macrophages are found within granulomatous structures in both animal and human models and the production of FM starts from the onset of infection by progressive accumulation of LBs[22]. Ongoing death of these cells causes lipid accumulation as caseous debris and, with time, this process induces cavitation and the eventual release of bacteria for further dissemination[23].

In an *in vitro* model of human tuberculous granuloma, it was shown that the virulent strains, *M. tuberculosis* and *M. avium* induce the rapid differentiation of phagocytes into foamy macrophages, whereas the avirulent *M. smegmatis* does not[17]. The presence of oxygenated mycolic acids in the former, but not in the latter strain was believed to induce this maturation of macrophages into FMs. Observation of *M. tuberculosis*-infected FMs by electron microscopy showed that the mycobacteria-containing phagosomes migrate towards host cell LBs, a process which culminates with the engulfment of the bacillus into LBs[17]. LB encapsulation provides the pathogen with ready access to nutrients in the form of fatty acids which is accumulated by the bacilli in the form of intracytoplasmic lipid inclusions. The foamy macrophages lose their phagocytic and bactericidal activities like the ability to develop a respiratory burst, which is a major intracellular bactericidal activity. The bacilli engulfed within LBs were also shown to slow down their replication and concomitantly acquire phenotypic resistance to at least two frontline drugs rifampicin and isoniazid[24]. Thus, residence in specialized lipid-rich cells, rather than in conventional macrophages is extremely beneficial for *Mtb* as the FMs form a secure reservoir for the tubercle bacilli for their long-term persistence in the human host.

Results from our laboratory reveals yet another level at which FMs contribute towards safeguarding the intracellular bacteria. This study was based on two apparently related observations, (a) virulent strains of *Mtb* actively suppress autophagic pathways[25] and (b) regulation of lipid homeostasis by autophagy, where basal autophagy mediates lipid turnover but an increase in the pool size of

intracellular lipid stores inhibits this process[26]. Taking cue from these findings, it was found that the accumulation of LBs in *M. tuberculosis* H37Rv-infected macrophages correlated with an inhibition of both autophagy and lysosome acidification[15]. Chemical inhibition of Acyl-CoA cholesterol acyltransferase 2 (ACAT2), an enzyme that catalyses synthesis of cholesteryl esters (a key constituent of LBs), inhibited LB formation in *Mtb*-infected macrophages. A direct result of these effects was the release of bacilli from their lipidic environment and their consequent co-localization with autophagosomes and/or lysosomes. In other words, inhibition of *Mtb*-induced LB formation shifted the host cellular response in favor of vesicular degradation of mycobacteria, leading to increased bacterial killing[15].

Our subsequent studies then identified the pathway by which intracellular *Mtb* manipulated the macrophage response to induce the foamy phenotype. Key to this process was the ability of virulent mycobacteria to induce the host macrophage into producing and secreting 3-hydroxybutyric acid (3HB). Interestingly, this molecule is a constituent of ketone bodies that are produced in the liver. These compounds are normally generated as a result of fatty acid oxidation and elevated levels are found in the blood of individuals with uncontrolled diabetes. In the case of *Mtb*-infected macrophages however, we found that 3HB production was a consequence of increased rates of glucose uptake by the host cell. This in turn led to accumulation of excess acetyl-CoA, which was then shunted towards the synthesis of 3-HB. Subsequent secretion of this compound caused feedback activation of a Gi-protein coupled receptor (GiPCR), GPR109A (ref. 15). GPR109A belongs to the subfamily of hydroxycarboxylic acid receptors and activation of this receptor results in an inhibition of adenylyl cyclases[27]. The consequent reduction in cellular cAMP levels led to attenuated protein kinase A (PKA) dependent signalling, as a result of which the turnover rate of TAGs was markedly reduced. It was this reduction in TAG lipolysis that caused a buildup of LBs within the *M. tuberculosis*-infected macrophage.

Interestingly, GPR109A was also identified as one of the hits in our earlier genome-wide RNAi screen against host factors that regulated mycobacterial growth in the macrophage[14]. Thus, this example serves to provide a glimpse of the elaborate mechanisms that *M. tuberculosis* has evolved in order to fine-tune functions of host macrophage to facilitate its own survival in the hostile intracellular milieu. Our future efforts are aimed at delineating how functions of the other proteins identified in the screen are modulated, during the crosstalk between the pathogen and the host cell.

A new virulence mechanism of the *M. tuberculosis* secretory protein, ESAT-6

The *Mtb* virulence factor, early-secreted antigenic target (ESAT-6) has received great attention in recent years. It is the prototype for a family of related proteins such as CFP-10 and CFP-7 (ref. 28). There are 23 ESAT-6 family members in *M. tuberculosis* strain H37Rv. ESAT-6 is secreted in a 1 : 1 heterodimeric complex with 10 kDa culture filtrate protein (CFP-10) by a secretion system called the ESX-1 system or type VII secretion system. The system is encoded by the region of difference 1 (RD1) of the mycobacterial genome and is conserved in several mycobacterial species including *M. tuberculosis*, *M. marinum* and *M. bovis*. However, repeated passage of *M. bovis* to obtain the vaccine strain BCG led to deletion of the RD1, resulting in attenuation[29]. More recently *M. microti*, another live TB vaccine strain, was found to be deficient in the synthesis of ESAT-6, owing to a different deletion in the RD1 region[30].

ESAT-6 has multiple virulence mechanisms, but the best studied is its role in membrane pore formation. In *M. marinum*-infected macrophages, it was shown that ESAT-6 can lyse the phagosomal membrane, allowing escape of the bacillus into the cytoplasm of the macrophage and subsequent pore formation in the cell membrane leading to spread[31]. ESAT-6 also contributes to the translocation of *Mtb* from the phagolysosomes to the cytoplasm in myeloid cells[32]. More recently, Kinhikar *et al.*[33] have shown that ESAT-6 is a laminin-binding adhesin of *Mtb*, that can cause contact lysis of both type 1 and type 2 pneumocytes. The damage to the alveolar epithelium allows *Mtb* to acquire a highly invasive phenotype *in vivo* and cytokine release by the pneumocytes could elicit enhanced migration of inflammatory cells to the alveoli thus contributing to granuloma formation. There are also reports suggesting that ESAT-6 or the CFP-10/ESAT-6 complex of virulent *M. tuberculosis* inhibits IL-12 and TNF-α production from infected macrophages and thus attenuates the innate immune response[34]. Together, these studies imply that ESAT-6 might contribute to mycobacterial virulence by having roles in cellular invasion, escape from phagolysosomes, cell-to-cell spread and dissemination of *Mtb*.

Work done in our laboratory identifies ESAT-6 as a critical mediator of FM differentiation, thus adding a new role by which this protein contributes towards the expression of mycobacterial virulence[15]. The activation of 3HB production by infected macrophages, which induces FM differentiation through the anti-lipolytic GPR109A, was found to be specific to virulent but not attenuated strains of *Mtb* and correlated with the ability to synthesize and secrete ESAT-6. Addition of the recombinant *Mtb* ESAT-6 protein purified from *E. coli* to THP-1 macrophages was also sufficient to induce 3HB production by the macrophages. However the ability to induce 3HB synthesis was not shared by its counterpart CFP-10. In looking at the mechanism as to how ESAT-6 induces FM differentiation, it was observed that glucose uptake by macrophages is accelerated in the presence of ESAT-6. This then translated into an increase in the acetyl-CoA to

oxaloacetate ratio in the macrophages favouring increased synthesis of the ketone body 3-HB, which then drives the FM differentiation. A schematic of the feedback mechanism deduced from this study is illustrated in Figure 1. More importantly, in the same study it was found that addition of exogenous ESAT-6 protein to macrophages infected with attenuated/avirulent strains of *Mtb* restored their ability to synthesize 3-HB, form the foamy phenotype and also improved the intracellular viability of these avirulent strains[15]. Thus this study gives credence to earlier reports indicating that mutations that abolish production or secretion of ESAT-6 confer an attenuated phenotype.

While the study carried out in our laboratory has identified ESAT-6 as a critical component for foamy macrophage differentiation, it is quite possible that this protein may act in conjunction with other *Mtb*-derived molecular components such as oxygenated mycolic acids[17], trehalose dimycolates[35] or other secreted proteins. And as discovered with ESAT-6, other molecular components of *M. tuberculosis* might also contribute to expressing the mycobacterial virulence in more ways than one, thus making it one of the most successful pathogens known in human history.

Drug resistance in TB and the current pipeline of anti-TB drugs

Treatment of TB consists of a standardized 6-month (or 8-month in previously treated TB cases) chemotherapy. This involves treatment with isoniazid, rifampicin, pyrazinamide and ethambutol in the intensive phase and with isoniazid and rifampicin in the continuation phase. Although resistance to anti-TB drugs has always presented a problem, this aspect has acquired a more serious and urgent dimension in recent years. Drug resistance in *M. tuberculosis* results from spontaneous and random mutations in the bacterial chromosome, which reduces the susceptibility to specific agents[36]. The prevalence of resistant clones in populations of wild type *M. tuberculosis* is extremely low with a mutational frequency of 1 in 10^6 bacilli for isoniazid and 1 in 10^8 bacilli for rifampicin[36]. The selective pressure that is exerted in the presence of the anti-mycobacterial agent, favours the selection and multiplication of the mutant bacilli. Clinically, drug resistance is divided into two types: primary resistance and acquired resistance. Primary resistance occurs when people get infected with a resistant strain of *M. tuberculosis* and acquired resistance develops during TB therapy, due to inappropriate use of anti-TB drugs, poor compliance and poor drug quality. TB drug resistance can be in the form of single drug resistant, multi-drug resistant (MDR-TB) which, is defined as resistance to isoniazid and rifampicin and extensively drug resistant (XDR-TB) which is defined as resistance to isoniazid, rifampicin, any fluoroquinolone and one of the second line injectable drugs (kanamycin, amikacin or capreomycin). In a study carried out recently it was found that the global proportions of new and previously treated TB cases showing multidrug resistance were 3.4% and 19.8% respectively[37]. Furthermore, XDR-TB has now been identified in as many as 77 countries globally and the proportion of MDR-TB cases with extensive drug resistance was 9.4% (ref. 37). The near-epidemic scenario that is now emerging in endemic regions due to the rapid spread of drug-resistant TB necessitates the immediate identification of new scaffolds that can translate into effective drugs and address this problem of drug resistance in TB.

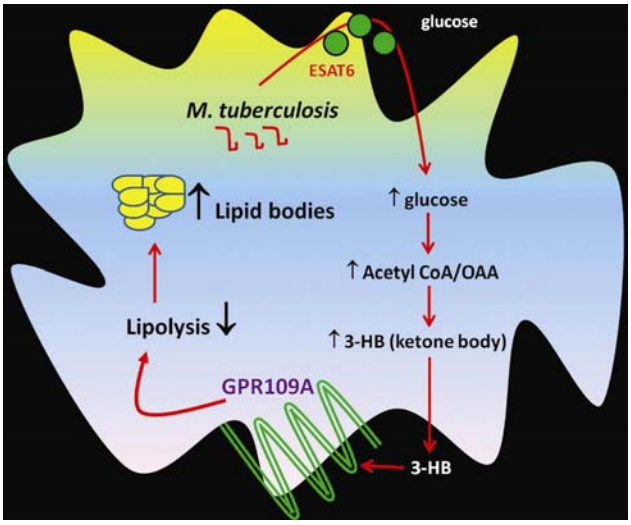

Figure 1. A schematic of the inferred mechanism for FM differentiation by virulent strains of *Mtb*. *Mtb* secretes the ESAT-6 protein inside the infected macrophage, which then stimulates glucose uptake by the infected cell. This likely enhances the rate of glycolysis, leading to a net accumulation of acetyl–CoA and an increased acetyl–CoA/OAA ratio which then favours the subsequent generation of 3HB through the HMG CoA cycle. The secreted 3HB activates GPR109A, causing inhibition of adenylyl cyclase activity and thus decreasing cellular cAMP levels. This attenuates the activity of PKA thereby decreasing lipolysis within the cell. This reduction in TAG turnover causes enhanced LB accumulation in the macrophage and appearance of the foamy phenotype[15].

Most of the drugs that are used in the first line of TB treatment were discovered way back during the 1950s and 60s. The rapid emergence of drug-resistant strains and the rise in cases of TB–HIV coinfections have heralded attempts towards discovery of new TB drugs. The fluoroquinolones gatifloxacin and moxifloxacin, which are commonly used for the treatment of respiratory tract infection are currently in phase-3 clinical trials for the treatment of TB. These agents specifically target the mycobacterial topoisemerase II DNA gyrase[38]. The compound TMC207, a novel diarylquinoline, was discovered in a whole-cell-screening exercise using *M. smegmatis*. This compound inhibits the proton transfer chain of the mycobacterial ATP synthase[39] and is currently being developed by Tibotec in collaboration with the TB alliance. Metronidazole, a drug used to treat infections caused by protozoa and anaerobic bacteria, is being used in a phase-2 study to explore the effect of addition of this compound to the standard second line drug regimen of TB. The bicyclic nitroimidazofuran, PA-824 has also been found

to display antitubercular activity[40]. The synthetic analogue of ethambutol, SQ109 is currently being evaluated in a phase-2a trial in adults with smear positive pulmonary TB[41]. The sulphur containing heterocyclic compounds called benzothiazinones (BTZ) which target the mycobacterial enzyme decaprenylphosphoryl-β-d-ribose 2′-epimerase (DprE1) are also being evaluated[42].

To complement the existing approaches we have adopted a strategy of targeting host factors that are coopted by the pathogen to ensure their intracellular sustenance. The underlying rationale here derives from the fact that adaptation and persistence of *Mtb* within the hostile environment of the host macrophage depends upon its ability to manipulate the host cellular machinery. Our reasoning therefore is that targeting host factors that govern such pathways should provide an alternate mode of therapy. Importantly, we anticipate that such an approach should also be capable of eliminating drug-resistant and latent infections, with minimal selective pressure on the pathogen to acquire new resistant phenoytpes. To this end, we first identified the host factors that are required for survival/persistence of intracellular mycobacteria. This was achieved by performing a genome-wide siRNA screen against human macrophage cells (THP-1) that had been infected with H37Rv, a virulent strain of *Mtb*. Through an iterative process we then shortlisted the 275 proteins identified, to a core subset of 74 host factors whose presence was found to be critical for survival of diverse clinical strains[14]. Subsequently, we also demonstrated 'proof-of-concept' that targeting such key host factors indeed provides a viable and potentially useful approach for the chemotherapy of TB[43]. Also relevant here is our confirmation of the fact that such a strategy was indeed equally effective against diverse *Mtb* genotypes and was also insensitive to the drug-sensitivity profile of the infecting strain. As discussed above, our subsequent interrogation of survival mechanisms adopted by *Mtb* highlighted GPR109A as an important target for further drug development. This was further supported by experiments in the murine model of TB infection where pharmacological inhibition of this receptor led to effective clearance of infection either with drug-sensitive, MDR and XDR strains of *Mtb*[15]. Our current efforts are focused on improving the drug-like properties of this inhibitor for further studies.

Conclusion

M. tuberculosis has been one of the most successful pathogens in human history. This should not come as a surprise given the plethora of mechanisms that this pathogen has evolved to establish its stable residence within the host macrophage. At least one facet of this successful strategy is represented by the ability of pathogen to induce the host macrophages to differentiate into the lipid-loaded foamy phenotype. This single act alone contributes substantially towards taming the aggressive anti-microbial character of the macrophage and transforming it into a secure survival niche for the bacilli. An additional ancillary benefit that the pathogen derives from this process is that of an increased threshold of drug-sensitivity. Although the significance of foamy macrophages in TB has been known for a long time, we have only recently begun to understand the role of *M. tuberculosis* in actively inducing these cells. The relevance of these cells as a reservoir for the pathogen is also being revealed. As a result of such findings, it is becoming increasingly clear that a mechanistic understanding of the biochemical pathways that mediate equilibration of *Mtb* within the host macrophage is important for the development of new and more effective strategies for therapy. One can readily imagine how complementing existing antibiotics with intervention strategies that compromise intracellular survivability of the pathogen can markedly improve the efficacy of treatment. While, at one level, this may help to address the problem of drug-resistance, the possibility of employing such complementary approaches to shorten the treatment duration also presents a strong likelihood.

Thus, while the spectre of TB as a global public health challenge continues to loom large, recent discoveries that are paving the way for alternate therapeutic strategies provide the grounds for optimism that this problem may at least be contained in the near future. For this promise to be realized, however, it is important that ongoing research and development efforts are backed by increased commitments from governmental and public funding agencies. This is because, while the costs of the drug discovery enterprise continue to escalate, anti-TB drug development is still viewed as a non-profitable venture from a purely commercial point of view.

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