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The Sculpting of the *Mycobacterium tuberculosis* Genome by Host Cell-Derived Pressures

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Abstract

Mycobacterium tuberculosis is an incredibly successful pathogen with an extraordinary penetrance of its target host population. The ability to infect many yet cause disease in few is undoubtedly central to this success. This ability relies on sensing and responding to the changing environments encountered during the course of disease in the human host. This chapter discusses these environmental cues and stresses, and explores how the genome of Mtb has evolved under the purifying selections that they exert. In analyzing the response of Mtb to a broad range of intracellular pressures it is clear that, despite genome down-sizing, Mtb has retained an extraordinary flexibility in central carbon metabolism. We believe that it is this metabolic plasticity, more than any of the virulence factors, that is the foundation for Mtb's qualities of endurance.

Introducing the host cell

The macrophage plays host to *Mycobacterium tuberculosis* (Mtb) for much of the duration of its infection cycle (1, 2). And, while the bacterium is not an obligate intracellular pathogen, the macrophage has undoubtedly played a dominant role in shaping the genome of Mtb.

The macrophage is an extremely plastic cell that fulfills multiple roles in the body. Macrophages are strongly influenced by their environment and the cytokines and chemokines released by cells in their vicinity. Tissue resident macrophages are non-inflammatory and maintain tissue homeostasis through the phagocytosis and degradation of dead cells. These resting or non-activated cells have extremely high proteolytic capabilities to facilitate breakdown of phagocytic cargo. In the face of infection the macrophage becomes activated through both innate and adaptive immune pathways. Innate immune activation is initiated by ligation of either transmembrane sensors, such as the toll-like receptors (TLRs), or cytosolic sensors, which includes the inflammasome or NALP3 sensors (3–6). Innate immune activation leads to enhancement of the microbicidal capacities of the macrophage, most notably the increased intensity of the superoxide burst. The production of reactive oxygen intermediates by the membrane-associated NADPH oxidase complex is a relatively short-lived event that is induced upon ligation of certain phagocytic receptors (7). Adaptive immune activation is predominantly due to the release of interferon- γ (IFN- γ) by CD4+ or CD8+ T-cells. Stimulation by IFN- γ leads to an extensive reprogramming of the

macrophage through the induction of “IFN- γ response genes” that impacts phagosome maturation, autophagy and the delivery of anti-microbial peptides, the superoxide burst and the expression of the inducible nitric oxide synthase (NOS2) (8–11). In mice, NOS2 is clearly the dominant IFN- γ -mediated microbial response that restricts the growth of Mtb. The up-regulation of these microbicidal activities is accompanied by a down-regulation in the degradative capabilities of the phagocyte (12). It is hypothesized that this down-regulation of proteolysis enhances the antigen-presentation capabilities of the cell by extending epitope half-life.

The macrophage’s role in host defense means that it is the cell type most likely to encounter microbes, placing considerable evolutionary pressure on pathogens such as Mtb to evolve a range of strategies to subvert killing by its potential host cell. The enduring nature of the infection, and the fact that most infections remain in a latent disease state, suggests a dynamic equilibrium that may move in favor of the pathogen should the host be perturbed by an immune-compromising situation such as age, malnutrition, or HIV. This chapter discusses the pressures within the host cell that have shaped the genome of Mtb.

Transcriptional profiling and transposon insertion-site mapping

Two methods of global genome analysis have had a major impact on our understanding on the genetic basis of Mtb’s survival in the macrophage. Transcriptional profiling enables simultaneous quantitation of all transcripts in the bacterium at any given time. While the data generated are correlative, the approach provides invaluable insights, most particularly into the metabolic pathways that are up-regulated in the intracellular environment. In contrast, transposon insertion-site mapping, or TraSH as it was named in the first study (13, 14), facilitates the functional interrogation of all conditionally-essential genes that are required for survival in a given context, such as in the macrophage or mice versus growth in rich medium.

In the original, full genome transcriptional analysis study published by Schnappinger and colleagues (15) the value of the approach to identify physiological themes was immediately apparent. The study was performed on resting and activated murine bone marrow-derived macrophages infected with Mtb for up to 48 hrs. The authors reported that Mtb perceives the phagosome as an environment that is DNA- and cell wall-damaging, and is replete in fatty acids rather than carbohydrates as a possible carbon source. The authors compared their data with similar profiling performed on *Salmonella*, where this bacterium appeared to favor gluconate as its primary carbon source. They hypothesized that Mtb infection may affect macrophage physiology in a manner that impacts nutrient availability in the endosomal system; a hypothesis, which has turned out to be a particularly insightful and is discussed in greater depth in the section on metabolism of intracellular Mtb.

The initial transposon-based screens performed on Mtb utilized signature-tagged mutagenesis (16–18), an approach developed first in *Salmonella* (19). It is difficult to assess the saturation of these screens, however both groups performing the screens identified loci involved in the synthesis and export of the cell wall lipid phthiocerol dimycocerosate (PDIM) as being critical to the maintenance of infection in the mouse. The first transposon

insertion-site mapping screen on an infection model was performed by Sasseti and Rubin on a murine infection (13, 14, 20). This approach allowed analysis of a much larger and complex input mutant pool and therefore had much greater genome coverage. The screen led to the generation of a list of 194 “functionally-essential” genes required for establishment and maintenance of infection in the mouse that were identified through their depletion from the output mutant pool, isolated post-infection. When the genes were clustered according to known, or annotated, function the largest gene cluster was associated with lipid metabolism, which is consistent with the transcriptional profiling analysis of Schnappinger and colleagues. Along a related vein, Lamichhane and colleagues used a sub-saturation mutagenesis approach to generate a defined library of transposon-insertion mutants to model the conditional essentiality of specific metabolic pathways (21, 22), and demonstrated the significance of polyketide, mycolic acid and fatty acid synthesis to Mtb in culture. This mutant collection was then interrogated in a mouse model and recapitulated the identification of genes involved in PDIM synthesis as being important to Mtb survival in a murine model of infection. The same collection of mutants has more recently been screened in an experimental primate infection model, which appears to exert a stronger selection pressure than the murine infection. Mutants defective in the transport of virulence factors, the synthesis and transport of cell wall intermediates, as well as DNA repair and sterol metabolism appeared particularly impaired for survival in macaques (23).

The “core” transcriptome of intracellular Mtb

Since the development of microarray, and more recently deep sequencing technology there have been several transcriptional profiles generated on Mtb under a variety of conditions ranging from drug pressure to host cell models, including both human and murine macrophages (24–27). In broad terms there is a strong degree of overlap across studies on intracellular bacteria that establishes lipid metabolism as a recurrent theme. However, variation in experimental conditions and the diversity of bacterial strains limit the power of comparison. In an attempt to reduce strain-specific limitations and to allow identification of a “core” intracellular transcriptional profile shared by all Mtb strains, Homolka and colleagues looked at the transcriptional profiles of 2 lab strains (CDC1551 and H37Rv) and 15 clinical isolates that represented the genetic diversity of the Mtb complex across the planet (28). The authors chose a single time point at 24 hrs infection of both resting and IFN- γ -activated macrophages, because this appears to be one of the more stressful time points in the infection process and induces maximal transcriptional response in the bacterium. Condition tree analysis of the transcriptional profiles was encouraging because the profiles segregated according to the genotype of the 17 strains, Figure 1. Analysis of shared transcriptional responses identified a core transcriptome of 280 genes of which 168 genes were universally-induced, and 112 genes were universally-repressed. The induction of expression of genes with roles in hypoxia, oxidative and nitrosative stress, cell wall remodeling and fatty acid metabolism across the panel of clinical strains confirmed previous studies using single strains. 1-way ANOVA analysis facilitated the identification of 719 genes whose expression was significantly modulated by macrophage activation. Responses to known intra-phagosomal stresses such as nitric oxide production (*dosR* regulon) and iron limitation (mycobactin synthesis operon) were evident amongst those up-regulated genes.

Amongst the genes more highly expressed in resting macrophages were many genes linked to protein synthesis and growth, and genes indicative of an iron replete environment, such as *bfrA* and *bfrB*. It was particularly interesting to note that markers of general stress response such as the heat shock proteins *grpE*, *groEL*, *groEL2*, and *groES* were all up-regulated in resting macrophages implying that these chaperones were required to sustain growth.

The intracellular growth characteristics of the different clinical isolates in murine macrophages showed considerable heterogeneity, with the West African strains consistently less “fit” and the most robust growth exhibited by the Beijing and Haarlem strains. There were also marked genotype- and strain-specific transcriptional signatures demonstrating that the genetic diversity did lead to distinct transcriptional responses when the panel of strains were interrogated under defined, common infection conditions.

Transitional physiological states in a prolonged experimental infection

More recently, Rohde and colleagues employed the same intensive profiling approach to delineate the different physiological states experienced by *Mtb* during an extended period of infection, 14 days, in macrophages (29). Analysis of *Mtb* colony-forming units (cfus) following experimental infection of macrophages revealed a reproducible trend. Immediately following infection, the initial inoculum clearly experiences extreme stress and many of the infecting bacilli are killed, Figure 2. The cfu count then stabilizes as the bacteria appear to undergo a period of adaptation to the intracellular environment, before the cfu number increases as the bacteria enter into a period of intracellular replication. These experiments were performed with *Mtb* CDC1551 strain, which shows little propensity to escape from the vacuole or to induce cell death in its host cell. To look at the relative replication rates of the bacteria during the infection, Rohde and colleagues exploited a “clock” plasmid developed by David Sherman’s laboratory (30). The plasmid is lost from bacteria at a fixed rate determined by the rate of bacterial replication. Intriguingly, when the rate of plasmid loss during the course of the infection was analyzed, it was determined that the bacteria were actually replicating fastest during initial infection when they were being killed, and the death rate diminished as bacterial replication slowed, Figure 2. These data indicate that rapid replication is inconsistent with intracellular survival.

Transcriptional profiling of changes in bacterial gene expression through these transitions was followed by isolation and microarray analysis of bacterial mRNA at 2 day intervals from day 0 to day 14. Analysis of the global transcriptional profile demonstrated that the largest change in expression, up and down, was observed at day 2 post-infection and that subsequently, the transcriptional activity trended towards baseline (1:1). However, within the changing profiles one can observe distinct patterns of gene clusters or regulons linked to known environmental cues, metabolic pathways, and stresses, Figure 2. The *dosR* regulon is of particular interest (31–34). These genes were up-regulated markedly in the first 2 days post-infection, the elevated level of expression was sustained until day 8, at which time expression was down-regulated to close to baseline. The period of up-regulation of transcription coincided with stress and adaptation while the period of down-regulation correlated with the restoration of growth.

Clearly the bacterium is responding to macrophage-derived pressures and these pressures are dynamic and change from the time of uptake by the phagocyte to the establishment of a productive infection.

***Mycobacterium* senses and responds to environmental cues**

Phagosome maturation represents a continuum, a series of environments that for the most part, become increasingly hostile as the phagosome acquires more of the characteristics of the lysosome. This is the niche that Mtb has evolved to exploit, and in order to do so it must sense and respond appropriately to environmental cues. Evolution appears to have generated responses that fall into two distinct categories. Those that are physiological imperatives that are linked directly to the environmental cue or stress, and “off-target” responses that confer an advantage to Mtb for reasons unrelated to the nature of the cue itself. The latter response appear linked predominantly to alterations in bacterial metabolism critical for intracellular survival.

There is convincing evidence that some strains of Mtb escape from their vacuole into the host cell cytosol with varying degrees of efficiency (35). Mtb mutants defective in the secretory system ESX-1 or in the secreted effector ESAT-6 are unable to access the cytosol and are attenuated in mice (36). Recent studies indicate that vacuolar egress leads to death of the host cell (37, 38). These data imply that existence in the cytosol of the host cell is likely to be a transient state that may be very important for the spread of the infection, but as the bulk of the intracellular, replicative phase appears to be intra-vacuolar this chapter focuses predominantly on Mtb’s strategies to survive within this membrane-bound compartment.

1. pH

Obviously it would be a great advantage for Mtb to be able to sense its intracellular environment and to modify its transcriptional profile to enhance its survival. The pH of the intracellular compartment in which the bacterium resides has long been known to constitute an important environmental cue to Mtb (39–41). The notable significance of pH is that it is an inevitable consequence of phagosome maturation (40, 42, 43). The more mature the phagosome the lower the pH. The pH of the Mtb-containing phagosome is around pH 6.4 (39, 42), which is lower than the neutral pH outside the cell but higher than the lysosomal pH (4.5–5.0). For Mtb, this means that the ability to quantify pH would act as an intracellular locator signal. Mtb possesses a *PhoPR* two component sensor/effector kinase homolog and mutants in this locus exhibit a marked drop in virulence to the extent that they have been exploited as experimental vaccine constructs (44).

In screening for Mtb mutants that were defective in resistance to pH stress, Vandal and colleagues identified Rv3671c, a membrane-bound protease required to maintain pH homeostasis of the bacterial cytosol (45). These data are consistent with several other screens that have identified mutations conferring hyper-sensitivity to acid pH in that the majority of these genes encode proteins associated with cell wall integrity.

Using transcriptional profiling Rohde and colleagues determined Mtb’s response to pH change in the macrophage by infecting murine macrophages with Mtb in the presence and

absence of the host proton ATPase inhibitor concanamycin A (27). Over the first 2 hrs post-invasion of the macrophage, Mtb up-regulated expression of 68 genes. In the absence of the pH drop, the increased expression of 30 of these 68 genes was no longer detectable. Amongst these genes was a locus, *aprABC*, that is unique to the tuberculosis complex and was probably acquired by horizontal gene transfer at the time the TB complex diverged from less pathogenic Mycobacteria (46). The first gene in the operon, *aprA*, behaves like a transcriptional regulator and mutants deficient in this gene show extensive alterations in transcriptional profile. Expression of *aprA* is PhoPR dependent and *aprA* regulates expression of genes linked to TAG and PDIM biosynthesis. When wild-type Mtb are exposed to low pH they show increased synthesis of PDIMs, most notably phthiodiolone and phthiocerol A, but this response is lost in the *aprA*-deficient mutant. In contrast, mutants lacking *phoP* are extreme over-producers of PDIMs at low pH indicating that the two regulators operate in the same pathway, but with *phoP* functionally upstream of *aprA*. These data appear consistent with the link between pH and cell wall integrity, however, recent data indicate that an increase in the production of methyl-branched cell wall lipids, such as PDIMs may also provide relief from other intracellular stresses associated with the major carbon sources exploited by intracellular Mtb. These data are discussed in depth later in this chapter.

In order to understand the link between environmental cues, such as pH, and the immune status of the host cell during the course of infection, Tan and colleagues generated reporter bacterial strains that exhibit conditional expression of GFP (47). The promoter upstream of the locus encoding Rv2390c and *rpfD* responds to both pH and chloride concentration as synergistic cues. This is significant because, as a phagosome matures, the chloride concentration increases with the drop in pH. Chloride is one of the counter ions that balance the activity of the proton ATPase responsible for phagosomal acidification (48). The induction of expression of GFP in Mtb was more marked in IFN- γ -activated compared to resting macrophages, which is consistent with increased phagosome maturation (40, 41). This reporter strain was then interrogated in a murine infection model in wild-type and IFN- γ -deficient mice, and the level of induction of GFP expression was found to be much lower in the immune-compromised animals. A second reporter strain was generated that encoded GFP driven by the *hspX* promoter. Expression of *hspX* is controlled through *dosRS*, which is strongly induced under conditions of low oxygen tension or in the presence of nitric oxide. The *hspX* reporter strain was shown to respond to both conditions in culture and exhibited strong induction upon infection of wild-type versus IFN- γ -deficient mice. Interestingly, the kinetics of expression of GFP differed in the two reporter strains. The chloride/pH responders showed high induction early, at 14 days, and reduced expression at later time points. In contrast, the *dosRS*-dependent reporter showed low induction early and strong induction at 28 days. The late induction of the *dosRS*-dependent reporter may correlate with the development of an acquired immune response and the expression of the inducible nitric oxide synthase, NOS2. These reporter strains are providing novel insights into the dynamic nature of the intracellular environment during the course of experimental infection in mice.

2. Reactive oxygen and nitrogen intermediates

As mentioned earlier, macrophages have the capacity to generate reactive oxygen and nitrogen intermediates. Superoxide production by the NADPH oxidase complex is increased in intensity in macrophages activated by IFN- γ (7). However, Mtb does not appear to be particularly sensitive to reactive oxygen intermediates or their downstream products. The course of infection in wild-type versus *p47^{phox}*-deficient mice was impacted minimally by the defect (49, 50). The absence of a phenotype indicates that Mtb is particularly adept at dealing with reactive oxygen intermediates, and there are several different resistance mechanisms that have been demonstrated. *katG* encodes a catalase-peroxidase-*peroxynitritase*, which processes H₂O₂ and a mutant lacking this gene was hyper-susceptible to H₂O₂ *in vitro* (51). This mutant was also seriously attenuated in wild-type mice and mice deficient in NOS2 but exhibited a comparable phenotype to wild-type Mtb in *gp91^{phox}*-deficient mice, indicating that the basis of the attenuation was dependent on the host *gp91^{phox}* NADPH oxidase. Mtb also possess two genes encoding superoxide dismutases, *sodA* and *sodC*, capable of converting superoxide to H₂O₂ (52). Mutants defective in each individual superoxide dismutase exhibited reduced virulence under different conditions but the iron-dependent SodA-deficient strains exhibited the strongest phenotype in a murine infection model (53). Finally, low-molecular weight thiols are important anti-oxidants in many biological systems, and Mtb synthesize mycothiol instead of glutathione (54). Deletion of the gene *mshA*, which encodes a glycosyltransferase that is the first step in mycothiol synthesis, renders the bacterium hyper-sensitive to oxidative stress (55), but these mutants exhibit minimal phenotype in murine infection models. These data all indicate that Mtb has evolved multiple, overlapping pathways of avoiding or mitigating the consequences of oxidative stress due to the action of the host NADPH oxidase complex.

This is in marked contrast to impact of NOS2 on controlling Mtb infection in mice. A NOS2-deficient mouse is almost as impaired in control of tuberculosis as an IFN- γ -deficient mouse (10), which, acknowledging the probable existence of compensatory microbicidal mechanisms, argues that nitric oxide and its products are the dominant means of immune-mediated control of Mtb infection in mice. The demonstration of NO production in humans under various conditions of infection argues comparable significance in human tuberculosis (56).

If nitric oxide and its products are the primary mechanism of immune-mediated control of Mtb it would be logical for Mtb to have evolved strategies to try to minimize the impact of these radicals on its survival. Mtb possesses an NADH-dependent peroxidase/*peroxynitrite* reductase that consists of four subunits; an alkylhydroperoxide reductase (*ahpC*), a thioredoxin-related oxidoreductase (*ahpD*), a dihydrolipoamide transferase (*dlaT*) and a lipoamide dehydrogenase (*lpd*) (57). *DlaT* and *lpd* are dual function proteins that also serve as the E2 and E3 subunits of pyruvate dehydrogenase (58), which generates acetyl-CoA. This enzyme complex serves to detoxify both reactive nitrogen and oxygen intermediates. Mtb deficient in *dlaT* exhibits a carbon source-dependent phenotype, growing well in fatty acids and poorly in glucose or glycerol, revealing the function of *dlaT* in the pyruvate dehydrogenase. This mutant was also hyper-susceptible to RNI *in vitro*, however the mutant

was avirulent in both wild-type and NOS2-deficient mice indicating that the phenotype was not related purely to detoxification of RNI (59).

In addition to the machinery for detoxification of RNI and ROI, Mtb also possess responses linked to the repair or degradation of proteins and DNA damaged by oxidative mechanisms. The sulfur of cysteine and methionine are known to be highly susceptible to oxidation, which leads to impairment of protein function. Mtb possesses two methionine sulfoxide reductases, *msrA* and *msrB*, capable of restoring oxidized methionine to its reduced state (60). Mtb deficient in *msrA* and *msrB* are hyper-susceptible to killing by acidified nitrite and hypochlorite but not by H₂O₂. Furthermore, the double mutant showed minimal phenotype in a murine infection model indicating that this pathway was not central to survival within the mouse.

Darwin and colleagues performed a transposon screen to identify mutants defective in resistance to RNI stress (61, 62). Amongst the mutants that were most susceptible were insertions in genes encoding components of the bacterial proteasome, including the proteasome ATPase *mpa* and the proteasome accessory factor *pafA*. Further analysis employing chemical inhibitors of proteasome function, and the characterization of mutants lacking the core proteasome genes *prcBA*, revealed that these bacteria were now highly-sensitized to RNI-mediated damage. The proteasome-deficient mutants were also attenuated in mice and the loss of virulence was restored in NOS2-deficient mice demonstrating that the phenotype correlated with the ability to produce RNI *in vivo*.

3. Metal Ions

The presence of metal ions in the endosomal-lysosomal continuum has been known for a while through both direct demonstration and the identification and characterization of different metal ion transporters. However, the role(s) that manipulation of metal ion concentration plays in controlling the growth of intracellular microbes is an emerging field. Cells can sequester trace metal ions from pathogens to deny them essential enzyme function, or deliver excess metal ions into phagosomes to combine with ROIs to generate highly toxic intermediates.

Wagner and co-workers utilized hard X-ray probe analysis to determine the concentration of 10 individual elements inside the phagosomes containing Mtb (63). They observed an early decrease in the intraphagosomal concentrations of calcium and potassium that was coincident with an increase in sulfur and chloride concentrations. These data on chloride concentrations match the recent reporter data of Tan et al. (47), and the further elevation of chloride concentration upon macrophage activation is consistent with increased maturation of the Mtb-containing phagosome.

They also documented extremely high concentrations of iron in the Mtb phagosome that reached maximal levels 24 hr post-infection (63). This demonstration of the Mtb-containing vacuole as an iron-replete environment is consistent with more recent transcriptional profiling that reports the up-regulation of expression of *bfrB*, which encodes an iron-chelating protein that protects and detoxifies the metal inside the bacterium. Homolka and colleagues, who generated the “universal” core intracellular transcriptome also reported up-

regulated expression of *bfrB* in infection of resting macrophages (28). In contrast, genes involved in the synthesis of the iron-acquisition molecule mycobactin (*mbtA-J*), which is produced in conditions of iron scarcity, were up-regulated in activated macrophages.

The natural resistance associated membrane protein (NRAMP-1/SLC11A1) has long been implicated in regulating Mtb infection through the manipulation of the concentration of Fe^{2+} or Mn^{2+} in the phagosome of the infected macrophage (64). Although there is still considerable controversy as to its mode of action, the transport of manganese and iron from the phagosome into the cytosol and out of the cell appears to provide the most plausible mechanism for NRAMP-1-mediated restriction of Mtb growth (65). This is consistent with the transcriptional response of Mtb and the known concentrations of iron in the phagosomes of resting versus activated macrophages.

Wagner also reported that macrophage activation leads to increases in the concentration of both copper and zinc in the Mtb-containing phagosomes (63). Several recent studies have demonstrated that these metal ions play important roles in controlling Mtb infection in its host cell. Excess copper is toxic to *E.coli* through destabilization of the Fe-S clusters in some bacterial enzymes and the displacement of iron or zinc in others. Mtb senses and responds transcriptionally to copper through the up-regulation of a range of genes encoding copper detoxification mechanisms, many of which are under control of the transcriptional regulator *csuR* (66, 67). Amongst the genes up-regulated is *ctpV* a putative cation transporting P_1 -type ATPase thought to be active in copper efflux (68–70). Mutants defective in *ctpV* show increased sensitivity to copper intoxication, and together with another copper transporter, *mctB*, are required for full virulence in mice. The macrophage controls the levels of copper in the phagosome through the activity of the transporters ATP7A and ATP7B. Their activity is increased in inflammatory conditions through the increased expression of Ctr1, a cytosolic copper-binding protein that acts as a chaperone and delivers the metal ion to the transporters (71), facilitating enhanced transport into the phagosome.

Zinc is delivered to the phagosome through the activity of several metal ion transporters including the SLC39/ZIP family, and the ZnT proteins of SLC/CDF family (72). These transporters liaise with the metal-chelating protein metallothionein to balance the cytosolic and vacuolar concentration of zinc. Expression of metallothionein is also up-regulated in inflammatory conditions. In a recent study by Botella and colleagues it was demonstrated that, much like copper, exposure of Mtb to zinc increases the expression of another P_1 -type ATPase, *CtpC* (73, 74). Mutants lacking the *ctpC* gene retain excess zinc and are growth-impaired. Transcriptional profiling of Mtb in macrophages revealed time-dependent up-regulation of expression of genes associated with zinc detoxication, *ctpC* and Rv3269, along with other P_1 -type ATPases associated with metal ion transport, *ctpG*, *ctpV* and *ctpF*. The intra-phagosomal and intra-bacterial accumulation of zinc was visualized at the electron microscopy level by a method developed by Stoltenberg and co-workers (75), and was shown to correlate with the immune status of the host cell (74), illustrated in Figure 3.

While the macrophage has evolved to exploit these metal ions to control intracellular infections, Mtb has evolved concomitantly to attempt to minimize their impact, predominantly from the expression of metal efflux pumps.

4. Antimicrobial peptides

The lysosomal system of macrophages contains anti-microbial peptides capable of killing a range of different bacterial pathogens. While it was known that the induction of autophagy leads to death of Mtb (11, 76), the mechanism(s) that lead to the actual demise of the bacteria was unknown. Alonso and colleagues demonstrated that the most active microbial peptide that could be isolated from the lysosomes of murine macrophage was a ubiquitin-derived peptide generated through the degradation of ubiquitinated cargo delivered to the lysosome through the autophagous pathway (77). The induction of autophagy enhanced both the production of the peptide and the delivery of live Mtb to the peptide-containing compartments, Figure 4. This observation was confirmed by Ponpauk and colleagues, who also identified peptides generated from the degradation of the autophagic chaperone Fau as contributing to the microbicidal activity of the autophagosome (78).

Purdy and coworkers have since studied a synthetic peptide, Ub2, based on a 12 amino acid, positively charged region of ubiquitin that has anti-Mtb activity (79–81). Using an assay based on the maintenance of cytosolic pH homeostasis in the bacterium they demonstrated that the peptide compromises membrane integrity. In a genetic screen conducted on *M. smegmatis*, the porin MspA was identified as a major susceptibility factor to killing by Ub2. Expression of the *M. smegmatis mspA* gene in Mtb rendered the bacteria exquisitely sensitive to Ub2 peptide-mediated killing and markedly reduced their ability to survive in macrophages. More recent studies indicate that the peptide adopts a β -sheet confirmation in artificial membranes that leads to loss of membrane integrity (79). Interestingly, Mtb has lost genes encoding MspA-like porins from its genome, which may represent an evolutionary step towards survival in an environment rich in anti-microbial peptides.

Recently, the mechanism underlying the mode of action of vitamin D (1 α ,25-dihydroxycholecalciferol) in the enhancement of killing of Mtb at the level of the infected macrophage has been resolved and shown also to rely on the up-regulated delivery of microbicidal peptides to Mtb-containing vacuoles through the process of autophagy (82, 83). Campbell and Spector have shown recently that treatment of human macrophages with physiological levels of vitamin D leads to an up-regulation in expression of the anti-microbial peptide cathelicidin and the induction of autophagy, which restricted the growth of both Mtb and HIV (84, 85). HIV infection leads to down-regulation of expression of Beclin-1 and microtubule-associated protein 1 light chain 3B (LC3B), which are required for autophagy, and vitamin D reverses this process. Treatment of the cells with RNAi targeting the mRNA encoding cathelicidin markedly reduced expression of the anti-microbial peptide and inhibited vitamin D-mediated killing of Mtb.

These studies demonstrate the importance of anti-microbial peptides in controlling Mtb infection, and also demonstrate that enhanced maturation of the Mtb-containing vacuole and delivery of the bacterium to the lysosome through the induction of autophagy is important in promoting this control.

5. Hypoxia, host tissue metabolism and the granuloma

The macrophage does not exist in isolation, and in an Mtb infection it is part of a complex tissue-remodeling program that leads to the generation of a granuloma (86, 87). In an immune-competent individual, the decision as to whether the infection will progress to active disease and transmission, or remain under immune control is made at the level of the granuloma. If the granuloma becomes fibrotic and necrotic it also becomes increasingly hypoxic and this drop in oxygen tension is part of a major realignment of host tissue metabolism that impacts on bacterial metabolism.

Transcriptional profiling of human TB granulomas isolated from infected lung tissue by laser-capture microdissection revealed a marked dysregulation in lipid metabolism with an up-regulation in genes involved in both lipid processing and lipid sequestration (88). The lipid sequestration phenotype was confirmed by immune-histology showing abundant expression of peripilin 2 or adipophilin, a protein associated with lipid droplet formation in foamy macrophages, in the phagocytes subtending the caseous center of the granuloma, Figure 5. Kim and colleagues isolated the caseum, performed lipid analysis on its contents and demonstrated that the major lipid species were cholesterol ester, cholesterol and triacylglycerol (88). As cholesterol from low-density lipoproteins (LDL) becomes esterified when it is incorporated into lipid droplets in macrophages, this suggests strongly that the accumulation of the caseum in TB granulomas most likely depends on the generation and subsequent death of foamy, lipid-laden macrophages. The foamy macrophage phenotype can be induced by hypoxia (89), by Mtb infection (90, 91) or by the exposure of macrophages to plastic beads coated in the bacterial lipid trehalose dimycolate (TDM) (88), which has been shown to be the most bio-active of the cell wall lipids released by intracellular Mtb (91–93). This latter response is likely due to chronic stimulation of pro-inflammatory pathways similar to those activated in atherosclerosis. Murine granulomas induced by TDM-coated beads exhibit many of the characteristics observed in human TB granulomas including neovascularization, foamy macrophage formation, fibrosis and extracellular matrix remodeling, and necrosis (92, 94, 95). Transcriptional analysis of the murine TDM granuloma reveals that 74% of the genes up-regulated in this model are also up-regulated in human TB granulomas implying that the response to TDM is a significant component of human tuberculosis (95). Comparable modulation of lipid metabolism is observed in infected macrophages in culture (90, 91). Mtb-infected cells show down-regulation of lipid hydrolysis and increased lipid retention.

TDM is recognized by the signaling receptor Mincle (96) and TLR2 (97, 98), however recognition by TLR2 requires the activity of the scavenger receptor MARCO to fulfill a tethering capacity. MARCO is expressed to low levels on resting macrophages but is highly up-regulated upon innate or adaptive immune activation.

The re-alignment of the host macrophage and the granuloma tissue has major impact on the metabolism of Mtb and its success as a pathogen. Many studies from transcriptional profiling of intracellular bacteria, to genetic screens of transposon libraries, have implicated the metabolism of host lipids in bacterial survival in its host. Pandey and Sassetti identified a locus, *mce4*, that functions as a bacterial cholesterol transporter (14, 99). Mutants defective

in this gene show a phenotype similar to that reported for *icl1*, the inducible isocitrate lyase (100), in their inability to sustain a chronic infection in mice. Mtb appears to rely on cholesterol as a significant carbon source inside its host cell, and this dependence is enhanced in immune-competent mice. The degradation of cholesterol generates a considerable amount of propionyl-CoA, which is potentially toxic to Mtb, Figure 6. Assimilation of propionyl-CoA is achieved through three possible routes (101). The Methyl Citrate Cycle (MCC), which is dependent on isocitrate lyase *icl1* functioning as a methylisocitrate lyase to generate succinate to feed into the TCA cycle. The Methyl Malonyl Pathway (MMP) that produces succinyl-CoA, which can also feed into the TCA cycle, or lastly, methyl malonyl-Co intermediates can be incorporated directly into methyl-branched polyketide lipids such as PDIMs, sulfolipids, and lipooligosaccharides. Recent data indicate that Mtb inside its host macrophages utilizes the synthesis of these cell wall lipids to relieve stress from propionyl-CoA intermediates (102). An *icl1* mutant, which lacks a functional MCC was attenuated inside murine macrophages in culture but could be rescued by either the addition of vitamin B12, which is a cofactor required for the MMP, or through the induction of a foamy macrophage phenotype by addition of oleate to the macrophages prior to infection (102), Figure 7. Oleate likely acts through expansion of the acetyl-coA pool following its degradation by β -oxidation, or directly as an acyl-CoA primer, to facilitate additional synthesis of methyl-branched lipids. The ability of Mtb to access and utilize the lipid stores of the host cell may also be visualized through the use of fluorescent fatty acid analogs (89, 90).

There is a satisfying sense of symmetry to these metabolic adaptations. The metabolism of the bacterium appears geared to exploit the altered metabolism of the host cell and tissue. The altered metabolism of the host can be induced by the Mtb cell wall lipids, and those cell wall lipids contain methyl branched lipids and act as a sink for potentially toxic cholesterol intermediates.

When bacterial metabolism is fueled primarily by fatty acids it is necessary for Mtb to synthesize sugars from intermediates generated in the TCA cycle. This process, gluconeogenesis, is initiated by phosphoenolpyruvate carboxykinase (*pckA*). Early experiments on *M. bovis* BCG demonstrated that *pckA* expression was up-regulated on growth on acetate and palmitate but not on glucose (103). Furthermore, *pckA*-deficient mutants showed impaired survival in macrophages and reduced virulence in mice. More recently Marrero and colleagues used a tet^r inducible-expression construct encoding *pckA*, to demonstrate that Mtb could only grow on fatty acids under conditions of induction of *pckA* (104). They went on to show that mutants of Mtb lacking *pckA* were attenuated in macrophages and mice.

More recently the same investigators have analyzed the phenotype of Mtb that is impaired in its ability to utilize glucose directly (105). The first step in glycolysis is the phosphorylation of glucose. Mtb encodes two functional glucokinase genes, one of which, *ppgk*, is required for normal growth on glucose while the other, *glkA*, appears dispensable. Metabolomic analysis revealed that both genes encode enzymes that facilitate entry of glucose into Mtb's central carbon metabolism and a double mutant was unable to utilize glucose as a substrate for growth or metabolism. The double mutant established an infection in murine lungs to

levels comparable to wild-type but was mildly attenuated when the infection transitioned into a chronic state. These data indicate that bacterial metabolism has to adapt during the course of an infection and that while mutants unable to utilize fatty acids are severely attenuated, those that are defective in glycolysis are impaired in survival in later stages of infection in the murine model.

At the heart of much of Mtb's metabolic flexibility is the enzyme isocitrate lyase, *icl1*. In early studies isocitrate lyase activity was shown to be required for growth on acetate and mutants of Mtb showed impaired growth on various fatty acid sources (100, 106). Mutants deficient in *icl1* also showed an attenuated phenotype in infection models that was most marked in activated macrophages, or in immune-competent mice once the infection was controlled. In keeping with our knowledge at the time it was thought that *icl1* functioned as the gating enzyme into the glyoxylate shunt that would enable Mtb to retain carbon while growing on fatty acids. Subsequently, McKinney and collaborators have shown that it is the ability of *icl1* to sustain the MMC for detoxification of propionyl-coA that is critical to the success of Mtb in a host environment (101, 107, 108). Most recently, Eoh and Rhee have used a metabolomics approach to demonstrate that *icl1* activity is also necessary under hypoxic conditions where the enzyme is critical to the sustained production of the flexible metabolic intermediate succinate, which is required to maintain membrane potential, ATP synthesis and for anaplerosis (109). The requirement for *icl1* activity is independent of fatty acid catabolism. The authors propose that this activity, coupled with the ability of Mtb to operate a reductive TCA cycle (110), enable the bacteria to generate ATP under low oxygen tension. The ability to adopt such a reduced yet sustainable metabolic state is likely critical to Mtb's capacity to maintain a subclinical infection for decades.

Concluding remarks

Mycobacterium tuberculosis is an incredibly successful pathogen with an extraordinary penetrance of its target host population. The ability to infect many yet cause disease in few is undoubtedly central to this success (111). This ability relies on sensing and responding to the changing environments encountered during the course of disease in the human host. This chapter discussed these environmental cues and stresses, and explored how the genome of Mtb has evolved under the purifying selections that they exert. In analyzing the response of Mtb to a broad range of intracellular pressures it is clear that, despite genome down-sizing, Mtb has retained an extraordinary flexibility in central carbon metabolism. We believe that it is this metabolic plasticity, more than any of the virulence factors, that is the foundation for Mtb's qualities of endurance.

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Figure 1. Genetic and transcriptome diversity of *M. tuberculosis* complex (MTC) clinical isolates (A) Radial neighbor-joining tree based on 24 loci MIRU-VNTR and 43 spacer spoligotyping showing the phylogenetic relationship of strains in this study. Three strains each from the 5 distinct lineages pathogenic to humans plus two sequenced reference strains (H37Rv and CDC1551) were chosen to represent the global diversity of MTC. (B) Condition tree of MTC clinical isolate transcription profiles *in vitro* during log phase growth in 7H9 medium relative to CDC1551 reference strain (three biological replicates). Expression profiles for genes passing quality filters (flagged as present in 42 of 48 samples) with differential expression in at least one strain (up or down $>1.5\times$ in at least 1 of 16) were clustered using the Spearman correlation. Each column represents the global transcription profile of a single strain. Genes were clustered vertically based on the distance measure. Red and blue indicate higher or lower gene expression than CDC1551 control, respectively. This figure is reproduced from Homolka et al. (28).

Figure 2. Life and death dynamics during long-term intracellular survival of *Mtb*

(A) Survival Assays. Resting murine bone-marrow derived macrophages were infected at low MOI (~1:1) with *Mtb* CDC1551. Viable CFU were quantified at day 0 and at 2 day intervals post-infection (p.i.) over a 14-day time-course by lysis of monolayers, serial dilution, and plating on 7H10 medium. Error bars indicate standard error of the mean from two independent biological replicates each consisting of three technical replicates per strain (total of 6 wells/strain). (B) Replication Clock Plasmid. The percentage of bacteria containing the pBP10 plasmid during growth in resting macrophages was determined by comparing CFU (mean $\pm$ s.d.) recovered on kanamycin vs. nonselective media (red). The cumulative bacterial burden (CBB) (black) was determined by mathematical modeling based on total viable CFU and plasmid frequency data. Data shown represents two independent experiments with each sample performed in quadruplicate (8 total wells/time point). (C) The "bottleneck" response. Temporal expression profiles of genes differentially regulated at Day 2 p.i., including genes from (A) that were up- (red) or down-regulated (blue) >1.5-fold (shown as ratio of signal intensity relative to control). Note the maximal change in transcript levels at day 2 p.i. followed by majority trending back towards control levels. (D) "Guilty by association" analysis. Genes regulated in synch with known virulence regulons – i.e. the DosR regulon – were identified by using a highly regulated member of this regulon, *hspX*, in place of synthetic profiles. This figure is reproduced from Rohde et al. (29).

Figure 3. Distribution of zinc within intraphagosomal *M. avium*

Bone marrow-derived mouse macrophages were infected with *M. avium* and treated for capturing of free zinc ions by an auto-metallography (AMG) procedure. Cells were then processed for EM observation. The zinc crystals formed during this procedure appeared as small dense deposits along the inner face of the phagosome membrane (arrows). The mycobacterial cytoplasm was devoid of zinc. Lysosomes (L) also displayed zinc crystals. Comparable images were observed in *Mtb*-infected macrophages. Scale bar = 0.5 μ m. This picture was provided by Dr. Chantal de Chastellier and was prepared as detailed in reference (74).

Figure 4. Immunoelectronmicroscopy of *M. tuberculosis*-infected macrophages

All sections were probed with mouse anti-ubiquitin (12 nm gold) and rat anti-LAMP1 (6 nm gold). (A) Untreated, infected macrophage demonstrating that the bacteria-containing vacuoles lack ubiquitin signal, and that the ubiquitin signal is predominantly associated with either the membranes (horizontal arrows) or the lumen (vertical arrowheads) of LAMP1-positive, dense lysosomes. (B) In cells treated by starvation for 2 hrs, the *M. tuberculosis*-containing vacuole has acquired dense, LAMP1-positive, flocculent matrix that is also positive for ubiquitin (arrowed) demonstrating delivery of lysosomal ubiquitin to the bacteria-containing compartment. The relative distribution of ubiquitin-associated label in *M. tuberculosis*-containing vacuoles in untreated and starved BMMΦ is detailed in fig 4D, with standard error bars. This figure is reproduced from Alonso et al. (77).

Figure 5. Peripilin 2/ADFP expression in human TB granulomas

Immunofluorescence signals were obtained for each granuloma (B) and the corresponding region from an H&E stained slide (A) are shown. Nuclei are seen in blue and antigens in red. The macrophages subtending the caseum of this fibrocaseous granuloma label strongly for peripilin2/ADFP expression. This figure is reproduced from Kim et al. (88).

Figure 6. Outline of the pathways relevant to C₃ metabolism

The metabolism of cholesterol, methyl-branched fatty acids and odd-chain length lipids will raise the intracellular levels of the C₃ compounds propionate or propionyl-CoA, which Mtb finds highly toxic. The bacterium has developed three different strategies to detoxify propionyl-CoA. Isocitrate lyase (*icl1*) activity has been suborned to fulfill the function of methylcitrate lyase in the last step of the methyl citrate cycle to generate the TCA cycle intermediate succinate. The methylmalonyl pathway has also been mobilized to metabolize propionyl-CoA to produce succinyl-CoA via the VitB12-dependent activity of methylmalonyl-CoA mutase (*mutAB*). Finally, intermediates from the methylmalonyl pathway can be incorporated directly into the abundant, methyl-branched lipids of the bacterial cell wall, such as PDIM and SL-1.

Macrophages : stained w/ BODIPY 493/503 after 2 days induction with oleate

Figure 7. Intracellular growth is restored to the *iclI* Mtb through the induction of oleate-containing lipid droplets in the infected cell

The survival of the *iclI* mutant is impaired in macrophages, however, growth could be restored by pre-loading the host cells with exogenous oleate, or by adding VitB12 to the cell culture medium to facilitate operation of the MMP. (A) Induction of macrophage lipid droplets, detected with BODIPY 493/503 (green), Mtb expressing pVV16-mCherry (*smc::mCherry*) (red), and nuclei (blue) in oleate-loaded macrophages versus untreated macrophages. In the absence of Mtb infection the lipid droplets are lost from the

macrophages in 48 hrs. However, in the presence of Mtb, the lipid droplets persist. (B) Bacterial CFUs were determined at 2 day intervals across an 8-day period for the *icl1* Mtb in untreated, control macrophages (triangle), in lipid droplet-containing, oleate-loaded macrophages (square), and in macrophage supplemented by the addition of VitB12 to the medium (diamond). Error bars indicate standard error of the mean from three representative replicates. This figure is reproduced from Podinovskaia et al (90) and Lee et al. (102).