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Host and bacterial factors that regulate *Mycobacterium tuberculosis* infection and persistence

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1. Introduction

Tuberculosis (TB) is one of the most common and deadliest infectious diseases caused by *Mycobacterium tuberculosis*. While the overall number of new TB cases in developed world has declined, TB is still a serious health threat. TB is transmitted by airborne droplet nuclei containing *M. tuberculosis* cells. After inhalation, the bacilli in the droplet nuclei are deposited within the terminal airspaces of the lung. Some of these cells are killed by the immune system or cause primary TB, while some become dormant. Approximately one-third of the world's human population is infected with *M. tuberculosis*, the majority of which is in the dormant state. The dormant bacilli are thought to halt active metabolism and persist without replication. Usually, these cells do not cause any symptoms in their host in what is called a latent infection. After the latency period, dormant *M. tuberculosis* cells become active when the host is weakened by factors such as stress, aging, malnutrition, and lowered immunity. Annually, approximately 2 million people die from TB worldwide, which contain caused by reactivation of previously infected *M. tuberculosis*.

M. tuberculosis has no environmental or animal reservoir, and it is believed to have coevolved with humans. Thus, the eradication of persisting dormant bacilli has been thought to be the most successful action against TB. However, current drugs for the treatment of TB have little effect on dormant *M. tuberculosis*. Currently, at least 6 months are required to treat TB patients. These lengthy treatments are costly in terms of patient time and money. Whereas, with abbreviated

treatment, *M. tuberculosis* cells that have developed mechanisms of evading the drugs can escape killing and proliferate as a resistant strain.

Before the discovery of anti-TB drugs, the attenuated *M. bovis* Bacillus Calmette–Guérin (BCG) vaccine was the only available therapy against TB. The BCG vaccine exhibits high efficacy against TB in newborns but variable efficacy against adult pulmonary TB and other mycobacterial diseases. It is also claimed that BCG does not prevent the establishment of latent TB or the reactivation of pulmonary TB in adults. Therefore, the development of a novel, effective, and improved BCG vaccination strategy is indispensable for the eradication of TB. One of the current attempts to develop a novel BCG strategy involves replacing the existing strain with a more effective vaccine. This is generally thought to demand an improved, attenuated mycobacterial vaccine strain obtained by reintroduction of important antigens into the existing BCG vaccine strain. Thus, it is important to identify the molecules and mechanisms that regulate the dormancy of mycobacteria for the development of novel, effective BCG-replacement vaccines. In this chapter, we introduce current studies of host and bacterial factors that regulate the dormancy of mycobacteria.

2. Slow growth, dormancy, longevity, and latent infection

Bacteria are simple prokaryotic single cell organisms, most of which replicate quickly compared to eukaryotic cells, which require 12–18 h for duplication. For example, the model organism *Escherichia coli* and the human pathogen *Streptococcus aureus* multiply within 0.5–1 h. By contrast, the doubling times of *M. tuberculosis* complex and *M. leprae* are 15–24 h and 2 weeks, respectively. These pathogens cannot compete with rapidly growing competitors for the struggle for survival. However, such a slow growth rate is suited to parasitism. Reflecting the growth rate, all mycobacterial diseases, excluding toxin-related-Buruli ulcer, are chronic and progress sluggishly.

Pathogenic mycobacteria survive in the host. *M. tuberculosis* have adapted to humans since the modern *Homo sapiens* evolved from its predecessor in east Africa and have spread across the world along

the movement of human being.[1,2] Even after being engulfed by phagocytic cells, such as macrophages, *M. tuberculosis* prevents the recruitment of vesicular proton-ATPase and in turn inhibits the acidification of phagosomes and lysosomal fusion.[3] A recent report suggested that several days after infection, *M. tuberculosis* enters the cytoplasm from phagosomes by utilizing a pore-forming toxin, ESAT6, and then multiplies.[4] Host immune responses cannot eradicate such virulent *M. tuberculosis* strains from the body. Instead, the host suppresses *M. tuberculosis* growth largely by the formation of granuloma, which blocks the oxygen supply to *M. tuberculosis*.

Most commonly, *M. tuberculosis* infection remains latent (over 90% of infections). Five to ten percent of those who initially control the infection later develop active disease at some point in their lifetimes (Figure 1). The development of disease is critical for preservation of the species because persisting *M. tuberculosis* will die upon the death of the host. Therefore, frequent disease progression is not beneficial, since the pathogen loses its harboring place. Mathematical models calculate that an approximate 5% rate of disease onset is optimal for species preservation of parasitic pathogens. Thus, it appears that *M. tuberculosis* maintains a desirable onset ratio, which may have facilitated the wide spread of this pathogen.

2.1. Models of dormancy in vitro

Although latent TB is clinically defined as a state in which the host exhibits no symptoms of disease, the conditions under which the dormant cells form and the actual state of the bacterium in human latency remain unknown. Based on the concept that dormancy is regarded as a stable but reversible and nonreplicating state, many scientists have attempted to mimic dormancy *in vitro*. As the morphology and staining pattern of persistent bacilli can be obtained by starving cells on nutrient-depleted agar plates,[5] starvation is one of the most extensively studied dormancy models. Loebel *et al.* investigated the effect of nutrient deprivation on the metabolism of persistent *M. tuberculosis* in a granuloma using a starvation model.[6] They found that nutrient deprivation resulted in a gradual shutdown of respiration to

minimal levels, but the bacilli remained viable and recovered when returned to a rich medium. Smeulders *et al.* observed that nutrient-deprived *M. smegmatis* remained viable for over 650 days and displayed increased stress resistance, mRNA stability, and an overall decrease in protein synthesis.[7] Another study using microarray and proteome analysis revealed that slowdown of the transcriptional apparatus, energy metabolism, lipid biosynthesis, and cell division occurred in response to starvation, suggesting that the cells adapted to the nutrient-starved state to maintain their viability.[8] Owing to its simplicity, ease of handling, and reproducibility, the starvation model may be useful for studying the metabolic activity of dormant bacilli.

Because tubercle bacilli are thought to persist in hypoxic areas such as granulomas and caseous lesions, oxygen depletion is also used to induce dormancy. Wayne established an *in vitro* model of dormancy by gradual oxygen depletion in *M. tuberculosis* cultures.[9] In this model, at least two stages of nonreplicating persistence were observed.[10] The first stage, designated as nonreplicating persistence 1 (NRP1), occurred when the declining dissolved oxygen level approached 1% saturation. When the oxygen content of the culture dropped below 0.06% saturation, the bacilli shifted to the second stage, designated as NRP2. NRP1, which is equivalent to a microaerophilic condition, was characterized by increased production of glycine dehydrogenase (GDH) and sustained ATP concentrations without a corresponding increase in the numbers of colony forming units or synthesis of DNA. On the other hand, marked decreases of GDH concentrations were observed in cells in NRP2 (equivalent to an anerobic condition). The upregulation of GDH production suggests that mRNA synthesis occurred at least during the NRP1 stage, and this enzyme is thought to provide a mechanism for regenerating essential nicotinamide adenine dinucleotide (NAD) as an adaptation to hypoxia. This model is now widely used to study the mechanism of dormancy and provides substantial information such as gene expression[11] or metabolism[12] in dormant mycobacteria.

Although the hypoxia model is used extensively, this model does not reflect the situation where *M. tuberculosis* cells survive the stress generated by surrounding activated macrophages, acidic pH, and high

concentrations of reactive oxygen intermediates. In an attempt to mimic additional host environmental conditions, other models such as treating *M. tuberculosis* cells with nontoxic levels of nitric oxide (NO) to model activated immune cells[13] or exposing cells to mildly acidic conditions that reproduce necrotic tissue[14] have been developed. In addition, combinations of such stresses were used recently to reflect more accurately the complex of host milieu in studies of mycobacterial responses.[15,16]

Although these *in vitro* models are artificial, there is some evidence that they accurately predict events in humans. For example, an RNA polymerase inhibitor, rifampin, is critical for short-course chemotherapy in humans[17] and is effective against latent TB.[18] Rifampin also possesses the unique ability to kill *M. tuberculosis* cells in many of these *in vitro* dormancy models. Similarly, metronidazole, a drug specific for anerobes, kills dormant tubercle bacilli under anerobic conditions, but it has no effect on actively growing aerobic cultures.[19] These phenomena suggest that the dormant cells in these models are not metabolically inert but maintain active transcription for select cellular processes. Thus, these models are likely to prove very useful in future studies.

2.2. Experimental models in animals

Animal models have played an important role in evaluating the effect of new anti-TB agents and vaccine candidates since Robert Koch first used mouse as an experimental model. Subsequently, a variety of animals have been used for TB research, and each animal model has its strengths and weaknesses.

The most preferred and commonly used animal models for studying the pathogenesis of *M. tuberculosis* is the murine model, owing to the advantages such as availability of immunologic reagents and low costs. However, it is claimed that the murine model differs from human TB in several ways. The most crucial difference is the ability of mice to tolerate relatively large numbers of bacilli within their lungs.[20] Further, unlike humans, they develop noncaseous granulomas in response to infection.[21] However, the immune response in mice has been

shown to have direct correlates in the human system, including the importance of $CD4^+$ T cells,[22,23] interleukin (IL)-12,[24,25] and tumor necrosis factor (TNF)-α.[26,27]

Guinea pigs are well suited to study airborne TB transmission due to their sensitivity to infection with a small number of *M. tuberculosis* bacilli.[28] This model has an advantage of replicating many aspects of TB in humans, such as lung tissue necrosis, loss of weight, and death caused by disease. Unlike the murine model, the granuloma formed in the guinea pig model contains Langhans multinucleated giant cells that can often be observed within human granulomas.[29] On the other hand, infected guinea pigs are unable to sustain the *M. tuberculosis* infection and rapidly succumb to disease.[30] This extreme and inherent sensitivity suggest that this model lacks the stage of latency. Although a latency model of guinea pigs has recently been published, it requires the use of a streptomycin-dependent strain and may not reflect the pathological condition of wild-type *M. tuberculosis* in a host.[31]

The rabbit model of TB has been extensively used by many researchers because it appears to resemble the pathology of TB infection in humans more closely because of the innate resistance of rabbits to *M. tuberculosis*.[32,33] *M. tuberculosis*-infected rabbits form organized granulomas with caseous necrotic cores. Recently, it was reported that rabbits serve as a model by which to study latent TB in humans, as defined by the low bacterial burden and lack of clinical symptoms.[34] However, unlike human latent TB, rabbits tend not to exhibit spontaneous reactivation unless they are experimentally immunosuppressed.[34] In addition, compared with murine and guinea pig models, rabbit models are expensive and require larger space to maintain. It is also claimed that the rabbit model has a disadvantage of paucity of commercial immunologic reagent relative to those available for mice.

Nonhuman primates such as the cynomolgus macaque may be more reliable experimental animals that reproduce human TB infection.[35-37] The macaque can be infected with a low-dose inoculum of *M. tuberculosis* by aerosol and manifest latent TB and reactivation as ob-

served in humans. In addition, granulomas in latent TB macaques are fibrotic and contain a caseous core that is similar to the lesion in humans.[38] Recent investigation demonstrated that all macaques challenged by low-dose aerosol exhibited evidence of infection by either the tuberculin skin test or lymphocyte proliferation assays to PPD. Roughly half of these macaques progressed to primary TB, whereas others developed a clinically latent infection. Moreover, about 40% of animals with latent TB spontaneously developed reactivated TB months after infection, whereas the others maintained a dormant state of infection.[36] Although macaques are costly and limit the number of potential experiments, they can be a useful model for the study of human latency.

2.3. Molecules that are involved in the regulation of dormancy

During long-term persistence in the host, *M. tuberculosis* cells are thought to change their surface antigen and metabolic pathways to adapt to the host immune system. Many studies have identified molecules that may be involved in dormancy using various approaches. Barry *et al.* identified a protein remarkably expressed under several stress conditions such as hypoxia and named it Acr or HspX.[39] Acr is a homolog of the small heat-shock protein α-crystallin and is induced under a variety of conditions such as in the stationary phase,[39] hypoxia[40], and by phagocytosis of macrophages.[41] Acr is a molecular chaperon and thus is thought to prevent protein degradation after infection in macrophages[42] and in the dormant state. The expression of Acr is controlled by a transcriptional factor, DosR, which is essential for initiating mycobacterial dormancy.[43] Expression of this protein is now used as a marker of dormancy regulation under hypoxia.

GDH was also identified as a protein induced in tubercle bacilli during adaptation to anerobic conditions as previously described.[10] A gene encoding NarX, a putative fused nitrate reductase, was found to be strongly induced in anerobic bacilli by a comparative Northern analysis performed by Hutter and Dick.[44] Other nitrate reductases, NarG, NarH, NarJ, and NarI, which are enzymes involved in nitrate respiration, were found to be important for the persistence of *M. tuber-*

culosis in the lungs, kidneys, and livers of mice.[45]

Histone-like protein (Hlp), one of the histone-like proteins in *M. smegmatis*, is present in low abundance in exponentially growing cells (about 120 molecules per cell) but is upregulated during cold shock or anerobiosis-induced dormancy.[46] Hlp is an ortholog of mycobacterial DNA-binding protein 1 (MDP1) as described in the middle of this chapter. Interestingly, one of the Hlp homolog, HupS in *Streptomyces coelicolor*, was found to be upregulated specifically during sporulation.[47] HupS is nucleoid-associated in spores and is considered to affect nucleoid architecture and the protection of spores. Hlp is also nucleoid associated and binds DNA *in vitro* with very high affinity. However, mutants lacking Hlp could grow normally and were not affected by cold shock[48] or oxygen depletion.[46] These results suggest that additional proteins must be involved in organizing the chromatin structure in mycobacteria such as Dps[49] and Lsr2.[50]

Transcriptional regulators are also regarded as playing a role in the responses of mycobacteria under environmental stresses. Sigma factors are reported to be important during sporulation in other bacteria. There are some sigma factors reported to be upregulated in dormant cells, although their role in dormant bacilli is unclear. For example, SigF was only observed in the slow growing pathogenic mycobacteria. It is also reported that the transcription of *sigF* increased after exposure to stresses such as entry into macrophages and in the stationary phase. However, a *sigF*-knockout mutant exhibited no differences in survival during microaerophilic or stationary-phase incubation. In addition, the mutant exhibited no loss of ability of growth within macrophages, although the *sigF* knockout could not produce Acr, which was thought to be important for its survival in macrophages.[42]

2.4. Dormancy and sporulation

Because *M. tuberculosis* can persist in the host for a long time, it has often been claimed that the bacilli may be able to form spores.[51,52] Recently, Ghosh *et al.* reported the possibility of mycobacteria forming spores.[53] They observed that spore-like structures were found in stationary cultures of *M. marinum* that germinated into vegetative cells

upon exposure to fresh medium. These "spores" exhibited many of the usual characteristics of well-known endospores, such as resistance to heat and the presence of dipicolinic acid. It was also revealed that old cultures of *M. bovis* BCG contained the same structures. However, another group, Traag *et al.*, protested against these results.[54] They performed genome sequence analysis of 15 mycobacterium genomes and found that they lack orthologs of many highly conserved genes diagnostic of endospore formation. They also failed to detect the presence of spore-like particles by light microscopy and by testing for heat-resistant colony forming units. They concluded that the possibilities of mycobacterial spore production under unknown conditions still remain, whereas spore-like inclusions previously reported have turned out to be neutral lipid bodies[55,56] or contaminated particles.[57] Further studies are required to investigate the mechanism of dormancy and the possibilities of spore formation.

3. The role of MDP1 in the regulation of mycobacterial growth and pathogenesis

Under tough competitive conditions, living organisms tend to maximize their rates of reproduction, while in relatively safe conditions they maximize competitive forces to survive in crowded ecological environments. Organisms that follow the latter pattern utilize a strategy of slow growth, low-frequency reproduction, and host maintenance that results in longevity. Mycobacteria resist bactericidal devices of the host organs or cells that eliminate other competitive organisms. Thus, mycobacteria are classified as organisms that use a slower pattern of growth, although this is uncommon among bacteria. Mycobacteria grow slowly and can downshift to a nonreplicating dormant state to persist for long periods. These characteristics are closely related with the clinical states of mycobacterial diseases. The coordination of growth is a central mechanism in biology, but in mycobacteria, its precise mechanism is still largely unknown. In this section, we present one of key proteins involved in the coordination of mycobacterial growth and longevity.

3.1. Identification of MDP1

One of the major factors responsible for the shift from a growing to a dormant state is considered low oxygen tension.[58] Because *M. tuberculosis* is an aerobic bacterium, hypoxia is sufficient to cause growth arrest.[59] Similarly, it was reported that NO[13] and carbon monoxide[60] induce mycobacterial dormancy depending on the inhibition of respiration. Overall, the protective immune response to *M. tuberculosis* infection induces a dormancy program *in vivo*.[61]

We postulated that regulation of gene expression is critical to coordinating growth and dormancy in mycobacteria. We proceeded to identify such proteins under the condition when Acr is expressed. We found that Acr is expressed when BCG is cultured on Sauton media.[62] When BCG was cultured on Sauton media, a 28-kDa protein was the most abundant cellular protein. Soon thereafter, we revealed that this protein possesses DNA-binding activity.[63] We purified this protein from BCG and also cloned the gene. Because this protein was distributed among mycobacteria,[64] we designated it mycobacterial DNA-binding protein 1(MDP1) (Figure 2). In the current DNA database, however, a gene encoding a homologous protein to MDP1 can be found in the *Nocardiaceae* genome as well.

The N- and C-terminal regions of MDP1 have similarity with the histone-like protein HU and eukaryotic histone H1, respectively. The structure of the bacterial chromosome is different from the chromatin of eukaryotic cells and is associated with bacterial Hlp. Histone-like protein coordinates the chromosomal structure and gene expression in bacterial cells. MDP1 can be categorized as an histone-like protein, and it might play pivotal roles in the fundamental process of DNA functions.

3.2. Orthologs of MDP1

Dick *et al.* found a protein that was remarkably upregulated in O_2-depleted dormant *M. smegmatis* (Wayne's model) and designated it Hlp.[65] Hlp is an ortholog of MDP1 in *M. smegmatis*. Furthermore, two groups identified MDP1 orthologs in *M. tuberculosis*[66] and *M. leprae*.[67]

Prasad *et al.* identified the protein as an immunodominant protein in healthy humans who are exposed to TB patients through a T-cell blot assay. They designated this protein as the Hlp of *M. tuberculosis* (Hlp_{Mt}), which is the same molecule as MDP1. *M. leprae* infects Schwann cells by interacting with the laminin-α 2 chain on the axon units.[68] Rambukkana *et al.* identified a protein adhesin in the cell wall fraction of *M. leprae* that binds to laminin. They designated it 21-kDa laminin-binding protein (LBP-21). LBP-21 is an ortholog of MDP1 in *M. leprae*. Hlp/MDP1 also localizes on the cell wall of mycobacteria and acts as an adhesin upon infection in nonprofessional phagocytes, such as lung epithelial cells, through interactions with glycosaminoglycans (GAGs).[69,70] MDP1 (HupB) is also observed in the extracellular biofilm of *M. ulcerans*.[71] However, Mukherjee *et al.* demonstrated that Hlp/MDP1 is associated with nucleoids but not with the cell wall.[72] MDP1/Hlp does not possess a secretion signal. Thus, it is not clear whether MDP1 in/on the cell wall originated from dead bacteria or was actively secreted by undiscovered machinery.

3.3. MDP1 suppresses macromolecular biosyntheses

Syntheses of macromolecules, such as DNA, RNA, and protein, are critical for the replication of cells. We examined the effect of MDP1 on macromolecular biosyntheses. The *in vitro* data demonstrated that MDP1 has suppressive activities on the syntheses of DNA, RNA, and proteins (Figure 3).[64]

The effect of MDP1 on the syntheses of DNA and RNA is dependent on its binding activity to nucleic acids, which is inhibited by exogenously added DNA. The synthetic peptide corresponding to the 31–50-amino acid region of MDP1 possesses strong DNA-binding activity.[73] Based on the sequence of the 31–50-amino acid region, we determined the DNA-binding motif to be TIxxAVxxGxxVTIxxFxxx (x can be substituted by alanine).[73] This motif was perfectly conserved in MDP1 orthologs but was not observed in any other nucleic acid-binding proteins in the database. MDP1 binds to GC-rich DNA, while almost all histone-like proteins bind to the AT-rich region. This discrepancy of binding to DNA is one of the differences between MDP1

and other bacterial histone-like proteins. The MDP1-specific motif interacted with poly dG and dC, suggesting the important role of this region in the interaction between MDP1 and DNA. Synthetic peptide-based studies also disclosed that the C-terminal 111–130- and 186–205-amino acid regions have DNA-binding activity, although their affinities are lower than that of the 31–50-amino acid region. The DNA-binding activities of these three regions may participate in the nucleic acid-binding activity of MDP1.

By contrast, our preliminary data showed that translational suppression by MDP1 was not inhibited by exogenously added DNA. We showed that MDP1 is a component of the purified 50S subunit of ribosomes,[63] suggesting that MDP1 localized on the ribosome similarly as nucleoids. Recombinant MDP1 lacking ribosome-binding activity fails to suppress translation. Thus, it is conceivable that translational suppression by MDP1 is mediated by direct binding of MDP1 to ribosomes.

3.4. MDP1 coordinates bacterial growth

Next, we examined the effect of MDP1 on the growth rate of bacteria. We expressed MDP1 in both *E. coli* and *M. smegmatis*. The growth of each bacterium was retarded by MDP1 expression (Figure 3). MDP1 (HupB) is presumably essential in slow growing mycobacteria, such as BCG and *M. tuberculosis*.[74,75] In 2008, Lewin *et al.* constructed an antisense plasmid targeting MDP1, which reduced the expression level of MDP1 by approximately 50% compared to that of parental BCG. They demonstrated that downregulation of MDP1 increased the growth of BCG in both culture media and macrophages.[76] Taken together, these reports suggest an important role of MDP1 in controlling the growth rate in slow growing mycobacteria.

As mentioned above, several groups including ours revealed that MDP1 localizes in/on the cell walls of mycobacteria.[69-71,77] Because MDP1 is retained in the cell wall but is not exported to the extracellular milieu, we hypothesized that MDP1 tightly bound to some unknown molecules in the cell wall. We found that MDP1 can bind glycolipids, such as trehalose 6-monomycolate (TMM).[78] TMM-derived mycolic

acids are transferred to other TMM to synthesize trehalose-6,6′-dimycolate and also to peptidoglycan-linked arabinogalactan to construct the inner layer of the envelope, which is critical for cell wall biogenesis in acid-fast bacilli.[79] Antigen 85 (Ag85) complex proteins (A, B, and C) are mycolyltransferases that catalyze the transfer of mycolic acids to TMM and arabinogalactan.[80,81] We found that MDP1 has a dual concentration-dependant regulatory activity on the mycolyltransferase functions of the Ag85 complex.[78] High doses of MDP1 suppressed mycolic acid transfer to trehalose. MDP1 accumulates in the cell wall fraction in the growth-retarded state and participates in the cessation of cell wall biogenesis by inhibiting mycolic acid transfer. Thus, MDP1 has a role to link cell wall biogenesis to the growth state.

3.5. The possible role of MDP1 in dormancy

MDP1 plays significant role in coordinating the growth of mycobacterial cells, and Hlp/MDP1 was found to be remarkably upregulated in the oxygen-depleted dormant state.[65] These reports suggested a significant role of MDP1 in the dormancy of mycobacteria. MDP1/HupB is presumably essential for slow growing mycobacteria,[74,75] but it can be dispensable in rapidly growing organisms, such as *M. smegmatis*.[65,82] Hlp-knockout *M. smegmatis* retained the ability to shift to the dormant state in response to oxygen depletion and acquired phenotypic resistance to isoniazid.[65] However, we recently found that dormant Hlp-KO *M. smegmatis* gradually loses viability during the dormant phase. Furthermore, Lewin *et al.* demonstrated that an approximate 50% reduction in MDP1 expression induced by an antisense plasmid remarkably influenced the protein expression and cell wall glycolipids constitutions in the oxygen-depleted dormancy model.[76] These results suggest that the biological role of MDP1 is much more significant in slow growers and has great impact on the viability of dormant mycobacteria.

3.6. Longevity-related enzymatic activity of MDP1

Mycobacteria have acquired long life spans. Dormant mycobacteria presumably live as long as their human hosts. Reactive oxygen

species (ROS) is one of the factors that promote the aging of cells.[83] Accordingly, the ability to detoxify ROS is related to aging. Iron is an essential metal for almost all living organisms.[84,85] The most active ROS is the hydroxyl radical, which is generated through the nonenzymatic Fenton reaction in the presence of H_2O_2 and Fe^{2+}. We recently discovered that MDP1 has ferroxidase activity and can prevent the Fenton reaction by converting Fe^{2+} to Fe^{3+}.[86] MDP1 is a major component in dormant mycobacteria. This newly identified enzymatic activity of MDP1 may contribute to the longevity of mycobacteria during the dormant state.

3.7. Antigenicity and vaccine efficacy

Prasad *et al.* reported that Hlp_{Mt}/MDP1 is a highly immunogenic antigen in *M. tuberculosis*-infected individuals.[66] We examined the antigenicity of MDP1 in mice and found that the immunogenicity of MDP1 is stimulated in the presence of DNA. Coadministration of MDP1 and CpG-DNA stimulated the production of proinflammatory cytokines, such as TNF-α and IL-6, from macrophages and induced host protection against *M. tuberculosis* infection.[87] Thus, MDP1 and CpG DNA are a disadvantageous combination for the parasitism of mycobacteria. Despite this fact, *M. tuberculosis* must produce MDP1 because it is essential to their survival and probably to their longevity. Based on this consideration, we think that the biological significance of antigens as well as their antigenicity should be considered in the development of TB vaccines and MDP1 is one of vaccine candidates against TB.

4. Conclusion and future perspectives

Instead of rapid multiplication, mycobacteria have acquired a slow growth pattern that promotes longevity. Host immunity affords a desirable noncompetitive environment for *M. tuberculosis* in human lungs, where this pathogen slowly grows or arrests growth, which prevents the rapid death of the host. The adaptation of mycobacteria to environmental stress may require the coordination of gene expression and metabolic activity. Given the complexity of human TB, both *in vivo* and *in vitro* models of latent TB models offer a vast resource for

vaccine development and evaluation. Deeper investigation of the mechanism of dormancy will lead to the improvement of anti-TB vaccination strategies.

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6. References

1. Hershberg R, Lipatov M, Small PM, *et al.* (2008) High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol 6:e311.
2. Wirth T, Hildebrand F, Allix-Beguec C, *et al.* (2008) Origin, spread and demography of the *Mycobacterium tuberculosis* complex. PLoS Pathog 4:e1000160.
3. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, *et al.* (1994) Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263:678-681.
4. van der Wel N, Hava D, Houben D, *et al.* (2007) *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 129:1287-1298.
5. Nyka W (1974) Studies on the effect of starvation on mycobacteria. Infect Immun 9:843-850.
6. Loebel RO, Shorr E,Richardson HB (1933) The influence of adverse conditions upon the respiratory metabolism and growth of human tubercle bacilli. J Bacteriol 26:167-200.
7. Smeulders MJ, Keer J, Speight RA, *et al.* (1999) Adaptation of *Mycobacterium smegmatis* to stationary phase. J Bacteriol 181:270-283.
8. Betts JC, Lukey PT, Robb LC, *et al.* (2002) Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol Microbiol 43:717-731.
9. Wayne LG (1976) Dynamics of submerged growth of *Mycobacterium tuberculosis* under aerobic and microaerophilic conditions. Am Rev Respir Dis 114:807-811.
10. Wayne LG,Hayes LG (1996) An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect Immun 64:2062-2069.

11. Voskuil MI, Visconti KC, Schoolnik GK (2004) *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. Tuberculosis (Edinb) 84:218-227.
12. Boon C, Li R, Qi R, *et al.* (2001) Proteins of *Mycobacterium bovis* BCG induced in the Wayne dormancy model. J Bacteriol 183:2672-2676.
13. Voskuil MI, Schnappinger D, Visconti KC, *et al.* (2003) Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. J Exp Med 198:705-713.
14. Fisher MA, Plikaytis BB, Shinnick TM (2002) Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes. J Bacteriol 184:4025-4032.
15. Kim SY, Lee BS, Shin SJ, *et al.* (2008) Differentially expressed genes in *Mycobacterium tuberculosis* H_{37}Rv under mild acidic and hypoxic conditions. J Med Microbiol 57:1473-1480.
16. Deb C, Lee CM, Dubey VS, *et al.* (2009) A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. PLoS One 4:e6077.
17. Blumberg HM, Leonard MK, Jr., Jasmer RM (2005) Update on the treatment of tuberculosis and latent tuberculosis infection. JAMA 293:2776-2784.
18. Polesky A, Farber HW, Gottlieb DJ, *et al.* (1996) Rifampin preventive therapy for tuberculosis in Boston's homeless. Am J Respir Crit Care Med 154:1473-1477.
19. Wayne LG, Sramek HA (1994) Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 38:2054-2058.
20. McCune RM, Jr., McDermott W, Tompsett R (1956) The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. J Exp Med 104:763-802.
21. Rhoades ER, Frank AA, Orme IM (1997) Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent *Mycobacterium tuberculosis*. Tuber Lung Dis 78:57-66.
22. Caruso AM, Serbina N, Klein E, *et al.* (1999) Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. J Immunol 162:5407-5416.
23. Scanga CA, Mohan VP, Yu K, *et al.* (2000) Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. J Exp Med 192:347-358.

24. Cooper AM, Callahan JE, Keen M, *et al.* (1997) Expression of memory immunity in the lung following re-exposure to *Mycobacterium tuberculosis.* Tuber Lung Dis 78:67-73.
25. de Jong R, Altare F, Haagen IA, *et al.* (1998) Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science 280:1435-1438.
26. Bean AG, Roach DR, Briscoe H, *et al.* (1999) Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. J Immunol 162:3504-3511.
27. Flynn JL, Goldstein MM, Chan J, *et al.* (1995) Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity 2:561-572.
28. Riley RL, Mills CC, O'Grady F, *et al.* (1962) Infectiousness of air from a tuberculosis ward. Ultraviolet irradiation of infected air: comparative infectiousness of different patients. Am Rev Respir Dis 85: 511-525.
29. Horwitz MA,Harth G (2003) A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect Immun 71:1672-1679.
30. Wiegeshaus EH, McMurray DN, Grover AA, *et al.* (1970) Host-parasite relationships in experimental airborne tuberculosis. 3. Relevance of microbial enumeration to acquired resistance in guinea pigs. Am Rev Respir Dis 102:422-429.
31. Kashino SS, Napolitano DR, Skobe Z, *et al.* (2008) Guinea pig model of *Mycobacterium tuberculosis* latent/dormant infection. Microbes Infect 10:1469-1476.
32. Lurie MB, Abramson S, Heppleston AG (1952) On the response of genetically resistant and susceptible rabbits to the quantitative inhalation of human type tubercle bacilli and the nature of resistance to tuberculosis. J Exp Med 95:119-134.
33. Dannenberg AM, Jr., Collins FM (2001) Progressive pulmonary tuberculosis is not due to increasing numbers of viable bacilli in rabbits, mice and guinea pigs, but is due to a continuous host response to mycobacterial products. Tuberculosis (Edinb) 81:229-242.
34. Manabe YC, Kesavan AK, Lopez-Molina J, *et al.* (2008) The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. Tuberculosis (Edinb) 88:187-196.
35. Walsh GP, Tan EV, dela Cruz EC, *et al.* (1996) The Philippine cynomolgus monkey (*Macaca fasicularis*) provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 2:430-436.
36. Capuano SV, 3rd, Croix DA, Pawar S, *et al.* (2003) Experimental *Mycobac-*

terium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human *M. tuberculosis* infection. Infect Immun 71:5831-5844.

37. Flynn JL, Capuano SV, Croix D, *et al.* (2003) Non-human primates: a model for tuberculosis research. Tuberculosis (Edinb) 83: 116-118.
38. Lin PL, Rodgers M, Smith L, *et al.* (2009) Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. Infect Immun 77:4631-4642.
39. Yuan Y, Crane DD, Barry CE, 3rd (1996) Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. J Bacteriol 178:4484-4492.
40. Cunningham AF, Spreadbury CL (1998) Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. J Bacteriol 180:801-808.
41. Monahan IM, Betts J, Banerjee DK, *et al.* (2001) Differential expression of mycobacterial proteins following phagocytosis by macrophages. Microbiology 147:459-471.
42. Yuan Y, Crane DD, Simpson RM, *et al.* (1998) The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. Proc Natl Acad Sci USA 95:9578-9583.
43. Boon C, Dick T (2002) *Mycobacterium bovis* BCG response regulator essential for hypoxic dormancy. J Bacteriol. 184: 6760-6767.
44. Hutter B,Dick T (1999) Up-regulation of narX, encoding a putative 'fused nitrate reductase' in anaerobic dormant *Mycobacterium bovis* BCG. FEMS Microbiol Lett 178:63-69.
45. Fritz C, Maass S, Kreft A, *et al.* (2002) Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect Immun 70:286-291.
46. Lee BH, Murugasu-Oei B, Dick T (1998) Upregulation of a histone-like protein in dormant *Mycobacterium smegmatis*. Mol Gen Genet 260:475-479.
47. Salerno P, Larsson J, Bucca G, *et al.* (2009) One of the two genes encoding nucleoid-associated HU proteins in *Streptomyces* coelicolor is developmentally regulated and specifically involved in spore maturation. J Bacteriol 191:6489-6500.
48. Shires K, Steyn L (2001) The cold-shock stress response in *Mycobacterium smegmatis* induces the expression of a histone-like protein. Mol Microbiol 39:994-1009.
49. Gupta S, Pandit SB, Srinivasan N, *et al.* (2002) Proteomics analysis of carbon-starved *Mycobacterium smegmatis*: induction of Dps-like protein. Protein Eng 15:503-512.

50. Colangeli R, Haq A, Arcus VL, *et al.* (2009) The multifunctional histone-like protein Lsr2 protects mycobacteria against reactive oxygen intermediates. Proc Natl Acad Sci USA 106:4414-4418.
51. Brieger EM, Glauert AM (1956) Sporelike structures in the tubercle bacillus. Nature 178:544.
52. Csillag A (1961) Spore formation and 'dimorphism' in the mycobacteria. J Gen Microbiol 26:97-109.
53. Ghosh J, Larsson P, Singh B, *et al.* (2009) Sporulation in mycobacteria. Proc Natl Acad Sci USA 106:10781-10786.
54. Traag BA, Driks A, Stragier P, *et al.* (2009) Do mycobacteria produce endospores? Proc Natl Acad Sci USA 107: 878-881.
55. Waltermann M, Steinbuchel A (2005) Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. J Bacteriol 187:3607-3619.
56. Garton NJ, Christensen H, Minnikin DE, *et al.* (2002) Intracellular lipophilic inclusions of mycobacteria *in vitro* and in sputum. Microbiology 148:2951-2958.
57. de Wit D, Mitchison DA (1993) DNA analysis demonstrates that mycococcus forms are not mycobacteria. Tuber Lung Dis 74:96-99.
58. Rustad TR, Sherrid AM, Minch KJ, *et al.* (2009) Hypoxia: a window into *Mycobacterium tuberculosis* latency. Cell Microbiol 11:1151-1159.
59. Wayne LG, Sohaskey CD (2001) Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu Rev Microbiol. 55:139-163.
60. Shiloh MU, Manzanillo P, Cox JS (2008) *Mycobacterium tuberculosis* senses host-derived carbon monoxide during macrophage infection. Cell Host Microbe 3:323-330.
61. Shi L, Jung YJ, Tyagi S, *et al.* (2003) Expression of Th1-mediated immunity in mouse lungs induces a *Mycobacterium tuberculosis* transcription pattern characteristic of nonreplicating persistence. Proc Natl Acad Sci USA 100:241-246.
62. Tabira Y, Ohara N, Ohara N, *et al.* (1998) The 16-kDa alpha-crystallin-like protein of *Mycobacterium bovis* BCG is produced under conditions of oxygen deficiency and is associated with ribosomes. Res Microbiol 149:255-264.
63. Matsumoto S, Yukitake H, Furugen M, *et al.* (1999) Identification of a novel DNA-binding protein from *Mycobacterium bovis* bacillus Calmette-Guérin. Microbiol Immunol 43:1027-1036.
64. Matsumoto S, Furugen M, Yukitake H, *et al.* (2000) The gene encoding mycobacterial DNA-binding protein 1 (MDP1) transformed rapidly growing bacteria to slowly growing bacteria. FEMS Microbiol Lett 182:297-301.