

Use of siRNA molecular beacons to detect and attenuate mycobacterial infection in macrophages

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Abstract

Tuberculosis is one of the leading infectious diseases plaguing mankind and is mediated by the facultative pathogen, *Mycobacterium tuberculosis* (MTB). Once the pathogen enters the body, it subverts the host immune defenses and thrives for extended periods of time within the host macrophages in the lung granulomas, a condition called latent tuberculosis (LTB). Persons with LTB are prone to reactivation of the disease when the body's immunity is compromised. Currently there are no reliable and effective diagnosis and treatment options for LTB, which necessitates new research in this area. The mycobacterial proteins and genes mediating the adaptive responses inside the macrophage is largely yet to be determined. Recently, it has been shown that the *mce* operon genes are critical for host cell invasion by the mycobacterium and for establishing a persistent infection in both *in vitro* and in mouse models of tuberculosis. The YrbE and Mce proteins which are encoded by the MTB *mce* operons display high degrees of homology to the permeases and the surface binding protein of the ABC transports, respectively. Similarities in structure and cell surface location implicate a role in cell invasion at cholesterol rich regions and immunomodulation. The *mce4* operon is also thought to encode a cholesterol transport system that enables the mycobacterium to derive both energy and carbon from the host membrane lipids and possibly generating virulence mediating metabolites, thus enabling the bacteria in its long term survival within the granuloma. Various deletion mutation studies involving individual or whole *mce* operon genes have shown to be conferring varying degrees of attenuation of infectivity or at times hypervirulence to the host MTB, with the deletion of *mce4A* operon gene conferring the greatest degree of attenuation of virulence. Antisense technology using synthetic siRNAs has been used in knocking down genes in bacteria and over the years this has evolved

into a powerful tool for elucidating the roles of various genes mediating infectivity and survival in mycobacteria. Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is rapidly gaining popularity.

Key words: Mammalian cell entry; Molecular beacons; siRNA; Mycobacterium tuberculosis; Macrophages

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Core tip: This review paper looks at the current status of research of the role of mammalian cell entry gene products in mediating cholesterol mediated latency of mycobacteria and the potential use of short-interfering RNA molecular beacons in detecting and attenuating mycobacterial infections.

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EPIDEMIOLOGY OF LTB

The year 2005 marked the 100th anniversary since Robert Koch received the Nobel prize for his work on tuberculosis (TB) and yet more than one hundred years later the World Health Organization (WHO) has reaffirmed its designation of TB as a global emergency^[1]. Tuberculosis still remains a pandemic, infecting one-third of the world's population and killing millions of people each year. Estimates are that a TB death occurs every minute. According to recent estimates of WHO, nearly 9 million people were infected with TB in 2012, including 1.3 million TB-related deaths worldwide^[2]. Incidentally, TB and reactivation of latent TB have turned out to be the leading causes of death for people who are infected with human immunodeficiency virus (HIV). At the same time, according to the Centers for Disease Control (CDC), there were 9945 TB cases reported in the United States in 2012^[3]. More than 80% of TB cases in the United States are from reactivation of latent TB infection^[4].

Tuberculosis is a disease that spreads from person to person through the air and is mediated by the pathogen *Mycobacterium tuberculosis* (MTB). The TB bacillus was discovered in 1882 and has been the subject of extensive research since then. There is still much to be learned about the nature of this organism, its virulent properties, and its response to host defenses. TB affects the lungs mainly, but can also have other target organs such as brain, spine and the kidneys. When a person with TB infection coughs or sneezes, droplets containing

MTB are released into the air and when another person breathes in the infected droplets, they can be infected. However, not everyone infected with TB bacteria becomes sick. There are two TB-related conditions that exist: latent TB (LTB) infection and active TB disease. Those that have latent TB infection do not feel sick or do not present with any symptoms. In LTB cases, even though they are infected with the mycobacterial pathogen, they do not have active TB disease. Overall, it has been shown that about 90% of the people infected with MTB will have LTB infection and 10% will eventually go on to have full-blown active TB at a later stage in their life^[5]. Nearly 50% of those who develop TB do so within the first two years of infection. This rate is even higher in immunocompromised individuals, such as those with HIV infection, where the risk of developing TB from LTB activation is significantly higher. Also of particular concern are those infected with drug-resistant TB (XDR TB)^[3].

Thus, this ancient human adversary continues to be a challenge in all aspects of medical care, from prevention to diagnosis and therapy.

NEED FOR IMPROVED DIAGNOSTIC AND THERAPEUTIC METHODS FOR LTB

Currently, there are no tests available to directly detect *in vivo* the presence of latent MTB in an affected individual and assessment of latent infection involves an imperfect approach of measuring the host immune response to mycobacterial infection^[4]. On the contrary, active TB infection is diagnosed by detecting MTB bacteria in clinical samples taken from patients. A positive diagnosis can be made only by culturing MTB from the specimen, even though the results from this may take four to eight weeks for conclusive answers. Other methods for diagnosing TB include chest X-rays, patient sputum smear microscopy, polymerase chain reaction (PCR) testing, immunological memory-based tests including the less specific purified protein derivative (PPD/tuberculin) skin test and more specific Interferon- γ release assays, phage amplification assays, solid and automated liquid cultures, as well as several tests for antibiotic resistance. These tests can only strongly suggest the presence of active TB or LTB as a diagnosis but they cannot confirm the presence of the bacteria in the body. Reliable and rapid diagnosis of latent TB is a major challenge in low socioeconomic areas, and even in parts of developed countries, especially in areas where immunodeficiency diseases like acquired immune deficiency syndrome are more endemic. In many cases, patients have to undergo time-consuming multiple testing before reaching an apparent diagnosis. This testing deficiency can be especially critical when trying to identify high-risk individuals for prophylactic regimen, and also for identifying and managing extrapulmonary TB sites in HIV co-infected patients^[6].

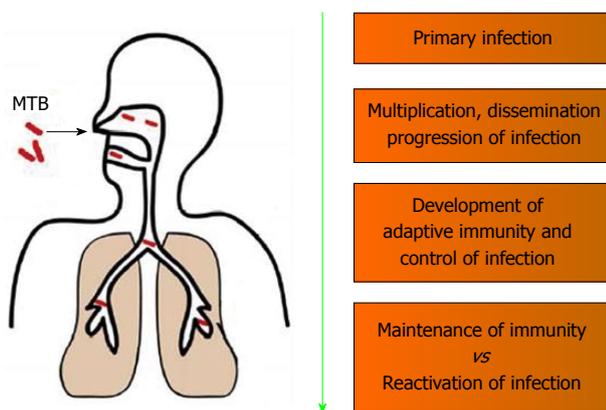


Figure 1 Spectrum of host immune responses against *Mycobacterium tuberculosis*. MTB: *Mycobacterium tuberculosis*.

Treatment for active TB cases consists of a combination of four first-line antibiotics for a period of two months, followed by two drugs for another four months^[7]. First line antibiotics consist of rifampicin, isoniazid, pyrazinamide, and ethambutol. These drugs are effective mainly in actively dividing bacilli and its effectiveness in treating LTB, where the bacilli are dormant, has not yet been proven. The treatment of LTB is usually long term with the intent of sterilizing the non-replicative or slowly replicating bacteria^[8]. If the particular mycobacterial strain is resistant to the first line drugs, then treatment is escalated for up to 18 mo with five lines of available drugs. Surgery is also performed as a last resort if treatment fails due to drug resistance. Since the chemotherapy regimen for active and latent TB infections usually spans many months, poor patient compliance rates is a major issue contributing to the emergence of resistant strains^[4]. There is a pressing need for rapid and inexpensive tests to confirm latent TB cases in order to manage this global epidemic.

Developing a direct MTB imaging screening tool for the asymptomatic population along with novel treatment strategies is vital to our fight against TB. This is especially true for high-risk categories with LTB such as drug users with unsanitary needles, healthcare workers in high risk and densely populated environments, the medically under-served poor and minority populations, children exposed to high risk adults, immunocompromised patients and patients on immunosuppressant drugs, and health care workers who serve these high risk populations^[9]. Developing a direct MTB imaging screening tool with combined therapeutic applications for the asymptomatic population is going to have vitally important and far reaching impact in the fight against TB.

SPECTRUM OF HOST IMMUNE RESPONSE AGAINST MTB AND DEVELOPMENT OF LTB

Humans are the only natural host of MTB and are highly

susceptible to MTB infections. Even a few (5-10) bacilli is capable of mediating a primary infection^[10]. The initial interaction of the MTB with the host involves alveolar macrophages, which is the only known cell type to harbor MTB *in vivo*^[11]. Upon coming in contact with MTB, the interaction of the host immune response with MTB can be divided into 4 general types of events^[12]: (1) Primary infection event involving the invading MTB; (2) Events that would promote the dissemination and progressions of the MTB infection; (3) Development of an adaptive immunity that would lead to the containment MTB infection; and (4) Interplay of protective immunity involved in latency vs immunologic compromises leading to reactivation of MTB infection (Figure 1).

Phagocytosis of the MTB by alveolar macrophages followed by its intracellular growth initiates the cascade of immune events of the primary infection^[13]. Activation of the components of the innate immunity, the recruitment of various classes of monocytes and lymphocytes to the site of infection, and the final development of specific immunity allow for the containment of infection (Figure 2).

The hallmark of latent TB is the granulomatous lung parenchymal lesions and their draining lymph nodes which is called the "Ghon complex". The events leading to the formation of granuloma begins when the MTB is inhaled into the lungs and the bacterium is phagocytosed by alveolar macrophages and dendritic cells. The infected cells release proinflammatory cytokines that help recruit more immune cells to the site of infection. The cytokines IL-12 and IL-18 from the infected cells induce natural killer cell activity, which in turn produce IFN- γ that help active macrophages to produce tumor necrosis factor- α and other microbicidal substances. Through the actions of these cytokines and chemokines, other immune cells are recruited leading to the formation of the granuloma^[14,15]. In the granuloma, the macrophages further differentiate into epithelioid cells and foamy macrophages and are surrounded by lymphocytes and an outer layer of fibroblasts and matrix proteins. The morphology of the lung granuloma is characterized by a central necrotic core surrounded by concentric layers of macrophages, epithelioid cells, multinucleated Langhans giant cells, and lymphocytes^[16,17]. Containment of MTB at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence (Figure 3). Successful containment of the pathogen to the site of the primary lesion results in latent infection, which appears in chest X-rays as calcified granulomatous lesions^[18].

The exact location of dormant MTB organisms in latent TB has not been elucidated^[18]. Studies have shown evidence of the presence of the pathogen in the normal tissue surrounding the granuloma necrotic centers^[19] which appears to be the preferred location of the pathogen during latency^[20].

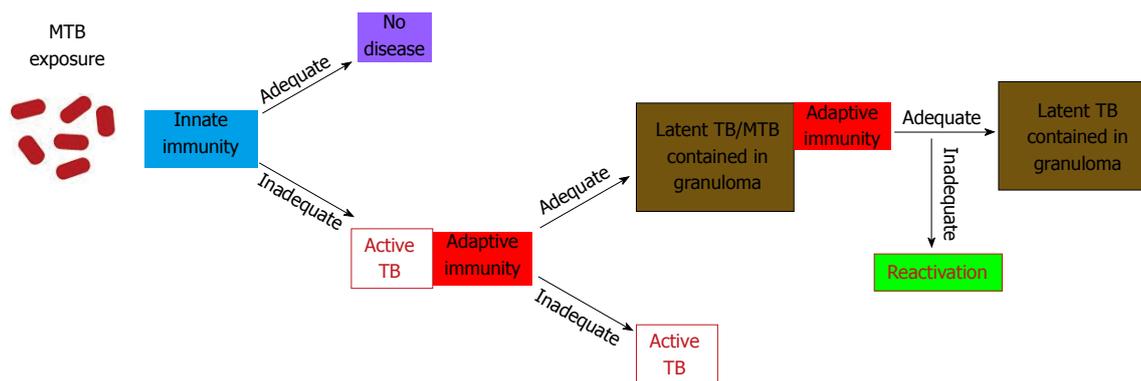


Figure 2 Latent tuberculosis pathogenesis and transmission profile. Most people are adequately protected by innate immunity against MTB infection, however, the infection progresses when the innate immune response levels are inadequate. The development of specific immunity leads to the containment of MTB in granulomas as asymptomatic latent infection. However, inadequate adaptive response at any time progresses to the reactivation and development of full-blown active tuberculosis. MTB: *Mycobacterium tuberculosis*.

A dynamic balance between the host immune response and the MTB pathogen is maintained during latency. Direct cross-talk between MTB and the host immune response occurs in a dense region surrounding the granuloma which is derived from lymphocyte infiltration^[21,22]. The granuloma has a central necrotic core which serves as nutritional source for persisting mycobacteria, surrounded by the thick leukocyte wall which prevents the spread of the mycobacteria. The leukocyte derived wall surrounding the granuloma is highly vascularized and facilitates the delivery of drugs against latent TB^[23].

POST-PHAGOCYTTIC MOLECULAR EVENTS FOLLOWING MYCOBACTERIAL ENTRY

Once entering the host, the ability of MTB to survive decades within the body of the host by subverting the host immune defenses is of continued intrigue and fascination. The precise mechanism of how the bacteria is able to achieve this long term dormancy leading to LTB is still unknown, however, recent advances in mycobacterial molecular biology have shed some light into these processes.

Macrophages make up the major component of the innate host defense, and they do this by pathogen recognition, ingestion and killing of foreign microbes that enters the body including pathogenic and non-pathogenic mycobacteria. The pathogenic mycobacteria have developed a number of strategies to subvert the host immune defenses and evade the destructive action of the macrophages, eventually surviving within this normally inhospitable cell for long periods of time thus resulting in the disease^[24]. The surviving bacteria within the macrophages can be in a latent state with stationary growth or, given the right conditions in an immunocompromised host, can switch to a metabolically active state that facilitates proliferation, dissemination and active disease.

Upon gaining entry into the body, most non-patho-

genic microbes get phagocytosed by the macrophage into a phagosome where the invading microbe gets exposed to high levels of reactive oxygen species and reactive nitrogen species. The phagosome then goes on to mature and fuse with the organelles of the endocytic pathway, thereby acquiring surface molecular markers which leads to the acidification of the phagosome to pH 5 as well as gaining hydrolytic enzymes that digest the invading microbe^[25,26] (Figure 4A). MTB, however, has developed several ways to evade attack by the macrophage and creates a favorable environment for replication (Figure 4B). This is mainly by inhibiting several aspects of phagosomal maturation, including fusion and fission events along the endocytic pathway and the recruitment of vacuolar H⁺-ATPases^[27,28]. The MTB carrying phagosome retains characteristics of an early phagosome with regard to its pH (about 6-6.5), presence of Rab5 (a Rho-GTPase directing endosomal trafficking and mediating fusion between phagosomes and other organelles), and continued access to other recycling endosomes^[29,30], but it lacks mature hydrolases^[31] and cathepsins, with interactions between the phagosome and the *trans* Golgi Network blocked^[32]. Several MTB products are believed to be inhibitors of phagosomal maturation, including components of the mycobacterial cell envelope such as lipoarabinomannan, trehalose dimycolate and sulpholipids, phosphatase SapM and kinase PknG^[29], and the secreted protein ESAT-6^[33]. The exact mechanism behind the inhibition of phagosomal maturation by the mycobacteria is yet to be elucidated.

Studies have shown that cholesterol is a necessary component for the uptake of the MTB into the macrophage *via* the complement receptors and for the inhibition of phagosomal maturation^[34] (Figure 4B). A host protein associated with the cell membrane called tryptophan-aspartate containing coat protein (TACO) is recruited and retained in the phagosomes harboring mycobacteria thereby preventing the bacterial delivery to lysosomes^[35]. TACO is an actin-binding protein seen associated with cholesterol rich regions of the host macrophage plasma membrane^[34]. The mycobacterium

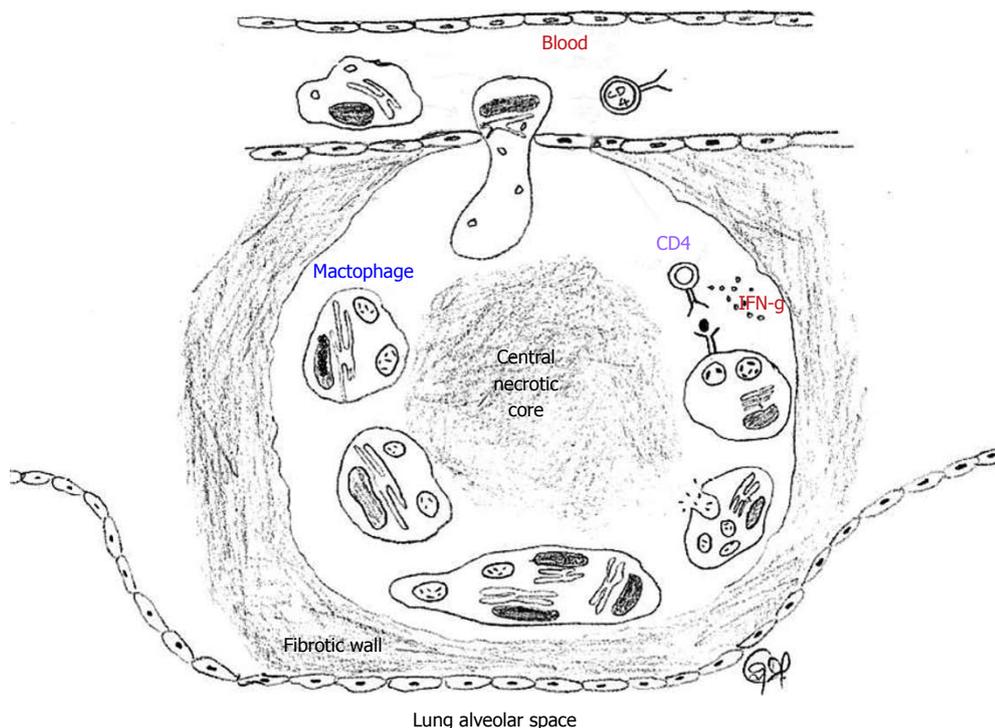


Figure 3 Tuberculosis granuloma. A granuloma sequesters MTB infected macrophages and is surrounded by immune cells, predominantly CD4⁺ helper T lymphocytes. Some infected macrophages fuse to form foamy giant cells. The infected macrophages and giant cells present antigens to T cells and activate them to produce a variety of cytokines and chemokines, and also kills the infected macrophage and the MTB. The chemokines also serve to recruit additional T cells to the granuloma from the circulating blood. IFN- γ activates the macrophages to kill the intracellular MTB by generating reactive oxygen species and reactive nitrogen species intracellularly. The center of the granuloma is filled with cell debris and both live and dead MTB spilled from dead macrophages (caseation), all of which form a central hypoxic necrotic core. A sheath of collagen fibers produced from lung fibroblasts surround the granuloma. MTB: *Mycobacterium tuberculosis*.

within the phagosome is somehow able to prevent the removal of the TACO coat protein which prevents the fusion of phagosome with lysosome^[35]. Moreover, it has been shown that TACO-mediated uptake of mycobacteria depends on cholesterol^[34,36,37]. The mycobacterial proteins and genes mediating these adaptive responses inside the macrophage is largely yet to be determined. MTB's unique ability to utilize cholesterol, a component of cell membranes, also plays a role in its persistence^[38]. In the nutrient-deficient intracellular environment, MTB adapts its metabolism by alternating between carbohydrate and fatty acid metabolism^[39]. Studies have shown that MTB utilizes cholesterol for its energy needs and for the biosynthesis of virulence-associated lipid phthiocerol dimycocerosate^[38]. A number of reports indicate that MTB metabolizes cholesterol during host infections and the metabolic products contribute to the long-term survival of MTB in the host^[38,40,41]. Furthermore, because the cholesterol catabolism pathway requires a large number of oxygenases, it should be no surprise that MTB infects the lungs where oxygen concentration is the highest^[42].

MCE OPERONS AND THEIR ROLE IN MYCOBACTERIAL INFECTION AND PERSISTENCE

Over the recent years, it was shown that a DNA fragment

from MTB cloned into *Escherichia coli* (*E. coli*) could mediate the latter's entry and survival in mammalian cells^[43] and was named as the mammalian cell entry (*mce*) operon. The *mce* operon genes have been shown to be important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection both *in vitro* and in mouse models^[44,45]. The analysis of the complete genome sequence of MTB in 1998^[46] showed that the *mce* operon is composed of a group of four homologous *mce* operons (*mce1*, *mce2*, *mce3*, and *mce4*). It was found that all the constituent *mce* genes in the four operons were arranged in an identical manner. Each of the operon contained eight genes, of which two genes preceding the *mce* genes are named *yrbEA* and *yrbEB* which encoded for integral membrane proteins and the six *mce* genes potentially encoding exported proteins (secreted or surface-exposed) thought to be important for the entry and survival of the pathogen in the mammalian cells^[46]. The four *mce* operons are widely seen throughout the genus *Mycobacterium*^[34] and the general organization of the genes in each of the four operons are shown in Figure 5.

The YRBE and MCE proteins encoded by the MTB *mce* operons have structural homology to the permeases and the surface binding protein components of the ABC transporters, respectively^[47]. The typical ABC permease contains six trans-membrane helices with the C-terminus located on the cytoplasmic side of the membrane

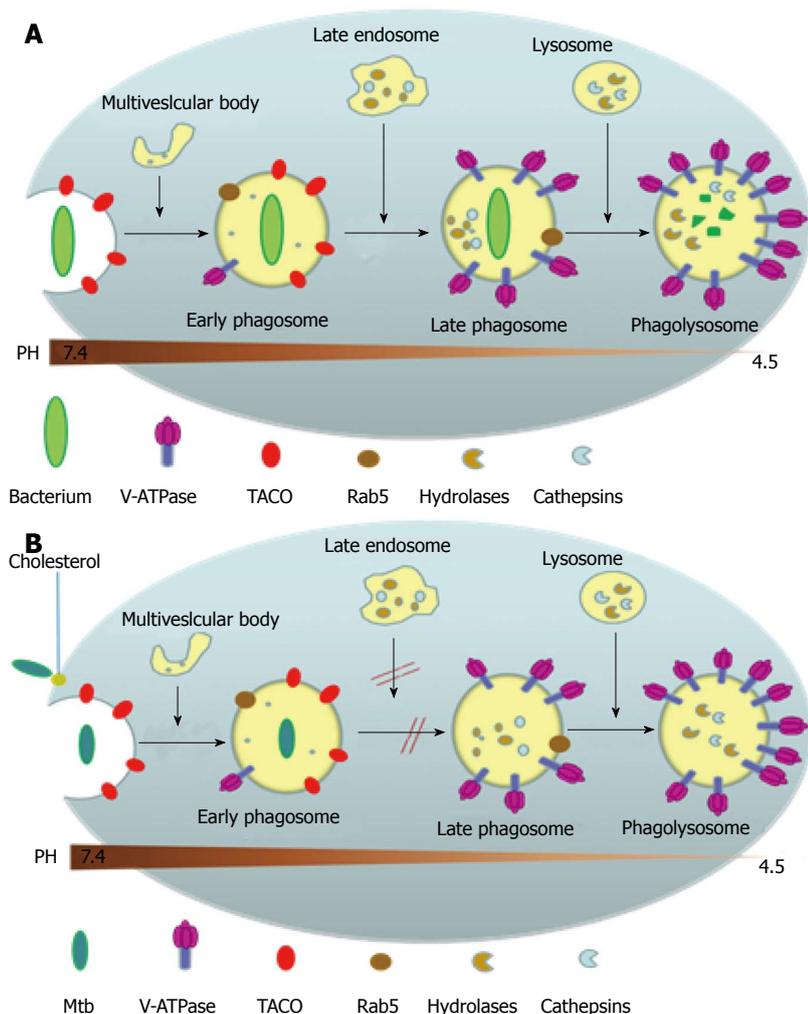


Figure 4 Phagosomal maturation or arrest following pathogen uptake (A) or *Mycobacterium tuberculosis* uptake (B), respectively. (A) shows that upon entering into the body, most non-pathogenic microbes are phagocytized by the macrophage into a phagosome which then goes on to mature by fusing with the vesicles of the endocytic pathway and to finally fuse with lysosomes. These phagosomes undergo acidification due to the presence of proton-ATPase molecules from vacuolar membranes and the lysosomes, and this increased level of acidification activates the lysosomally derived acid hydrolases, cathepsins and other enzymes, along with reactive oxygen and nitrogen intermediates, to destroy the pathogen. Phagocytosis also initially triggers the recruitment of TACO around the particle to be ingested, as a result of the latter's initial association with cell cortex microtubules, but is released prior to the lysosomal delivery of the bacteria; (B) Shows the effect of *M. tuberculosis* on phagosome maturation. Cholesterol serves as a docking site for the mycobacteria and its cell surface receptor there by facilitating its phagocytosis at cholesterol-rich regions. Cholesterol plays a crucial role in not only the entry of mycobacteria into macrophages but also mediates the phagosomal association of TACO (Coronin 1), a coat protein associated with cholesterol-rich regions which is actively retained on the phagosomal membrane housing the mycobacteria through a yet unknown mechanism, which prevents the degradation of the mycobacteria in the lysosomes. TACO: Tryptophan-aspartate containing coat protein.



Figure 5 The organization of the *mce* operons. The operon structures of the four *mce* operons with their constituent genes are shown. The grey arrows represent *yrbE* genes and the blue arrows represent the *mce* genes.

(Figure 6A). The YRBE permease contains five or six transmembrane segments outside the C-terminus and the orientation of the N-terminal transmembrane helix may be either cytoplasmic or outside (Figure 6B), suggesting a transmembrane transport role^[47,48].

The MCE protein, including MCE1A, 3A and 4A, but not MCE2A, make up a patch of 275-564 amino

acid residues, with the hydrophobic stretch at the N-terminal anchored in the membrane, after folding and modification^[47], along with a 22 amino acid "invasion domain" near the C-terminal exposed outside the membrane^[49,50] (Figure 6B). These characteristics are consistent with their cell surface location and proposed role in cell invasion and immunomodulation^[51], however,

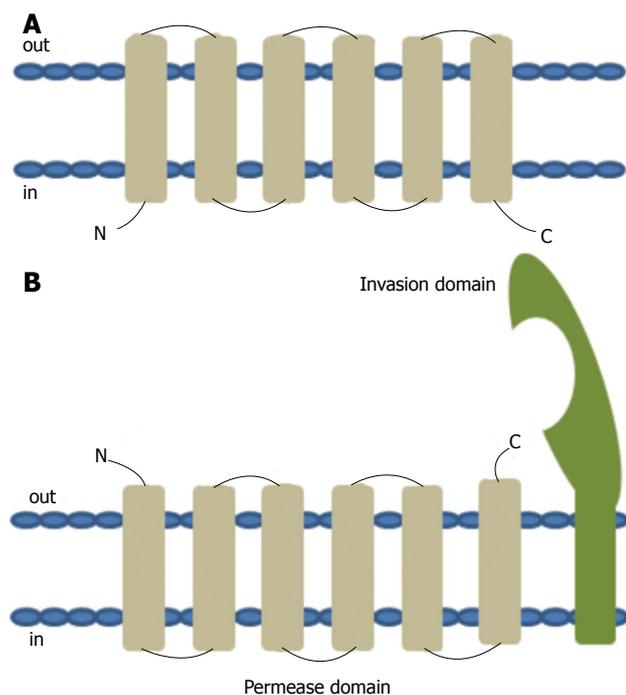


Figure 6 Topology of typical ATP binding cassette transmembrane permease (A) and the predicted topology of YRBE permease and MCE invasion domain (B). ATP binding cassette (ABC) transport systems in both prokaryotes and eukaryotes consists of four domains, two cytoplasmic ABC domains and two hydrophobic membrane spanning permease domains (A). Each of the membrane spanning domains typically contains 6 hydrophobic transmembrane segments anchored in the cell membrane with the C- and N-termini located towards the cytoplasmic side. ABC transporters in prokaryotes that function as importers also typically require additional extracytoplasmic helper proteins called 'substrate binding proteins' for function. Each of the *mce* operons contains two YRBE domains that have structural homology to the permease domain of the ABC transporters (B). Each YRBE permease contains five or six transmembrane segments with the C-terminal end located on the extracytoplasmic side and the N-terminal end being either cytoplasmic or extracellular. The MCE protein, MCE4A, consists of a patch of 400 amino acid residues, with a hydrophobic stretch anchored in the cell membrane and a 22-amino acid invasion domain exposed outside to the membrane, which is predicted to be structurally similar to the substrate binding domain of ABC importers.

the mechanism of interaction between YRBE and MCE proteins is not yet clear^[52].

Phylogenetic studies have shown that the MCE proteins share between 30%-70% amino acid identity to their inter-operon counterparts but only 16%-26% identity with other MCE proteins encoded by the same operon. For example, there is 61% identity between MCE1A and MCE2A, however, it falls to 25% identity between MCE1B and MCE1C^[53,54]. Because of the multiple *mce* operons in the genome, it is proposed that they may have redundant or time dependent activities. This possibility is supported by the temporal transcriptional expression differences during different stages of *in vitro* growth between *mce1* in comparison to *mce3* and *mce4*^[55]. Differences are also seen with *in vivo* growing bacilli in that even though *mce1*, *mce3*, and *mce4* transcripts are detectable up to 24 wk post infection in rabbit lung tissue, only *mce4* transcript is detectable 16 wk post infection in guinea pig spleen^[55].

The expression of *mce2* was not detected under any conditions tested^[55].

Studies using *mce* operon deletion or disruption mutants of MTB have demonstrated varying effects with the different *mce* operons. Some studies have shown that disrupting *mce* operons lead to attenuation^[56,57], while others have shown some degree of hypervirulence for the host MTB following the mutations^[58,59]. Deletion of *mce* operons 3 and 4 attenuated MTB virulence in infected macrophages^[60].

The *mce4A* gene is the first among the six *mce* genes in the *mce4* operon that is studied the most. Studies showed that the MCE4A protein is not only important for host cell invasion but also for survival of the MTB pathogen in human macrophages^[61,62]. Individual *mce1*, *mce2*, *mce3*, and *mce4* mutants administered intranasally or intravenously in mice have shown to result in lower bacterial burdens and slower mortality of the infected mice, with *mce4* operon deletion showing the greatest effects on MTB virulence^[56,60]. The route of infection was also shown to be having an effect on the attenuation results in one study^[56]. Hypervirulence among *mce1* mutants have been demonstrated in two separate studies when administered intranasally, intravenously, or intraperitoneally^[58,59]. The deletion mutants in all the above studies, however, were not identical in the nature of their deletions and had variations in their deletion sequences that possibly led to different polar effects on downstream genes, which may explain the discrepancy in the results of some of the deletion mutant studies.

Apart from its possible role in mediating host infection, it is thought that at least some of the MCE4 proteins form an outer membrane channel that mediates cholesterol entry into the cell, thereby enabling uptake by the mycobacterium of host lipids vital for its survival during the prolonged latent infection^[63]. Transposon Site Hybridization studies have shown that certain MTB genes involved in the lipid metabolism was genetically linked to the *mce4* operon genes^[60]. The *mce4* has been shown to encode a cholesterol transport system that enables the mycobacterium to derive both carbon and energy from the host membrane lipids and also for possibly generating sterol metabolites mediating its long-term survival within the macrophage^[38], with the *mce4* expression progressively increasing as the latency phase advances^[64].

ANTISENSE TECHNOLOGY IN BACTERIA

Hundreds of bacterial encoded short interfering RNAs (siRNA) have been reported over the past decade^[65-67]. Majority of these siRNAs act by binding to their target mRNAs to bring about the repression. They fall into two major categories: some are encoded at locations farther away from the target gene (trans-acting) and others are encoded by DNA strand complementary to the target gene (cis-acting). The trans-acting siRNAs

generally share only limited complementarity with their target gene and thus is prone to have off target effects. Trans-acting siRNAs are by far the most characterized bacterial siRNAs and have been shown to usually require the chaperon protein Hfq for base pairing^[68]. The cis-encoded siRNAs, or anti-sense RNAs, have perfect complementarity with their target gene and thus have more extensive and stronger base pairing. Among the reported bacterial antisense RNAs, some are short (siRNA), with around 100 nucleotides in length and are usually encoded by plasmids or bacteriophages, while some are chromosomally encoded and are longer, in some cases overlapping entire genes or corresponding to the 5' or 3' extension of the protein coding region of the mRNA. The 5' untranslated region of the *mogR* mRNA in *Listeria monocytogenes* overlaps 3 genes on the opposite strand involved in the flagellar synthesis and serves as an example of chromosomally encoded long antisense RNAs^[69]. The binding region of antisense RNA on the target mRNA can also vary and may be located in the 5' end, 3' end, the central region, or the entire coding region.

Antisense RNAs in the bacterial cell have been shown to repress many detriments to the cell such as transposons and toxic proteins. One of the first antisense RNA to be discovered in bacteria was the RNA-OUT of the transposon Tn10, which was shown to inhibit transposition by preventing the translation of transposase mRNA^[70]. Antisense RNAs are also seen encoded opposite transposase genes in *Salmonella enterica*^[71,72], *Caulobacter crescentus*^[73], and *Listeria monocytogenes*^[69]. Thus a critical role of bacterial antisense RNA, as in eukaryotes^[74], appears to be the inhibition of transposition. There is increasing evidence that antisense RNAs downregulate the expression of toxic proteins^[75,76]. It has been found that most of these repressed proteins are hydrophobic, small with less than 50 amino acids, and toxic at higher levels. An example of this tight repression of one such toxic protein is seen in *E. coli*, where the low levels of SymE protein is maintained by the LexA repressor of the save our souls response, the SymR antisense RNA and the Lon protease^[77]. Some of the antisense RNAs to transposases and toxic genes, such as SymR, are expressed constitutively in the cell^[77].

Studies have shown that antisense RNAs can positively and negatively regulate the expression of various transcriptional regulators and other metabolic and virulence proteins in bacterial systems. For example, The 109 nucleotide *GadY* antisense RNA of *E. coli* overlaps the intergenic region of the dicistronic *gadXW* mRNA which encodes two transcription regulators of the acid stress response genes and enhanced transcription of the *GadY* gene which leads to cleavage of *gadXW* mRNA into *gadX* and *gadW* transcripts, leading to positive regulation (increased expression) of those genes^[78,79]. And on the other hand, in the nitrogen-fixing cyanobacterium *Anabaena sp.* PCC 7120, the approximately 2200 nucleotide *alr1690- α -furA* antisense RNA spans the

entire *alr1690* coding region and extends through the gene encoding the ferric uptake transcriptional regulator, *FurA*, into its promoter and regulator regions and it helps decrease *furA* expression and translation, thereby acting as a negative regulator of iron absorption and nitrogen metabolism^[80]. Similar regulatory RNAs controlling metabolic responses to environmental effects have been reported in many other bacterial systems^[81-87].

Another recently discovered phenomenon is the antisense-mediated gene regulatory switch in the bacteria called the "excludon". This comprises a gene locus encoding an unusually long antisense RNA that spans divergent genes or operons with related or opposing functions. In such a regulatory system, the antisense RNA can inhibit the expression of one operon while functioning as an mRNA for the adjacent operon, there by acting as fine-tuning regulatory switches in bacteria^[88].

Antisense RNA also regulates the expression of various structural and virulence factors in different bacteria. For instance, the 1200 nucleotide *AmgR* RNA which is encoded opposite the *Salmonella enterica mgtCBR* operon is responsible for the bacteria's virulence and survival in macrophages^[89]. A number of other antisense RNAs modulating virulence and regulating host-pathogen interactions have been discovered in a variety of bacterial species over the years^[90-97]. Antisense RNAs have also been found to impact other benign structural components including flagellar synthesis in *Rhizobium*^[98], *H. pylori*^[99], *L. monocytogenes*^[69] and *S. enterica*^[100].

The first complete experimental confirmation of short antisense RNAs in mycobacteria was published in 2009, which revealed 5 trans-acting and 4 cis-acting siRNAs in MTB H37Rv in the context of pH and oxidative stress^[101]. By the end of 2013, a total of more than 200 endogenous antisense RNAs were experimentally identified in various mycobacteria, including 70 in MTB^[102-110], 90 in *M. Bovis*^[103,104,111], 9 in *M. avium*^[112], and 44 in *M. smegmatis*^[102-104,113]. From these recent studies, a stronger connection between mycobacterial pathogenesis and the levels of expression of the antisense RNAs have emerged but many new questions about their potential pathogenic vs housekeeping functions remain to be answered. The lack of identification of an Hfq homolog in mycobacteria prevents the current approach of coimmunoprecipitation, making the study of the role of antisense RNAs all the more difficult in this genus^[102]. Pandey *et al.*^[114] have proposed an alternative protein, Rv2367, as a potential RNA chaperon in place of Hfq^[114], however, studies are ongoing in this direction to find a functionally-equivalent chaperon or to get around this issue^[113]. Also, the role of mycobacterial antisense RNAs in regulating transposition is not as clear as in other bacterial systems like *E. coli*.

Antisense RNAs can repress or modulate expression of target genes by a variety of mechanisms, including transcription interference, transcription attenuation,

degradation by endo- or exonucleases, or by blocking ribosome binding. When inducing transcription interference, the transcription of antisense RNA from one promoter hinders the RNA polymerase from either binding or extending the target gene transcript from the opposite strand^[115,116]. This type of interference occurs only in Cis and does not involve base pairing. In transcription attenuation, the binding of the antisense RNA to the target RNA causes a conformational change creating a terminator structure in the mRNA leading to its premature termination of transcription^[117]. Antisense RNAs can affect target mRNA stability by stimulating or inhibiting its degradation. When employing endo- or exonucleases for gene regulation, the antisense RNA, upon binding to its target mRNA, induces or blocks a ribonuclease target site within the mRNA or can indirectly block the binding of the ribonuclease at a distant site. In many bacterial systems, two major endoribonucleases have been identified, RNase III which cleaves double stranded RNA into two with different stabilities than the original transcript^[118-121], and RNase E, a component of the multi-protein degradasome complex which cleaves single stranded RNA and interacts with Hfq and globally affect mRNA stability^[119,122]. It is not precisely clear how the antisense RNA modulates RNase E activity, but the proposed mechanisms include the donation of its 5' monophosphate to stimulate RNase E activity or physically block the RNase E recognition site by basepairing to downregulate activity^[123]. Other ribonucleases in bacteria have also been identified with more specialized functions, including RNase G (a non-essential paralog of RNase E)^[124,125], RNase P^[126,127], RNase LS^[128], RNase Z^[129,130], RNase H^[131], RNase J1/J2^[132], and the recently characterized RNase Y^[133]. Many of these ribonucleases have already been characterized in various Mycobacteria, including MTB and *M. smegmatis*^[134-142]. Apart from these mechanisms, it has also been found that some antisense RNAs can physically block mRNA expression by binding to the Shine Dalgarno sequences of their target mRNA and prevent ribosome binding^[66,77,80], or they may indirectly modulate expression by altering the target mRNA conformation^[67]. Finally, antisense RNAs can exhibit dual functions by acting as mRNAs and antisense RNAs or cis- and trans- acting RNAs^[78].

Two general mechanisms have been proposed for base-pairing in antisense RNAs. The first type is a single-step mechanism in which the antisense RNA makes initial contact with the target mRNA to form a duplex^[143]. The second type is a multi-step system in which the initial duplex formed is stabilized by a protein, followed by the formation of the more stable complete duplex^[144,145]. In many cases of base pairing for the antisense RNA, a stem-loop structure is found to be important, along with a "pyrimidine-uracil-any nucleotide-purine" U-turn motif^[146].

Synthetic antisense RNAs are generally delivered

either by expressing the antisense transcript from a gene introduced into the cell or by direct delivery of antisense oligonucleotides. Degradation of the antisense transcripts can be a problem for both these delivery approaches, however, this issue is mitigated by using sequences that form more stable hair-pin structures with paired ends^[147]. In order to increase the stability and uptake, antisense RNAs have been modified in many ways, including the addition of peptide nucleic acid (PNA) or alternating 2'O-methyl to their backbones, switching ribose rings to morpholine rings (PMO), switching internucleoside bonds with phosphorothioates (PS-ODNs), or by conjugating cationic peptides to PNAs and PMOs^[148-150].

Antisense technology using synthetic siRNAs have been used as a powerful tool in knocking down genes in prokaryotes (and also in eukaryotes), including hepatitis G virus^[151], influenza virus^[152], picornavirus^[153], and *Trypanosoma brucei*^[154]. When targeted to essential genes, siRNAs inhibit growth of *E. coli*^[148,149,155,156], *S. enterica*^[157], *Staphylococcus aureus*^[158], *M. smegmatis*^[159] and MTB^[61,160]. Antisense RNAs have been successfully used for the study of bacterial growth and metabolism since this approach allows conditional knock down of target genes^[161-165]. It has also been used for the study of various putative virulence factors in bacteria^[166,167]. Antisense technology has helped in identifying new antibiotics^[168,169], antibiotic targets^[170,171], sensitizing bacteria to antibiotics^[171-173], and to elucidate the mechanism of action of potential new drugs^[171].

Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is gaining popularity. Molecular beacon based short interfering RNA (MB siRNA) has recently been proven to be a powerful tool for therapeutic gene silencing because of its specificity, broad applicability, and high efficiency^[174-176]. The on/off signals produced by the fluorophore/quencher pair depends on the conformational state of the MB (Figure 7). In the absence of the target mRNA, the stem brings the quencher in close proximity with the fluorophore and turns the fluorescence off with high quenching efficiency *via* fluorescence resonance energy transfer (FRET). In FRET, the energy from the donor chromophore is transferred to acceptor quencher nearby thus resulting in the absence of fluorescence. If the quencher and the fluorophore are far apart (following hybridization of the beacon to its target) then the quencher molecule will not be able to absorb the energy from the donor fluorophore. This would result in an increase in fluorescence. This technology has been used to detect mRNA expression in cells as well as for the detection and knockdown of telomerase expression in human breast cancer cells^[177], *BMP4* mRNA in hedgehog signaling^[178], aromatase mRNA in breast cancer cells^[179], and for the detection and attenuation of *mce4a* mediated *M. smegmatis* infection in macrophages as discussed in section below^[155,180].

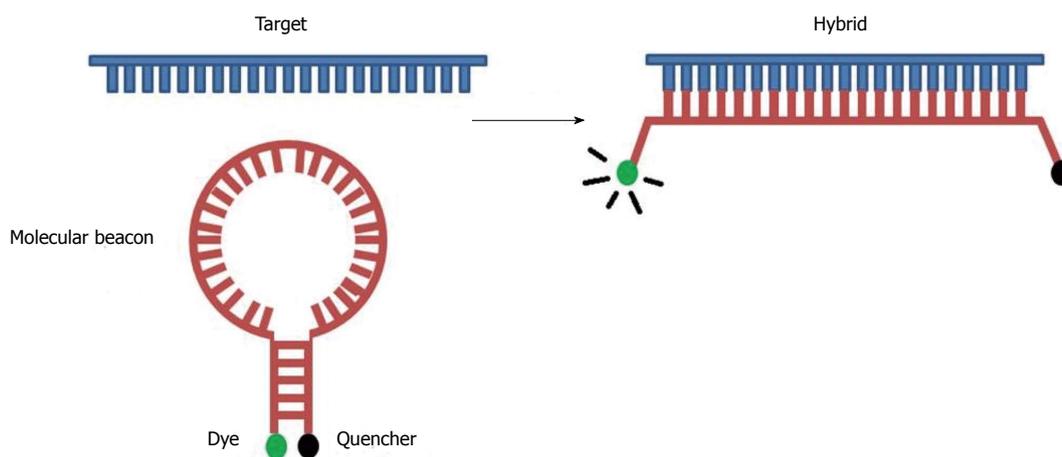


Figure 7 Molecular beacon technology. Molecular beacons are hairpin shaped antisense RNAs that does not fluoresce in the absence of mRNA binding (left) while the fluorescence increases when the beacon hybridizes with its target mRNA (right).

***M. SMEGMATIS* AS AN *IN VITRO* MODEL FOR STUDYING MYCOBACTERIAL INFECTION AND PERSISTENCE**

The *mce* operons are widely seen throughout the genus *Mycobacterium* and a homolog of *mce4* has been confirmed in the mycobacterial species *M. smegmatis*^[64,181]. Even though *M. smegmatis* is non-pathogenic, previous studies have shown that it can survive and multiply within macrophages in a pathogen-like manner by manipulating the host cell during initial stages by delaying phagosomal acidification and recruitment of V-ATPase^[28,182], thus making it a suitable model to study *mce4* operon mediated invasion and intracellular mycobacterial survival.

STUDIES IN OUR LAB ON THE ROLE OF MOLECULAR BEACONS IN DETECTING AND ATTENUATING MYCOBACTERIAL INFECTION IN MACROPHAGES

In order to determine the ability of a siRNA molecular beacon to detect and attenuate mycobacterial infection in macrophages, we first conducted experiments towards determining the most infective gene in the *mce4* operon. Because of the slow growth rate of MTB and also due to the high degree of homology between *mce4* operons of mycobacteria^[181], the *mce4* operon of the rapid growing *M. smegmatis* was selected for our studies. Using gene specific primers with the reverse primer for each set excluding the termination codon, each of the *mce4* genes, *mce4A*, *mce4B*, *mce4C*, *mce4D*, and *mce4F* were PCR amplified, cloned into the prokaryotic expression vector *pTrcHis2-TOPO* and stably expressed in *E. coli*. Western blot analyses with monoclonal antibodies against c-myc and 6xHis showed that the MCE4 proteins were expressed in host *E. coli*. Next we conducted invasion assays in MCF7

breast cancer cells using *E. coli* clones expressing the *M. smegmatis* genes and the results showed that *mce4A-F* conferred virulence to its host *E. coli*. However, *mce4A* appeared to confer the earliest virulence to its host *E. coli* and the virulence was found to be sustained during the entire invasion period (72hr)^[180]. We later, repeated the cloning experiments using the *mce4* operon genes of MTB, by PCR amplifying each of the *mce4A-F* (Figure 8), cloning into TOP10 *E. coli* using the prokaryotic expression vector *pTrcHis2-TOPO*, and performing invasion assay using MCF7 breast cancer cells. Our results showed that, as with *M. smegmatis*, the *mce4A* gene conferred the greatest degree of virulence to its host *E. coli* (Figure 9). Therefore, *mce4A* was selected as the target gene for designing a molecular beacon antisense RNA.

The *mce4A* antisense molecular beacon RNA was designed to have a stem-loop structure, with the nucleotides in the stem complementary to each other to form a 5-base pair double stranded stem and the loop consisting of 20 nucleotides that are complementary to a region of the target *mce4A* mRNA in *M. smegmatis*. Also, conjugated to the 5' and 3' ends of this molecular beacon are the fluorophore 5' TYE™ 665 and quencher Iowa Black RQ-SP (fluorophore quencher for 500-700 nm spectrum), respectively. This molecular beacon design combined both detection and therapeutic capabilities^[177-179]. The rationale is that in the absence of the target *mce4A* mRNA, the molecular beacon remains in its hairpin form while in the presence of its target mRNA the 20 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their target *mce4A* mRNA and the stem to its complementary pairs on the opposite ends of the target sequence. The hybridization potential of the loop to its target, based on the number of nucleotides within it (20 vs 5), will be greater than that of the strands for the stem. Hybridization of the loop to the *mce4A* mRNA will separate the fluorophore from the quencher thus inducing fluorescence (detection) and degradation (therapeutic) of the mRNA.

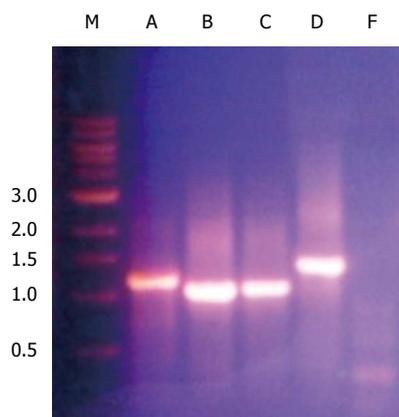


Figure 8 Polymerase chain reaction amplification of *mce4A*, *mce4B*, *mce4C*, *mce4D* and *mce4F* of *Mycobacterium tuberculosis*. *Mce4* operon genes were polymerase chain reaction amplified from *M. tuberculosis* H37Rv using gene specific primers and resolved on 1% agarose gel. The forward primer spanned the first 21 nucleotides from the beginning of the open reading frame and the reverse primer covered 21 nucleotides spanning the complementary strand to the 3' end of the gene. The termination codon was omitted so that the product, MCE4A-F, will be expressed with a 6XHis tag and a myc tag.

Since the mycobacterium utilizes the product of *mce4A* for entry in to macrophages and for its survival using host cholesterol for carbon and energy source transported through the MCE4 transporters^[41,45,62,183], the degradation of the *mce4A* mRNA will lead to its reduced survival. Our studies first tested the ability of the *mce4A* siRNA to detect its target *mce4A* mRNA in *M. smegmatis* and in macrophages infected with *M. smegmatis* and the results show that the molecular beacon siRNA detected its target in *M. smegmatis* and in macrophages infected with *M. smegmatis*. Thus, we were able to show that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* that facilitates the detection of mycobacterial infection in macrophages.

Tests were carried out to test the ability of this siRNA molecular beacon to not only detect but also attenuate mycobacterial infection in macrophages. Towards this end, we used a green fluorescent protein (GFP) expressing lentiviral vector, *piLenti-siRNA-GFP*, to successfully transduce and stably express the *mce4A* siRNA molecular beacon construct in macrophages infected with either *E. coli* expressing *mce4A* gene (*E. coli-4A*) or *M. smegmatis*. Using confocal imaging and Western blot analyses with anti-GFP antibodies, we were able to demonstrate stable expression of siRNA up to 48 h post transduction and infection using the GFP reporter.

After confirming the expression of the GFP protein by fluorescence imaging and Western blot analyses, invasion assay was carried out to assess the effect of *mce4A* siRNA on mycobacterial infection in macrophages. For this, differentiated U937 macrophages were transduced with *piLenti-siRNA-GFP* phage for 24hrs followed by infection with *E.coli-4A* or *M. smegmatis*

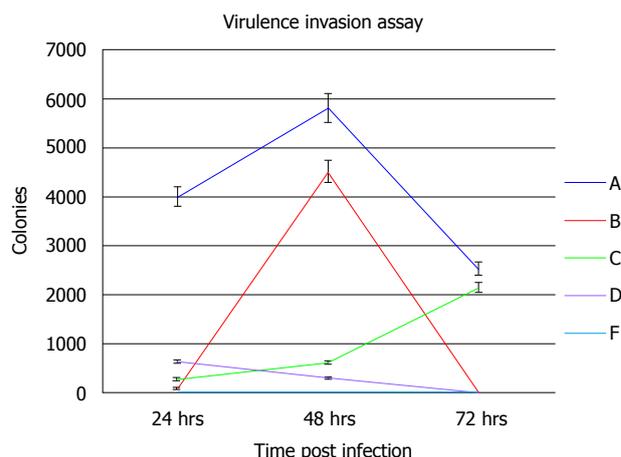


Figure 9 Mtb-MCE4 proteins confer virulence to *Escherichia coli*. *Escherichia coli-Mtbmce4* clones were used to infect 2×10^6 MCF7 epithelial cells at an MOI of 10:1 for 2 h. The level of infection was assessed by counting bacterial colony numbers at 24 , 48 and 72 h post-infection ($n = 3$).

for 3 h, and incubation for 0, 3, 6, 24, and 48 h, respectively. The cells were extensively washed and lysed in 0.1% Triton-X 100 lysis buffer and the lysates were plated on either LB agar containing 100 μ g/mL ampicillin for *E. coli-4A* or 7H11 media for *M. smegmatis*. The degree of attenuation of *E. coli-4A* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 0%, 77%, 59.6%, and 99.7%, respectively. The degree of attenuation of *M. smegmatis* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 94.8%, 70.3%, 98.9%, and 93.4%, respectively. Thus, our results showed that the *mce4A* siRNA was able to significantly attenuate both *E. coli-4A* and *M. smegmatis* infection in macrophages^[159].

Separate set of experiments were conducted to further test the hypothesis that the *mce4A* siRNA molecular beacon can attenuate the *mce4A* mRNA levels in *E. coli* expressing *mce4A* gene within infected macrophages. For this, reverse transcription polymerase chain reaction analysis was performed on lysates from differentiated U937 cells which were transduced with the *piLenti-siRNA-GFP* phage for 24 h, followed by infection with *E. coli-4A* for 3 h and incubation for 0, 3, 6, and 24 h. The cells were washed and lysed and the intracellular bacteria were isolated and washed at each time point of incubation. The bacterial sample from each of the time points were lysed and the mRNA was isolated and purified using DNase 1 enzyme treatment. Reverse transcripts were generated using RTPCR and the cDNAs were amplified using gene specific primers for *M. smegmatis mce4A* and *E. coli 16S* rRNA gene as internal control. The degree of attenuation of *mce4A* mRNA levels was compared between 3, 6, and 24 h against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively using densitometry gel analysis. Our results thus showed that *mce4A* siRNA was able to attenuate *mce4A* levels within infected

macrophages as opposed to *E. coli* 16S rRNA internal positive control and the degree of attenuation of *mce4A* mRNA levels in *E. coli*-4A was found to be significant.

Thus, we have successfully demonstrated that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* which can be used to both detect and attenuate mycobacterial infection in macrophages.

Antisense oligonucleotides, considered the pharmacology of the future^[184], interact with their mRNA targets with greater specificity and binding affinity than traditional drugs to their protein targets. Recent advances have enhanced their hybridization to target mRNA, reduced their overall toxicity with decreased susceptibility to cellular nucleases. The lung provides an excellent target for direct antisense oligonucleotide delivery by inhalation, thereby achieving a bolus dose directly to the target site. Cationic lipids in the lung surfactants enhance oligonucleotides entry into cells^[185]. Penetration of the inhaled oligonucleotides into deeper tissues of the lung has been established by autoradiogram, surgical dissection and receptor quantification studies^[186]. Further studies to test the hypothesis that *mce4* siRNA respirable molecular beacons can localize and attenuate mycobacterial infection in pulmonary granulomas in animal models will take the fight against TB a long way in eradicating this versatile human pathogen.

CONCLUSION

The association of the *mce* operons, especially that of *mce4*, with mycobacterial invasion and latency is no longer considered casual and with strong evidences emerging over the recent years it can now be considered as a potent mediator of *MTB* infection and survival in its only human host. The *mce* invasion domain is equipped to mediate the entry and localization of the bacteria in the host macrophages at cholesterol rich regions creating cholesterol-associated protein coated phagosomes, thereby creating an ingenious mechanism for subverting the immune defenses. Another paradigm to the mycobacterial saga was added by the discovery that the MCE associated protein, YRBE4 transporters, in conjunction with the MCE4 domains, transport cholesterol into the cell for its energy and carbon needs, which then possibly generates metabolites that can further mediate its latency in the host. Strategies like identifying the level of infectivity of individual *mce* operon genes and designing efficacious drugs like molecular beacon siRNAs against *mce* targets can aid in the simultaneous detection and eradication of this elusive human pathogen.

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