Details of the research work for which the Sun Pharma Research Fellowship is claimed.
The focus of my research laboratory is (i) Delineating the novel mechanisms of pathogenesis of *Mycobacterium tuberculosis*, (ii) identifying novel small molecules with antitubercular activity, and (iii) developing new TB vaccine candidates. Despite significant progress, understanding the fundamental molecular mechanisms underlying mycobacterial adaptation and reactivation in the host remains a challenging area for further research. *Mycobacterium tuberculosis*, for instance, reprograms its metabolic responses to adapt to various host-associated stress conditions such as hypoxia, acidic conditions, and nutrient starvation. This metabolic reprogramming leads to a slowdown in bacterial metabolism, enabling *M. tuberculosis* to persist in the host in a non-replicating dormant state. The precise regulatory mechanisms that drive these adaptations during latency and disease reactivation still remain unknown. Among the various metabolic processes, our lab has focused on investigating the role of enzymes involved in inorganic polyphosphate (PolyP) metabolism and toxin-antitoxin (TA) systems in the pathophysiology of *M. tuberculosis*.

Research Article 1: Chugh S, Tiwari P, Suri C, Gupta SK, Singh P, Bouzeyne R, Kidwai S, Srivastava M, Rameshwaram NR, Kumar Y, Asthana S and **Singh R***. Polyphosphate Kinase -1 regulates bacterial and host metabolic pathways involved in pathogenesis of *Mycobacterium tuberculosis*. **Proceedings of the National Academy of Sciences**, 2024, 121(2), e2309664121.

Inorganic polyphosphate (PolyP), an inorganic linear polymer consisting of hundreds of phosphate residues, is synthesized by Polyphosphate kinase – 1 (PPK-1). Previously, we have shown that mycobacteria accumulate PolyP at later stages of growth and upon exposure to different stress conditions and drugs. Also, we have reported that PolyP deficiency results in attenuated growth of *M. tuberculosis in vivo* and also enhances its susceptibility to isoniazid (INH). Studies have shown that dysregulation in PolyP levels impaired *M. tuberculosis growth* in stress conditions, inside macrophages, and host tissues. However, a precise understanding of the modulation of mycobacterial pathogenesis by PolyP still remained unclear. In a recently published study from the laboratory in **PNAS, 2024**, we have performed experiments to delineate the mechanisms by which PolyP deficiency regulates the pathogenesis of *M. tuberculosis*. To identify PolyP-interacting proteins from *M. tuberculosis*, pull-down experiments were performed using biotinylated $PolyP_{700}$. We identified 8 $PolyP_{700}$ interacting proteins from mid-log and late-log phase *M. tuberculosis* cultures. Interestingly, the most commonly identified $PolyP_{700}$ interacting proteins belonged to the Acyl CoA carboxylases family of enzymes. These included AccA1, AccD1, AccA2, AccD2, AccD4, AccD5, and AccE5. Interestingly, many of these interacting proteins require ATP for their cellular functions. These observations suggest that PolyP might be able to substitute for ATP in their enzymatic activity. In our bio-layer interferometry (BLI) experiments, we observed that purified $(His)_6$-AccA1, $(His)_6$-AccD1 and $(His)_6$-PPK-1 exhibited a simple 1:1 binding with biotinylated $PolyP_{700}$. Studies have shown that AccA1-AccD1 form a multisubunit complex and is involved in (i) the leucine degradation pathway and (ii) the carboxylation of acetyl-CoA to malonyl-CoA, which serves as a precursor for fatty acid biosynthesis. We also performed molecular docking experiments to identify the key residues involved in the interaction of AccA1 and AccD1 with $PolyP_{700}$. Based on these findings, we speculate that PolyP levels might regulate the activity of the AccA1-AccD1 enzyme complex and contribute to lipid biosynthesis in *M. tuberculosis*. In addition to these enzymes, we observed that PolyP interacted with key enzymes involved in the translation and transcription of *M. tuberculosis*. Further, to understand the mechanisms by which PolyP deficiency contributes to mycobacterial pathogenesis, we compared the transcription profiles of mid-log phase cultures of wild-type and Δ*ppk-1* mutant (PolyP

deficient) strains. We noticed that the expression of 247 and 183 transcripts was decreased and increased, respectively, in Δ*ppk-1* mutant strain compared to the parental strain. Intriguingly, the mutant strain had reduced transcript levels of genes encoding for enzymes involved in phthiocerol dimycocerosates (PDIM) biosynthesis (*pps* gene cluster, *ppsABCDE*) or components of type VII secretion systems. Previously, studies have demonstrated that phosphoglucokinase from *M. tuberculosis* prefers PolyP as the phosphoryl donor for converting glucose to glucose-6-phosphate. As expected, Δ*ppk-1* mutant strain displayed a growth defect in the 7H9 medium containing glucose as the carbon source. Glucose-6-phosphate is a key intermediate in central carbon metabolism and is a precursor for trehalose biosynthesis. In agreement, the levels of glucose-6-phosphate intermediate belonging to the TCA cycle, trehalose metabolism, and amino acids were significantly reduced in the Δ*ppk-1* mutant strain relative to the parental strain in the glucose-containing medium. Also, the mutant strain exhibited reduced virulence-associated lipids such as TDM and TMM compared to the parental and complemented strains.

In agreement with the reduced levels of CFP-10 and PDIMs, we observed increased phagosome-lysosome fusion in macrophages and decreased Type I IFN signalling in lung tissues of Δ*ppk-1* mutant strain infected mice relative to the wild-type strain infected animals. ESX-1 also enhances bacterial intracellular multiplication by promoting tissue necrosis and mobilizing intracellular Ca^{2+}. In concordance, we also noticed reduced expression of transcripts that encode proteins implicated in calcium signalling and cell death in the lung tissues of Δ*ppk-1* mutant strain infected mice. Several studies have shown that *M. tuberculosis*-infected macrophages in granulomas accumulate lipid droplets and differentiate into foamy macrophages. Foamy macrophages represent a favourable niche for the intracellular multiplication and dissemination of *M. tuberculosis*. It has also been reported that *M. tuberculosis*-infected macrophages release mycobacterial cell wall lipid-containing vesicles (such as TDM and oxygenated mycolic acids) and induce the formation of foamy macrophages. Consistent with the reduced levels of PDIMs, TMM, and TDM, we found that transcript levels of markers associated with forming foamy macrophages were reduced in Δ*ppk-1* mutant strain animals. The reduced amount of neutral lipid content in THP-1 macrophages, as well as in lung tissues of Δ*ppk-1* infected guinea pigs, further substantiates our observations from the RNA-seq experiment. Studies have demonstrated that strains harbouring deletions in proteins involved in mycolic acid biosynthesis or the ESX-1 secretion system elicit a weaker T_{H1} immune response during infection. As expected, the levels of various inflammatory cytokines and chemokines were significantly lower in the lung tissues of mutant strain-infected mice relative to wild-type strain-infected mice.

Previous studies have shown that different microorganisms require PPK-1 for stress adaptation, virulence, and persistence. These observations indicate that PPK-1 is an attractive target for identifying small molecule inhibitors with a novel mechanism of action. We have screened a collection of 1280 pharmacologically active compounds belonging to the Sigma Lopac 1280 library to identify PPK-1 inhibitors. The preliminary screening performed at 100 μM concentration resulted in the identification of 60 compounds that inhibited PPK-1 enzymatic activity by at least 50%. Among these, we shortlisted 11 molecules that were either FDA-approved or are in trials for clinical use against other diseases. We next determined the interaction of the remaining small molecules with PPK-1 using microscale thermophoresis. Among the identified molecules, raloxifene hydrochloride (raloxifene) showed the highest binding with PPK-1. We also observed that raloxifene inhibited both ATP and polyP synthesis activity associated with PPK-1 in a dose-dependent manner. Raloxifene is a selective estrogen receptor modulator and is being used in the treatment of osteoporosis and invasive breast cancer. Additionally, we showed that raloxifene

also inhibited the activity of PPK-1 homologs from *E. coli* and *K. pneumoniae*. Also, the binding patterns of raloxifene with PPK-1 homologs of *E. coli*, *K. pneumoniae*, and *M. tuberculosis* were similar. Computational studies revealed that Glu289 of *M. tuberculosis* is critical for the interaction of raloxifene with PPK-1. We observed that in APO protein, Glu289 is involved in forming an intra-hydrogen bond network with Arg310 and Phe462, and this network is destabilized by the binding of raloxifene. Previously, we have shown that PolyP deficiency results in attenuation and enhanced susceptibility of *M. tuberculosis* against INH. In agreement with our earlier observations, we demonstrated that raloxifene was able to inhibit *M. tuberculosis* growth in macrophages and mice and also enhance INH's intracellular activity. We also reported that the intracellular activity of recently approved drugs, bedaquiline and pretomanid against *M. tuberculosis,* was also enhanced when combined with raloxifene. These observations suggest that PPK-1-specific inhibitors, in combination with existing drugs, may shorten the duration of chemotherapy. Since small molecules targeting PPK-1 protein inhibit a novel mechanism of action, raloxifene also inhibited the growth of INH-resistant *M. tuberculosis* strain in THP-1 macrophages. To the best of our knowledge, this is the first comprehensive study to delineate the mechanisms by which PolyP contribute to pathogenesis and identify small molecule inhibitors against the PPK-1 enzyme from *M. tuberculosis*. The identified small molecules may be combined with existing TB drugs to eradicate both drug-susceptible and drug-resistant bacterial populations in different metabolic states. Taken together, we demonstrate that the PPK-1 enzyme regulates the expression of virulence-associated genes, lipid biosynthesis and utilization of glucose as a carbon source in *M. tuberculosis*. The decreased levels of these effector proteins and lipid molecules might result in an increase in the fusion of phagolysosome, along with a decrease in necrosis and dissemination to the cytosol. This also results in reduced formation of foamy macrophages and minimal inflammatory and T_{H1} response in mice (Fig. 1). Since PPK-1 is a highly conserved enzyme, the identified PPK-1 specific inhibitors may possess broad-spectrum *in vivo* activity.

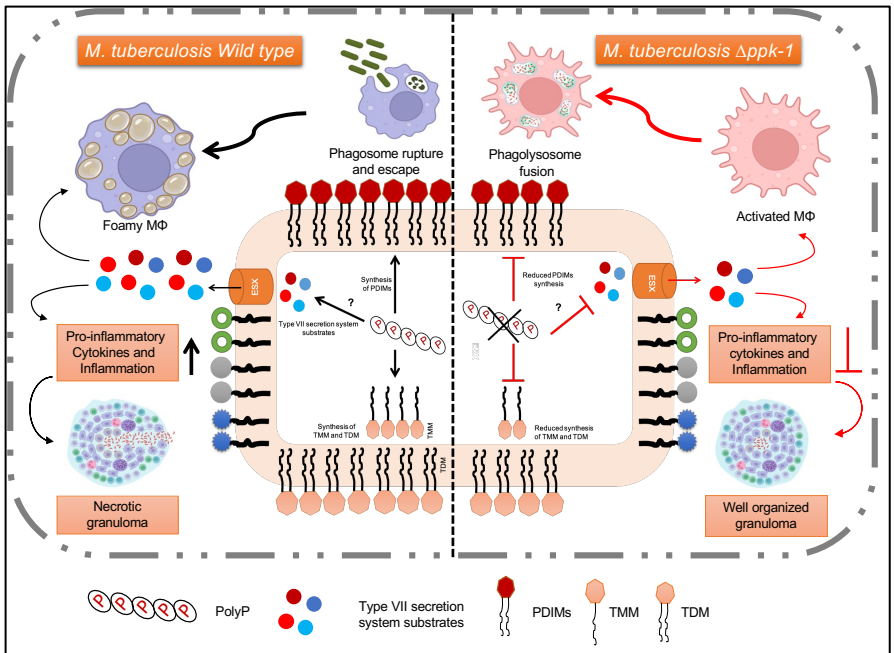

Figure 1: PolyP acts as a regulator of bacterial and host metabolic pathways implicated in mycobacterial pathogenesis. *M. tuberculosis* virulence factors such as PDIMs and ESX-1 induce phagosome rupture and necrosis and promote bacterial pathogenesis in host tissues. These virulence factors, along with TMM and TDM, trigger the formation of lipid-laden foamy macrophages. These foamy macrophages provide a favourable environment and support bacterial nutrition and persistence. The secretion of cytokines, such as type I interferon and TNF-α, contribute to tissue inflammation and damage, resulting in disease dissemination. In the present study, we show reduced levels of various virulence-associated factors such as ESX-1 effectors, PDIMs, TMM and TDM in mid-log phase cultures of Δ*ppk-1* mutant strain. The reduced levels of these virulence factors are most likely to be associated with (i) increased phagosome-lysosome fusion in macrophages, (ii) reduced Type-I IFN signalling, (iii)inflammation and formation of foamy macrophages and (iii) faster clearance of Δ*ppk-1* mutant strain from host tissues.

Research Article 2: Gosain TP, Chugh S, Rizvi ZA, Chauhan NK, Kidwai S, Thakur KG, Awasthi A and **Singh R***. *Mycobacterium tuberculosis* strain with deletions in *menT3* and *menT4* is attenuated and confers protection in mice and guinea pigs. **Nature Communications,** 2024, 15 (1): 5467.

Toxin-antitoxin (TA) systems are small genetic elements that are prevalent in most prokaryote genomes. TA systems consist of two genes that encode for a stable toxin and an unstable antitoxin. TA systems have been categorized into eight types based on the nature of antitoxin (protein or RNA) and the mechanisms by which toxin activity is neutralized. The genome of *Mycobacterium tuberculosis* encodes for an extensive repertoire of TA systems. The detailed phylogenetic and bioinformatic analysis revealed that the *M. tuberculosis* genome encodes for $\geq$ 90 TA systems, which are highly conserved among the members of the *M. tuberculosis* complex. Most of these belong to various subfamilies of type II TA systems, and toxins belonging to type II TA systems inhibit *M. tuberculosis* growth by cleaving either mRNA, tRNA, rRNA, degrading NAD^+ or inhibiting DNA gyrase activity or by ADP ribosylation of single-stranded DNA. Additionally, subsets of TA systems exhibit differential expression patterns upon *M. tuberculosis* exposure to stress conditions. Many of these systems are dispensable for the survival of *M. tuberculosis* in stress conditions, thereby indicating that these modules might function cumulatively. The genome of *M. tuberculosis* also encodes for four proteins belonging to the DUF1814 family of nucleotidyl transferases. These proteins, MenT1, MenT2, MenT3 and MenT4, share sequence homology with toxins from type IV TA systems. Previous studies have shown that overexpression of MenT toxins results in growth inhibition by preventing aminoacylation and tRNA charging. In another recent paper published in **Nature Communications**, 2024, we performed experiments to investigate the contribution of MenT3 and MenT4 in *M. tuberculosis* pathophysiology.

Using IPTG and anhydrotetracycline inducible vector, we showed that the overexpression of MenT3 and MenT4 resulted in growth inhibition of both *E. coli* and *M. tuberculosis*. We also showed that mutation of active site residues abrogated the growth inhibition activity associated with these toxins. The relative abundance and upregulation of a subset of TA systems in response to stress conditions and drugs suggest that these function in a cumulative manner to enable *M. tuberculosis* to adapt to these conditions. To comprehend the role of MenT3 and MenT4 in the physiology of *M. tuberculosis*, we constructed Δ*menT3,* Δ*menT4* single mutant and Δ*menT4*Δ*T3* double mutant strains using temperature-sensitive mycobacteriophages. We observed that MenT3 and MenT4 are mutually redundant, but both MenT3 and MenT4 contribute cumulatively to the adaptation of *M. tuberculosis* upon exposure to oxidative stress. Complementation of the double mutant strain with *menT3* partially restored the growth defect associated with the double mutant strain upon exposure to oxidative stress. We also demonstrate that the survival of parental, Δ*menT3,* Δ*menT4* and Δ*menT4*Δ*T3* strains were similar after exposure to other conditions such as nitrosative, nutritional, or acidic. Previously, we reported that TA systems or toxins belonging to the type II subfamily are essential for *M. tuberculosis* to establish infection in mice or guinea pigs. Here, we report that Δ*menT4*Δ*T3* strain exhibited attenuated growth at both acute and chronic stages of infection in guinea pigs compared to the wild-type strain. As observed in the case of other TA mutant strains, the growth defect associated with the Δ*menT4*Δ*T3* strain was more prominent in spleens and during the chronic stage of infection in guinea pigs. In agreement with bacterial burdens, H&E-stained lung sections from guinea pigs infected with wild type strain displayed increased cellular infiltration and severely reduced alveolar spaces. This increased cellular

infiltration indicates severe inflammation and pathology in lung tissue sections of wild-type strain-infected guinea pigs at both time points. The histologically stained lung sections of Δ*menT4*Δ*T3* infected guinea pigs demonstrated intact lung architecture and large alveolar spaces at both time points. Taken together, we demonstrate that simultaneous deletion of *menT3* and *menT4* resulted in attenuation of *M. tuberculosis* growth in guinea pigs.

Transcriptomics is widely used to gain a better understanding of the mechanisms for the attenuation of bacterial pathogens. Next, we compared the transcriptional profiles of mid-log phase cultures of wild type and Δ*menT4*Δ*T3* strain to identify the differentially expressed pathways among these two strains. Using a 2.0-fold cut-off and P_{adj}-*value* of $\leq$ 0.05, we observed that transcripts encoding for 36 and 51 proteins were increased and decreased, respectively, in Δ*menT4*Δ*T3* compared to the parental strain. Bacterial RNA sequencing revealed that transcript levels of genes encoding for proteins involved in stress adaptation and virulence were reduced in the double mutant strain's mid-log phase cultures compared to the parental strain. Therefore, we hypothesized that the *in vivo* attenuated phenotype of the mutant strain might be associated with the reduced expression of these proteins. We also compared the growth of wild type and Δ*menT4*Δ*T3* strain in aerosol-infected Balb/c mice at 4 and 8- weeks post-infection. In agreement with guinea pig data, we noticed that Δ*menT4*Δ*T3* was attenuated for growth in aerosol-infected mice at 4- and 8-week post-infection compared to the parental strain. We could not restore this growth defect in the lungs and spleens of infected animals upon complementation of the double mutant strain with either *menT3* or *menT4*. These observations suggest that both MenT3 and MenT4 contribute cumulatively to the ability of *M. tuberculosis* to establish infection in host tissues. To further unravel the plausible mechanisms associated with the *in vivo* growth defect of the mutant strain, we also compared the transcriptional profiles obtained from lung tissues of animals infected with either wild type or Δ*menT4*Δ*T3* strain. Detailed analysis of the RNA-seq data revealed that the levels of transcripts encoding for proteins involved in calcium signalling, apoptosis and autophagy were increased in Δ*menT4*Δ*T3* infected animals relative to parental strain infected animals. We also noticed that the expression of proteins involved in inflammatory response was reduced in animals infected with the double mutant strain compared to parental strain-infected animals. The data suggests that the coordinated execution of these pathways might be associated with the observed growth defect of the mutant strain in host tissues.

Studies have shown that immunization of animals with live attenuated *M. tuberculosis* strains provides long-term protection against challenge with virulent strains as these strains closely mimic the antigenic repertoire of the infectious agent. Given that Δ*menT4*Δ*T3* was significantly attenuated for growth in guinea pigs, we next evaluated whether immunization with this strain imparts protection against the *M. tuberculosis* challenge in C57BL/6 mice. We observed that the protection conferred by immunization of mice with Δ*menT4*Δ*T3* and BCG in lung tissues was comparable. We demonstrated that immunization of mice with Δ*menT4*Δ*T3* imparted better protection than BCG in spleens at 10 weeks post-infection. In agreement with mice protection data, we show that immunisation of guinea pigs with Δ*menT4*ΔT3 resulted in better protection against *M. tuberculosis* challenge. The protection imparted upon immunization with Δ*menT4*ΔT3 at 4 weeks post-infection was significantly greater than that seen in BCG-immunized guinea pigs. However, the levels of protection were similar in Δ*menT4*ΔT3 and BCG-immunized guinea pigs at 8 weeks post-challenge. Interestingly, although the bacterial loads in lung tissues of Δ*menT4*ΔT3 and BCG-immunized guinea pigs were similar, analysis of H&E-stained lung sections revealed larger alveolar spaces and minimal cellular infiltration in lung sections of Δ*menT4*ΔT3 immunized guinea pigs in comparison to BCG immunized animals. The levels of protection seen in C57BL/6

and guinea pigs after immunization with Δ*menT4*ΔT3 were similar to those reported for other attenuated vaccine candidates such as Δ*leuD* (in guinea pigs) or Δ*panCD* (in guinea pigs) or Δ*RD1*Δ*panCD* (in C57BL/6 mice).

Several studies have shown that antigen-specific T_H1 response is desired to confer protection against the *M. tuberculosis* challenge. Previous studies have demonstrated that protective immunity against TB depends on an acquired cellular immune response involving T-cell subsets, and a T_H1-type response is considered favourable in imparting protection against *M. tuberculosis*. In agreement, we observed a significant increase in the frequency of IFN-γ and T-bet expression in $CD4^+$ and $CD8^+$ T cell compartments in Δ*menT4*Δ*T3* immunized mice compared to BCG-immunized mice. This T_H1 skewed response might be associated with the increased protection of Δ*menT4*Δ*T3* against *M. tuberculosis* challenge in spleen tissues. Further, we also evaluated the antigen-specific effector and memory T cell response in immunized mice. We noticed increased activated memory T cell response in Δ*menT4*Δ*T3* immunized mice compared to BCG immunized or naive mice. Given that memory responses are desirable for vaccine-induced long-lasting protection, we speculate that increased activated memory T cell response in Δ*menT4*Δ*T3* immunized mice might be able to confer protection against relapses as well. We also observed a significant increase in the secretion of T_H1 cytokines such as IFN-γ, TNF-α and IL-2 in culture supernatants of PPD-stimulated splenocytes from Δ*menT4*Δ*T3* immunized mice, in agreement with the flow cytometry data. Studies have shown that IFN-γ and TNF-α are important for effectively controlling *M. tuberculosis* infection. IL-2 has also been shown to stimulate the growth of B-cells, T-cells and NK cells and is essential for cellular immunity and granuloma formation in *M. tuberculosis* infection. We propose that increased amounts of these cytokines in culture supernatants from PPD-stimulated splenocytes from Δ*menT4*Δ*T3* immunized mice might contribute to its ability to impart protection against the *M. tuberculosis* challenge.

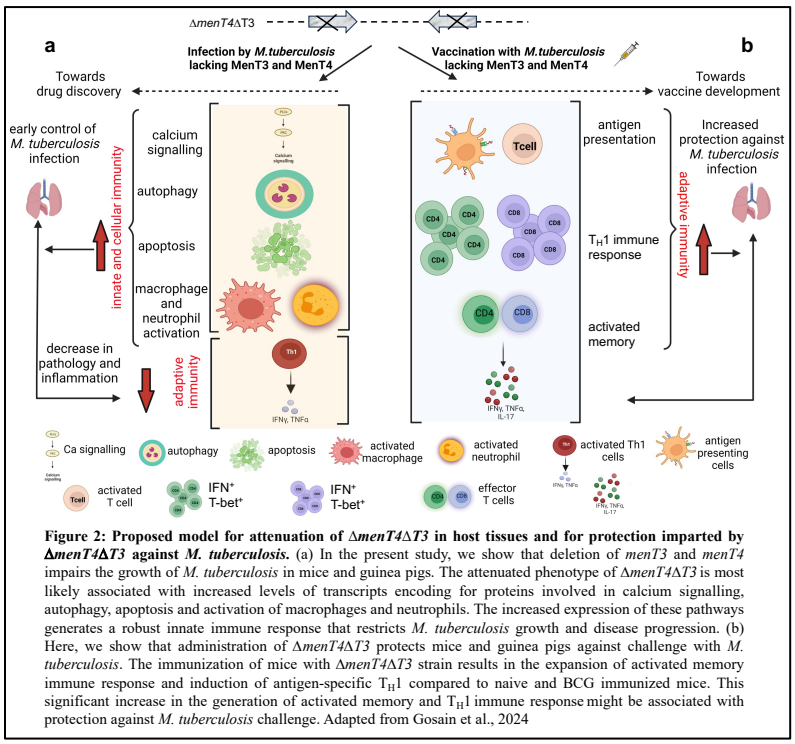

Figure 2: Proposed model for attenuation of Δ*menT4*Δ*T3* in host tissues and for protection imparted by Δ*menT4*Δ*T3* against *M. tuberculosis*. (a) In the present study, we show that deletion of *menT3* and *menT4* impairs the growth of *M. tuberculosis* in mice and guinea pigs. The attenuated phenotype of Δ*menT4*Δ*T3* is most likely associated with increased levels of transcripts encoding for proteins involved in calcium signalling, autophagy, apoptosis and activation of macrophages and neutrophils. The increased expression of these pathways generates a robust innate immune response that restricts *M. tuberculosis* growth and disease progression. (b) Here, we show that administration of Δ*menT4*Δ*T3* protects mice and guinea pigs against challenge with *M. tuberculosis*. The immunization of mice with Δ*menT4*Δ*T3* strain results in the expansion of activated memory immune response and induction of antigen-specific T_H1 compared to naive and BCG immunized mice. This significant increase in the generation of activated memory and T_H1 immune response might be associated with protection against *M. tuberculosis* challenge. Adapted from Gosain et al., 2024

Overall, this study revealed that although MenT3 and MenT4 are dispensable for *in vitro* growth, these toxins function in a cumulative manner and are essential for *M. tuberculosis* to establish disease in mice and guinea pigs. We also show that immunization of mice and guinea pigs with Δ*menT4*ΔT3 is able to impart protection against *M. tuberculosis*. We propose that the protection observed in Δ*menT4*Δ*T3* immunized animals is most likely associated with increased antigen-specific T_H1-based and activated memory immune response (Fig. 2). Future studies include unmarking and construction of Δ*menT4*Δ*T3*-based multiple-allele mutant strains (such as

panCD, *leuD*, *metX,* etc.), and these strains would be evaluated for safety and efficacy studies in compliance with the Geneva consensus.

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