Characterization of a secretory hydrolase from *Mycobacterium tuberculosis* sheds critical insight into host lipid utilization by *M. tuberculosis*

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*Mycobacterium tuberculosis* causes tuberculosis in humans and predominantly infects alveolar macrophages. To survive inside host lesions and to evade immune surveillance, this pathogen has developed many strategies. For example, *M. tuberculosis* uses host-derived lipids/fatty acids as nutrients for prolonged persistence within hypoxic host microenvironments. *M. tuberculosis* imports these metabolites through its respective transporters, and in the case of host fatty acids, a pertinent question arises: does *M. tuberculosis* have the enzyme(s) for cleavage of fatty acids from host lipids? We show herein that a previously uncharacterized membrane-associated *M. tuberculosis* protein encoded by Rv2672 is conserved exclusively in actinomycetes, exhibits both lipase and protease activities, is secreted into macrophages, and catalyzes host lipid hydrolysis. In light of these functions, we annotated Rv2672 as mycobacterial secreted hydrolase 1 (Msh1). Furthermore, we found that this enzyme is up-regulated both in an *in vitro* model of hypoxic stress and in a mouse model of *M. tuberculosis* infection, suggesting that the pathogen requires Msh1 under hypoxic conditions. Silencing Msh1 expression compromised the ability of *M. tuberculosis* to proliferate inside lipid-rich foamy macrophages but not under regular culture conditions *in vitro*, underscoring Msh1’s importance for *M. tuberculosis* persistence in lipid-rich microenvironments. Of note, this is the first report providing insight into the mechanism of host lipid catabolism by an *M. tuberculosis* enzyme, augmenting our current understanding of host lipid utilization by *M. tuberculosis*.

*Mycobacterium tuberculosis*, the pathogen that causes the debilitating disease tuberculosis (TB) 3 in humans, predominantly infects the wide varieties of alveolar macrophages. One of the ways by which the host immune system fights *M. tuberculosis* infection is by mounting an inflammatory reaction that results in the formation of granulomas (1, 2). However, as the pathogen has co-evolved with the human hosts for millennia or even longer, it has developed diverse strategies to survive inside the host lesions and to evade immune surveillance (3). A subset of the invading pathogens utilize the host granulomas to its advantage, surviving within these structures for a prolonged time and resurrecting upon sensing a weakened host immune system (4, 5). During this period of latency, the bacterium realigns its metabolism and reduces its growth rate to withstand the multitude of stresses inflicted upon it by the host (6). Such an altered state with slow metabolic activity of the quiescent population also imparts tolerance to primary anti-TB drugs (7).

Earlier studies have shown that *M. tuberculosis* requires the utilization of host lipids/fatty acids as a major nutrient source for its persistence (8–11). The pathogen has a gene cluster, *mce4*, that encodes a cholesterol import system (8) and a family of fatty acid transporters (12). In the recent years, studies revealed that *M. tuberculosis* accumulates triacylglycerol (TAG) in hypoxic environments (9, 10). The bulk of these accumulated TAGs are synthesized by bacterial TAG synthase utilizing fatty acids, including host-derived fatty acids. Of note, within granulomas of TB patients, a distinctive population of lipid-laden macrophages, designated foamy macrophages, contains TAG-filled lipid droplets (13). These lipid droplets likely provide lipid-rich microenvironments for *M. tuberculosis*.

However, it remains unclear how some host lipids are cleaved before their fatty acid components are imported into the bacteria. A substantial answer addressing this will help better our understanding of latent TB biology.

In an effort to address this question, we chose to study the hydrolytic enzymes that were perceived to be secreted from *M. tuberculosis*. In this respect, a literature survey led to the finding of a report suggesting that *M. tuberculosis* harbors approximately half a dozen such enzymes (14). Subsequently,
most of these proteins were studied in detail by other groups (15–18). However, none of these molecules are associated with the host lipid catabolism. In the present work, using a multi-scale approach that comprises in silico, in vitro, and ex vivo studies, we show that a mycobacterial gene, Rv2672, of hitherto unknown function encodes a secretory protein that exhibits both protease and lipase activities, catalyzes the hydrolysis of host lipids, and is up-regulated in M. tuberculosis in an in vitro model of latency and a mouse model of M. tuberculosis infection. Based on these functions, we propose its name as mycobacterial secreted hydrolase 1 (Msh1). Furthermore, we show that msh1 is not essential for M. tuberculosis growth in culture medium in vitro; however, an ex vivo study in foamy macrophages with wild-type and Rv2672 knockdown (KD) strains showed the importance of this enzyme in facilitating growth of the pathogen in lipid-rich hypoxic environments.

Results

Msh1 is conserved only in actinomycetes and contains two hydrolytic domains

To map the pervasiveness of Msh1 and to derive insight into the structural features it encompasses, bioinformatics approaches were used. A search, using BLASTP, to identify the homologs of Msh1 in the currently available sequence databases showed that Msh1 is conserved only in actinomycetes, particularly among mycolic acid-containing taxa such as Corynebacterium, Mycobacterium, and Nocardia (Fig. 1A). Mycolic acid-containing bacteria represent a unique phyletic cluster in the evolutionary classification of actinomycetes, which encompasses several pathogenic strains that pose a threat to human health. Furthermore, a comparative amino acid sequence analysis of Msh1 revealed that it shares as high as ~60% identity with its homologs in the genus Mycobacterium.
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(Fig. 1A). However, it shares a moderate (<30%) identity with homologs from other higher organisms. Msh1 comprises 528 amino acids that include an N-terminal signal peptide followed by a lipobox motif, LAAC, at positions 27–30 (Fig. 1B). A search in the conserved domain database (19) to find whether Msh1 possesses conserved functional domain(s) revealed that Msh1 contains two α/β-hydrolase domains, one (Domain A) with putative protease functionality and the other (Domain B) a putative lipase (Fig. 1B).

Msh1 is a secretory hydrolase

The presence of a signal peptide in Msh1 with a lipobox motif prompted us to investigate different subcellular fractions to determine its localization, which will further help in identifying its physiological substrate(s) and determining its plausible biological function. For this purpose, recombinant Msh1 was prepared by overexpressing Msh1 in Mycobacterium smegmatis mc²¹⁵⁵ system followed by purification to a high degree of homogeneity (Fig. 2, A and B). A search in the conserved domain database (19) to find whether Msh1 possesses conserved functional domain(s) revealed that Msh1 contains two α/β-hydrolase domains, one (Domain A) with putative protease functionality and the other (Domain B) a putative lipase (Fig. 1B).

Msh1 exhibits both lipase and protease activities

To obtain the first insight into the function of Msh1, in vitro assays were set up to examine lipase and protease activities as predicted by the bioinformatics studies. Proteolytic activity was examined using a universal protease substrate, β-casein (Sigma), and different concentrations of the Msh1. Msh1 cleaved the substrate into smaller size fragments (Fig. 2D), confirming that it indeed exhibits a protease activity.

Furthermore, to determine whether Msh1 hydrolyzes esters, an enzymatic assay was performed using p-nitrophenyl palmitate (NPP) (C₁₆:0) as the substrate (Fig. 2E). The enzyme activity follows Michaelis-Menten kinetics with $K_{m}$, $V_{max}$, and $K_{cat}$ values were calculated from a Lineweaver-Burk plot. Each point on the plot represents an average of triplicate experiments. Error bars represent S.D. F, specific activities of Msh1 with triglycerides tributyrin and triolein as substrates were similar to a known Pseudomonas lipase (PML; Sigma), confirming its role as a lipase. In contrast, no activity of porcine liver esterase (PLE) was observed with these substrates, which further substantiates specific lipase activity in Msh1. Each data point represents an average of four replicates. G, orlistat significantly inhibited Msh1 lipase activity. Msh1 was incubated with the desired triglyceride substrate, tributyrin or triolein, for 1 h at 37 °C, and activity was measured in the absence or presence of 20 μM orlistat, a lipase inhibitor.

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Figure 2. Isolation, localization, and kinetics of Msh1. A, affinity chromatogram profile of the recombinant Msh1. The peak shows the elution of the protein using 300 mM imidazole. B, SDS-PAGE analysis of the purification fractions of the recombinant Msh1. Protein was washed and eluted with 80 and 300 mM imidazole, respectively. C, immunoblot of the endogenous Msh1 in M. tuberculosis H37Rv and its homolog MSMEG_2786 in M. smegmatis mc²¹⁵⁵ depicting their subcellular localization. Msh1 was detected in culture filtrate, whereas the homologous protein in M. smegmatis was not. GroEL1 and antigen 85 confirmed the purity of the fractions. Culture filtrate was concentrated 50 times. The immunoblot is representative of three independent experiments. D, proteolytic activity of Msh1 against β-casein at 37 °C for 2 h. The proteolytic activity of Msh1 increased proportionately with its concentration. E, assessment of the lipase activity of Msh1 with different concentrations of the substrate p-nitrophenol palmitate. $K_{m}$ (3898 ± 298 μM) and $V_{max}$ (1.5 ± 0.16 μM min⁻¹) values were calculated from a Lineweaver-Burk plot. Each point on the plot represents an average of triplicate experiments. Error bars represent S.D. F, specific activities of Msh1 with triglycerides tributyrin and triolein as substrates were similar to a known Pseudomonas lipase (PML; Sigma), confirming its role as a lipase. In contrast, no activity of porcine liver esterase (PLE) was observed with these substrates, which further substantiates specific lipase activity in Msh1. Each data point represents an average of four replicates. G, orlistat significantly inhibited Msh1 lipase activity. Msh1 was incubated with the desired triglyceride substrate, tributyrin or triolein, for 1 h at 37 °C, and activity was measured in the absence or presence of 20 μM orlistat, a lipase inhibitor.
values of $3898 \pm 298 \, \mu M$, $1.5 \pm 0.16 \, \mu M\, min^{-1}$, and $274 \pm 34 \, s^{-1}$, respectively. Values of these kinetic parameters lie within the range of those of the reported lipases (20, 21). To ascertain whether Msh1 is indeed a lipase, we performed assays with lipase-specific substrates tributyrin (composed of butyric acid (C4:0) and glycerol) and triolein (made up of oleic acid (C18:1) and glycerol) that are unfavorable for hydrolysis by esterases. The results (Fig. 2F) of these experiments clearly demonstrated that Msh1 exhibits significant levels of hydrolysis of these substrates, confirming its function as a lipase. Moreover, the inhibition of Msh1 lipase activity by orlistat, a well known lipase inhibitor, further validated our claim (Fig. 2G). We then showed that Domain A exhibits protease activity, but the plausible lipase activity of Domain B could not be tested as the required amount of protein could not be prepared because of poor overexpression of this domain. However, as a whole enzyme, Msh1 exhibits both lipase and protease activities.

**Msh1 hydrolyzes macrophage lipids and is up-regulated under hypoxic stress conditions**

After establishing that Msh1 is secreted and exhibits distinctive lipase activity, we next asked whether Msh1 hydrolyzes host-derived lipids. To assess this, total lipids were extracted from macrophages RAW 264.7 and incubated with Msh1 for 2 or 4 h at 37 °C followed by analysis of hydrolyzed products using TLC (Fig. 3A). As evident from Fig. 3A, Msh1 does exhibit hydrolytic activity against host-derived lipids. Of all the spots corresponding to different lipids belonging to the host lipid pool, the one with an $R_f$ of 0.15 was diminished significantly compared with other spots. Furthermore, we tested the activity, if any, of a well characterized lipoprotein lipase from *Pseudomonas* species (Sigma) on macrophage lipids. The results showed that, unlike Msh1, the *Pseudomonas* lipoprotein did not exhibit a noticeable hydrolytic activity against macrophage lipids (Fig. 3B). However, it showed lipase activity for NPP as mentioned earlier (Fig. 2F). These observations suggested that indeed the mycobacterial Msh1 has specificity toward lipids derived from its natural host, macrophages.

Hypoxia has long been perceived as a prominent environmental factor that triggers metabolic realignment in bacilli, often inducing latency, quiescence, and increased lipid metabolism (22, 23). Based on the above results, we analyzed whether the expression levels of Msh1 are altered in an *in vitro* Wayne model of hypoxia (23). Briefly, *M. tuberculosis* was grown in Sauton’s medium with limited aeration in sealed vials for 14 days to gradually establish hypoxic conditions followed by analysis of Msh1 expression in both whole-cell lysates and culture filtrate by anti-Msh1 immunoblotting. In agreement with our hypothesis, expression levels of Msh1 were significantly elevated in both fractions, further substantiating the role of this enzyme under hypoxic conditions (Fig. 3, C and D).

**Msh1 promotes bacillary growth inside foamy macrophages**

The host responds to *M. tuberculosis* infection by inducing the formation of a granuloma, a collection of inflammatory cells including lipid-loaded foamy macrophages, to contain myco-
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**Figure 4. Msh1 plays a significant role in the survival of M. tuberculosis in foamy macrophages.**

*Panel A:* Immunoblot showing whole-cell lysates of RAW264.7 macrophage cells that were either left uninfected, infected with *M. tuberculosis* for 12 and 24 h (1:10 m.o.i.), or infected subsequent to oleate and cholesterol treatment (infected + foamy). Elevated levels of Msh1 were observed under the hypoxic conditions of foamy macrophages. Actin served as a loading control for macrophage cells, and GroEL1 was used as a loading control for *M. tuberculosis*. Data are representative of three independent experiments. *Panel B:* Generation of the Rv2672 KD strain of *M. tuberculosis* by CRISPR interference technology. Levels of Rv2672 in ATc-treated and untreated cultures of Rv2672 KD were examined by both quantitative RT-PCR (top) and Western blotting (bottom) and compared. As shown, ATc treatment resulted in ∼85% suppression of Rv2672 transcripts, whereas no change was observed in the expression of the control gene sigA. Simultaneously, expression of dcas9 was also analyzed in these cultures to ascertain its induction by ATc. Average -fold expression values from two different experiments are shown. Error bars denote S.D. Similar results were obtained with anti-Msh1 Western blotting, which indicated 90% reduction in the expression levels of Msh1 in Rv2672 KD compared with control. The image shown is representative of two different blots. *Panel C:* Comparison of growth profiles of wild-type H37Rv and Rv2672 KD strains of *M. tuberculosis* in vitro in culture medium. Similar growth profiles of both H37Rv and Rv2672 KD indicate that Msh1 is dispensable for in vitro growth of *M. tuberculosis*. Shown are the average A_{OD_{600}} values from three different measurements. Error bars represent S.D. *Panel D:* Dot plot representing a reduction in bacillary load of Rv2672 KD as compared with wild-type H37Rv in foamy macrophages (M\_foamy). However, in normal macrophages, no significant difference was observed, indicating the specific role of Msh1 in the survival of *M. tuberculosis* in foamy macrophages. *Panel E:* Immunoblots depicting an increase in expression of Msh1 in the whole-lung lysates of H37Rv-infected BALB/c mice. Maximum expression was observed in day 56 lysates with β-actin serving as a loading control. The blots are representative of six biological replicates.

bacterial pathogens under hypoxic, nutrient-limited environments. However, *M. tuberculosis* is able to survive inside granulomatous lesions for long-term periods using nutrients, particularly lipids, derived from the host (24). Our study reveals that Msh1 is a secretory protein, hydrolyzes host-derived lipids, and is overexpressed under hypoxia. Together, these observations strongly indicate a possible role of Msh1 in the intracellular survival of *M. tuberculosis*. To test our hypothesis, we analyzed Msh1 expression and proliferation of *M. tuberculosis* in both foamy and normal macrophages.

Infection of both macrophage types was performed with *M. tuberculosis* at an m.o.i. of 10 followed by analysis of Msh1 levels using anti-Msh1 immunoblotting. Notably, an elevated level of Msh1 was observed in foamy macrophage whole-cell extracts compared with its normal counterpart and 24 h postinfection (Fig. 4A). Subsequently, we examined the role of Msh1 in the proliferation of *M. tuberculosis* within the foamy macrophages. For this, an Rv2672 KD strain of *M. tuberculosis* was generated (Fig. 4B). No significant difference was observed in bacterial growth of the wild-type and the Rv2672 KD strains in culture medium (Fig. 4C). Furthermore, normal and foamy macrophages were infected separately with wild-type and Rv2672 KD strains of *M. tuberculosis* at an m.o.i. of 10. Bacillary load was examined by plating macrophage lysates prepared 12 and 24 h postinfection, respectively. Interestingly, bacterial loads of the native and the Rv2672 KD strains were largely similar in normal macrophages, whereas there was a significant difference in bacterial counts of Rv2672 KD relative to the control strain in lipid-rich foamy macrophages, particularly at the 24-h time point (Fig. 4D). Overall, these results clearly suggest that the presence of Msh1 augments the ability of *M. tuberculosis* to survive in lipid-rich intracellular environments.

**Expression of Msh1 in M. tuberculosis is up-regulated in mouse model of infection**

To decipher the expression profile of Msh1 in the granulomatous lesions in vivo, we analyzed its expression in mouse lungs following *M. tuberculosis* infection. BALB/c mice were infected with wild-type *M. tuberculosis* by the aerosol route, and lungs were harvested after 28 and 56 days of infection. Subsequently, expression levels of Msh1 were checked by Western blotting using lung homogenates. As depicted in Fig.
E, a significant level of Msh1 was observed in mouse lung homogenates 56 days postinfection when granulomas were distinctly visible. Msh1 was up-regulated by ~12-fold with respect to day 1 as analyzed using ImageJ (25). These results are in agreement with our previous observations, clearly indicating that Msh1 is important for M. tuberculosis to meet its energy requirement at latent stages of intracellular growth.

Discussion

M. tuberculosis has infected 2 billion of the global population in the form of latent infection. Reactivation presents a major problem in curbing TB infections, particularly in high-burden nations. Although the current drug regimen is effective against actively replicating bacteria, this set of drugs is proven ineffective to control the increasing burden of TB cases due to their inability to eradicate latent infections.

Over the course of many millennia, M. tuberculosis has developed strategies to outsmart the agents of host armamentarium, a notable one being hypoxia. For survival under such environments that essentially reduce the aerobic efficiency, the pathogen tends to use host-derived lipids/fatty acids as a major source of nutrition (24). Therefore, delineating the molecular mechanism underlying host lipid hydrolysis is an important area to address. The present study, which dissects the function of a novel enzyme, Msh1, of M. tuberculosis, sheds the first insight into the underlying mechanism of this phenomenon, which is relevant in many aspects.

The finding that Msh1 is conserved exclusively in actinomycetes suggests that these organisms have developed a unique common trait during the course of evolution, potentially to survive in harsh host environments. Msh1 is a bifunctional enzyme with distinct protease and lipase domains. The present study deciphered that M. tuberculosis does require Msh1 for its growth in a hypoxic macrophage model. Because survival in these environments requires host lipids (24), the pertinence of Msh1 lipase activity is understood; however, the relevance of protease activity in Msh1 needs to be examined. Moreover, the up-regulation of Msh1 in a hypoxic macrophage model and a mouse model of TB infection clearly demonstrated that M. tuberculosis requires Msh1 for its persistence in stress conditions, such as granulomas filled with lipid-loaded macrophages.

On the basis of the results of the present study combined with available literature data, we propose a model (Fig. 5)
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Table 1
Sequences of the primers used in the study

| Primer                  | Sequence                          |
|-------------------------|-----------------------------------|
| 2672_full_length (forward) | 5′-CACCCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| 2672_full_length (reverse) | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Domain A (forward)       | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Domain A (reverse)       | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Domain B (forward)       | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Domain B (reverse)       | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Rv2672_RT_f             | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |
| Rv2672_RT_r1            | 5′-CACCATATGCACTATCATCATATCATCTAGACTGGAACATGAG-3′ |

depepting how M. tuberculosis likely manages to use host lipids for its advantage. Primarily, in hypoxic stress situation, it uses Msh1 to hydrolyze host lipids and takes up the lipid-derived fatty acids into its cytoplasm through fatty acid transport proteins. The import of these fatty acids replenishes the bacillary cytoplasmic lipid reservoir. Furthermore, it is likely that M. tuberculosis re-esterifies these fatty acids into TAG mainly by its intracellular TAG synthase and/or likely uses these fatty acids to generate other lipids. In conclusion, the functional characterization of Msh1, which provides the first-ever insight into the host lipid catabolism by an M. tuberculosis enzyme, augments the current understanding of the persistence strategies latent M. tuberculosis uses. These results together suggest that Rv2672 is a potential molecular drug target. Abolishing its function by small molecule compounds could open up new strategies to combat latent M. tuberculosis. Furthermore, the trait that Rv2672 is overexpressed in hypoxic environments opens up the possibility that it could be a useful biomarker for latent TB diagnosis. Future studies to address whether M. tuberculosis contains additional factor(s) involved in this important metabolic process would shed further insight into the host lipid catabolism.

Experimental procedures

In silico analysis tools

The amino acid sequence of Msh1 was obtained from UniProt and analyzed for the presence of, if any, conserved protein domains using the Pfam database (26). Secondary structure elements and functional domains were predicted using Jpred4 (27) and the NCBI conserved domain database (19). The amino acid sequence was scanned for signal peptide and lipobox motif using SignalP 4.0 (28) and DOLOP (29) servers, respectively. Sequence alignments were performed using the multiple sequence alignment tool ClustalX (30). Phylogenetic and evolutionary analyses were conducted using MEGA6 (31). The evolutionary tree was inferred using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in units of number of amino acid substitutions per site.

Bacterial strains and macrophage cell line

Escherichia coli strain DH5α (Invitrogen) was grown in Luria-Bertani (LB) (Difco) broth at 37 °C with constant shaking at 180 rpm for 12 h. Selection of E. coli DH5α was carried out using appropriate antibiotics (50 μg ml⁻¹ kanamycin, 100 μg ml⁻¹ ampicillin, and 150 μg ml⁻¹ hygromycin) in LB broth or an LB agar plate. M. smegmatis mc² 4517, M. smegmatis mc²155, and M. tuberculosis H37Rv strains were grown in Difco Middlebrook 7H9 broth liquid medium enriched with 10% albumin-dextrose-catalase, 0.05% Tween 80 (Sigma-Aldrich), and 0.2% glycerol (Sigma-Aldrich) at 37 °C with constant shaking at 180 rpm. 7H10 agar medium (BD Biosciences) supplemented with oleic acid-albumin-dextrose-catalase (OADC) was used for plating and streaking for the selection of the transformants using the appropriate combination of antibiotics, 50 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ hygromycin. For experiments that required culture filtrate sample, mycobacterial strains were cultured in chemical medium (Sauton’s medium).

Macrophage culturing

Mouse macrophage cell line RAW264.7 was obtained from ATCC and maintained in RPMI 1640 medium (Gibco) supplemented with 200 μg ml⁻¹ glutamine, 100 units ml⁻¹ penicillin-streptomycin, 50 μg ml⁻¹ neomycin, and 10% fetal bovine serum (Gibco) at 37 °C in the presence of 5% CO₂. For infection experiments, cells were expanded in medium without antibiotics.

For passage, the medium was aspirated, and the cells were treated with 1× trypsin-EDTA at 25 °C for 15 min after which cells were pipetted and aspirated, pelleted at 1000 rpm for 5 min, resuspended in 1 ml of complete RPMI 1640 medium, and transferred to a fresh T75 flask or 24-well culture plates. All experimental setups were carried out in 24-well culture plates with each well containing 10⁵ cells (counts were made using trypan blue and a cytometer).

For generation of activated macrophages, the cells in 6-well plates were treated with 200 ng/ml LPS, and further experiments were carried out 12 h post-treatment. Foamy macrophages were generated using established methods (32). Briefly, the cells in 2 ml of medium in 6-well plates were treated with 400 ng/ml sodium oleate and 40 ng/ml cholesterol, and further experiments were carried out 12 h post-treatment.

Preparation of expression constructs, overexpression in M. smegmatis, and purification

Various expression constructs of full-length Rv2672, signal peptide-truncated, and Domain A (nucleotides 270–1116) and Domain B (nucleotides 1122–1560) of Rv2672 were prepared in a similar manner. Briefly, the target gene was PCR-amplified using M. tuberculosis H37Rv genomic DNA as the template with specific forward and reverse primers (Table 1). The linear amplicon was then purified and cloned into the entry vector pENTR-D-TOPO (Invitrogen) according to the manufacturer’s manual, and the entry clone was transformed into DH5α. The plasmid containing the gene was isolated from the positive col-
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Mycobacterial cultures grown in 7H9 or Sauton's medium were harvested by centrifugation at 10,000 × g. To obtain culture filtrate, chemical medium (Sauton’s) was concentrated using a centrifugal unit (Millipore) after harvesting cells. M. smegmatis cultures were lysed using a cell disruptor system (Constant Systems, UK) at 4 °C, whereas the M. tuberculosis cultures were lysed using a bead beater (BioSpec). Further isolation of cell wall and cell membrane fractions was carried out using differential centrifugation (33). For the subcellular localization study of the endogenous protein, mycobacterial cell lysate was first centrifuged at 10,000 × g for 30 min and then centrifuged at 100,000 × g for 2 h at 4 °C to obtain the cell envelope fraction comprising both cell wall and cell membrane compartments. For secreted fraction analysis, the chemical medium was collected after harvesting the bacterial cells at 10,000 × g for 30 min at 4 °C. The medium then was concentrated using a centrifugal unit followed by acetone precipitation.

Biochemical assays

The protease activity of Msh1 was tested against the universal protease substrate β-casein (Sigma). Test experiments were performed to determine the optimum temperature and pH for protease activity of Msh1. A reaction mixture of 0.65% (w/v) of β-casein in 50 mm Tris, 100 mm NaCl, pH 7.5, buffer was treated with a concentration gradient (0.25–5.0 μM) of Msh1 for 1 h at 37 °C. The progression of the reactions was stopped by adding 2 M NaOH and heating at 95 °C with SDS protein loading dye, and the reactions were analyzed by SDS-PAGE.

Lipase activity was assayed using the substrate NPP (Sigma) as described elsewhere (34, 35). The activity was performed at pH 8 at 37 °C using an established spectrophotometric method. Briefly, 1 mm NPP was emulsified using 0.2% gum arabic in aqueous buffer, 50 mm Tris, 100 mm NaCl, by brief sonication and incubated with 20 μM purified enzyme at 37 °C for 2 h. The formation of the product nitrophenol was measured at 405 nm, and activity was assayed by calculating the amount of nitrophenol released from nitrophenol esters. One unit of enzyme activity was defined as the amount of enzyme that can release 1 μM nitrophenol/min at 37 °C. Also we verified the lipase activity of Msh1 with different substrates such as 1,3-di(butanoyloxy)propan-2-yl butanoate (tributyrin) (MP Bioscience) and 2,3-bis[(Z)-octadec-9-enoyloxy]propyl (Z)-octadec-9-enolate (triolein) (Alfa Aesar). Briefly, the substrates triolein and tributyrin were emulsified using 2% gum arabic by mechanical triturating. The reaction mixture contained 1 mm substrate emulsion, 2 μM Msh1, 50 mm Tris, and 100 mm NaCl, pH 8. The mixture was incubated for 2 h at 37 °C. The free glycerol liberated from lipid hydrolysis was measured using free glycerol reagent (Sigma) at 540 nm, and corresponding activity was measured based on a standard curve obtained for glycerol and free glycerol reagent. The possibility of showing activity due to impurities was nullified by measuring a blank using substrate without enzyme that was kept in the same conditions. For the inhibition study, different concentrations of the lipase inhibitor orlistat were also added to the reaction mixture.

Subcellular fractionation

Polyclonal antibodies against Msh1 were raised at an in-house animal facility using New Zealand White strain rabbits aged 15 weeks. Purified Msh1 (1 mg/ml) was emulsified with adjuvant (Freund’s incomplete adjuvant) at a 1:1 ratio using a glass syringe connected to a three-way stopcock. The injection was administered subcutaneously with a maximum of 500 μg of Msh1 per site, and the maximum number of injection sites was two. Four booster immunizations were carried out with an interval of 2 weeks to get maximum serum antibody titer. A blood serum was collected retro-orbitally before every booster dose to collect serum antibodies generated against Msh1. Serum was prepared after incubating the blood sample at 37 °C for 5–10 min followed by centrifugation at 3500 rpm for 15 min at 4 °C. Anti- Msh1 serum was collected and stored at −80 °C.

Antibody generation

Polyclonal antibodies against Msh1 were raised at an in-house animal facility using New Zealand White strain rabbits aged 15 weeks. Purified Msh1 (1 mg/ml) was emulsified with adjuvant (Freund’s incomplete adjuvant) at a 1:1 ratio using a glass syringe connected to a three-way stopcock. The injection was administered subcutaneously with a maximum of 500 μg of Msh1 per site, and the maximum number of injection sites was two. Four booster immunizations were carried out with an interval of 2 weeks to get maximum serum antibody titer. A blood sample was collected retro-orbitally before every booster dose to collect serum antibodies generated against Msh1. Serum was prepared after incubating the blood sample at 37 °C for 5–10 min followed by centrifugation at 3500 rpm for 15 min at 4 °C. Anti- Msh1 serum was collected and stored at −80 °C.
**Lipase activity on host lipids**

Total lipids of macrophages were isolated following the method of Bligh and Dyer (36). First, the lipids were isolated from 1 $\times$ 10⁶ RAW cells using chloroform:methanol (2:1) solvent. Extracted lipids were resuspended in chloroform and used for the lipase assay. The samples were incubated with 2 $\mu$M Msh1 for 4 and 6 h. Then the lipid portion was precipitated with chloroform:methanol. The organic phase was dried, and the lipid was used for further analysis. The product mixture was analyzed by TLC in hexane:diethyl ether:formic acid (40:10:1 by volume) as the solvent system. Linolenic acid (Sigma) was spotted as an external control. Quantitative lipids were monitored by charring with iodine vapors.

**In vitro hypoxia model**

The Wayne model of in vitro hypoxia for *M. tuberculosis* was generated as described in Wayne and Sohaskey (23) except for a minor modification; i.e., protein-free Sauton’s medium was used to facilitate the detection of secreted endogenous Msh1 in the culture filtrate. *M. tuberculosis* cultures were grown in tightly capped bottles with a ratio of head space air to medium of 0.5 and regular stirring at 15 rpm. The culture was grown for 14 days, and then culture filtrate and cell lysate fractions were harvested using the protocol described earlier. The supernatant obtained after harvesting the bacterial cells served as the culture filtrate fraction. The collected medium was concentrated and precipitated with acetone, and the resulting sample was used for the immunoblotting.

**Macrophage infection**

Culturing and staining of macrophage were conducted as mentioned elsewhere (38). Briefly, *M. tuberculosis* culture was grown to an $A_{600}$ of 0.6, and on the day of infection, macrophage cells were plated at 10⁶/well in a 24-well culture plate. Briefly, cells were washed with PBS followed by addition of antibiotic-free RPMI 1640 medium. The cells were then infected with *M. tuberculosis* at an m.o.i. of 10. After 4 h, non-phagolysed bacteria were washed three times with PBS, and adherent monolayers were replenished with antibiotic-free culture medium. At 12, 24, and 36 h postinfection, medium was removed, and monolayers were lysed with 0.05% SDS. Serial dilutions ($10^{-6}$–$10^{-1}$) were plated on agar plates (Middlebrook 7H10 with 10% OADC enrichment; Difco). cfu were counted after incubation at 37°C for 21 days.

**Construction of Rv2672 knockdown strain**

The *M. tuberculosis* Rv2672 KD strain was generated using the clustered regularly interspersed short palindromic repeats (CRISPR) interference approach as described in an earlier study (37). Briefly, Rv2672–specific complementary oligonucleotides, Rv2672_UP (5″–GCCCGAGTGGTCCGCAACC-3″) and Rv2672_DN (5″–CCCGCGTGCATCGGCAATGC-3″) targeting 115–135 bases from the start codon were designed so that after annealing the corresponding double-stranded DNA bears SpII and AclII overhangs. The target sequence was cloned in Hyg<sup>R</sup> replicative plasmid pGmra at the same sites upstream of the Cas9 handle sequence. Subsequently, mycobacteria harboring Kan<sup>R</sup> plasmid pTetInt-dcas9 were transformed with the recombinant pGmra-Rv2672c, and colonies were obtained on 7H11-OADC containing 50 mg ml<sup>−1</sup> hygromycin and 25 mg ml<sup>−1</sup> kanamycin after 3 weeks of incubation. The resulting strain was termed Rv2672 KD. *M. tuberculosis* harboring pTetInt-dcas9 and empty plasmid pGmra were simultaneously used as controls. Conditional expression of Rv2672 in both the control and Rv2672 KD strains of *M. tuberculosis* was analyzed in the presence of ATc as reported previously. The primers used for real-time PCR are given in Table 1.

**Animal infection**

Wild-type *M. tuberculosis* H37Rv strain was grown to an $A_{600}$ of 0.6–0.8, and 6–8-week-old BALB/c mice were challenged with 200 cfu through aerosolic route to establish an infection. Mice were sacrificed at different time points (days 1, 28, and 56), and lungs were harvested and homogenized. These homogenized samples were further lysed by bead beating and used for Western blotting experiments.

**Statistical analysis**

Statistical analysis was carried out using two-tailed Student’s t test.

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Characterization of a secretory hydrolase from *M. tuberculosis*

Note added in proof—Dr. Divya Arora was inadvertently omitted as an author on the version of this article that was published as a Paper in Press on May 17, 2017. This error has now been corrected.

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