

Supplementary Material

Supplementary Materials and Methods

***M. smegmatis* phagocytosis assay**

Microscopic visualization of *M. smegmatis* phagocytosis was performed as previously described [1]. Briefly, Msmg-2387 or Msmg-EV was stained with SYTO-9 (Thermo Scientific, USA) before infection. THP-1 or RAW 264.7 macrophages were then infected with SYTO-9-stained recombinant *M. smegmatis* at an MOI of 10. At 4 hrs post-infection, the macrophages were washed and fixed in a 4% paraformaldehyde solution for 0.5 hrs. Next, the macrophages were permeabilized with 0.2% (v/v) Triton X-100 solution for 20 min and stained with DAPI solution for 8 min in the dark. The images were obtained through the fluorescence microscope.

Analysis of the subcellular localization of Rv2387 protein in recombinant *M. smegmatis*

Subcellular fractionation was performed as previously described [2]. In brief, recombinant *M. smegmatis* strains were grown in medium at 37 °C to an OD₆₀₀ of 0.6-0.8. Bacteria were harvested by centrifugation. Culture supernatants were filtered and concentrated 100-fold to obtain bacterial culture filtrate (CF). The whole cells were sonicated and centrifuged at 3,000 g for 30 min to obtain the whole cell lysates (WCL) from the supernatant. WCL was centrifuged at 27,000 g for 30 min. The pellet from this centrifugation step was considered the cell wall fraction (CW), and the supernatant was supposed to be the cell membrane and cytosolic (CM+Cy) fractions. All centrifugation steps were performed at 4 °C.

Equal amounts of protein from fractionated samples were subjected to Western blot analysis. The Myc-tagged Rv2387 protein was detected by an anti-Myc antibody (CTS).

GroEL, which served as a cytosol marker protein of mycobacteria [3], was detected by an anti-His primary antibody (CTS).

Supplementary Figures

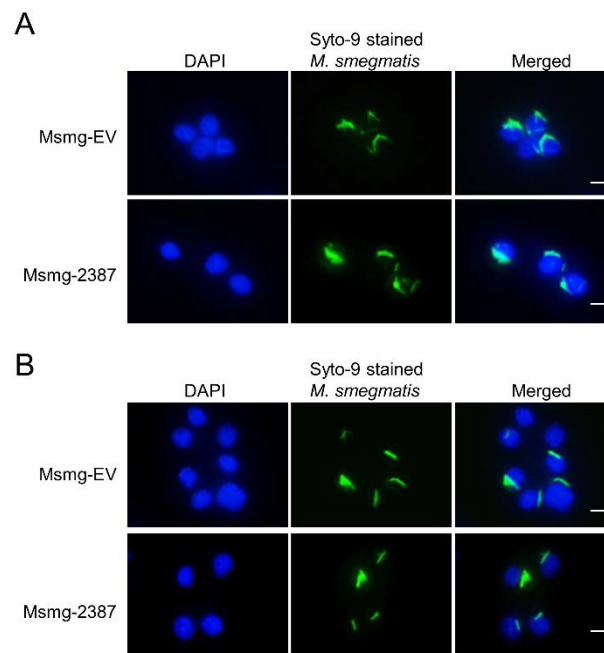


Figure S1. Microscopic visualization of *M. smegmatis* phagocytosis. THP-1 macrophages (A) or RAW 264.7 macrophages (B) were infected with SYTO-9-stained Msmg-2387 and Msmg-EV. Internalization of recombinant *M. smegmatis* by macrophages was visualized through a fluorescent microscope. The scale bars are equivalent to 5 μ m. Similar results were obtained in two independent experiments.

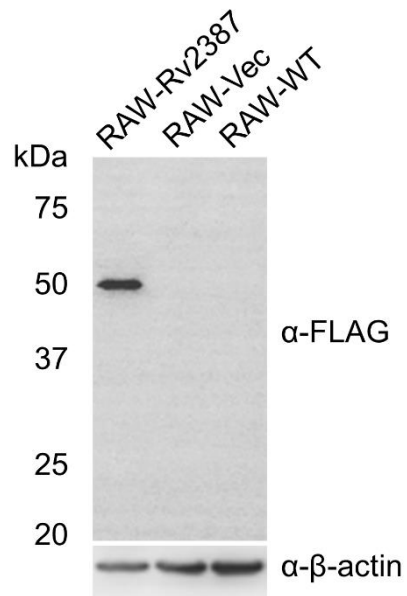


Figure S2. The expression of Rv2387 in RAW-Rv2387 cells. RAW-WT, RAW-vector, or RAW- Rv2387 cells were lysed in the sample buffer. Cell lysates were separated by SDS-PAGE, and proteins were detected with an anti-Flag antibody (Sigma).

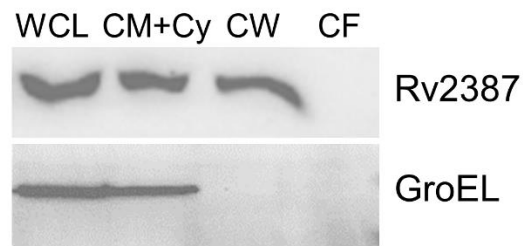


Figure S3. Subcellular localization of Rv2387 in MS_Rv2387. Equal amounts of protein from whole cell lysates (WCL), cytoplasmic membrane and cytoplasm (CM+Cy), cell wall (CW), and culture filtrate (CF) from MS_Rv2387 were separated by SDS-PAGE and proteins were detected with an anti-Myc antibody. Native GroEL was detected as a cytoplasmic control.

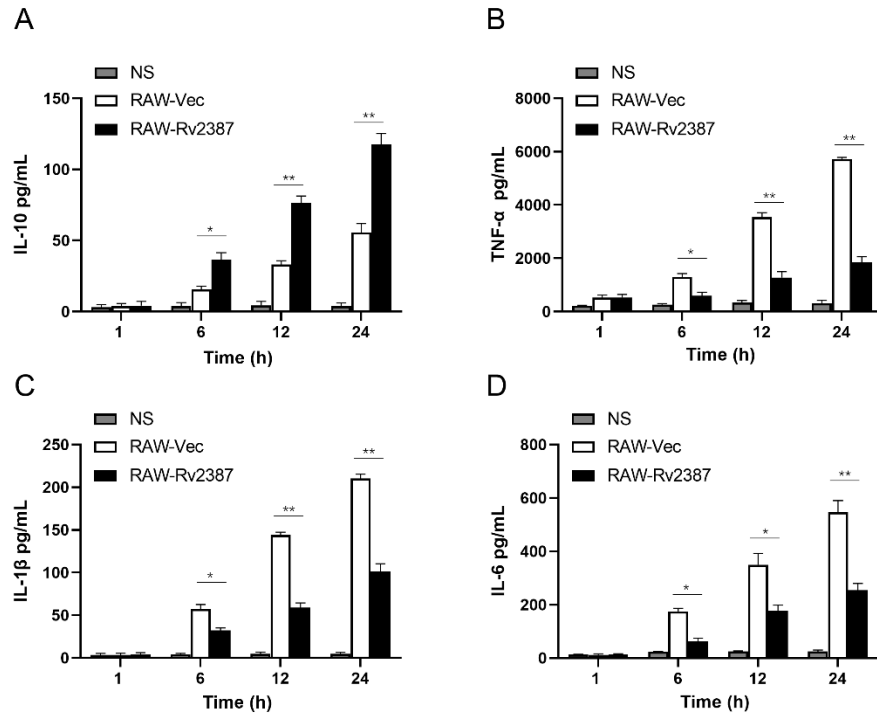


Figure S4. The expression of IL-10, TNF- α , IL-1 β , and IL-6 in the RAW264.7 stable cells treated with TLR-2 agonist. RAW-Vec and RAW-Rv2387 macrophages were left unstimulated (NS) or treated with TLR2 agonist Pam3CSK4 (200 ng/mL). Culture supernatants were collected and subjected to an ELISA analysis to monitor the secretion of IL-10 (A), TNF- α (B), IL-1 β (C), and IL-6 (D). Data are means \pm SD of technical triplicate from one representative of two independent experiments. (* $p < 0.05$, ** $p < 0.01$).

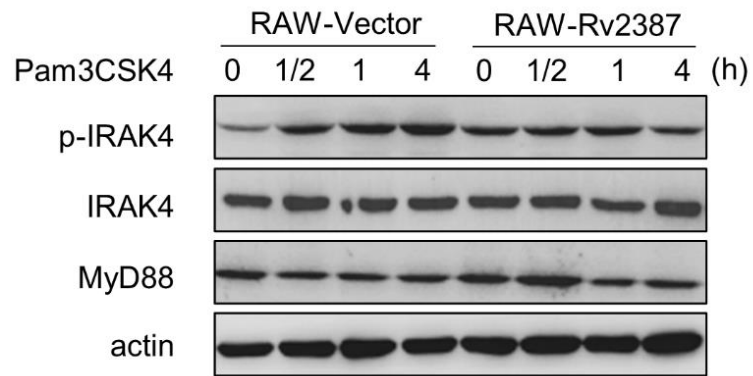


Figure S5. Rv2387 blocked the activation of IRAK4 in RAW-Rv2387 macrophages.

Murine RAW-Vec and RAW-Rv2387 macrophages were treated with TLR2 agonist Pam3CSK4 (200 ng/mL) at different time points. Infected macrophages were lysed in RIPA buffer and subjected to immunoblot analysis to detect the protein expression of phosphorylated or total IRAK4, MyD88, and β -actin. Similar results were obtained in two independent experiments.

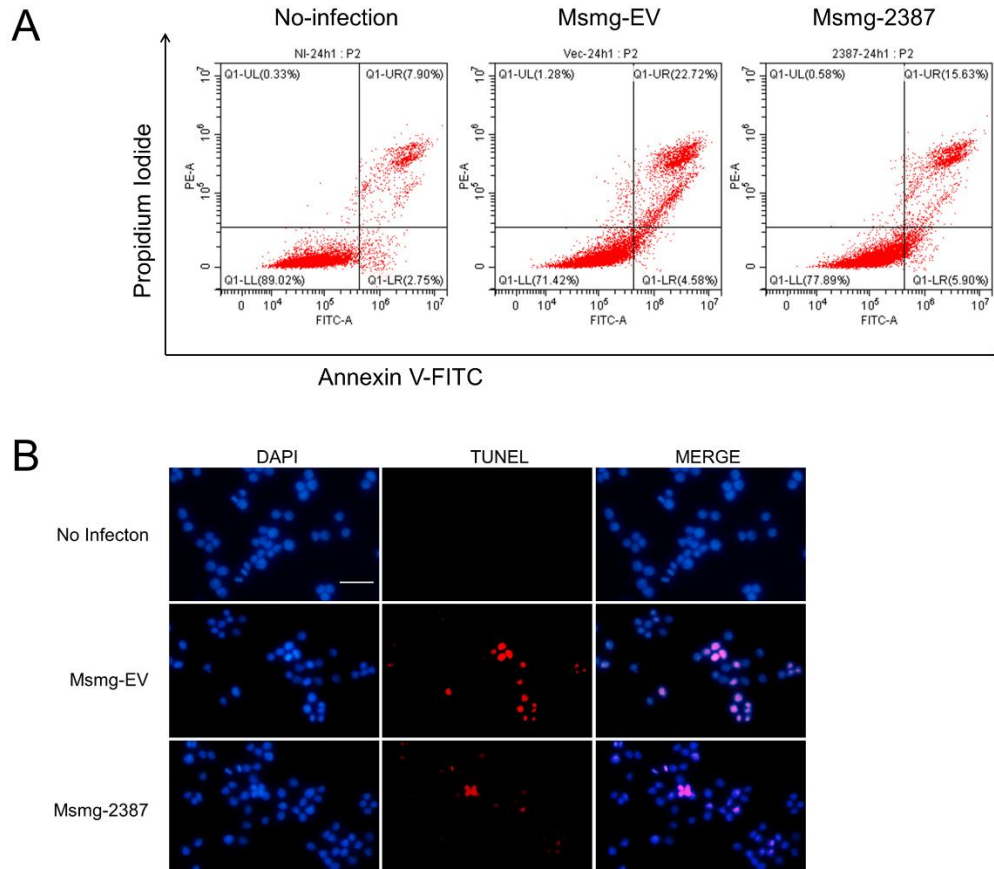


Figure S6. Rv2387 inhibited mycobacteria-induced macrophage apoptosis in BMDMs.

A. a representative FACS profile corresponding to Fig. 7B. B. BMDMs were infected with either engineered Msmg-2387 or Msmg-EV strains for 24 hrs. DNA fragmentation in infected macrophages was detected via TUNEL staining analysis. The scale bars are equivalent to 50 μ m. Similar results were obtained in two independent experiments.

Reference

1. Arora, S.K.; Naqvi, N.; Alam, A.; Ahmad, J.; Alsati, B.S.; Sheikh, J.A.; Kumar, P.; Mitra, D.K.; Rahman, S.A.; Hasnain, S.E.; et al. Mycobacterium smegmatis Bacteria Expressing *Mycobacterium tuberculosis*-Specific Rv1954A Induce Macrophage Activation and Modulate the Immune Response. *Front Cell Infect Microbiol.* **2020.** *10*, 564565.
2. Li, W.; Zhao, Q.; Deng, W.; Chen, T.; Liu, M.; Xie, J. *Mycobacterium tuberculosis* Rv3402c Enhances Mycobacterial Survival within Macrophages and Modulates the Host Pro-Inflammatory Cytokines Production via NF-Kappa B/ERK/p38 Signaling. *PLoS One* **2014**, *9*, e94418.
3. Bashiri, G., Perkowski, E.F.; Turner, A.P.; Feltcher, M.E.; Braunstein, M.; Baker, E.N. Tat-Dependent Translocation of an F(420)-Binding Protein of *Mycobacterium tuberculosis*. *PLoS One* **2012**, *7*, e45003.