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Regulation of *M. tuberculosis* cell envelope composition and virulence by Regulated Intramembrane Proteolysis

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Abstract

M. tuberculosis (Mtb) infection is an ongoing global health crisis that kills 2 million people each year¹. Although the structurally diverse lipids of the Mtb cell envelope each have nonredundant roles in virulence or persistence²⁻⁷, the molecular mechanisms regulating cell envelope composition in Mtb are undefined. In higher eukaryotes, membrane composition is controlled by site two protease (S2P) mediated cleavage of sterol regulatory element binding proteins (SREBPs)^{8,9}, membrane bound transcription factors that control lipid biosynthesis. S2P is the founding member of a widely distributed family of a membrane metalloproteases^{10,11} that cleave substrate proteins within transmembrane segments¹². Here we show that a previously uncharacterized Mtb S2P homolog (*Rv2869c*) regulates cell envelope composition, *in vivo* growth, and *in vivo* persistence of Mtb. These results establish that regulated intramembrane proteolysis (RIP) is a conserved mechanism controlling membrane composition in prokaryotes and establish RIP as a proximal regulator of cell envelope virulence determinants in *M. tuberculosis*.

Despite the well established role of S2P in lipid metabolism in higher eukaryotes, prokaryotic S2P family members characterized to date control sporulation in *Bacillus subtilis* (SpoIVFB)^{13,14}, the periplasmic stress response in *E. coli* (YaeL)^{15,16}, and cell polarity¹⁷. To examine the physiologic role of RIP in Mtb, we searched the Mtb genome for Site two protease homologs with the signature HExxH zinc chelation active site motif and the LDG C terminal motif both present within predicted transmembrane domains. Through this approach, we identified a S2P homologue (*Rv2869c*) in the Mtb chromosome which has not been characterized previously. Figure 1a shows the hydropathy plots of human S2P, YaeL and *Rv2869c*. Although the amino acid identities between the three proteins are low (16-22%), the conserved HExxH and F/LDG motifs and transmembrane topology establish *Rv2869c* as an intramembrane cleaving protease (iCLIP)¹².

To characterize the function of *Rv2869c* in mycobacteria, we deleted this gene from the chromosomes of *M. bovis* BCG and *M. tuberculosis* Erdman by specialized transduction¹⁸. Figure 1b shows the genomic location of *Rv2869c* between *dxr* and *gcpE*, two genes in the non-mevalonate pathway of isoprenoid biosynthesis^{19,20}. Southern blotting (Figure 1c) confirmed successful replacement of *Rv2869c* with a Hygromycin resistance cassette in both BCG and *M. tuberculosis*, demonstrating that, in contrast to YaeL in *E. coli*, *Rv2869c* is not an essential gene for *M. tuberculosis* viability.

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Competing interests statement

The authors declare that they have no competing financial interest.

When grown on solid media, the *Rv2869c* null mutant displayed altered colony morphology. In pathogenic mycobacteria, the colonial and microscopic morphology of cording has long been associated with virulence and is dependent on multiple cell envelope lipids 2,5. Both the BCG and *Mtb* $\Delta Rv2869c$ strains lacked cording, as measured by colonial morphology (Figure 2A) and by microscopic examination of Auramine-Rhodamine stained bacilli (Figure 2B). Wild type cording was restored to the $\Delta Rv2869c$ strain by a wild type copy of *Rv2869c*, confirming that loss of *Rv2869c* function was responsible for the noncording phenotype (Figure 2). Alanine substitution mutations in the conserved HExxH (H21A) and FDG (D341A) motifs, which are required for proteolytic activity of RIP proteases^{10,13,16}, did not restore wild type colony morphology (Fig 2C). These results demonstrate that the proteolytic activity of *Rv2869c* regulates cell envelope composition in pathogenic mycobacteria. These results suggested that RIP is a conserved mechanism of membrane composition control in prokaryotes.

The SREBP pathway regulates multiple pathways of lipid biosynthesis, including cholesterol and fatty acids²¹. To directly ask whether *Rv2869c* regulates lipid composition in *Mtb*, we analyzed the extractable and esterified mycolic acids of the cell envelope of wild type cells, $\Delta Rv2869c$ cells, and complemented mutant cells by quantitative thin layer chromatography (TLC) of ¹⁴C acetate labeled cells in the presence or absence of detergent in the culture medium. In the esterified mycolic acids, we observed similar quantities of the three major mycolic acids in all strains regardless of culture conditions (figure 3A, top panel). In contrast, while wild type cells maintained extractable mycolic acid synthesis after shift to detergent free media (Figure 3A, bottom panel), $\Delta Rv2869c$ cells downregulated alpha mycolate synthesis by 4.6fold, methoxymycolates by 3.5 fold, and ketomycolates by 2.3 fold. In addition, $\Delta Rv2869c$ cells upregulated synthesis of a slow migrating lipid near the origin of the TLC plate by 6 fold compared to no Tween conditions. This lipid was absent from wild type or complemented mutant extractable lipids (Figure 3A, bottom panel). These results indicated that *Rv2869c* regulates the composition of extractable mycolic acids in the cell envelope in response to changes in membrane fluidity but has no role in the covalently esterified mycolates of the cell wall. To examine whether *Rv2869c* also regulates other classes of cell envelope lipids, we examined the composition of phosphatidylinositol mannoside (PIM). Two dimensional TLC of chloroform-methanol extracts revealed typical pattern of PIM2 and PIM6 with differing degrees of acylation^{22,23}. We found no difference between mutant and wild type cells in PIM2 species (Figure S1, spots 1 and 2) but observed a two fold decrease in the abundance of a PIM6 subspecies (spot 4) in mutant but not wild type or complemented mutant cells.

To ask whether *Rv2869c* controls cell envelope composition through transcriptional regulation, we compared the transcriptional profiles of the wild type and $\Delta Rv2869c$ strains in the presence or absence of detergent using an *M. tuberculosis* whole genome oligonucleotide microarray. We found that *Rv2869c* was both a positive and negative transcriptional regulator of multiple lipid biosynthetic and lipid degrading genes. Consistent with the lipid analysis, *Rv2869c* mediated complex transcriptional regulation of mycolic acid biosynthetic genes in response to detergent, including *pks13*, *kasB*, *accD6*, *fatty acid synthase*, *fabG1*, *fabG2*, and two lipid desaturases (figure 3B and supplementary data). Cluster analysis grouped the mycolic acid biosynthetic genes *kasB*, *pks13*, and *accD6* together but indicated that other components of the pathway such as *fabG1/fabG2* were divergently regulated by *Rv2869c* (Figure 3, S2, supplementary tables). Other lipid biosynthetic genes were positively regulated by *Rv2869c*, including multiple genes involved in PDIM synthesis (*mas*, *ppsA*, *drvB*). Absence of *Rv2869c* resulted in strong overexpression of a lipase (*lipP*) and an epoxide hydrolase (*ephC*) and strong underexpression of *rpfC* (Tables S1,S2), three genes putatively involved in lipid or cell wall degradation²⁴. Taken together, these data demonstrate that the lipid perturbations in the cell envelope of the *Rv2869c* mutant resulted from altered transcriptional control of diverse lipid anabolic and catabolic pathways.

An emerging model in Mtb pathogenesis is that the extractable lipids of the cell envelope act as direct effectors of pathogenesis either to modulate host immune responses or alter intracellular trafficking 2,3,7,25-29. Some of these lipids are regulated by *Rv2869c*, suggesting that RIP might be a proximal regulator of critical cell envelope pathogenesis determinants. To test this idea, we examined the *Rv2869c* mutant in the mouse model of aerosol Mtb infection. Despite identical inocula one day after infection, the *Rv2869c* mutant was impaired for bacterial growth during the acute phase of infection such that mutant bacterial titers in the lung after three weeks of infection were approximately 100 fold lower than wild type (Figure 4a). In mice, wild type Mtb persists at constant titer in the lung for the life of the animal. Surprisingly, we found that *Rv2869c* was also required for this persistence phase of the infection. $\Delta Rv2869c$ organisms were progressively eliminated from the lung such that by 22 weeks after infection, the number of bacilli in the lung infected with *Rv2869c* mutant was 10,000 fold lower than wild type. This *in vivo* phenotype was due to loss of *Rv2869c* function and not a polar effect on neighboring genes because the wild type growth and persistence phenotypes were restored by a plasmid expressing *Rv2869c* (Figure 4b). In the liver, *Rv2869c* organisms were completely cleared by 22 weeks of infection (Figure S3). Gross and microscopic examination of infected lungs revealed a dramatic attenuation of granulomatous histopathology in the mutant infected lungs (Figure 4c-d).

While S2P mediated RIP of SREBPs is a well described mechanism controlling eukaryotic membrane composition, S2P family members in prokaryotes characterized to date regulate stress responses^{15,16,30}, sporulation¹³, and cell polarity¹⁷. The present study implicates RIP in controlling the lipid composition of the complex mycobacterial cell envelope. Consistent with the role of other RIP proteases in controlling transcription, the observed changes in cell envelope lipids were associated with transcriptional dysregulation of lipid biosynthetic and degradative genes. Further mechanistic studies will determine whether *Rv2869c* directly cleaves membrane bound transcriptional regulators or controls membrane composition through cleavage of other membrane proteins that control membrane composition.

The recent sequencing of the *M. tuberculosis* genome has stimulated rapid progress in the identification of virulence determinants, many of which are involved in cell envelope biosynthesis. While each of these individual lipid species serves distinct pathogenetic function, the cell envelope dysregulation in the *Rv2869c* mutant affected all phases of murine infection including both *in vivo* replication and *in vivo* persistence. These results suggest that *Rv2869c* controls multiple cell envelope based virulence determinants. As such, this study identifies regulated intramembrane proteolysis as an attractive target for Mtb drug development. Further characterization of mycobacterial RIP will yield important information about the regulation of the unique cell envelope of Mtb and the molecular mechanisms that allow Mtb to persist despite host immunity.

Methods

Media and strains

Mtb strain Erdman was grown at 37°C in 7H9 (broth) or 7H10 (agar) (Difco) media supplemented with 10% OADC, 0.5% glycerol, 0.05% Tween 80 (broth), and where appropriate Hygromycin (Roche) at 50 µg/ml. BCG strains were grown similarly except ADS supplement was substituted for OADC. Strain names are as follows: MGM307=BCG Pasteur *Rv2869c::hyg*; MGM309=Mtb Erdman *Rv2869c::hyg*; MGM336= MGM307+pHMG121; MGM350=MGM309+pHMG121.

Construction of *Rv2869c* null mutant by allelic change and complementation

Gene disruption was performed by specialized transduction of *Rv2869c::hygR* cassettes using temperature-sensitive mycobacteriophages as described previously^{2,18}. The *Rv2869c::hyg* null allele included the first 6 nucleotides of *Rv2869c* (5') and the last 26 nucleotides of *Rv2869c* (3') flanking a Hygromycin resistance cassette. After 4 weeks of selection on Middlebrook 7H10 agar medium containing 50 µg/ml hygromycin, *hyg^R* transductants were genotyped by at the *Rv2869c* locus by Southern blotting as described previously².

For complementation of the *Rv2869c* mutant, we reconstructed the *Rv2869c* operon with a truncated 55 amino acid in frame fusion of *Rv2870c* followed by the intact *Rv2869c* gene expressed from its native promoter 5' of *Rv2870c*. This complementation construct was used to transform MGM307 and MMG309 to kanamycin resistance.

Preparation and analysis of mycolates

Extractable lipids were prepared by the Folch method, dried under nitrogen, and dissolved in diethyl ether. TLC was performed on Adsorbosil silica HPTLC plates (Alltech) and spots visualized with 20% sulfuric acid in ethanol and charring. For PIM analysis, total lipids were developed with CHCl₃/CH₃OH/H₂O (60:30:6) as first dimension and CHCl₃/CH₃COOH/CH₃OH/H₂O (40:25:3:6) as second dimension. For analysis of Mtb bound and extractable mycolic acids, the indicated strains were labeled with 1- ¹⁴C Acetic Acid and mycolic acids prepared described previously² from chloroform methanol extracts (extractable) and cell pellets (cell wall bound). TLC development conditions were 3 developments Hexanes:Ethyl Acetate 95:5. Radio TLCs were imaged by phosphorimaging and quantitation of individual spots was performed on a FUJI FLA-5000 and Image Gauge software (Version. 4.1, FUJIFILM).

Animal Infections

Before infection, exponentially replicating bacteria ($A_{600} = 0.3$) were washed in PBS containing 0.05% Tween 80, and sonicated to disperse clumps. For aerosol infection, mice were exposed to 8×10^7 CFU of the appropriate strain in a Middlebrook Inhalation Exposure System (Glas-Col). This dose of bacteria delivers 100 CFU per animal. Bacterial burden was determined by plating serial dilutions of lung, spleen or liver homogenates onto 7H10 agar plates. Plates were incubated at 37°C in 5% CO₂ for 3-6 weeks prior to counting colonies. For histologic analysis, organs were fixed in 10% normal buffered formalin, embedded in paraffin, and 6 µm sections were stained with hematoxylin and eosin (H&E stain).

Microarray analysis

An Mtb whole genome Microarray was produced using the Operon Tuberculosis whole genome oligo set, version 1.0 (Operon). Details of probe preparation, hybridization and microarray data has been deposited in GEO under accession GSE2561. This complete array was printed on Corning UltraGaps Coated slides using a Microgrid TAS arrayer (Genomic solutions) in duplicate such that each slide contains two complete copies of the array. Hybridizations were performed on biologic triplicates (+Tween, 6 total spots per gene) or duplicates (-Tween, 4 total spots per gene) and included one dye reversal experiment for each condition. Spot quantitation was performed in GenePix 4.0 and data analysis was performed in GeneSpring 7.0 using intensity dependent Lowess normalization. Note that overexpression of *Rv2868c* and *Rv2867c* were excluded from the list of regulated genes due to the possibility that this observation was a polar effect of the hygromycin marker.

Active site mutagenesis of Rv2869c

Site-directed mutagenesis on Rv2869c was performed by the overlapping extension mutagenesis method on pHMG121. Substitutions H21A or D340A were generated with mutagenic primers HMG123 [5'–CCCACATGTGACCACATTCTGGCCAGGGCCACCGAAATCAGG–3'] and HMG124 [5'–CCTGATTTTCGGTGGCCCTGGCCGAATGTGGTTCACATGTGGG–3'] or HMG127 [5'–GCGACGGCAATATGGCCGCCGGCGAACGGCAGCAACG GCAG–3'] and HMG128 [5'–CTGCCGTTGCTGCCGTTCTGCCGGCGGCCATATTGCC GTCGC–3'], respectively. The fragment 5' of the H21A mutation was a 176-bp product of the primers HMG109 and HMG123, and the 3' fragment was a 451-bp product of primers HMG124 and HMG112. In a third PCR the overlapping fragments were mixed and amplified into a 588-bp DNA using the primer HMG109 and HMG110. The 5' fragment for D340A mutation a 553-bp product with the primers HMG113 and HMG127, and the 3' fragment was amplified as a 214-bp product with the primers HMG113 and HMG110. In a third PCR the overlapping fragments were mixed and amplified into a 736-bp DNA using the primer HMG113 and HMG110. Fragments containing the point mutations were sequenced completely and subcloned in pHMG121 at the *Xba*I and *Eco*RI for H21A and *Eco*RI and *Hind*III site for D340A, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. WHO. Global Tuberculosis Control: Surveillance, Planning, Financing. World Health Organization; Geneva, Switzerland: 2004.
2. Glickman MS, Cox JS, Jacobs WR Jr. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. *Mol Cell* 2000;5:717–27. [PubMed: 10882107]
3. Reed MB, et al. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* 2004;431:84–7. [PubMed: 15343336]
4. Cox JS, Chen B, McNeil M, Jacobs WR Jr. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* 1999;402:79–83. [PubMed: 10573420]
5. Gao LY, et al. Requirement for kasB in *Mycobacterium mycolic acid* biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. *Mol Microbiol* 2003;49:1547–63. [PubMed: 12950920]
6. Dubnau E, et al. Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Mol Microbiol* 2000;36:630–7. [PubMed: 10844652]
7. Rao V, Fujiwara N, Porcelli SA, Glickman MS. *Mycobacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule. *J Exp Med* 2005;201:535–43. [PubMed: 15710652]
8. Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 2000;100:391–8. [PubMed: 10693756]
9. Sakai J, et al. Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. *Cell* 1996;85:1037–46. [PubMed: 8674110]
10. Rawson RB, et al. Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol Cell* 1997;1:47–57. [PubMed: 9659902]

11. Duncan EA, Dave UP, Sakai J, Goldstein JL, Brown MS. Second-site cleavage in sterol regulatory element-binding protein occurs at transmembrane junction as determined by cysteine panning. *J Biol Chem* 1998;273:17801–9. [PubMed: 9651382]
12. Weihofen A, Martoglio B. Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. *Trends Cell Biol* 2003;13:71–8. [PubMed: 12559757]
13. Rudner DZ, Fawcett P, Losick R. A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. *Proc Natl Acad Sci U S A* 1999;96:14765–70. [PubMed: 10611287]
14. Cutting S, Roels S, Losick R. Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. *J Mol Biol* 1991;221:1237–56. [PubMed: 1942049]
15. Alba BM, Leeds JA, Onufryk C, Lu CZ, Gross CA. DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev* 2002;16:2156–68. [PubMed: 12183369]
16. Kanehara K, Ito K, Akiyama Y. YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev* 2002;16:2147–55. [PubMed: 12183368]
17. Chen JC, Viollier PH, Shapiro L. A membrane metalloprotease participates in the sequential degradation of a *Caulobacter* polarity determinant. *Mol Microbiol* 2005;55:1085–1103. [PubMed: 15686556]
18. Bardarov S, et al. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* 2002;148:3007–17. [PubMed: 12368434]
19. Kuzuyama T, Takahashi S, Takagi M, Seto H. Characterization of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic amino acid residues. *J Biol Chem* 2000;275:19928–32. [PubMed: 10787409]
20. Altincicek B, et al. GcpE is involved in the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. *J Bacteriol* 2001;183:2411–6. [PubMed: 11274098]
21. Horton JD, et al. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci U S A* 2003;100:12027–32. [PubMed: 14512514]
22. Gilleron M, Quesniaux VF, Puzo G. Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guérin and *mycobacterium tuberculosis* H37Rv and its implication in Toll-like receptor response. *J Biol Chem* 2003;278:29880–9. [PubMed: 12775723]
23. Kremer L, et al. Characterization of a putative alpha-mannosyltransferase involved in phosphatidylinositol trimannoside biosynthesis in *Mycobacterium tuberculosis*. *Biochem J* 2002;363:437–47. [PubMed: 11964144]
24. Cohen-Gonsaud M, et al. The structure of a resuscitation-promoting factor domain from *Mycobacterium tuberculosis* shows homology to lysozymes. *Nat Struct Mol Biol* 2005;12:270–3. [PubMed: 15723078]
25. Glickman MS, Jacobs WR Jr. Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. *Cell* 2001;104:477–85. [PubMed: 11239406]
26. Schaible UE, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med* 2003;9:1039–46. [PubMed: 12872166]
27. Rhoades E, et al. Identification and macrophage-activating activity of glycolipids released from intracellular *Mycobacterium bovis* BCG. *Mol Microbiol* 2003;48:875–88. [PubMed: 12753183]
28. Vergne I, et al. *Mycobacterium tuberculosis* phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Mol Biol Cell* 2004;15:751–60. [PubMed: 14617817]
29. Hingley-Wilson SM, Sambandamurthy VK, Jacobs WR Jr. Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. *Nat Immunol* 2003;4:949–55. [PubMed: 14515128]

30. Schobel S, Zellmeier S, Schumann W, Wiegert T. The *Bacillus subtilis* sigmaW anti-sigma factor RsiW is degraded by intramembrane proteolysis through YluC. *Mol Microbiol* 2004;52:1091–105. [PubMed: 15130127]

Figure 1.

Rv2869c is a nonessential intramembrane cleaving protease (iCLIP) of pathogenic mycobacteria.

- Hydropathy plots of human S2P, *E. coli* YaeL, and Mtb *Rv2869c* where positive numbers on the y axis indicate hydrophobic residues plotted against amino acid number on the x axis. Arrowheads indicate the conserved sequences (HExxH and F/LDG) within predicted transmembrane domains typical of intramembrane cleaving proteases.
- Map of the *Rv2869c* genomic region in wild-type and the *Rv2869c* mutant. Restriction sites and probe location are indicated.
- Southern blot of genomic DNA from indicated strains probed with the DNA fragment indicated in (b) showing allelic exchange at the *Rv2869c* locus in both *M. tuberculosis* and *M. bovis* BCG.

Figure 2.
Rv2869c and its proteolytic activity are required for mycobacterial cording

- a. Single colonies of the indicated BCG strains at 5x magnification. Scale bar indicates 2 mm.
- b. Auramine-Rhodamine stained smears of the indicated *M. tuberculosis* strains examined by fluorescence microscopy at 400 x magnification.
- c. Conserved HExxH and FDG motifs of Rv2869c are required for complementation of the mutant cording phenotype. The *M. bovis* BCG Rv2869c null mutant was complemented either with the wild type Rv2869c, Rv2869c(H21A), or Rv2869c (D340A) and single colonies were assessed for return of wild type colony morphology.

Figure 3. Rv2869c transcriptionally regulates extractable mycolic acid composition of the mycobacterial cell envelope.

- a.** Radio thin layer chromatogram of mycolic acid methyl esters (MAME) and Fatty acid methyl esters (FAME) in the presence (+Tween80) or absence (-Tween80) of detergent. The top panel shows lipids covalently bound to the cell wall (esterified) while the bottom panel shows extractable lipids. The 3 major mycolic acid subtypes (alpha, methoxy, and ketomycolates) are labeled in the bottom panel.
- b.** Rv2869c controls cell envelope composition through positive and negative transcriptional control of lipid anabolic and catabolic genes. Lipid related genes significantly regulated in the Rv2869c mutant as determined by microarray analysis in detergent (+Tw) or short term detergent withdrawal (-Tw) conditions. Color bar depicts ratio of mutant/wt.

Figure 4.

Rv2869c is required for both Mtb replication and persistence in mice.

a and b. Lung bacterial loads plotted (log scale) from mice infected by aerosol with wild type Mtb (black bar), the $\Delta Rv2869c$ strain (open bar), and the complemented mutant (gray bar) in two separate experiments. The horizontal line indicates the limit of detection of the assay and error bars are SD.

c. Gross pathology of infected lungs at 20 weeks from wild type (top), Rv2869c mutant (middle), or complemented mutant (bottom)

d. Hematoxylin and eosin-stained lung tissue from C57BL/6 mice infected with wild type (upper panels) and Rv2869c mutant (lower panels) at 6 (left panels) and 22 weeks (right panels) after infection.