Mycobacterium tuberculosis Alters the Metalloprotease Activity of the COP9 Signalosome

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<td>DOI</td>
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**Mycoberium tuberculosis** Alters the Metalloprotease Activity of the COP9 Signalosome

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L.B. and S.J.R. contributed equally to this work.

**ABSTRACT** Inhibition of apoptotic death of macrophages by Mycobacterium tuberculosis represents an important mechanism of virulence that results in pathogen survival both in vitro and in vivo. To identify *M. tuberculosis* virulence determinants involved in the modulation of apoptosis, we previously screened a transposon bank of mutants in human macrophages, and an *M. tuberculosis* clone with a nonfunctional Rv3354 gene was identified as incompetent to suppress apoptosis. Here, we show that the Rv3354 gene encodes a protein kinase that is secreted within mononuclear phagocytic cells and is required for *M. tuberculosis* virulence. The Rv3354 effector targets the metalloprotease (JAMM) domain within subunit 5 of the COP9 signalosome (CSN5), resulting in suppression of apoptosis and in the destabilization of CSN function and regulatory cullin-RING ubiquitin E3 enzymatic activity. Our observation suggests that alteration of the metalloprotease activity of CSN by Rv3354 possibly prevents the ubiquitin-dependent proteolysis of *M. tuberculosis*-secreted proteins.

**IMPORTANCE** Macrophage protein degradation is regulated by a protein complex called a signalosome. One of the signalosomes associated with activation of ubiquitin and protein labeling for degradation was found to interact with a secreted protein from *M. tuberculosis*, which binds to the complex and inactivates it. The interference with the ability to inactivate bacterial proteins secreted in the phagocyte cytosol may have crucial importance for bacterial survival within the phagocyte.

Tuberculosis (TB), an ancient bacterial disease, is still one of the common infectious causes of death worldwide. *Mycobacterium tuberculosis*, the etiological agent of TB, is an intracellular organism capable of survival and replication in human macrophages. These phagocytic cells are considered the first line of immune defense responsible for the killing of many bacterial pathogens. However, *M. tuberculosis* has as its primary habitat macrophages. Studies on the interaction between *M. tuberculosis* and host cells over the last decade have revealed a limited number of pathogen-derived effector molecules that directly modulate diverse macrophage killing processes. Following phagocytosis by macrophages, *M. tuberculosis* actively subverts phagolysosome biogenesis by secreting the effectors ESAT-6/CFP10 and SecA1/2, which block phagolysosome fusion and ATP hydrolysis, respectively (1, 2). *M. tuberculosis* also secretes the lipid phosphatase SodA in the inhibition of apoptosis.

The *M. tuberculosis* infection process is characterized by the formation of an acidified phagosome, which results in the activation of the autophagic pathway (3–5). Study of trehalose dimycolate of *M. tuberculosis* strongly indicates that this glycolipid is involved in the impairment of phagosome trafficking at an early endosomal stage (5). Furthermore, *M. tuberculosis* is able to survive in phagocytic cells by avoiding proteolytic degradation by the autophagic pathway (6). Conversely, when autophagy is stimulated by starvation, sirolimus, or gamma interferon, *M. tuberculosis* phagosomes are acidified and delivered to lysosomes, resulting in significant reduction of viable bacteria (7). Many *M. tuberculosis* effectors involved in the autophagy process are yet to be elucidated; however, some bacterial virulence effectors, such as ESAT-6/CFP-10, have been implicated in controlling autophagy (8). The secreted enhanced intracellular survival (Eis) protein has also been suggested to play an essential role in modulating host innate responses and autophagy-mediated cell death via a reactive oxygen species-dependent pathway (9).

If macrophages fail to eradicate the intracellular pathogen via autophagy or other mechanisms, host cells will undergo apoptosis as another strategy to contain the infection. However, substantial work in vitro and in vivo has revealed that macrophages infected with virulent strains of *M. tuberculosis*, in contrast to macrophages infected with an attenuated strain, exhibit less apoptosis (10, 11). The nucleotide gene has been implicated in the suppression of host cell apoptosis (12). Infection with *pknE* or *secA2* deletion mutants of *M. tuberculosis* induces greater apoptosis upon macrophage infection than wild-type *M. tuberculosis* (13, 14). When the *secA2*−/− clone is complemented with secreted superoxide dismutase A (sodA), the antiapoptotic phenotype is restored, implicating SODA in the inhibition of apoptosis.

We previously identified several genes associated with the an-
tiapoptotic behavior of *M. tuberculosis* (15). We demonstrated that *M. tuberculosis* is capable of blocking the extrinsic pathway of apoptosis by secreting the Rv3654c and Rv3655c effectors, which alter the caspases’ posttranscriptional events (15). We also identified the secreted Rv3364c protein, which inhibits caspase-1 activation and consequently host cell apoptosis (pyroptosis) through suppression of the enzymatic activity of cathepsin G (16). In the present study, we characterized the function of the Rv3354 gene and demonstrated for the first time the novel virulence mechanism of *M. tuberculosis* in which the secreted Rv3354 exploits the host ubiquitylation system by altering COP9 signalosome function to limit the degradation of *M. tuberculosis* effector proteins.

RESULTS

Characterization of the Rv3354 gene knockout mutant. The 2G2 mutant (Fig. 1A), which lacks the ability to inhibit macrophage apoptosis, was identified from a transposon bank of *M. tuberculosis* mutants (15). Sequencing analysis revealed that transposon insertion at the 105-amino-acid (aa) site disrupted proper translation of Rv3354 (Fig. 1B). Bioinformatic analysis of the Rv3354 protein revealed domains of DUFF732 (unknown function) and PKc_MEK1 (the catalytic domain of the dual-specificity protein kinase mitogen-activated protein kinase/extracellular signal-regulated kinase 1 [MAPK/ERK1]). Using the sequenced-based prediction for secreted proteins and SignalP 4.1, the presence of a 32-aa signal peptide and export via the Sec system were predicted for Rv3354. Complementation of the 2G2 mutant (Rv3354*) restored the antiapoptotic phenotype (Fig. 1C). We next examined 2G2 for survival in THP-1 cells. *In vitro* studies revealed no difference between growth of *M. tuberculosis* H37Rv wild type (WT) and growth of 2G2 in liquid culture medium (Fig. 1D); however, the Rv3354 knockout clone showed a significant decrease in growth within macrophages (Fig. 1E). The viability was fully recovered by complementing 2G2 with the functional Rv3354 gene.

![FIG 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4447851/fig1.png)

**FIG 1** Inactivation of the *M. tuberculosis* Rv3354 gene. (A) Genetic organization of the Rv3354 gene in *M. tuberculosis* strain H37Rv. (B) The signal peptide, predicted domains, and Tn5367 insertion site in the Rv3354 protein. (C) Apoptosis was analyzed in THP-1 cells infected with WT, 2G2, and 2G2 (Rv3354*) in a cell death detection ELISAPLUS assay (Roche). Results represent means ± standard errors of the means of three independent experiments. ***, P < 0.001; *, P < 0.05, for the significance of differences between 2G2 and WT. (D) *In vitro* growth of WT, 2G2, and 2G2 (Rv3354*) in aerated 7H9 medium. (E) Infection and impaired growth of 2G2 in THP-1 cells. WT, 2G2 and 2G2 (Rv3354*) were used at an MOI of 10:1. The significance of differences between 2G2 and WT survival, recorded with bacterial CFU at days 3 and 5 of infection, was P < 0.01 (**).
β-lactamase reporter enzyme (Bla) and a mammalian fluorescence resonance energy transfer (FRET)-based probe which can be used to monitor Bla enzyme activity in cultured cells. The positive-control bla+ vector with a full-length β-lactamase gene, the negative-control bla-deficient vector lacking a signal sequence of the bla gene important for β-lactamase enzyme secretion, and the experimental vector containing an Rv3354:bla-deficient fusion were transformed into the M. tuberculosis -lactam-sensitive PM638 clone. Bla protein secretion was monitored in THP-1 cells via hydrolysis of the FRET-based β-lactamase substrate CCF2-AM by using fluorescence microscopy (Fig. 2A) as well as a cytofluorometer (Fig. 2B). The infection rate of M. tuberculosis-infected THP-1 cells was found to be similar in all groups (Fig. 2C).

**Effector Rv3354 interacts with the metalloprotease (JAMM) domain of CSN5.** To identify the host protein(s) targeted by the effector Rv3354, we performed a yeast two-hybrid screen with a human universal cDNA library and with the M. tuberculosis target protein as the bait. Out of 2 × 10^7 transformants screened, 21 clones grew in the absence of tryptophan (-Trp), leucine (-Leu), histidine (-His), and adenine (-Ade). Eighteen clones were further eliminated through screening on 125 ng/ml aureobasidin plates in the presence of 5-bromo-4-chloro-3-indolyl-α-galactopyranoside (X-α-Gal) and absence of Ade, His, Leu, and Trp. Three interacting positive clones were sequenced and, two out-of-frame clones were further eliminated. A 48- to 261-aa coding sequence of CSN5 was identified as an interacting partner with Rv3354, and at 68- to 154-aa JAMM domain sequence was detected (Fig. 3A). Further studies using full and reverse constructs of the bait and prey targets confirmed a positive interaction between JAMM and Rv3354 (Fig. 3B, panels a and b). When we mutated JAMM (mJAMM) in the amino acid sequences essential for its metalloprotease activity, neither bait nor pray constructs showed a positive interaction, suggesting that JAMM is a specific target for the M. tuberculosis effector.

**The effector Rv3354 colocalizes and binds to CSN5 in THP-1 cells.** To demonstrate direct binding of Rv3354 to JAMM, we performed a yeast two-hybrid screen with a human universal cDNA library and with the M. tuberculosis target protein as the bait. Out of 2 × 10^7 transformants screened, 21 clones grew in the absence of tryptophan (-Trp), leucine (-Leu), histidine (-His), and adenine (-Ade). Eighteen clones were further eliminated through screening on 125 ng/ml aureobasidin plates in the presence of 5-bromo-4-chloro-3-indolyl-α-galactopyranoside (X-α-Gal) and absence of Ade, His, Leu, and Trp. Three interacting positive clones were sequenced and, two out-of-frame clones were further eliminated. A 48- to 261-aa coding sequence of CSN5 was identified as an interacting partner with Rv3354, and at 68- to 154-aa JAMM domain sequence was detected (Fig. 3A). Further studies using full and reverse constructs of the bait and prey targets confirmed a positive interaction between JAMM and Rv3354 (Fig. 3B, panels a and b). When we mutated JAMM (mJAMM) in the amino acid sequences essential for its metalloprotease activity, neither bait nor prey constructs showed a positive interaction, suggesting that JAMM is a specific target for the M. tuberculosis effector.

**TABLE 1 Comparison of the level of apoptosis and intracellular M. tuberculosis growth in infected human MDM**

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<tr>
<th>Infection</th>
<th>% Apoptosis</th>
<th>CFU/ml of lysate</th>
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<tr>
<td></td>
<td>1 day</td>
<td>3 days</td>
</tr>
<tr>
<td>WT</td>
<td>8 ± 3</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>2G2</td>
<td>11 ± 3</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>2G2 (Rv3354)</td>
<td>9 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>No infection</td>
<td>3 ± 1</td>
<td>5 ± 3</td>
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a Approximately 5 × 10^5 macrophages were infected with M. tuberculosis at an MOI of 10. 

b The results represent the means ± SD of two assays and were determined in an ELISA.
FIG 3  In vitro interaction between Rv3354 and CSN5 proteins. (A) Predicted JAMM domain and mutated sites in the CSN5 protein. The amino acids Glu77 and Arg106, responsible for metalloprotease activity of CSN5, were replaced with Ala and Gly by using a site-directed QuikChange mutagenesis kit according to the manufacturer’s protocol (Stratagene). (B) The yeast two-hybrid interaction of Rv3354 with the host target protein. (a) The full-length CSN5 and, mainly, its JAMM domain showed a positive interaction with Rv3354. (b) Reverse screening of the Rv3354 interaction with JAMM or mJAMM. (C) Subcellular localization of Rv3354 and the JAMM motif of CSN5 in THP-1 cells. dtTomato:Rv3354 and ZsGreen:JAMM viral particles were coexpressed transiently in THP-1 cells for 24 h. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm. (D) Coimmunoprecipitation of Rv3354 and CSN5 proteins. The recombinant 6×His:CSN5 or mutated version of a protein was incubated with purified M. tuberculosis proteins overexpressing Rv3354 with Flag tag. The bound proteins were captured with His columns and subjected to IB with 6×His and Flag antibodies.
analyzed bacterial and host protein colocalization within macrophages by using a lentiviral transfection system (Fig. 3C) and performed a coimmunoprecipitation assay (Fig. 3D). Following infection of THP-1 cells with lentiviral particles of the fusions tdTomato:Rv3354 and ZsGreen:JAMM or ZsGreen:mJAMM, samples were analyzed with fluorescence microscopy. ZsGreen:JAMM-expressing cells showed granular fluorescence in the cytosol and mainly colocalized with dtTomato:Rv3354-expressing cells, as shown in the merged image in Fig. 3C. Conversely, mutated ZsGreen:mJAMM-expressing cells showed diffuse and uniform cytoplasmic staining with the Rv3354 protein. The control interaction between tdTomato and ZsGreen:JAMM displayed a dispersed location as well.

We also performed coimmunoprecipitation of Flag-tagged Rv3354 (Flag:Rv3354) with 6×/H11003 HN-tagged CSN5 (6×/H11003 HN:CSN5). Recombinant 6×/H11003 HN:CSN5 with or without mutations in JAMM was expressed in the pET system. The Flag:Rv3354 fusion was overexpressed in pMV261 and transformed into M. tuberculosis. Bacteria were lysed at the mid-log growth phase, and the cleared protein fraction was incubated with the recombinant 6×/H11003 HN:CSN5 protein. The control interaction between tdTomato and ZsGreen:JAMM displayed a dispersed location as well.

Rv3354 binding to JAMM alters the function of Cullin-based E3 ubiquitin ligases. To examine if the Rv3354 interaction with CSN5 leads to any modifications of the host protein, we performed Western blot analysis of CSN5 on whole-cell lysates of THP-1 macrophages infected with the WT, 2G2 mutant, or a complemented clone. We did not observe any cleavage or changes in the molecular mass of CSN5 (Fig. 4A). Next, we asked whether the interaction of Rv3354 with the metalloprotease domain altered CSN5 function. JAMM directly binds to the Nedd8 protein, a key facilitator of ubiquitin-protein isopeptide ligase (E3) complex assembly, and it cleaves Nedd8 from Cul-RING E3 ligases, a process known as deneddylation (17). This process is essential for efficient recycling progression by E3 enzymes. The Western blot analysis of cullin1 (Cul1), cullin3 (Cul3), and associated Nedd8 protein was carried out in WT-, 2G2- or 2G2 (Rv3354/H11001)-infected macrophages. As shown in Fig. 4B, while immunoblotting (IB) of Cul1 and Cul3, derived from 2G2-infected THP-1 cells, revealed a nearly complete deneddylation of Cul1 and Cul3 at all time points, the wild-type and complemented 2G2 infections partially blocked the deneddylation process. The loss of Nedd8 was apparent in the positive-control group, which was cells treated with 50 μM N-ethyl-carbamidoadenosine (NECA) (Fig. 4B).

Modification of Cul3-based E3 ubiquitin ligase activity mediates caspase-8 inhibition during M. tuberculosis infection. The cul3-based E3 ligase interaction with DISC (death-inducing signaling complex) has been demonstrated to induce Cul3-mediated polyubiquitination of caspase-8, promoting its full activation and apoptosis (18). To examine whether alteration in the Cul3 deneddylation process by M. tuberculosis results in changes of caspase-8 polyubiquitination and, alternatively, its activation, we performed a caspase-8 immunoprecipitation assay with extracts of THP-1 cells infected with either WT or 2G2 followed by Western blotting with caspase-8 or ubiquitin (Ub) antibody. The results indicated that the amount of ubiquitinated caspase-8 in WT-infected cells markedly decreased at 48 h compared with 2G2 infection (Fig. 4C, panels a and b). The amount of activated p18

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**FIG 4** Downstream pathways of CSN5 affected by Rv3354. (A) THP-1 cells were infected with WT, 2G2, and 2G2 (Rv3354*), and after 24 h of infection cell lysates were immunoblotted and probed with an anti-CSN5 antibody. (B) THP-1 cells were treated with 50 μM NECA or infected for up to 24 h with WT, 2G2, and 2G2 (Rv3354*), and deneddylation of Nedd8 was assessed by IB using anti-Cul1 and Cul3 antibodies. (C) Caspase-8 polyubiquitination in 2G2-infected cells. THP-1 cells, infected with WT or the 2G2 mutant for up to 48 h, were lysed in sample buffer containing 1% Triton X-100 and cleared by centrifugation. Samples were subjected to caspase-8 immunoprecipitation under denaturing conditions by using an agarose-conjugated primary antibody and analyzed via caspase-8 (a), Ub (b), or p18 (c) IB.
(caspase-8) was significantly lower in WT-infected cells than in 2G2-infected cells at both 24 h and 48 h postinfection (Fig. 4C, panel c).

**Rv3354 demonstrates protein kinase activity.** Bioinformatic analysis of the Rv3354 protein revealed a catalytic domain of the MAP/ERK1 protein on the C terminus. To determine if Rv3354 possesses any kinase activity, phosphotransferase activity was measured by ADP production. Briefly, ATP, as a kinase substrate, was added to the recombinant 6×HN:Rv3354 protein (200 μg/ml) or bacterial or host total protein extracts (650 μg/ml; positive controls). As shown in Fig. 5A, the experimental wells that contained purified Rv3354 produced significantly more ADP than the reaction buffer (negative control) alone, demonstrating that when ATP is a substrate, the Rv3354 protein possesses kinase activity.

**CSN5 has a reduced level of phosphorylation in the absence of Rv3354.** To evaluate the effect of Rv3354, as a potential protein kinase, on CSN5 activity, we analyzed CSN5 phosphorylation during *M. tuberculosis* infection. Figure 5B shows increasing amounts of CSN5 at 4 h postinfection in WT- and 2G2 (Rv3354*)-infected macrophages, while 2G2-infected cells had significantly lower CSN5 protein levels at 4 h and 8 h postinfection. Over time, the phosphorylation of CSN5 markedly decreased in H37Rv- and 2G2 (Rv3354*)-infected cells, whereas CSN5 phosphorylation levels increased in 2G2 mutant-infected cells at 24 h postinfection (Fig. 5B).

**Modulation of CSN5 function most likely protects *M. tuberculosis* effectors from Ub degradation.** The most-characterized biological process that the COP9 signalosome regulates via JAMM and CSN5 is the cleavage of the ubiquitin-like protein Nedd8 from cullins. The neddylation and deneddylation of Cul-containing E3 ligases. Furthermore, protein-protein interaction studies identified the JAMM motif within CSN5 as an interacting partner for Rv3354. CSN5 is implicated in diverse biological functions, including apoptosis (23). In particular, when CSN5, an essential subunit for CSN metalloprotease activity, is deleted from the COP9 complex, it leads to apoptotic cell death in *vivo* (23). In addition, CSN5 can stably exist independently from the complex.

**DISCUSSION**

Host-driven apoptotic death of macrophages during bacterial infection appears to be an essential aspect of innate immunity aimed at the elimination of niche cells on which bacteria rely for replication and survival (19). Modulation of apoptosis is one of the mechanisms whereby *M. tuberculosis* avoids death by macrophages (20). Less is known about the *M. tuberculosis* virulence factors that are involved in control of the apoptosis process, although *M. tuberculosis* antiapoptotic phenotypes in host cells have been well documented (11, 19, 20) and de novo protein synthesis has been suggested to control an antiapoptotic activity in both macrophages and epithelial cells (11). We identified an *M. tuberculosis* transposon clone with a nonfunctional Rv3354 gene as deficient in the inhibition of macrophage apoptosis and observed that Rv3354 is required for *M. tuberculosis* virulence and survival in macrophages. By constructing the Rv3354 signal sequence fusion with the β-lactamase reporter gene and utilizing the mammalian cell-based reporter assay, we demonstrated Rv3354 protein secretion within THP-1 phagocytic cells. Complementary to our findings, other groups have shown Rv3354 protein export in culture filtrates of *M. tuberculosis* H37Rv and experimentally confirmed its secretion via a phoA reporter system in *vitro* (21, 22). Furthermore, protein-protein interaction studies identified the metalloprotease (JAMM) motif within CSN5 as an interacting partner for Rv3354. CSN5 is implicated in diverse biological functions, including apoptosis (23). In particular, when CSN5, an essential subunit for CSN metalloprotease activity, is deleted from the COP9 complex, it leads to apoptotic cell death in *vivo* (23). In addition, CSN5 can stably exist independently from the complex.
As an independent apoptotic mechanism from CSN, CSN5 can enhance apoptosis via activation of transcription factor E2F1 (23). CSN5 regulates the cullin-ring ligase (CRL) families of ubiquitin E3 complexes by cleaving (via de neddylation) a ubiquitin-like protein, Ned, from the cullin ring. Many bacterial pathogens have developed diverse strategies of interference with the host ubiquitin-proteasome system and, in several cases as a consequence, alter the apoptosis process (24, 25). Evidence suggests that effector proteins such as NleG (Escherichia coli O157:H7), LubX and SidH (Legionella pneumophila), SopA and SbpH2 (Salmonella), and IpaH3 (Shigella) mimic and function as E3 ubiquitin ligases in the host (24). In contrast, effectors like SseL (Salmonella Shigella) mimic and function as E3 ubiquitin ligases (27). In some cases, as a part of the intracellular activation mechanism of effector proteins, for example, with ExoU (Pseudomonas), the host ubiquitin is used as a cofactor for enzymatic activation (27).

In the current study, we characterized the Rv3354 virulence effector that is required for M. tuberculosis survival in macrophages. The underlying mechanism of the Rv3354-CSN5 interaction as a cause of apoptosis remains unclear; however, an indirect mechanism cannot be excluded at this time. Based on our work, we can conclude that M. tuberculosis alters the CSN function and limits E3 ligase activity via Rv3354, and this possibly prevents the ubiquitin-mediated protein degradation of M. tuberculosis effector proteins. This finding adds another layer of complexity to M. tuberculosis virulence and provides insight into future research that will help to elucidate a novel mechanism of M. tuberculosis pathogenicity.

**MATERIALS AND METHODS**

**Antibodies and systems.** The primary antibodies against CSN5, Cul1, Cul3, caspase-8, Ub, PSF, β-actin, phosphoserine/threonine/tyrosine of human origin, and Flag and 6His probes were purchased from Santa Cruz Biotechnology. All chemicals were obtained from Sigma. The GenBlazer cell-based assay loading kit was from Life Technologies. The yeast two-hybrid Normalized Mate and Plate universal human library, the yeast transformation system, and the bait, prey, and control vectors were obtained from Clontech. The Lenti-X lentiviral expression system was also purchased from Clontech.

**Cell and bacterial cultures.** The THP-1 human monocye cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Lonza) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 2 mM l-glutamine. To promote maturation and adherence, cells were treated with 100 mg/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich) and then seeded at 80% confluence into 75-cm² tissue culture flasks, 24-well plates, or chamber glass slides, as needed. After 24 h, cells were replenished with new medium and incubated for an additional 48 h for cell differentiation. The M. tuberculosis 2G2 mutant, 2G2 (Rv3354Δ), and wild-type H37Rv, purchased from ATCC, were grown until mid-exponential phase in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase, 0.2% glycerol, and 0.05% Tween 80. Where appropriate, kanamycin was added at a concentration of 200 μg/ml. M. tuberculosis cells were homog-
enized to remove clumps, and only dispersed inocula were used in infection experiments. The viable counts of inocula were determined by serial dilution and plating on 7H11 agar with 10% oleic acid-albumin-dextrose-catalase. Human MDM were obtained from the blood of volunteers by using protocols approved by the Institutional Human Subject Committee. The purification, culture, and maturation of these cells were described previously (34). Cells were cultured in RPMI 1640 medium supplemented with 10% autologous serum. In all experiments, macrophages were infected at a multiplicity of infection (MOI) of 10. CFU were calculated at different times postinfection. In another set of experiments, apoptosis was measured via a cell death detection enzyme-linked immunosorbent assay (ELISAPLUS; Roche) or cells were cleared and analyzed by Western blotting for selected host proteins.

**Complementation of 2G2.** The PCR-generated 390-bp coding fragment of Rv3354 was cloned into the mycobacterium shuttle vector pMV261-ApRII containing the apramycin resistance marker. The resulting vector was electroporated into 2G2. Transformants were plated on agar plates containing 200 µg/ml of apramycin and screened for positive clones by PCR using apramycin primers (15).

**β-Lactamase assay for protein secretion.** A 32-aa coding sequence of the Rv3354 gene was cloned into the mycobacterium-E. coli shuttle vector pLDG13 downstream of the G13 promoter of Mycobacterium tuberculosis and upstream of the bla gene lacking the first 69-bp signal sequence. The resulting vector was transformed into the blaC knock out strain PM638 of *M. tuberculosis* H37Rv. Approximately 10^8 TPH-1 cells were seeded into 96-well plates or in 2-chamber slides and infected with *M. tuberculosis* clones expressing the full-length bla gene, the bla-deficient mutant missing the signal sequence, or the Rv3354:bla-deficient fusion. After a 1-h incubation at 37°C in 5% CO₂, extracellular bacteria were removed by washing wells with Hanks’ balanced salt solution. Infected control and experimental monolayers were loaded with the cell-permeable dye CCFF2-AM using the manufacturer’s protocol (Life Technologies). Readings were recorded with a Tecxan Infinite 200 microplate reader with two filter sets with excitation at 405 ± 20 nm and emission at 460 ± 40 nm or excitation at 405 ± 20 nm and emission at 530 ± 30 nm. Fluorescence micrographs were captured with a Leica DM4000B microscope.

**Matchmaker Gold yeast two-hybrid screening.** The Rv3354 gene was cloned in frame with the GAL4 DNA binding domain of pGBK7. The resulting pGBK7:Rv3354 vector was transformed into Saccharomyces cerevisiae strain Y2HGold following the manufacturer’s instructions (Clontech). The normalized yeast two-hybrid universal human library, fused with the GAL4 activation domain of the pGADT7 vector and stored in the Y187 yeast strain, was purchased from Clontech. The interaction between pGBKT7-53 and pGADT7-T served as a positive control, otherwise indicated. The significance level was determined by using Student’s t test. A P value of <0.05 was considered statistically significant.

**Lentiviral gene transfer and coexpression.** To enhance the efficient cotransduction and expression of bacterial and host proteins in TPH-1 cells, we used the lentiviral expression system (Clontech). The coding sequence of Rv3354 was cloned into the pLXV-dtTomato-C1 (dtTomato) vector. *Homo sapiens* first-strand cDNAs from total RNA were synthesized using the SuperScript III first-strand synthesis system (Life Technologies). The JAMM sequence of the CSN5 gene was amplified from the synthetic cDNA and cloned into the pLVX-ZsGreen1-C1 vector. Generated lentiviral constructs were transduced into THP-1 cells with or without 20 mg/ml of X-

**REFERENCES**


has a role in the nitric oxide stress response.

Mycobacterium tuberculosis


http://dx.doi.org/10.1172/JCI31947.


http://dx.doi.org/10.1172/JCI31947.

Rv3364c suppresses caspase-1 and pyroptosis


M. tuberculosis

phorylation of human vacuolar protein sorting 33B. Cell Host Microbe

2010.

ZW, Cho SN, Kim JM, Friedman RL, Jo EK.

2008. Delay of phagosome maturation by a

cellular effectors. J. Immunol. 2008. Protein kinase E of

Mycobacterium H37Rv.

Mycobacterium tuberculosis

infection causes different levels of apoptosis and necrosis in

tuberculosis-H37Rv. Microbes and Infection. 2003.22:


D, Seeger M, Dubiel W.


bination with IL-2, but not IFN-gamma, is associated with macrophage


1988. Tumor necrosis factor, alone or in com-

motes its degradation. Biochim. Biophys. Acta

2008. The COP9 signalosome: more than a


Ebel C, Meylan PR.

j.tibs.2008.09.004.

M. tuberculosis

infection causes different levels of apoptosis and necrosis in


http://dx.doi.org/10.3349/ ymj.2009.50.1.1.


analysis of exported proteins from Mycobacterium tuberculosis H37Rv.


Ansell SM, Pethe K, Russell DG, Purdy GE.


http://dx.doi.org/10.1371/journal.ppat.0030003.


2008. Protein kinase E of

Mycobacterium H37Rv.

Mycobacterium tuberculosis

infection causes different levels of apoptosis and necrosis in


http://dx.doi.org/10.3349/ ymj.2009.50.1.1.


analysis of exported proteins from Mycobacterium tuberculosis H37Rv.


