Secreted *Mycobacterium tuberculosis* Rv3654c and Rv3655c Proteins Participate in the Suppression of Macrophage Apoptosis

Lia Danelishvili¹, Yoshitaka Yamazaki², Jeannie Selker³, Luiz E. Bermudez¹,4

¹ Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, United States of America, ² Department of Infectious Diseases and Laboratory Medicine, School of Medicine, Shinshu University, Matsumoto, Japan, ³ Institute of Molecular Biology, University of Oregon, Eugene, Oregon, United States of America, 4 Department of Microbiology, College of Science, Oregon State University, Corvallis, Oregon, United States of America

### Abstract

**Background:** Inhibition of macrophage apoptosis by *Mycobacterium tuberculosis* has been proposed as one of the virulence mechanisms whereby the pathogen avoids the host defense. The mechanisms by which *M. tuberculosis* H37Rv strain suppress apoptosis and escapes human macrophage killing was investigated.

**Methodology/Principal Findings:** The screening of a transposon mutant bank identified several mutants, which, in contrast to the wild-type bacterium, had impaired ability to inhibit apoptosis of macrophages. Among the identified genes, Rv3659c (31G12 mutant) belongs to an operon reminiscent of type IV pili. The Rv3654c and Rv3655c putative proteins in a seven-gene operon are secreted into the macrophage cytoplasm and suppress apoptosis by blocking the extrinsic pathway. The operon is highly expressed when the bacterium is within macrophages, compared to the expression level in the extracellular environment. 
Rv3654c recognizes the polypyrimidine tract binding Protein-associated Splicing Factor (PSF) and cleaves it, diminishing the availability of caspase-8. While *M. tuberculosis* inhibits apoptosis by the extrinsic pathway, the pathogen does not appear to affect the intrinsic pathway. Inactivation of the intrinsic pathway by pharmacologic agents affects *M. tuberculosis* and induces cell necrosis. Likewise, inactivation of PSF by siRNA significantly decreased the level of caspase-8 in macrophages.

**Conclusion:** While *M. tuberculosis* inhibits the extrinsic pathway of apoptosis, it appears to activate the intrinsic pathway leading to macrophage necrosis as a potential exit strategy.

### Citation

Danelishvili L, Yamazaki Y, Selker J, Bermudez LE (2010) Secreted *Mycobacterium tuberculosis* Rv3654c and Rv3655c Proteins Participate in the Suppression of Macrophage Apoptosis. PLoS ONE 5(5): e10474. doi:10.1371/journal.pone.0010474

### Editor

Delia Goletti, National Institute for Infectious Diseases L. Spallanzani, Italy

### Received

December 16, 2009; Accepted March 29, 2010; Published May 4, 2010

### Copyright

© 2010 Danelishvili et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Funding

This work was supported by an Agricultural Experiment Station grant and by the National Institute of Allergy and Infectious Diseases grant # R21-A1064018. The mass spectrometric sequencing was supported in part by the National Institute of Environmental Health Science National Institute of Health Grant P30 ES00210. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Competing Interests

The authors have declared that no competing interests exist.

* E-mail: Luiz.Bermudez@oregonstate.edu

### Introduction

*Mycobacterium tuberculosis* is an intracellular pathogen infecting primarily mononuclear phagocytes. The bacterium has developed intricate strategies to evade killing mechanisms of phagocytes [1]. Once *M. tuberculosis* is inhaled and deposited in the alveolar space, it infects alveolar macrophages, prevents phagosome acidification and subsequent fusion of the vacuole with lysosomes [2,3]. Macrophages control bacterial growth by a number of mechanisms, including the production of reactive oxygen intermediaries and nitric oxide (NO) [4]. *M. tuberculosis* blocks the delivery of nitric oxide synthase (iNOS) to the vacuole membrane and consequently avoids the toxic effects of NO [5]; although NO still has some impact on the bacterial viability, as evidenced by the increase in bacterial survival in iNOS knockout mice [5]. Autophagy, a repair mechanism of eukaryotic cells, frequently associated with cell starvation, has also recently been shown to eliminate intracellular bacteria through the delivery of ubiquitin-derived peptide to the mycobacterial vacuoles [6,7]. A number of reviews have explored the role of autophagy in host defense against infectious agents [8]. Autophagy is connected through several possible pathways with apoptosis [8]. It is plausible to assume that, when infected macrophages fail to kill *M. tuberculosis* by autophagy, the alternative host strategy, apoptosis, is used to eliminate the intracellular bacteria [9]. *M. tuberculosis* has the ability to suppress macrophase apoptosis using active mechanisms, with the aim of blocking the expression of apoptosis-associated genes or stimulating anti-apoptosis pathways [10,11]. The importance of apoptosis as a host-defense strategy has also been studied in vivo [12]. In one of these studies, while macrophages isolated from mice with resistant *sst1* (super-susceptibility to tuberculosis-1) locus undergo apoptosis in response to *M. tuberculosis* infection, macrophages of *sst1*-susceptible mice die after infection and show widespread necrosis [12]. A recent study suggested that both H37Rv and H37Ra strains of *M. tuberculosis* disrupt the mitochondrial outer membrane, but only the virulent H37Rv induces significant mitochondrial transmembrane potential loss [13]. Other studies have concentrated their observations on...
focused aspects of the pathways [9,11], but none thus far have used gene knockout mutants to dissect the possible mechanisms involved. Only recently, a report by Velmurugan and colleagues attempted to investigate a H37Rv mutant clone deficient in suppression of apoptosis [14].

The underlying mechanisms by which virulent M. tuberculosis inhibits apoptosis in macrophages remain largely unknown. Macrophages undergo apoptosis by at least two different pathways. The extrinsic pathway, in which released tumor necrosis factor-alpha (TNF-α), activates caspase-8. The adaptor proteins TRADD, RIP-1 or FAADD (TRADD in the case of TNF-α stimulus), stimulate the downstream caspases, through the type I (extrinsic) or type II (intrinsic) pathways [15,16,17]. Recent work by Kundu and colleagues showed that M. tuberculosis-induced apoptosis is mediated by p38 and ASK-1 (apoptosis signal-regulating kinase 1), and that the pathogen also induces degradation of FLIPs, which, when ubiquitinated, blocks TNF-α-mediated apoptosis [18]. In contrast, Chen and colleagues described that the virulent H37Rv strain stimulates LxA4, which inhibits PGE2 production. PGE2 protects mitochondrial membranes from damage and inhibits necrosis [19].

The discordant findings in the literature, regarding M. tuberculosis inducing or inhibiting apoptosis, may be related to a large variety of factors, such as cell types, strain phenotype and phases of infection when the studies are carried out. Because of the inconsistency, we decided to investigate the ability and mechanism by which virulent M. tuberculosis H37Rv strain suppresses apoptosis and escapes killing by human macrophages, by using the screening of a transposon library to identify mutant(s) that do not inhibited the apoptosis. Previous studies establish that inhibition of apoptosis by M. tuberculosis is seen in U937, THP-1 and human monocyte-cleaved macrophages [10]. In the present study, we demonstrate that M. tuberculosis is capable of blocking the extrinsic pathway of apoptosis by secreting effectors proteins that interfere with caspases' post-transcriptional events.

Materials and Methods

Bacterial strain, cell culture and infections

Mouse-passed, virulent Mycobacterium tuberculosis strain H37Rv (ATCC 25618) was cultured for 3 weeks in liquid Middlebrook 7H9 medium or Middlebrook 7H10 agar plates supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC) enrichment (Hardy Diagnostics, Santa Maria, CA). Bacteria were harvested at mid-log phase and adjusted to 1 x 10^7 cells/ml in Hank’s Balanced Salt Solution (HBSS, Invitrogen). Kanamycin sulfate was added to all media at a final concentration of 200 μg/ml, where appropriate. Human monocytic cell line U937 (ATCC CRI-1593.2) was used for all assays and was grown in RPMI-1640 (GIBCO Laboratories) supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were seeded at 80% confluence into 75 cm^2 tissue culture flasks, in 96-well plates or chamber glass slides, as needed. U937 macrophage monolayers were treated with 500 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) for 8 h to promote maturation and adherence and then monolayers were replenished with a new medium. After additional 24 h incubation, cells were infected with M. tuberculosis H37Rv or transposon mutants at MOI of 10. Infected and uninfected macrophages were collected at different time points for cytotoxicity assay, caspase activity or microarray assays. The percentage of phagocytized bacterium (wild-type or mutants) was determined as lactate dehydrogenase (LDH) release from necrotic macrophages compare with control. Selected wells were used for detection of macrophage apoptosis. Necrosis, as determined as lactate dehydrogenase (LDH) release from necrotic cells, was measured using the CytoTox 96 non-radioactive cytotoxicity assay (Promega), as previously described [10].

Cytotoxicity assays

Cell Death Detection ELISA®PLUS and TUNEL assays (Roche) were used for detection of macrophage apoptosis. Necrosis, determined as lactate dehydrogenase (LDH) release from necrotic cells, was measured using the CytoTox 96 non-radioactive cytotoxicity assay (Promega), as previously described [10].

Real-time PCR procedures and transcriptional analysis for M. tuberculosis genes

U937 macrophages were exposed to M. tuberculosis H37Rv for 30 min or infected for 3 h with MOI 100 bacteria:1 cell. Both bacteria exposed to 7H9 broth and RPMI medium, for either 30 min or 3 h, were used as controls. Bacterial RNAs from control and experimental (exposed and intracellular) samples were extracted and processed for Real-time PCR, as previously described [23]. To determine whether the Rx3654c–Rx3660c locus produced a transcript containing seven genes, we arbitrarily chose approximately 1000 bp size intergenic regions of Rx3654c–Rx3657c, Rx3657c–Rx3659c and Rx3659c–Rx3660c and performed RT-PCR using intracellular bacterial RNA (Table S1).
Caspase-8 activity

FLICE/Caspase-8 fluorimetric protease assay (Chemicon, Temecula, CA) was used to determine the changes in caspase-8 activity, 72 h following both M. tuberculosis wild-type and mutant infection of human macrophages. Treatment of U937 cells with human recombinant TNF-α protein (10^3 U/ml) was used as a positive control. The assay was performed directly into 96-well tissue culture plate. Briefly, cells were resuspended in 50 μl of chilled lysis buffer, incubated on ice for 10 min, and 50 μl of 2× reaction buffer containing 10 mM DTT was added. Each sample was mixed with 0.5% v/v (Chemicon) was added to each well for 5 min at 37 °C. The assay was performed directly into 96-well tissue culture plate. Briefly, cells were resuspended in 50 μl of chilled lysis buffer, incubated on ice for 10 min, and 50 μl of 2× reaction buffer containing 10 mM DTT was added. Each sample was mixed with 0.5% v/v (Chemicon) was added to each well for 5 min at 37 °C.

Immunostaining of cytochrome C

The U937 cells were cultured in duplicate, using eight-chamber slides (NUNC). Macrophages infected with M. tuberculosis wild-type or with the mutant 31G12 for 72 h were washed three times with HBSS and fixed in 4% paraformaldehyde for 1 h. Cells were permeabilized with 1% Triton X-100 phosphate-buffered saline (PBS) for 5 min and incubated with mouse monoclonal antibody against cytochrome C (1:500, PharMingen, San Diego, CA). Macrophages were then washed and incubated with Texas Red-labeled goat anti-mouse IgG (1:1000, Molecular Probe, Eugene, OR) for 1 h.

Transfection experiments

pLDRv3654c, pLDRv3655c, pLDRv3656c, pLDRv3657c, pLDRv3659c, or the empty pDsRed1-C1 vector were transformed into U937 macrophages with Lipofectamine 2000 transfection reagents using the manufacturer’s protocol (Invitrogen).

Western blot analysis of M. tuberculosis-secreted proteins

The pJAM2:Rv3654c and pJAM2:Rv3655c plasmid constructs were introduced into M. tuberculosis and kanamycin-resistant colonies.

| Experiment | Target | Primers | PCR product (bp) |
|------------|--------|---------|-----------------|
| Complementation: | Rv3659c | 5’ ttggattcatgcctcgccgaccgaa 3’  F 1059 | |
| | Rv3656c–Rv3659c | 5’ ttggattcatgctcgccgaccgaa 3’  F 2700 | |
| | Rv3654c–Rv3659c | 5’ ttggattcatgcctcgccgaccgaa 3’  F 3334 | |
| Real-time PCR: | Rv3654c | 5’ atggtggtgacatccagat 3’  F 200 | |
| | Rv3656c | 5’ atggtggtgacatccagat 3’  F 200 | |
| | Rv3659c | 5’ atggtggtgacatccagat 3’  F 200 | |
| Transcriptional analysis: | Rv3654c–Rv3657c | 5’ cggcgccgctgccgtaaa 3’  F 960 | |
| | Rv3657c–Rv3659c | 5’ cggcgccgctgccgtaaa 3’  F 1000 | |
| | Rv3659c–Rv3660c | 5’ cggcgccgctgccgtaaa 3’  F 800 | |

doi:10.1371/journal.pone.0010474.t001

Table 1. Sense (F) and Antisense (R) primers.
grown in M63 medium \((7.6 \times 10^{-2} \text{M} \text{NH}_4\text{SO}_4, 0.5 \text{M} \text{KH}_2\text{PO}_4, 5.8 \times 10^{-6} \text{M} \text{FeSO}_4, 7\text{H}_2\text{O, pH 7})\) supplemented with 1 mM \(\text{MgSO}_4\), 0.5% Tween-80 and 2% succinate for non-induced cultures or 2% succinate and 2% acetamide for induced cultures. Bacteria were grown for 21 days, after which cells were harvested, washed two times and used for infection of U937 macrophages.

Macrophages were infected with \(M. \text{tuberculosis}\) pJAM2:Rv3654c, or pJAM2:Rv3655c, induced and non-induced clones for 48 h. Cell cultures were lysed with 0.05% SDS and centrifuged at 3,000 \(g\) for 15 min to remove bacterial pellet from the suspension. Pre-cleared cell lysates were incubated with His.tag primary agarose conjugate antibodies, overnight at 4°C. Samples were resolved by electrophoresis on 12.5% SDS-PAGE gels, transferred to nitrocellulose membranes and blocked overnight with 5% blocking reagent. The secondary mouse horseradish peroxidase-linked whole (IgG) antibody was used at a dilution 1:5000. Reactivity was assessed with Hyperfilm ECL detection reagents (Amersham) followed by autoradiography.

His-tagged pull-down assay

Macrophage lysates were prepared and labeled as previously described [22], mixed with 1 mg of purified His.tag-Rv3654c or His.tag-Rv3655c proteins and 60 \(\mu\text{l}\) of His.tag agarose conjugate rabbit polyclonal IgG beads (500 g/ml with 25% AG). The samples were incubated at 4°C overnight, spun down, washed three times with PBS and processed for electrophoresis. The pulled-down proteins were subjected for In-Gel Tryptic Digestion (PIERCE, Rockford, IL) and sequencing by electrospray ionization mass spectrometry (ESI-MS/MS).

Mass Spectrometric sequencing

Proteins were analyzed at Environmental Health Science Center (EHS) Mass Spectrometry Facility, Oregon State University, Corvallis, by chromatography and electrospray ionization mass spectrometry (ESI-MS/MS). Tandem MS was performed with ESI, and mass spectra were acquired by using quadrupole–time-of-flight

### Table 2. Transposon-based list of \(M. \text{tuberculosis}\) genes related to increased apoptosis during infection of macrophages.

| Clone | \(M. \text{tuberculosis}\) H\(_3\)R\(_v\) Gene | Product description |
|---|---|---|
| **Lipid metabolism** | | |
| 2F6 | Rv0129c or flpC | Secreted antigen 85-C FBPC |
| 9D6 | Rv0166 or fadDS | Probable fatty-acid-CoA ligase fadDS. Involved in lipid degradation. |
| 27G1/30H10 | Rv0468 or fadB2 | 3-Hydroxybutyryl-CoA dehydrogenase. Butyrate/Butanol-producing pathway |
| 4G2 | Rv2246 or kasB | 3-oxoacyl-[Acyl-carrier protein] synthase 2. Involved in fatty acid biosynthesis. |
| 24F10 | Rv3801c or fadD32 | Probable fatty-acid-CoA synthetase |
| 29D1 | Rv3825c or psk2 | Polyketide synthase |
| **Information pathways** | | |
| 4E3 | Rv0041 or leuS | Leucyl-TRNA synthetase. Involved in translation mechanism. |
| 2B9 | Rv0058 or dnaB | Replicative DNA helicase. Participates in initiation and elongation during chromosome replication. |
| **Cell wall and cell processes** | | |
| 9B9 | Rv1476 | Possible membrane protein |
| 8B6 | Rv2690c | Conserved integral membrane alanine, valine and leucine rich protein |
| 30G12 | Rv2729c | Conserved integral membrane alanine, valine and leucine rich protein |
| 22C12/23D1 | Rv3823c or mmpL8 | Probable conserved integral membrane transport protein |
| **Intermediate metabolism and respiration** | | |
| 41H7 | Rv1285 or cysD | Sulfate adenylyltransferase subunit 2. Involved in sulfate activation pathway. |
| 40D11 | Rv1286 or cysN | Bifunctional enzyme: Sulfate adenylyltransferase (subunit 1) |
| 3E4 | Rv1304 or atpB | ATP synthase a chain ATPB. Key component of the proton channel. |
| 24D1 | Rv1436 or gap | Probable Glyceraldehyde 3-phosphate dehydrogenase |
| 40H7 | Rv1437 or pgk | Phosphoglycerate kinase |
| 41H4 | Rv1880c or cyp140 | Probable cytochrome p450 140 cyp140. Contains cytochrome p450 cysteine heme-iron ligand signature. |
| 43A2/45F4 | Rv2454c | Putative oxidoreductase (beta subunit). |
| 8H10 | Rv3518c or cyp142 | Probable cytochrome p450 142 cyp142. Contains cytochrome p450 cysteine heme-iron ligand signature. |
| 50F4 | Rv3545c or cyp125 | Probable cytochrome p450 125 cyp125. They oxidize a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. |
| 4G3 | Rv3859c or gdhB | Probable involved in glutamate biosynthesis. |
| **Conserved hypotheticals** | | |
| 18E11 | Rv2567 | Conserved hypothetical alanine and leucine rich protein. |
| 23G6/30G7 | Rv2751 | Conserved hypothetical protein |
| 2G2 | Rv3354 | Conserved hypothetical protein. |
| 20A11 | Rv3365c | Conserved hypothetical protein. Contains neutral zinc metallopeptidases, zinc-binding region signature. |
| 31G12/39H7 | Rv3659c | Conserved hypothetical protein |

doi:10.1371/journal.pone.0010474.t002
(Q-TOF) Global Ultima system from Micromass (Manchester, UK), operated with a spray voltage of 3.5 kV. Digested protein samples were mixed 1:1 (v/v) with 0.1% formic acid, 0.005% trifluoroacetic acid, and 3% acetonitrile in H2O (solvent A). A Symmetry 300 C 18 trap from Dionex (Sunnyvale, CA) and 75-m inner-diameter PicoFrit column from New Objective (Woburn, MA), packed in-house with Jupiter C 5 from Phenomenex (Torrance, CA), were used for the ESI experiments. The LC program consisted of a gradient from 3% to 33% B over 60 min, to 70% B at 65 min, and finally 95% B from 70 to 80 min. Solvent B contained 0.1% formic acid and 0.005% trifluoroacetic acid in 80% acetonitrile. Data-dependent MS/MS was generated using a 0.5-sec MS survey scan and 2.5-sec MS/MS scans on the three most abundant peaks found in the survey scan. A database search was performed using Mascot (Matrix Science, London, UK) and IPI human (Bioinformatics Solutions, Inc., PEAKS studio, Canada) software.

PSF and ALK proteins
The anti-polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF) antibody (Santa Cruz Biotechnologies, Inc.) and FITC-conjugated mouse anti-human NPM-ALK/ALK (Anaplastic Lymphoma Kinase) antibody (ALK1, BD Pharmingen) were used for fluorescence microscopy studies. Briefly, 4×10^5 cells were seeded on eight-chamber slides and infected or not with rhodamine-labeled wild-type or 31G12 bacteria. One well was transfected with either pLDRv3654c or pLDRv3655c plasmid to investigate the expression of either PSF or ALK proteins, respectively. After 48 h of infection, cells were fixed for 1 h in 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 min and blocked in 2% bovine serum albumin, followed by a 2 h incubation with rabbit monoclonal antibody against PSF (1:500) or FITC-conjugated monoclonal ALK1 antibody (1:500). Fluorescein-labeled goat anti-rabbit IgG (1:2000, Molecular Probe, Eugene, OR) was used as secondary antibody for PSF protein. Results were confirmed by Western blot analysis of immunoprecipitated samples (experimental and control) for PSF protein.

Inactivation of PSF by siRNA
To examine the role of PSF in caspase-8 activation, transfection of macrophages with PSF siRNA (Santa Cruz) was performed using...
Infections and Treatment | % Apoptosis/200 cells (TUNEL assay) | U937 (after 5 days)
---|---|---
No bacteria | 12.5±5 | 
Treated with TNF-α | 82±1 | 
Infected with wild-type | 36.5±2 | 
Infected with 31G12 | 78.5±2 | 
Infected with complement pLD31G12-1 (Rv3659c) | 75±4 | 
Infected with complement pLD31G12-2 (Rv3656c-Rv3659c) | 80±2 | 
Infected with complement pLD31G12-3 (Rv3654c-Rv3659c) | 49±3 | 
Infected with wild-type and treatment with TNF-α | 40±1 | 

Macrophage monolayers were treated with 10^7 U/ml (1 ng/ml) of human recombinant TNF-α.

\(^{1}\)p<0.05 compared to apoptosis with no bacteria.

\(^{2}\)p<0.05 compared to macrophage treated with TNF-α.

\(^{3}\)p<0.05 compared to monolayer infected with wild-type H37Rv.

\(^{4}\)p<0.05 compared to monolayer infected with the strain 31G12 or treated with TNF-α.

doi:10.1371/journal.pone.0010474.t003

Changes in the extrinsic and intrinsic pathways of apoptosis

It was observed that, while recombinant TNF-α-treated and 31G12-infected macrophages showed caspase-8 activation, significant suppression of caspase-8 activation was observed in both macrophages infected with wild-type bacterium and with the complemented 31G12 strain containing the pLD31G12-3 construct (Figure 2A).

Using mitochondrial permeability and cytochrome C assays (Figure 2, B and C) as indicators of the activation of the intrinsic pathway, we observed that there was no differential effect between the wild-type and the knockout bacterium on macrophage apoptosis. Figure 2B shows that the lipophilic cation dye is accumulated in the mitochondria of uninfected cells (bright red fluorescence). The mitochondrial membrane was damaged in M. tuberculosis H37Rv- and 31G12-infected macrophages, where the dye had diffused in the cytoplasm giving bright green fluorescence.
Immunolabeling of cytochrome C in uninfected macrophages showed a granular pattern; whereas, a diffuse cytoplasmic staining was observed in macrophages infected with \textit{M. tuberculosis} wild-type or 31G12 mutant. The pattern was similar to the control cells treated with staurosporine (Figure 2C).

Resistance to TNF-\(\alpha\)-mediated apoptosis during \textit{M. tuberculosis} infection

To investigate whether the putatively secreted proteins in the operon were capable of inhibiting macrophage apoptosis by the extrinsic pathway, U937 cells were transfected with pDsRed1-C1 plasmid, containing \textit{Rv3654c}, \textit{Rv3655c}, \textit{Rv3656c}, \textit{Rv3657c} or \textit{Rv3659c} genes, and evaluated for caspase-8 activation and nuclear fragmentation following treatment with human recombinant TNF-\(\alpha\). \textit{M. tuberculosis} putatively secreted the proteins \textit{Rv3654c}, \textit{Rv3655c} significantly suppressed TNF-\(\alpha\)-induced apoptosis (Figure 3A), which could not be observed in cells transfected with \textit{Rv3657c} and \textit{Rv3659c}. Components of type IV pili apparatus, or the transmembrane gene-encoding protein \textit{Rv3656c}. The findings were confirmed by quantification of apoptotic cells (Figure 3B).

Secretion of \textit{Rv3654c} and \textit{Rv3655c}

To investigate whether \textit{Rv3654c} and \textit{Rv3655c} were secreted into the macrophage cytoplasm, we fused both proteins with 6-His-tag and over-expressed them in \textit{M. tuberculosis} using pJAM2 vector. Infected macrophages were lysed by day 2, separated from the intracellular bacteria and processed for Western blot analysis. \textit{Rv3654c} and \textit{Rv3655c} proteins were detected in the macrophage cytoplasmic fraction (Figure 3C).

Host proteins interacting with \textit{M. tuberculosis} secreted proteins

His-tagged pull-down assay (Figure 4A) and mass spectrometric analysis (Table 4) of host cell proteins interacting with \textit{M.
In macrophages, the bacterium cleaves PSF protein, which was not seen in phagocytic cells infected with the 31G12 mutant (Figure 4C).

Using the interference siRNA system, we then inactivated PSF to identify its role in caspase-8 activation. As observed in Figures 5A and 5B, the expression of PSF was significantly reduced in macrophages transfected with siRNA. Figures 5C and 5D show that inactivation of PSF leads to a significant reduction of caspase-8 protein in macrophages.

ALO17, a protein of unknown function, has recently been described as a novel fusion partner of ALK [24]. We used fluorescent labeling of ALK to examine whether it formed fusion with ALO17. ALK was not expressed in M. tuberculosis-infected macrophages, as determined by fluorescence microscopy and RT-PCR using primers designed for the fused transcripts, as previously described [25]. Real-time PCR was also performed for the expression of ALO17. During M. tuberculosis wild-type infection of macrophages, ALO17 gene showed 5.3-fold higher expression over the uninfected control.

**Survival in macrophages**

To verify the differences in macrophage survival during 31G12 and the wild-type bacterium infection, intracellular bacteria were cultured from detached (apoptotic) and attached macrophages at two different time points. The results (Table 5) show that, while the wild-type bacterium grows within attached macrophages, the 31G12 mutant loses viability over time in apoptotic macrophages, demonstrating attenuated phenotype.
Intrinsic pathway of apoptosis and macrophage necrosis

Since *M. tuberculosis* is known to exit macrophages by inducing cell necrosis, we decided to examine whether pharmacological inhibition of macrophage apoptosis by the intrinsic pathway could have any impact on the phagocytic cell necrosis induced by *M. tuberculosis*.

In agreement with previously published data, *M. tuberculosis* H37Rv infection was associated with reduced macrophage necrosis after 1 and 3 days of infection compared with the level of apoptosis at the same time points (data not shown); however, *M. tuberculosis* triggered a greater level of necrosis than apoptosis at later time points (Figure 6A). To identify whether *M. tuberculosis*-induced necrosis of macrophages is related to the intrinsic apoptotic pathway, host cells were treated with irreversible caspase-9 inhibitor Z-LEHD-FMK.TFA (Sigma) and levels of cytotoxicity were determined by 3, 5, 7 and 9 days post-infection. *M. tuberculosis* H37Rv-infected macrophages did not undergo necrosis after blocking the intrinsic pathway with the caspase-9 inhibitor (Figure 6B). The data indicate that inhibition of the intrinsic pathway of apoptosis at later time points had direct correlation to increased levels of necrosis during *M. tuberculosis* infection of U937 cells.

### Discussion

*M. tuberculosis* is a pathogen that evolved several different strategies to survive within host cells. Among them are the ability to impair nitric oxide delivery to the phagosome [5], the prevention of phagosome-lysosome fusion [2,3], and the capacity to interfere with autophagy [7]. Macrophage apoptosis is a host mechanism of innate immunity that has been shown to eliminate *M. tuberculosis* [13]. A number of studies, however, have suggested that *M. tuberculosis* infection of macrophages either induces or blocks apoptosis [10,14], which apparently correlates with strain virulence and microbial burden and certainly reflects the different models used. We have evaluated apoptosis and necrosis caused by *M. tuberculosis* H37Rv in two cell lines (U937 and THP-1) and in primary human monocyte-derived macrophages [10]. In our hands, *M. tuberculosis* infection of the three macrophages above was associated with inhibition of apoptosis by the extrinsic pathway but did not interfere in a significant manner with the intrinsic pathway.

### Table 4. Amino acid sequences of macrophage proteins interacting with *M. tuberculosis* Rv3654c and Rv3655c.

| Protein (Accession number) | Sequence |
|----------------------------|----------|
| 76kDa Splicing factor, proline- and glutamine-rich; Polypyrimidine tract-binding protein-associated-splicing factor (PSF); UniProt P23246 | FGQGAGPVGQGPRGMGP |
| 175kDa ALK lymphoma oligomerization partner on chromosome 17 (ALO17); UniProt Q9HCF4 | QFPAEHGWKESLLGDMEWRTK |

doi:10.1371/journal.pone.0010474.t004
In the majority of the bacteria, however, the function of this chromosomal region is currently unknown. Our findings suggest a novel function for the region that contains genes encoding for four secretion apparatus structural proteins, one transmembrane protein, and two secreted proteins, which interfere with macrophage apoptosis.

The secreted proteins Rv3654c and Rv3655c interact with host PSF and ALO17, respectively, and as consequence, interfere with the extrinsic pathway of apoptosis, as demonstrated by transfecting macrophages with the bacterial proteins. PSF is a factor essential for pre-mRNA splicing [24]. PSF has been shown to regulate apoptosis by influencing caspase activity at the translational level. During cell apoptosis, PSF remains intact and phosphorylated, which is not expected of a splicing factor, suggesting a possible secondary function. In fact, we were able to demonstrate that inactivation of PSF leads to significant decrease of caspase-8 expression. Our study also shows that the wild-type bacterium cleaves PSF after 48 h of infection, in contrast with the 31G12 mutant, making it the probable mechanism of inactivation. In fact, the work by Shav-Tal and colleagues suggests that PSF is sensitive to proteolysis and can be degraded [27]. Alternative explanation for the decrease of PSF would be related to the phosphorylation of PSF, which occurs following macrophage apoptosis. It could make at least some of the protein undetected by antibodies.

Rv3655c recognizes ALO17, a protein usually associated with ALK (anaplastic lymphoma kinase) [25]. Various fusion partners of ALK and ALO17 have been described, all of them with anti-apoptotic function of cells [28]. Once ALK is activated in the cytoplasm, it migrates into the nucleus and phosphorylates NIPA (nuclear interacting partner of ALK) protein. NIPA protein has been found to inhibit PI3-kinase/AKT anti-apoptotic signaling pathway and then inhibit caspases. Although we could not show the ALK-ALO17 interaction, we demonstrated that the ALO17 gene is highly expressed in macrophages, after infection with M. tuberculosis H37Rv.

| Table 5. Mutants with impaired ability to inhibit apoptosis have an attenuated phenotype in macrophages. |
|---------------------------------|---------------------------------|
| Bacterial Strain | Macrophage | CFU/10^5 macrophages |
|------------------|------------|----------------------|
|                  | 2 days     | 5 days               |
| H37Rv            | adherent   | 2.6±0.5×10^5         |
|                  |            | 3.1±0.4×10^6         |
|                  | detached   | 2.8±0.5×10^6         |
| 31G12            | adherent   | 1.9±0.5×10^5         |
|                  |            | 2.8±0.6×10^5         |
|                  | detached   | 1.2±0.5×10^5         |
|                  |            | 2.6±0.2×10^5         |

*p<0.01 compared with the number of bacteria in attached macrophages.

*p<0.01 compared with the number of intracellular 31G12 mutant at day 5.

NA: Not Available. The number of detached macrophages was small and accurate quantification was not possible.
Apoptosis is now considered as one of the host mechanisms of innate immunity. Intracellular pathogens, which overcome many of the killing strategies of phagocytic cells, such as reactive oxygen and reactive nitrogen products, antimicrobial peptides and autophagy, had to evolve strategies to survive the apoptosis of the host cell. Because host cells have many triggering mechanisms for apoptosis, and at least three apoptotic pathways are currently known, we can assume that M. tuberculosis had to adapt to all in order to remain viable within macrophages. By screening a transposon mutant library, we identified several deficient mutants that very likely have different or complementary mechanisms to suppress apoptosis. The overlapping functions of bacterial genes point to the importance of the strategy, which, in addition, influence the outcome of infection. Our results also unveil a plausible important strategy of the pathogen. By inhibiting the translation phase of the extrinsic pathway of apoptosis, other proteins co-expressed with caspases may remain intact to perform potentially significant functions, some of them still unknown. In addition, the level of inhibition is the most efficacious one, not depending on gene regulation, which can be complex. The fact that M. tuberculosis does not seem to significantly suppress apoptosis by the intrinsic pathway is one of the reasons for much of the diverse results in the field. In fact, if one does not use mutants to understand the effect on the pathway, conflicting results are very likely. Our findings suggest that M. tuberculosis acts in different ways to manipulate apoptosis in macrophages. Suppression and, possibly, stimulation of apoptotic pathways by the pathogen depend on the phase of the host cell infection and have important roles in the outcome of the condition, as demonstrated in this study and elsewhere [29]. This complex interaction can also be responsible for some of the discrepancies among the published results. Data from in vivo observation supports that clinical strains capable of inhibiting apoptosis are more virulent [29]. The finding that M. tuberculosis uses the intrinsic pathway of apoptosis as a strategy to induce macrophage necrosis explains, at least in part, the fact that the pathogen appears to inhibit apoptosis selectively through the extrinsic pathway. It is interesting that the bacterium uses the activation of the intrinsic pathway during a very defined phase of infection to induce necrosis. One can propose that there is some advantage to following this particular strategy, although we currently cannot understand it. We do know that the proteins ESAT-6 and CFP-10 appear to participate in the process of macrophage necrosis [30,31]. M. tuberculosis is now accepted as a pathogen that escapes the macrophage during a phase of the intracellular life, probably infecting other cells. All the evidence thus far supports the idea that the pathogen triggers necrosis or some lesion of the macrophage surface [32]. The genes linked to this process and the mechanisms are still under investigation. Other pathogens, such as Legionella pneumophila, inhibit macrophage apoptosis by targeting pro-death members of the Bcl-2 protein family [33]. In this case, SidF, a Legionella protein, binds to Bcl-rambo and BNIP-3, interfering with the intrinsic apoptotic pathway.

Our model agrees with the model described by Chen and colleagues [13] that the suppressive effect of M. tuberculosis infections on apoptosis occurs in the extrinsic but not in the intrinsic pathway. We also found that M. tuberculosis uses the intrinsic pathway to trigger necrosis. Future investigation will attempt to enhance our understanding of the complex interaction of M. tuberculosis with different apoptotic pathways.

Supporting Information

Table S1
Found at: doi:10.1371/journal.pone.0010474.s001 (0.04 MB DOC)

Acknowledgments

We would like to thank Brian Arbogast for assistance with mass spectrometric sequencing and analysis. We thank Denny Weber for help in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: LD LB. Performed the experiments: LD YY JS LB. Analyzed the data: LD JS LB. Contributed reagents/materials/analysis tools: YY JS. Wrote the paper: LD LB.

References

1. Ting LM, Kim AC, Cattamanchi A, Ernst JD (1999) Mycobacterium tuberculosis inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 163: 3898–3906.

2. Sturgill-Koszycki S, Schlesinger PH, Chakrabarty P, Haddix PL, Collins HL, et al. (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263: 678–681.
14. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, et al. (2007) Apoptosis in macrophages: phagosome maturation is blocked by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem 272: 13326–13331.

15. Boldin MP, Grouchov TM, Goltsev YY, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protein, in Fas/APO-1- and TNF receptor-induced cell death. Cell 83: 603–615.

16. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, et al. (1997) Suppression of the TNF receptor-induced cell death. Cell 85: 803–815.

17. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, et al. (2004) Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity 20: 21–32.

18. Muppidi JR, Tschopp J, Siegel RM (2004) Signaling by the TNF family member death agonist TRAIL: mechanisms of cellular necrosis but minimal apoptosis in murine macrophages. J Exp Med 205: 2791–2801.

19. Chen M, Drizazghi M, Gan H, Shin DS, Hong S, et al. (2000) Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. J Exp Med 205: 2791–2801.

20. Li Y, Milner E, Wu M, Petroisky M, Bermudez LE (2005) A Mycobacterium avium PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. Cell Microbiol 7: 539–548.

21. Tamme R, Camp E, Kortschak RD, Lardelli M (2006) Nonspecific, nested suppression PCR method for isolation of unknown flanking DNA. Biotechniques 18: 599–599, 902.

22. Daneshvili L, Li V, Stang B, Harrill M, Girillo S, et al. (2007) Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc Natl Acad Sci U S A 104: 11030–11035.

23. Deretic V, Singh S, Master S, Harris J, Roberts E, et al. (2006) M. avium infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cell Microbiol 8: 649–660.

24. Deretic V, Singh S, Master S, Harris J, Roberts E, et al. (2006) Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cell Microbiol 8: 719–727.

25. Davis AS, Vergne I, Master SS, Kyei GB, Chua J, et al. (2007) Mechanism of TNF-induced macrophage apoptosis. Nat Immunol 10: 693–699.

26. Davis AS, Vergne I, Master SS, Kyei GB, Chua J, et al. (2007) Mechanism of TNF-induced macrophage apoptosis. Nat Immunol 10: 693–699.

27. Shav-Tal Y, Cohen M, Lapter S, Dye B, Paton JG, et al. (2001) Nuclear relocalization of the pre-mRNA splicing factor PSF during apoptosis involves hyperphosphorylation, masking of antigenic epitopes, and changes in protein interactions. Mol Biol Cell 12: 2320–2340.

28. Ouyang T, Bai RY, Bassermann F, von Klitzing C, Klumpen S, et al. (2003) Identification and characterization of a nuclear interacting partner of anaplastic lymphoma kinase (NIPA). J Biol Chem 278: 30028–30036.

29. Park JS, Tamayo MH, Gonzalez-Juarrero M, Orme IM, Ordway DJ (2006) Virulent clinical isolates of Mycobacterium tuberculosis grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. J Leukoc Biol 79: 80–86.

30. MacGurn GA, Raghavan S, Stanley SA, Cox JS (2003) A non-RDI1 gene cluster is required for Stu mutant in Mycobacterium tuberculosis. Mol Microbiol 57: 1653–1663.

31. Tan T, Lee WL, Alexander DC, Grinstein S, Liu J (2006) The ESAT-6/CFP-10 secretion system of Mycobacterium marinum modulates phagosome maturation. Cell Microbiol 8: 1417–1429.

32. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, et al. (2007) Autophagy is a defense mechanism inhibiting BCG and mycobacteria as a host. J Biol Chem 282: 1653–1663.

33. Banga S, Gao P, Shen X, Fiscus V, Zong WX, et al. (2007) Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. Proc Natl Acad Sci U S A 104: 5121–5126.