Mycobacterium tuberculosis Serine Protease Rv3668c Can Manipulate the Host–Pathogen Interaction via Erk-NF- κ B Axis-Mediated Cytokine Differential Expression

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Tuberculosis caused by *Mycobacterium tuberculosis* (MTB) remains a serious global public health concern. About one-third of the global population has been latently infected with this pathogen. MTB proteases are important virulence factors and involve in subverting the host immunity. MTB protease Rv3668c was implicated in drug action and dormancy by Gene Expression Omnibus data. To define the role of Rv3668c in pathogen–host interaction, we constructed recombinant strain *Mycobacterium smegmatis*–Rv3668c (*Ms-Rv3668c*). The resultant strains were used to challenge the human macrophage cell line U937. The cytokine levels and the survival of recombinants and macrophages were monitored. The results showed that recombinant *Ms-Rv3668c* specifically upregulated the secretion of proinflammatory cytokines TNF- α , IL-1 β , and IL-6 and downregulated the secretion of anti-inflammatory cytokine IL-10 by U937 cells, consistent with the upregulated transcription of TNF- α and IL-1 β . Rv3668c recombinants demonstrated prolonged survival within the U937 cells and accelerated the death of the host cells. Inhibitor experiments showed that the ERK-NF- κ B axis was involved in the Rv3668c-triggered TNF- α and IL-1 β changes. These results provided evidence for the engagement of Rv3668c in the interaction between *Mycobacterium* and host.

Introduction

ACCORDING TO THE WORLD HEALTH ORGANIZATION (WHO) report, tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains a major public health threat. One-third of the global population has been latently infected with MTB. There are about 2 million deaths of active TB annually. Effective novel therapeutics are urgently needed to tackle the inexorable increase of multi- and extensively drug-resistant strains, together with HIV coinfections.

The serine proteases from several bacteria and viruses were well-established factors involved in the invasion of mammalian cells, including macrophages (Ohol and others 2010; Muttucumaru and others 2011). MTB serine proteases, such as MycP1 and Rv3610c, are well-documented virulence factors (Zhao and Xie 2011). RIP metalloprotease Rv2869c (Rip1) is an MTB virulence determinant by altering cell envelope composition (Makinoshima and Glickman 2005). PepA (Rv0125), a putative secreted serine protease, selectively stimulates peripheral blood mononuclear cells to proliferate and secrete gamma interferon (Skeiky and others 1999). Proteases play pivotal roles in the

cellular homeostasis and the recruitment of specific cellular proteins in other pathogens (Butler and others 2006). *Porphyromonas gingivalis* can use its protease to degrade the host proteins for nitrogen source (Armstrong 2006). Pathogen proteases contribute to virulence by cleaving host proteins, including immunoglobulins, complement components, and the extracellular matrix proteins (Hu and others 2010). *Haemophilus influenzae* IgA1 protease cleaves human IgA1 to evade the host immune attack (Fernaays and others 2006). *Chlamydia trachomatis* secretes three proteases (CPAF, Tsp, cHtrA) into host cells in order to manipulate host signaling and complete the intracellular growth cycle (Zhong 2011).

As a predicted serine protease, no report exists concerning the role of Rv3668c in the pathogen–host interaction. As an exceedingly successful typical intracellular pathogen, MTB can maneuver its secreted molecules to maximize the survival odds (Malen and others 2007). Several lines of evidence suggested an important role for MTB Rv3668c: a predicted signal sequence in Rv3668c and the presence in the MTB H37Rv culture filtrates, highly conserved among MTB, *M. leprae*, *M. bovis*, and *M. avium* paratuberculosis (Cerdeira-Maira and Darwin 2009), orthologs of DegS, and the

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SIP of the SigE proteolytic cascade of *Escherichia coli* (Ohol and others 2010). Rv3668c is inducible and potentially involved in persistence. Browsing the Rv3668c expression profiling under various treatments in Gene Expression Omnibus found that capreomycin, a peptide antibiotic for multi-drug resistance TB, can suppress its expression about 1.5-fold (Johansen and others 2006). The upregulation of Rv3668c in *dosR* mutant suggested that it might be repressed by dormancy-related transcription factor DosR. The differential expression of Rv3668c between H37Rv and clinical isolates implicates a role in the virulence variation among strains.

Macrophages are the primary residence of intracellular MTB. Cytokines secreted by macrophages are important for the outcome of infection. Upon TLR2 triggering by *Mycobacterium* spp., including *Mycobacterium smegmatis*, the adaptor protein MyD88 can be recruited to the receptor complex, followed by the interaction between the IL-1R-associated kinase and TNF receptor-associated factor 6, culminating in the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF- κ B), and differential cytokine expression (Akira 2003). To define whether Rv3668c functions through these pathways, MTB Rv3668c was expressed in *M. smegmatis*, a fast-growing nonpathogen widely used as a surrogate host for pathogenic mycobacteria (Daim and others 2011). The human macrophage cell line U937 was used as *in vitro* model in our recombinant *M. smegmatis* challenge.

Materials and Methods

Bioinformatics analysis

Rv3668c protein sequence feature was found using the protein-protein BLAST (BLASTP) program comparison of predicted proteins from MTB H37Rv and other mycobacteria. Eighteen orthologs of Rv3668c protein sequences were downloaded from the NCBI website, including *Mycobacterium bovis* AF2122/97 (Sequence ID: NC_002945.3), MTB H37Rv (Sequence ID: ref|NP_218185.1|), MTB SUMu005 (Sequence ID: gb|EFP29033.1|), MTB T46 (Sequence ID: gb|EFD11259.1|), *M. kansasii* (Sequence ID: NZ_CM000636.3), *M. marinum* M (Sequence ID: ref|YP_001853417.1|), *M. ulcerans* Agy99 (Sequence ID: ref|YP_907742.1|), *M. gilvum* PYR-GCK (Sequence ID: ref|YP_001132649.1|), *M. vanbaalenii* PYR1 (Sequence ID: ref|YP_956203.1|), *M. megmatitis* str MC2 155 (Sequence ID: ref|YP_890400.1|), *M. sp.* JLS (Sequence ID: ref|YP_001073452.1|), *M. sp.* MCS (Sequence ID: ref|YP_641970.1|), *M. avium* subsp. *paratuberculosis* K-10 (Sequence ID: ref|NP_959340.1|), *M. intracellulare* ATCC 13950 (Sequence ID: ref|YP_005335953.1|), *M. parascrofulaceum* ATCC BAA-614 (Sequence ID: gb|EFG77477.1|), *M. leprae* TN (Sequence ID: ref|NP_302490.1|), *M. abscessus* (Sequence ID: gb|ANAR01000017.1|), and *M. avium* 104 (Sequence ID: |NC_008595.1).

Bacterial strains, media, and growth conditions

M. smegmatis strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5% glycerol and 0.05% Tween 80, or on Middlebrook 7H10 agar supplemented with 0.5% glycerol at 37°C. When necessary, the growth media were supplemented with the antibiotic ampicillin (50 μ g/

mL) and kanamycin (25 μ g/mL for *Mycobacteria* and 50 μ g/mL for *E. coli*).

Rv3668c serine protease protein purification and activity assay

To determine protease activity, recombinant Rv3668c proteins were prepared in *E. coli* BL21, and purified. In brief, the PCR products were ligated to the pMD19-T simple vector, and then subcloned into pET32a (+) and pGEX-6p-2. Two recombinant strains, BL21-pET32-Rv3668c and BL21-pGEX-6p-2-Rv3668c, were constructed. Fusion protein expression was induced by isopropyl- β -D-thiogalactoside (IPTG) at a final concentration of 0.1 mM. Cells were sonicated. The supernatant was purified using Ni-NTA Superflow Cartridge (Qiagen) and Glutathione Sepharose 4B (GE), respectively. The purity of the recombinant protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The glutathione-S-transferase (Gst) fusion protein GST-Rv3668c was determined by SDS-PAGE and Western blotting. Primary monoclonal anti-bodies against GST were developed in mouse, secondary monoclonal antibodies horseradish peroxidase (HRP)-conjugated IgG were developed in goat (Tian gen), and bands were visualized by enhanced chemiluminescence. To detect Rv3668c protease activity, azocasein was used as substrate (Sigma), which can be degraded by all known proteases. As previously described (Vazquez Peyronel and Cantera 1995), the reaction was started with 1 mL of enzyme dilution in water. After 20 min, 1 mL of 10% TCA was added to terminate the reaction, followed by incubation for 10 min at 37°C and centrifugation for 20 min at 2,200 $\times$ g. The absorbance of the supernatant was determined at 440 nm.

The construction of recombinant *M. smegmatis*

Molecular techniques, including gene amplification, plasmid construction, and construction recombinant *M. smegmatis*, have been described previously (Pandey and others 2009). The full-length *rv3668c* gene was amplified by PCR using genomic DNA of the sterilized MTB H37Rv strain. The PCR fragments were purified and digested by *EcoRI/BamHI*. The construct was ligated into modified pNIT shuttle vector and electroporated into *M. smegmatis* mc²155. The pNIT-plasmid was a gift from University of Massachusetts Medical School Prof. Christopher M. Sasseti (Pandey and others 2009) and modified by our lab. Preparation of competent *M. smegmatis* and electroporation were performed as previously described (Pandey and others 2009). The recombinant plasmids pNIT and pNIT-Rv3668c were transformed into *M. smegmatis* by electroporation and colonies were selected on 7H10 agar plates supplemented with the antibiotic kanamycin (25 μ g/mL); further culture in 7H9 liquid media was conducted whenever necessary. The successfully transformed *rv3668c* gene recombinant *M. smegmatis* was confirmed by PCR, the positive clone was named *Ms-Rv3668c*, and the transformed vector strain was named *Ms-vec*. When the recombinant *M. smegmatis* cultured in 7H9 liquid medium reached OD₆₀₀=0.8–1.0, inducer ϵ -caprolactam was added to a final concentration of 28 mM and shaken at 110 rpm for 24 h (Pandey and others 2009). An aliquot of 2 mL was centrifuged with 12,000 rpm for 1 min, and the cell pellet was washed three times with prechilled phosphate buffered saline (PBS) and resuspended

in 10 mL 200 μ L PBS for sonication. Next, 36 μ L of 5 $\times$ SDS loading buffer was added to 60 μ L cell suspension with 4 μ L β -mercaptoethanol. The cell suspension was boiled at 100°C for 10 min and centrifuged at 14,000 rpm for 5 min, and then proteins were analyzed by SDS-PAGE and Western blotting.

Subcellular localization of the Rv3668c protein

Subcellular localization of the Rv3668c protein was determined using the method previously described (Dong and others 2012). Briefly, the cells were lysed by sonication for 30 min, and intact cells and cell debris were removed by centrifugation at 3,000 $\times g$ for 5 min. The supernatant was subjected to ultracentrifugation at 27,000 $\times g$ for 40 min at 4°C. The supernatant was considered the cell membrane and cytosol fractions, and the pellet from this centrifugation step was considered the cell wall fraction. The protein from each fraction was subjected to Western blotting using monoclonal antibodies against Myc (dilution, 1:500; Tian gen) to confirm Rv3668c expression.

Equal amounts of the protein from *Ms-Rv3668c* were loaded to each lane and *Ms-vec* as a negative control. Primary monoclonal antibodies against Myc were developed in mouse, secondary monoclonal antibodies HRP-conjugated IgG were developed in goat (Tian gen), and bands were visualized by enhanced chemiluminescence. Native GroEL was detected as a cytoplasmic control (Daleke and others 2012). Native GroEL was detected using an anti-His mouse primary antibody (Tian gen). The anti-His antibody recognizes a string of endogenous histidines in *M. smegmatis* GroEL (Bashiri and others 2012). The secondary antibody was goat anti-mouse conjugated to HRP (Tian gen).

Macrophage infection with recombinant M. smegmatis

Human macrophage-like U-937 cell line was a gift from Prof. Yu Chao (Chongqing Medical University). Cells were cultured in RPMI-1640 (Hyclone) medium containing 2 mM L-glutamine, supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin, and 100 U/mL streptomycin. U-937 cells were allowed to adhere and differentiate in the presence of Phorbol-12-myristate-13-acetate (PMA) (PMA/TPA) (100 ng/mL) at 37°C in a humidified atmosphere of 5% CO₂ for 48 h (Wei and others 2000). The cells were then washed three times with sterilized PBS. About 1 $\times 10^6$ differentiated U937 cells were infected with *M. smegmatis* transformants using the multiplicities of infection (MOI) of 10:1, and incubated for 4 h at 37°C under 5% CO₂. After the time allowed for phagocytosis, cells were washed three times with sterilized PBS to remove extracellular bacteria and then incubated again with a complete medium for 24 h, each sample has three replicates in this experiment, and the culture supernatants were removed and stored at -80°C for assay.

Assay for lactate dehydrogenase release

Culture supernatants were harvested after infection of differentiated U937 macrophages with *Ms-vec* or *Ms-Rv3668c* for 6, 24, or 48 h. Lactate dehydrogenase (LDH) activity in the culture supernatants was measured with an LDH cytotoxicity detection kit assay (Promega) according to the manufacturer's instructions (Daim and others 2011). The percentage of

LDH release was calculated as follows: percentage release = 100 $\times$ (experimental LDH release - spontaneous LDH release)/(maximal LDH release - spontaneous LDH release). Each sample set has three repeats in this experiment.

Measurement of the growth of recombinant bacteria in vitro and in vivo

Ms-vec and *Ms-Rv3668* were cultured as mentioned above, and each sample was repeated three times. OD₆₀₀ value was determined every 4 h until the mycobacteria reached logarithmic phase. The inducer ϵ -caprolactam was added, and OD₆₀₀ value was monitored every 4 h.

To examine the intracellular invasion, growth, and survival of bacteria, *M. smegmatis* and cells were cultured as previously described (Master and others 2008). We lysed the macrophages with 1 mL of PBS with 0.5% Triton-X-100 and plated cell lysates at serial dilutions onto 7H10 agar at 6, 24, 48, and 72 h postinfection. Colony forming units (CFUs) were enumerated 3 days postplating.

Semi-quantitative reverse-transcription (RT)-PCR and quantitative real-time PCR quantification of the expression of cytokines after challenge

Total RNA was extracted using TRIzol Reagent according to the manufacturer's instructions and then treated with DNase I (Invitrogen). RNA quantification was performed on a NanoDrop ND-100 spectrophotometer (Thermo). The transcription levels of the genes encoding TNF- α , IL-1 β , BNIP3-P1, Bcl-rambo, and suppressor of cytokine signaling-3 (SOCS3) were detected by one-step semi-quantitative reverse-transcription PCR using kit (Tian gen). The primer sequences are listed in Table 1. PCR cycling conditions were as follows: reverse transcription of 30 min at 50°C and 94°C for 2 min, followed by 30 cycles of 30 s at 94°C for denaturation and 1 min at 65°C for annealing and extension 10 min at 65°C. All RNA was purified and first-strand cDNA synthesized using 2 μ g of total RNA Moloney murine leukemia virus reverse transcriptase and random primer according to the manufacturer's instructions (Roche). Quantitative real-time PCR was performed on the Real-Time PCR Detection System (Bio-Rad), and using SYBR Select Master Mix reagents (Life Technologies). The relative quantities of the gene tested per sample were calculated against the β -actin mRNA using the CT formula as previously described (Liu and others 2006).

Cytokine assay

Culture supernatants were harvested from macrophages infected with recombinant *M. smegmatis* at intervals 6, 20, 32, 48, and 72 h, respectively. Concentrations of TNF- α , IL-1 β , IL-6, and IL-10 in the culture supernatants were determined with ELISA kits (eBioscience). Standard curves for these cytokines were obtained using recombinant standard proteins provided along with the kits.

Assay for extracellular signal-regulated kinase p38, ERK, and NF- κ B regulation of the cytokine secretion

All inhibitors used in the study were purchased from Sigma. AG490 inhibits STAT3 activation, and the concentration is 60 μ M in this study (Al-Shami and Naccache 1999; Eriksen

TABLE 1. THE PRIMERS USED

Primer	Primers name	Sequence (5'–3')	Comment
Rv3668c (Link PET-32 vector)	Forward	TAC <u>GAATTCC</u> GATATGACGGGAGC	Restriction site (<i>Eco</i> RI) product length 699 bp
	Reverse	TATA <u>AAGCTTT</u> CAGCCCAGCCAAC	Restriction site (<i>Hind</i> III) product length 699 bp
Rv3668c (Link PNIT vector)	Forward	TAC <u>GAATTCC</u> GATATGACGGGAGC	Restriction site (<i>Eco</i> RI) product length 699 bp
	Reverse	G <u>CGGATCCA</u> ACTCTGGCCGGTACC	Restriction site (<i>Hind</i> III) product length 699 bp
BNIP3-P1	Forward	GGGCTCCTGGGTAGAACTG	Product length 538 bp
	Reverse	AAAGGTGCTGGTGGAGGTT	
Bcl-rambo	Forward	TGCTGTGGCTTCTGTG	Product length 150 bp
	Reverse	GGGCTGGATTTCTCAA	
TNF- α	Forward	GGCGGTGCTTGTTCTT	Product length 187 bp
	Reverse	GCTACAGGCTTGTCACCTG	
β -actin	Forward	ACACTGTGCCCATCTACG	Product length 153 bp
	Reverse	TGTCACGCACGATTTCC	
IL-10	Forward	ATGCCTTCAGCAGAGT	Product length 174 bp
	Reverse	CTGGGTCTTGTTCTC	
IL-1 β	Forward	CCAGTGAAATGATGGCTTAT	Product length 150 bp
	Reverse	TGTAGTGGTGGTCCGAGA	
capase-1	Forward	GAAGGTACAATAAATGGCTTAC	Product length 133 bp
	Reverse	GAATAACGGAGTCAATCAAA	

Forward and reverse represent sense and antisense primers, amplifying a fragment of the gene from the H37Rv genome or total RNA of *Mycobacterium smegmatis* infection of macrophages. Restriction sites are underlined in the sequence, and the corresponding enzymes are listed in parentheses.

FIG. 1. *Mycobacterium tuberculosis* (MTB) Rv3668c is a conserved hypothetical secreted protein. **(A)** The Rv3668c protein is predicted to be an outer membrane protein with a small intracellular sequence, a short transmembrane region, and a large extracellular sequence. **(B)** Molecular phylogenetic tree inferred from Rv3668c of MTB H37Rv and other orthologs in other mycobacteria. Numbers on branches are confidence percentages obtained from 100 bootstraps. Scale bar, 5 substitutions in 100 nt. The tree was constructed by software MEGA4.0. **(C)** The alignment was generated by the program Vector NTI. MTB SUMu005 and MTB T46 lack of region compared with MTB H37Rv are boxed. The secondary structure of Rv3668c was obtained online. H, helix; E, strand.

FIG. 1. (Continued).

and others 2001). SB 202190 is an inhibitor of p38 MAP kinase, and the concentration is 10 μ m (Song and others 2010). PD 98059 is a specific inhibitor of the activation of MAPK kinase (MAPKK), and the concentration is 20 μ m (Chen and others 2005). TPCK blocks the cytokine-induced activation of NF- κ B, and the concentration is 30 μ m (Kengatharan and others 1996). Macrophages were pretreated with three inhibitors for 1 h before challenge with the two recombinant strains. The culture supernatants were harvested 24 h after infection, and ELISA was performed to determine the concentrations of secreted TNF- α and IL-1 β . About 5×10^5 differentiated U937 cells were infected with *M. smegmatis* transformants using an MOI of 10:1 in this study.

Statistical analysis

All data were analyzed using Student's two-tailed *t*-test. In all figures, the asterisks denote range of *P* values (**P* < 0.05, **0.01 > *P* > 0.001, ****P* < 0.001, ns = not significant) as determined by the significance. Each determination was repeated at least three times.

Results

Rv3668c is a secreted protease by bioinformatics analysis

Rv3668c is a nonessential gene (Griffin and others 2011) present in the culture filtrate of MTB H37Rv (de Souza and

others 2011). A putative N terminal signal sequence with 99.9% confidence supported its secreted identity. Rv3668c protease is predicted to be an outer membrane protein with a small intracellular sequence, a short transmembrane region, and a large extracellular sequence by TMHMM Server V2.0 software on line (Fig. 1A). These data suggest that Rv3668c might be associated with the pathogenesis of MTB.

Rv3668c is highly conserved among pathogenic *Mycobacteria* by phylogenetic analysis (Fig. 1B and C). Rv3668c from MTB SUMu005 and MTB T46 are evolutionarily close (Fig. 1B), and we found that the two strains lack up to 60 aa from its C terminus in comparison with H37Rv (Fig. 1C).

Rv3668c crystal structure is currently not available. Structures from three homology proteases can be found: bacterial chymotrypsin from *Cellulomonas bogoriensis*, *Thermobifida fusca* Protease A, and serine proteinase from *Streptomyces fradiae* ATCC 14544 crystal structure with identities ranging from 30% to 29%. These data suggested that Rv3668c is a serine protease.

Purified recombinant Rv368c protein and serine protease activity assay

Two prokaryotic expression systems for Rv3668c were constructed. The results of PCR and sequencing confirmed the *rv3668c* gene clone successfully (data not shown). The expression of the target recombinant protein was confirmed

FIG. 2. The purified recombinant Rv3668c protein and serine protease activity. (A) Silver-stained SDS-PAGE with the recombinant Rv3668c protein after IPTG-induced expression. Rv3668c: lane 1, protein molecular weight marker; lane 2, *Escherichia coli* BL21 containing pET-32 vector as the control; lane 3, *E. coli* BL21 containing pET-32-Rv3668c without IPTG induction as the control; lane 4, *E. coli* BL21 containing pET-32-Rv3668c with IPTG induction for 4 h. Relevant molecular mass markers are shown on the left in kilodaltons (kDa). (B) Coomassie blue-stained SDS-PAGE with recombinant His-tag-Rv3668c protein: lane 1, protein molecular weight marker; lane 2, *E. coli* BL21 containing pET-32-Rv3668c without induction as the control; lanes 3 and 4, precipitate and supernatant of sonicated BL21 with pET-32-Rv3668c, respectively; lane 5, His-tag-Rv3668c purified protein. (C) Coomassie blue-stained SDS-PAGE with recombinant GST-tag-Rv3668c protein: lane 1, protein maker; lanes 2 and 3, *E. coli* BL21-GST uninduced and induced recombinant *E. coli* as the control; lanes 3 and 4, BL21-GST-Rv3668c uninduced and induced recombinant *E. coli* strains; lanes 5 and 6, GST-tag-Rv3668c purified protein; lanes 7 and 8, Western blotting of GST-tagged Rv3668 purified protein (lane 2). *E. coli* BL21 induced recombinant *E. coli* as the control. (D) The proteolytic activities of Rv3668c protein assay using His-tag-Rv3668c purified protein. Azocasein was the substrate. The enzyme activity of 1 unit was defined as an increase of 0.1 at OD440 nm. PBS is used as the negative control. Data are representative of three independent experiments with similar results. PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacryl amide gel electrophoresis.

by SDS-PAGE and Western blotting (Fig. 2). The purified His-tagged recombinant protein was ready for further enzymatic characterization. Rv3668c purified after over-expression in *E. coli* exhibited this activity, using azocasein (Sigma) as a substrate as previously described (Imamura and others 2008). Figure 2D shows that Rv3668c has proteolytic activity at 1.6 unit/mL. The enzyme activity of 1 unit was defined as an increase of 0.1 at D440 nm. Three bands can be visualized in the GST-tag-Rv3668c protein. This is the result of degradation using anti-GST antibody against 26 kDa (Fig. 2C and D). Same result can be found in the His-tagged recombinant protein. One reason might be the self-degrading activity of the recombinant protease.

Subcellular localization of the Rv3668c protein in *Ms-Rv3668c* strain

To investigate the effects of Rv3668c on the U937 cell response to bacterial infection, we generated two recombinant *M. smegmatis* strains, *Ms-vec* and *Ms-Rv3668c*: *Ms-vec* contained the pNIT-Myc plasmid, and *Ms-Rv3668c* contained the recombinant plasmid pNIT-Myc-Rv3668. PCR confirmed that the *rv3668* gene was successfully electroporated into *M. smegmatis* mc²155 (data not shown). Total proteins of recombinant *M. smegmatis* were gained

after ϵ -caprolactam induction for 24 h. We added the Myc polypeptide tag in pNIT plasmid, so Rv3668c can be expressed as a fused protein with myc. Furthermore, Western blotting with the anti-myc antibody detected the presence of expressed 28-kDa Rv3668c protein bands in total cell lysates from the recombinant *M. smegmatis*, while no similar bands existed in *M. smegmatis* control. The Rv3668c protein was observed in the *Ms-Rv3668c* strain cell wall fraction. The cytosolic control protein GroEL2 was not detected in the cell wall fraction (Fig. 3). These data showed that the Rv3668c protein from MTB was successfully expressed in *M. smegmatis* and thus can be used for further analysis.

The effect of the Rv3668c *M. smegmatis* recombinant on the viability of human macrophages

In vitro studies revealed no difference between *Ms-vec* and *Ms-Rv3668c* in 7H9 cultures (Fig. 4A). U937 human macrophages were infected with *Ms-vec* and *Ms-Rv3668c* at an MOI of 10:1. As revealed by CFU assays, we found that the *Ms-Rv3668c* infected group showed greater number of bacteria at 24 and 48 h. On the third day, many cells died (Fig. 4B). Whether this was caused by the enhanced replication or augmented survival of the bacterium within

FIG. 3. Western blotting confirmed the expression of Rv3668c located at the *M. smegmatis* cell wall. All lanes were probed with the anti-myc antibody. Equal amounts of total proteins were used. Rv3668c's predicted molecular mass was 23 kDa, and the myc 5-kDa myc tag expressed by recombinant *M. smegmatis* strains was added. As expected, no Rv3668c was detected in total lysates of *Ms-vec*. Native GroEL was detected using an anti-His mouse primary antibody as a cytoplasmic control. The cytosolic control protein GroEL was not detected in the cell wall fractions. Lane 1, cell membrane and cytosol fractions of *Ms-vec*; lane 2, cell wall of *Ms-vec*; lane 3, cell membrane and cytosol fractions of *Ms-3668c*; lane 4, cell wall of *Ms-3668c*.

macrophages remains to be determined. We further found that no *in vitro* growth kinetic difference can be found for both recombinant strains (Fig. 4A).

To determine whether Rv3668c was able to affect the death of macrophages infected with *M. smegmatis*, viability of the

infected macrophages was determined using an LDH release assay (Promega) to ensure that the release of Rv3668c and vesicles containing Rv3668c into the tissue culture supernatant was not because of the lysis of infected macrophages. Percentage of LDH activity is shown in Fig. 4C. At 24 and

FIG. 4. (A) Comparison of the growth kinetics of two *M. smegmatis* strains. *In vitro* growth of *Ms-vec* (◆) and *Ms-Rv3668c* (□) in 7H9 cultures. (B) The CFUs of the survived strains in infected U937 cells in three time points. Ten-fold serial dilutions of the homogenates were plated onto Middlebrook 7H10 agar plates. The plates were incubated at 37°C for 3 days, and the colony number was counted. The bacterial number in the cells was calculated as log₁₀ CFUs per cell. (C) The percentage of dead U937 cells infected by recombinant *M. smegmatis* at 6, 24, and 48 h. Bars show the mean $\pm$ SD of three independent experiments. (D) Reverse transcription PCR detected two proapoptotic factors, BNIP3 and Bcl-rambo transcription, 24 h after infection. Data were generated from three independent experiments. Top: representative gel image of three independent replicates is shown, and the amplified products were resolved by electrophoresis on 1.0% agarose gels and visualized by ethidium bromide staining. Bottom: the relative densities of expression levels were analyzed by densitometry using software Quantity One. All densitometry values were normalized to those of β -actin mRNA. (*P < 0.05).

48 h, macrophages infected by *M. smegmatis* containing Rv3668c showed higher LDH release amount than the control group with appreciable difference: 22.7% and 45% for 24 h, and 82.7% and 86% for 48 h. Experimental results showed that the *Ms-Rv3668c* strain-infected U937 group promoted the death of a large number of cells compared with the *Ms-vec* strain-infected group in 24 h. Recombinant *M. smegmatis* in U937 triggered the death of cells. The LDH release showed that Rv3668c can induce cell death at the MOI of 10 BNIP3 and that Bcl-rambo can promote cell death in the study of *Legionella pneumophila* (Banga and others 2007). It is well established that MTB actively modulates host apoptotic pathways (Persson and others 2008). Transcription of a number of prodeath genes, including Bcl-rambo, BNIP3, and many caspases, also was elevated in response to the intracellular growth of the bacterium. The induction of prodeath genes poses a problem for infected cells because these proteins sensitize cells to environmental insults, which, during MTB infection, can be represented by intracellular growth of the bacterium or the translocation of toxic substrates into host cells. We speculated that Rv3668c might exert its function via BNIP3 and Bcl-rambo. Based on the results of Fig. 4D, only the Bcl-rambo transcriptional level was affected by Rv3668c instead of BNIP3. Here we speculated that Rv3668c might facilitate the survival of intracellular pathogen dependent on Bcl-rambo.

Rv3668c protease involved in the differential expression of cytokines in macrophage

The SOCS3 protein is known to play important roles in the regulation of proinflammatory responses during myco-

bacterium-macrophage interactions (Imai and others 2003; Vazquez and others 2006). In this study, the mRNAs of TNF- α and SOCS3 were monitored by semi-quantitative RT-PCR. Quantitative real-time PCRs were used to measure the expression of TNF- α , IL-1 β , and IL-10 after infection for 24 h. The expression of TNF- α and IL-1 β in U937 cell was upregulated by *Ms-Rv3668c* instead of *Ms-vec* challenge (Fig. 5A). *Ms-Rv3668c* downregulated the mRNA levels of IL-10 (Fig. 5B). We found that SOCS3 can somehow modulate the expression of IL-10. Our data are consistent with previous reports using LPS to induce IL-10, where SOCS3 is usually upregulated. This study is to investigate mRNA expression of proinflammatory and anti-inflammatory cytokines in U937.

Assay for cytokine production

To determine whether intracellular bacterial growth rates are involved in the differential cytokine production as transcriptional levels, ELISA was used to confirm the concentrations of the four cytokines at the various time points postinfection. The cell group was infected with the same volume of medium. For TNF- α and IL-1 β , no statistically significant difference can be found at the first time points (6 h) between the *Ms-vec* and *Ms-Rv3668c* infections (Fig. 6). However, there was a marked increase in the concentration of TNF- α , IL-1 β , and IL-6 between 18 and 72 h postinfection. At 6 h after *Ms-3668c* infection, the production of the anti-inflammatory cytokine IL-10 was found to be dampened.

Based on the results described above, we seek to investigate the molecular events underlying the *Ms-Rv3668c*-

FIG. 5. (A) The transcriptional levels of cytokines secreted by U937 cells infected by *Ms-Rv3668c* at 24 h. Total RNA was purified and subjected to semi-quantitative RT-PCR analysis for assessment of TNF- α and SOCS3 expression. Top: representative gel image of three independent replicates is shown. Bottom: the relative densities of the expression levels were analyzed by densitometry using software Quantity One. All densitometry values were normalized to those of β -actin mRNA. (B) TNF- α , IL-1 β , and IL-10 transcriptional levels were determined by qPCR. Values represent averages of three biological replicates, each with two technical replicates. Total RNA was isolated from infected bacterium U937 cells after 24 h. The β -actin gene of U937 cell was determined as an internal control of all the RT-PCRs. Bars show the mean $\pm$ SD of three independent experiments. (* $P < 0.05$).

FIG. 6. (A, C, D) ELISA analysis of TNF- α , IL-6, and IL-10 secreted by U937 cells after being infected by recombinant *M. smegmatis* at 6, 20, 32, 48, and 72 h. U937 cells infected with *Ms-vec* were used as a positive control and uninfected macrophages served as a negative control. PMA-differentiated U937 cells were used as a control in both experiments. Target cytokine was negligible from noninfected cells. About 1×10^6 PMA-differentiated U937 macrophages were used in this study. Macrophages were infected with *Ms-vec* and *Ms-Rv3668c* at an MOI of 10. (B) ELISA analysis of IL-1 β secreted by infection of macrophages with *Ms-Rv3668c* or *Ms-vec* for 6, 24, or 48 h. All data are shown as mean $\pm$ SD of triplicate wells. Significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

induced differential level of pro- and anti-inflammatory responses. Here we present evidence that the MTB serine protease Rv3668c enhances TNF- α secretion by enhancing the activity of SOCS3 (Fig. 5A). Upregulation of TNF- α , IL-1 β , and IL-6 indicated a proinflammatory response, and downregulation of IL-10 suggested a weakened anti-inflammatory response by 24 h after *Ms-Rv3668c*. Based on above results, we speculated that a tip of the balance between proinflammatory during infection might change the outcome of infection.

Next, using qRT-PCR, we examined the mRNA expression of caspase-1. The results show that *Ms-Rv3668c* enhanced IL-1 β secretion by enhancing the activity of caspase-1 (Fig. 5B).

Rv3668c regulating ERK and NF- κ B levels in macrophages

It is well established that MAPK cascades (JNK, ERK, and p38) and NF- κ B cascades are the major signaling pathways involved in the LPS-induced macrophage activation (Guha and Mackman 2001). To determine whether these pathways are affected by Rv3668c expression to alter the cytokine secretion, we used three inhibitors as previ-

ously described. The activation of p38 and ERK was established for the transcription TNF- α gene in macrophages infected with *M. smegmatis* (Daim and others 2011). To investigate whether the activation of MAPKs might be affected in macrophages infected with *Ms-Rv3668c*, ELISA was performed to assess the altered levels of TNF- α and IL-1 β within the infected macrophages with the inhibitors added. Figure 7A and B shows a lower transcriptional level of activation of the TNF- α and IL-1 β genes in macrophages infected with *Ms-Rv3668c*. This might be because of a lower level of activation of NF- κ B, ERK, and p38. In the two infected groups, the U937 cell line secreted TNF- α and IL-1 β remarkably down-regulated with the addition of ERK and NF- κ B inhibitors compared to solvent control. Interestingly, IL-1 β secretion increased after the addition of P38 inhibitors of the MAPK pathway. Thus, Rv3668c can manipulate the apoptotic status of infected cells at different levels, ranging from controlling some major pathways to the specific inhibiting of inflammatory signaling proteins.

NF- κ B activity was required for the *Ms-Rv3668c*-induced activation of ERK1/2 and p38 MAPK. These data suggest that mycobacterial Rv3668c induces proinflammatory responses through the crosstalk between the NF- κ B, ERK, and MAPK pathways within macrophages.

FIG. 7. Cytokines secreted by U937 cells treated with four inhibitors after infected by *Ms-vec* and *Ms-Rv3668c* at 24 h. AG490 (a JAK/STAT inhibitor, 60 μ M), TPCK (an NF- κ B inhibitor, 30 μ M), PD 98059 (an ERK1/2 inhibitor, 20 μ M), or SB202190 (a p38 inhibitor, 20 μ M) was added at the indicated concentrations. DMSO (Sigma) was added to the cultures at 0.1% (v/v) as a solvent control. PBS was used as a negative control. About 5×10^5 PMA-differentiated U937 macrophages were pretreated for 1 h with these four inhibitors, and then macrophages were infected with *Ms-vec* and *Ms-Rv3668c* at an MOI of 10. Culture supernatants were harvested after 24 h of infection, and the concentrations of TNF- α (A) and IL-1 β (B) in the culture supernatants were determined by ELISA. Shown are means and standard deviations of triplicate measurements of one representative experiment out of three. Significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Discussion

MTB Rv3668c protease has an N-terminal signal sequence, suggesting that it may be translocated into the periplasmic region via a sec-dependent secretion pathway.

FIG. 8. Proposed model of signaling cascade-mediated, *Ms-Rv3668c*-induced U937 cell activation. Treatment of U937 cells with *Ms-Rv3668c* activates Erk activity, which results in an increase in NF- κ B activity, leading to increased production of TNF- α , IL-1 β , and IL-6 and a subsequent proinflammatory response *in vitro*. Transcription factor NF- κ B and its upstream activator ERK play key roles in this response.

MTB express a variety of virulence factors, such as virulence-associated secretion systems (CFP-10) or pore-forming toxins (ESAT-6), that disrupt host cell systems or manipulate cellular signaling pathways to allow the establishment of infection. Rv3668c, a putative serine protease by bioinformatics, might disrupt the macrophage cytokine production. Five mycobacterium serine proteases (MycP1-5) contain the same catalytic triad (Asp, His, Ser) in MTB (Brown 2000). MycP1, one of the important members of the family that localize to the cell wall/membrane, is required for the growth in mice during acute infection and can target EspB, a substrate of ESX-1 required for secretion (Sun and others 2004). The cell wall localization of the protease Rv3668c supported its role in the host interaction (Fig. 3). The *Ms-Rv3668c* strain showed enhanced survival within U937 macrophages (Fig. 4B). It is fascinating to expect a similar role for MTB serine protease ES-31. Previous studies have reported that adding the anti-ES-31 serine protease antibody strongly inhibited MTB growth as observed by significantly decreased CFUs (Upadhye and others 2009).

Macrophages play important roles in innate immunity against microbial infection. An efficient immune response against the intracellular pathogen MTB is critically dependent on the rapid detection of the invaded pathogen by the innate immune system and coordinated activation of the adaptive immune response. TNF is a pleiotropic cytokine that plays an important role in the outcome of mycobacterial infections by controlling the expression of a wide range of cytokines and chemokines (Flynn and Chan 2001). TNF is required for the control of acute MTB infection in a mouse model (Flynn and others 1995) and plays a critical role in granuloma formation (Flynn and Chan 2001; van Crevel and others 2002). In our study, significant greater amounts of

TNF- α released from U937 macrophage cultures infected with *Ms-Rv3668c* compared with the control. IL-1 β has a central role in this balance during infection with MTB as both a mediator of antimycobacterial immunity and a driver of tissue damage. Large amounts of TNF are released in response to lipopolysaccharide, together with IL-1 and IL-6. Pro-IL-1 β transcription is initiated upon NF- κ B activation. However, a second trigger is required for the release of IL-1 β (Petrilli and others 2007).

Once in the cells, these effectors manipulate various cellular signaling pathways and promote the secretion of some cytokines such as IL-1 β . Interestingly, IL-1 β secretion was increased by adding P38 inhibitors in the MAPK pathway (Fig. 7). We speculated that the IL-1 β secretion may be through other pathways, or negatively regulated by P38. The cysteine protease caspase-1 is responsible for the proteolytic processing and secretion of IL-1 β (McCoy and others 2010).

MTB prevents the inflammasome activation, probably by secreting a putative metalloprotease Zmp-1 (Rv0198c) and inhibiting the stimulation of caspase-1-dependent apoptosis (Master and others 2008). Our data showed that *Ms-Rv3668c* upregulated caspase-1 transcription, consistent with the previous finding that caspase-1 activation is enhanced by macrophage apoptosis (Mizutani and others 1991). SOCS3 is a negative regulator of IL-6 signal transduction. SOCS3 expression in bone marrow cells upregulated IL-6 upon exposure to lipopolysaccharide or tumor-associated factors (Zhang and others 2009).

NF- κ B is a family of transcriptional activator. NF- κ B has been shown to regulate many genes involved in immune and inflammatory responses (Zang and others 2012). IL-10 is thought to play pivotal roles in the regulation of host antimicrobial immunity (Samuel and others 2007). In view of the data presented above, PMA differentiated U937 macrophages highly expression the IL-10. PMA is often employed in biomedical studies to activate the signal transduction enzyme protein kinase C (Castagna and others 1982; Blumberg 1988). So we speculated that PMA stimulated the secretion of IL-10. Macrophage phagocytosis of MTB is accompanied by the activation of NF- κ B and secretion of inflammatory mediators that play an important role in granuloma formation and immune protection during MTB infection. The findings from this study indicate that during the initial encounter of the macrophage, the Rv3668c protein might be involved in eliciting an immediate proinflammatory immune response. A better recognition of MTB serine proteases and their roles in the host immune response to the pathogens may help us better understand the immunological processes induced by MTB.

Addition of ERK and NF- κ B pathway inhibitors significantly downregulated the macrophage-secreted TNF- α and IL-1 β . MAPKs differ significantly with their trigger cues, downstream molecules, and functions (Schaeffer and Weber 1999). In the study, we found that *Ms-Rv3668c* stimulated U937 TNF- α and IL-1 β are dependent on the activity of ERK and NF- κ B activity. This discovery indicates that ERK is involved in the signaling of Rv3668c-stimulated human monocyte activation. The possible pathway is shown in Fig. 7. A more precise cascade remains to be defined. SOCS3 is known to predominantly target the STAT family of proteins to attenuate proinflammatory signaling (Imai and others 2003; Gao and Ward 2007). In our study, we found

that SOCS3 expression was upregulated (Fig. 5A). One possibility is that the effect of SOCS3 rests also on signaling other than STAT signaling. JNK and p38 MAPK can activate transcription factors Elk-1 and ATF2 (Schaeffer and Weber 1999). Rv3668c, as a serine protease, may be used by MTB to target host proteins for manipulating host signaling pathways. Whether Rv3668c can degrade other relevant molecules such as ubiquitin, deubiquitinating enzyme, phosphorylase involved in the ERK, and NF- κ B signaling remains elusive.

Ms-Rv3668c can modulate the secretion of TNF- α , IL-1 β , and IL-6; induce U937 cell death; and enhance the survival of the bacteria. The data confirmed the crucial role of Rv3668c in the interaction between *Mycobacterium* and host. An outline of this cascade is proposed in Fig. 8. However, many details remain to be determined. Further work is needed to establish whether inhibitors of Rv3668c can alter the outcome of *Mycobacterium* infection.

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Author Disclosure Statement

No competing financial interests exist.

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