**Mycobacterium tuberculosis** hypoxic response protein 1 (Hrp1) augments the pro-inflammatory response and enhances the survival of **Mycobacterium smegmatis** in murine macrophages

Changfeng Sun, Guoping Yang, Jinning Yuan, Xuan Peng, Chunxi Zhang, Xiaoqian Zhai, Tao Luo and Lang Bao*

**Abstract**

**Purpose.** The DosR/DosS two-component regulatory system of **Mycobacterium tuberculosis** regulates the expression of numerous genes under stress conditions and is important for the long-term survival of **M. tuberculosis** in the host. The rv2626c gene of **M. tuberculosis** is one of the most strongly induced transcripts of the dormancy regulon. This study focused on the immunological effects and possible function of Rv2626c in maintaining mycobacterial survival under various stress conditions.

**Methodology.** We heterologously expressed the Rv2626c protein in **Mycobacterium smegmatis** by constructing a recombinant strain Ms_rv2626c. The viability of Ms_rv2626c was evaluated both in vivo and ex vivo. Different stress conditions, including acidified sodium nitrite, malachite green, low pH, SDS and lysozyme, were used to evaluate the effect of Rv2626c on bacterial resistance. An in vitro assay using a macrophage infection model was utilized to investigate the potential effect of Rv2626c to alter the immune response of host cell and its associated pathways. The effect of Rv2626c on cell necrosis was also explored.

**Results.** The expression of Rv2626c-enhanced *M. smegmatis* survival under hypoxia and nitric oxide stress in vitro, and this enhancement was maintained within macrophages and in mouse tissues. In addition, macrophages infected with *M. smegmatis* expressing Rv2626c showed significantly higher interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) expression, as well as a higher level of cell necrosis, compared with the control.

**Conclusion.** *M. tuberculosis* protein Rv2626c plays a significant role in stimulating macrophages to provoke a pro-inflammatory response and in mycobacterial survival during infection.

**INTRODUCTION**

Tuberculosis (TB) remains a serious threat to global public health. In 2015, there were approximately 9.6 million new cases of TB and 1.5 million TB-related deaths [1]. It is estimated that approximately one-third of the world’s population is currently latently infected with **Mycobacterium tuberculosis**, and in 5–10% of people this latent infection will develop into an active disease during their lives [2]. The persistence of **M. tuberculosis** is a major challenge for effective TB therapy, as conventional treatment is mainly targeted against replicating bacilli. The mechanisms allowing **M. tuberculosis** to enter a persistent state, retain viability during latency and be reactivated from latency remain largely unknown. Understanding such dynamic interactions between the host and pathogen is crucial for developing effective anti-tuberculosis strategies.

To adapt to various environmental stressors and/or physiological stresses invoked by the host, such as hypoxia, reactive oxygen, nitrogen intermediates and acidic pH [2, 3], **M. tuberculosis** has evolved multiple factors, including molecular chaperones, protein-modifying and -degrading enzymes, accessory sigma factors and two-component systems [4]. Among these, two-component systems represent an efficient mechanism utilized by bacilli to rapidly sense and respond to a variety of in vitro or in vivo stresses [5]. The genome of **M. tuberculosis** reference strain H37Rv encodes 11 complete paired two-component signal systems (TCSSs), 2 orphan histidine kinases (HKs) and 6 orphan response regulators (RRs) [6]. Of these, the
DosR/DosS two-component regulatory system is one of the best-studied regulatory systems, and it has been found to be involved in the response of bacilli to hypoxia, nitric oxide, ascorbate and carbon monoxide stresses, while it is thought to be crucial for the long-term survival of the bacilli in the host [7, 8]. The DosR/DosS system regulates the expression of 48 genes, among which rv2626c has one of the most strongly induced transcripts under hypoxia or NO exposure stresses [7, 8]. The DosR/DosS system is one of the best-studied regulatory systems, and it has been found to be involved in the response of bacilli to hypoxia, nitric oxide, ascorbate and carbon monoxide stresses, while it is thought to be crucial for the long-term survival of the bacilli in the host [7, 8].

Studies have shown that the hypoxia-induced dormancy response is established and maintained by the regulator DosR and ATP-related proteins such as Rv2623 and Rv2626c [13–15]. Rv2626c, also called hypoxic response protein 1 (Hrp-1), is predicted to contain two cystathionine-β-synthase (CBS) domains [16], which could potentially attach to a wide range of other protein domains, and possibly have regulatory roles in sensitizing proteins to adenosyl carrying ligands, or be involved in AMP/ATP binding in these proteins [17, 18]. However, experimental evidence is still lacking for the biological function of the Rv2626c protein [12, 19].

**Mycobacterium smegmatis** is a fast-growing non-pathogenic mycobacterium species and is related to *M. tuberculosis*. Although *M. smegmatis* cannot persist as long as *M. tuberculosis* in intracellular cultures or animal models of tuberculosis, many studies have shown that *M. smegmatis* can survive in host cells or in vivo for a certain period of time [20, 21]. Therefore, *M. smegmatis* is a widely accepted model for studying the function of potential virulence genes of *M. tuberculosis*. Furthermore, several studies have used this model to study the potential persistence-associated factors of *M. tuberculosis* [20–22]. As a first step in assessing the potential function of the *M. tuberculosis* protein Rv2626c in the current study, we took advantage of the lack of rv2626c or orthologue genes in *M. smegmatis mc2 155* and generated a recombinant strain (Ms_rv2626c) that expresses the rv2626c-encoded protein, to study its functions. The ability of Ms_rv2626c to survive inside macrophages and within tissues was assessed in an in vitro infection model of mouse macrophages and in a medium-dose intraperitoneal (i.p.) mouse infection model, respectively. In addition, the effect of Rv2626c on the resistance of *M. smegmatis mc2 155* to various antibacterial conditions and macrophage cytokine response was also investigated.

**METHODS**

**Bacterial strains, macrophages and growth conditions**

*Escherichia coli* strains DH5α and BL21 were used for DNA cloning and protein expression procedures, respectively, and were grown in Luria–Bertani (LB) broth or on LB agar plates at 37°C. *M. smegmatis mc2 155* strains were grown on Middlebrook 7H10 (M7H10) agar supplemented with 10% ADS [0.5% (w/v) bovine serum albumin fraction V, 0.2% (w/v) dextrose, 140 mM NaCl] and 5% (v/v) glycerol, or in enriched Middlebrook 7H9 broth (M7H9, Difco) supplemented with 10% ADS, 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 at 37°C. The ANA-1 murine macrophages (Boster) were cultured at 37°C in a humidified atmosphere with 5% (v/v) CO2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS, Hyclone).

**Generation of a recombinant *M. smegmatis* strain expressing Rv2626c**

The rv2626c gene was polymerase chain reaction (PCR)-amplified from M. tuberculosis H37Rv genomic DNA using the forward primer 26-c-F (5’-GGAATTCATGACCACGCAGCCGACATCAT-3’), containing an EcoRI site (underlined), and the reverse primer 26-R1 (5’-GGCTGCTTGGTGTTCCGTGGCGGGGCCCAG-3’), containing five 5’ His codons. The approximately 450 bp PCR product was cloned into the vector pMD18-T (Takara), generating the recombinant plasmid p5H-rv2626c. One additional His codon was introduced at the C-terminus of the aforementioned fragment using the same forward primer and a second reverse primer, 26-c-R2 (5’-CCCAAGCCTCTAGTGTTGTTCTGGTGTTGGTCG-3’), containing one termination codon and a HindIII site (underlined). The resultant PCR product containing the 6x-His-tag codons was inserted into the cloning sites of pMV361, generating pMV361-rv2626c. The plasmid pMV361 is a mycobacterial expression vector that contains the hsp60 promoter and a selectable kanamycin resistance marker (aph). The vector stably integrates once into the mycobacterial genome at the non-essential attB site as a result of the presence of the phage-derived attB and int genes. Electrocompetent *M. smegmatis* cells were electropropated with the recombinant plasmid pMV361-rv2626c or the empty pMV361 into *M. smegmatis* with an electroporator 2510 (Eppendorf) according to a standard protocol [23, 24]. Transformants were screened on M7H10 plates supplemented with 25 μg kanamycin ml−1 and incubated for 3 to 5 days.

**Extraction of RNA**

RNA isolation was performed using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. To remove contaminating DNA, RNA samples were treated with RNase-free DNaseI (Invitrogen). The concentration of total RNA was measured using a SmartSpec Plus spectrophotometer and standard cuvettes with a 1 cm path length (Bio-Rad) according to the manufacturer’s instructions. RNA samples proceeded to downstream applications or were stored at −70°C.

**Detection of rv2626c gene expression in *M. smegmatis***

To detect the transcription of rv2626c, synthesis of single-stranded cDNA was performed using 200 ng of total RNA.
as a template and random hexamer primers using the RevertAid First Strand cDNA synthesis kit (Thermo) in a S1000 Thermal Cycler (Bio-Rad) according to the manufacturer’s instructions. A 2 µl aliquot of each cDNA sample was used for PCR amplification using the primers RT-26F (5'-TTGCGAACACGAGACGC-3') and RT-26R: (5'-AGCAGATTGCTTGACGAAC-3'), which are specific for the rv2626c gene (336 bp), or with primers aph-F (5'-TC TTCCGACATCAAGCA-3') and aph-R (5'-CACCGAGG CATTTCCAATA-3'), which are specific for the aph gene (470 bp). PCR products were visualized in 1.5 % (w/v) agarose gels (Difco).

To detect Rv2626c protein levels, protein samples were prepared as previously described [25] and subjected to SDS-PAGE. The His-tagged Rv2626c protein was detected using a mouse anti-6×His tag antibody (NeoBioscience) and an HRP-conjugated goat anti-mouse IgG antibody (NeoBioscience). Signals were detected using the DAB-Plus reagent set (Invitrogen) according to the manufacturer’s instructions.

**In vitro growth of the *M. smegmatis* strains**

Single-cell suspensions of *M. smegmatis* strains were prepared as previously described [26] and inoculated in triplicate with a starting OD_{600} of approximately 0.02. Inoculated cultures were grown in a shaker incubator at 200 r.p.m. at 37 °C, with aliquots taken at various time points for the measurement of OD_{600}.

**Growth of *M. smegmatis* strains under anaerobic conditions**

Recombinant *M. smegmatis* strains were subjected to slow withdrawal of oxygen as previously described [27, 28]. Briefly, bacteria were inoculated at a concentration of 1 × 10^7 c.f.u. ml⁻¹ (initial OD_{600} of 0.02) in sealed bottles to maintain a head-to-space ratio of 0.5, as defined by the Wayne model; 1.5 µg methylene blue ml⁻¹ served as an indicator of oxygen levels. The cultures were then stirred gently at 120 r.p.m. on a magnetic stirring platform at 37 °C. All cultures were prepared in triplicate and independent tubes were processed for each strain at each time point to prevent the introduction of oxygen into the growth medium during sampling. After the methylene blue indicator turned from blue to colourless, the bacteria were serially diluted at various time points and aliquots were plated onto 7H10 agar plates for c.f.u. determination.

**Measurement of the sensitivity of *M. smegmatis* strains to malachite green, nitric oxide, SDS, lysozyme and low pH**

To investigate the tolerance of recombinant *M. smegmatis* under different stress conditions, bacterial suspensions were adjusted to a concentration of 1 × 10^7 c.f.u. ml⁻¹. To assess the effect on *M. smegmatis* cell wall permeability by malachite green, equal numbers of Ms rv2626c or Ms_vec were plated onto 7H10 plates with or without 1 mg malachite green l⁻¹. To test the susceptibility of strains to nitrosative stress, Ms rv2626c or Ms_vec was incubated at pH 5.4 with 5 or 10 mM NaNO₂ for 2 or 4 h. To test the susceptibility of *M. smegmatis* strains to SDS and lysozyme, these were treated with 0.1 % (w/v) SDS or 2500 µg lysozyme ml⁻¹ for various times. For the pH stress assays, cells were harvested, washed with M7H9 (pH 3 or 5.4) and then resuspended at a concentration of 1 × 10^7 c.f.u. ml⁻¹ in 5 ml M7H9 (pH 3 or 5.4) for 3, 6 or 9 h. At certain time points, 100 µl samples were removed to determine the viable bacteria.

**Determination of antibiotic susceptibility**

Drug sensitivity was tested by resazurin microplate assay (REMA), and the final drug concentrations were as follows: 0.01 to 16 µg ml⁻¹ for isoniazid, 0.05 to 50 µg ml⁻¹ for rifampin, 0.01 to 16 µg ml⁻¹ for ofloxacin and 0.24 to 500 µg ml⁻¹ for ampicillin. Triplicate wells were inoculated for each concentration of antibiotics. Approximately 5 × 10^5 c.f.u. well⁻¹ were exposed to the twofold serial dilutions in 96-well plates, with the control wells containing only bacteria and medium. The plates were incubated at 37 °C for 3 days and then 0.02 % (w/v) resazurin was added, after which the minimum inhibitory concentration (MIC) was recorded as the minimum concentration at which no colour change was visualized after 48 h. The MICs were also determined on M7H10 plates containing twofold dilutions of the antibiotics. The MIC was defined as the lowest concentration that inhibited more than 99 % of the bacteria.

**Intracellular survival assay**

Intracellular viability of the Ms rv2626c was determined using macrophage infection studies. ANA-1 macrophages were seeded at a density of 1 × 10⁶ cells well⁻¹ in 12-well culture plates. Cells were incubated overnight, washed twice with RPMI-1640 medium and then infected with Ms rv2626c or Ms_vec at a multiplicity of infection (m.o.i) of 1 : 10 macrophages to bacteria. After being incubated for 4 h, the supernatants were removed and the wells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) to remove extracellular bacteria, and then fresh RPMI-1640 medium was added. Cells were harvested post-infection at 6, 24, 48 or 72 h and were lysed with PBS solution supplemented with 1 % (v/v) Triton X-100 (Sigma). Lysates were 10-fold serially diluted with sterile water and plated onto M7H10 plates. Viable intracellular bacterial counts were enumerated after the plates had been incubated for 5 days. For gamma interferon (IFN-γ) activation experiments, macrophages were treated with 100 IU of recombinant murine IFN-γ ml⁻¹ (Sigma) 16–20 h prior to infection.

**Purification of the recombinant Rv2626c protein**

Recombinant His-tagged Rv2626c (rRv2626c) was expressed in *E. coli* BL21 (DE3) according to a published protocol [10]. Briefly, the open reading frame (ORF) of the rv2626c gene was PCR-amplified from pMV361-rv2626c using the forward primer r26c-F (5'-GGAAATTGGATGACGACCG-3'), containing an EcoRI site (underlined), and the reverse primer r26c-R (5'-CACCTGATTGCGACCGAC-3'), and then the product was cloned into the bacterial expression vector pET-22b (+) (Invitrogen). The protein was purified by His-tag affinity chromatography on a HisTrap
FF column (GE Healthcare) and eluted in elution buffer containing 200 mM imidazole. The purified rRv2626c was dialysed against PBS (pH 7.4) and then purified with a TOXINer Eraser endotoxin removal kit (GenScript) to remove any endotoxin contamination before filter sterilization. Protein concentrations were determined using the BCA protein assay reagent kit (Thermo) and samples were stored at −70 °C until further use. To examine the effect of endotoxin or lipopolysaccharide (LPS) in the Rv2626c protein preparation, Rv2626c protein was heated for 30 min at 99 °C before being added to the macrophage cultures.

**Assays for cytokines and nitric oxide production**

ANA-1 cells were infected with Ms_rv2626c or Ms_vec at an m.o.i of 10 for 6 or 24 h. The concentration of TNF-α, IL-6, IL-1β, IL-12p70, IL-1 and IFN-γ in the culture supernatants was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience), and NO was assayed using the Griess reagent system (Promega). At 24 h post-infection, total RNA was extracted from macrophages as previously described [29]. The DNase-treated total RNA (1 µg) was transcribed into cDNA using an oligo (dT)18 primer with the RevertAid First Strand cDNA synthesis kit. Real-time PCR was performed on the CFX96 touch q-PCR system (Bio-Rad) with 2x SYBR Select Master Mix for CFX (Thermo) using the following primer pairs: (IL-1β) (5'-CAACGAATACCCAAA-GAGAAGA-3') (forward) and (5'-ATTAGAAACAG TCCAGCCCATAC-3') (reverse); (IL-6) (5'- TCTCTCTC TGCAAGAGACT-3') (forward) and (5'-TTGATCTTCT GAAAGGACT-3') (reverse); (TNF-α) (5'-CATGAGCAACA GAAACATGATCCG-3') (forward) and (5'-CTGAGCCA CCTAGAAAATGTGAGG-3') (reverse); (iNOS) (5'- TTCCATGCTAATGCGAAAGGTCA-3') (forward) and (5'-CAGTCTCCATCCCATAATGTGCT-3') (reverse); and (β-actin) (5'-CATCTGGCCCTCACTGTCACCAC-3') (forward) and (5'-ACTGGCTGTGCCTACCGTTC-3') (reverse). β-actin was used as an internal control for normalizing gene expression. Analyses of the relative gene expression data were performed with Bio-Rad CFX manager software using the −ΔΔCT method, with relative changes of gene expression expressed as 2−ΔΔCT.

To determine the effects of pharmacological inhibitors on Rv2626c-induced cytokine production, ANA-1 cells were incubated for 1 h with or without an inhibitor of p38 (SB203580, 20 µM), nuclear factor (NF)-κB (Bay 11–7082, 10 µM) or extracellular signal-regulated kinases (ERK) 1/2 (U0126, 20 µM) before being stimulated with purified rRv2626c protein. As a control, 0.1% (v/v) dimethyl sulfoxide (DMSO) was added to some samples. After 24 h treatment, the concentrations of TNF-α, IL-1β and IL-6 were measured by ELISA.

**Measurement of the release of lactate dehydrogenase from macrophages**

Cells and culture supernatants were harvested after the infection of ANA-1 macrophages with or without *M. smegmatis* strains at an m.o.i of 10 for 6, 24 and 48 h. Lactate dehydrogenase (LDH) activity was assayed with the LDH cytotoxicity detection kit (Takara) as described in the manufacturer’s instructions. The percentage of LDH release was calculated according to a published method [30].

**In vivo infection in mice with recombinant *M. smegmatis* strains**

For the murine infection experiments, female BALB/c mice aged 5 to 6 weeks (body weight 18–25 g) were infected with *M. smegmatis* strains as described previously [20]. Each of the mice received a 100 µl (i.p.) injection of 1×10⁷ c.f.u. of Ms_vec or Ms_rv2626c. Five infected mice from each group were sacrificed at 3, 6 or 9 days, and the lungs, spleens and livers were aseptically harvested and homogenized in PBS. Organ homogenates were serially diluted and plated on M7H10 agar, and the survival of the *M. smegmatis* strains was determined by counting bacterial c.f.u. after incubation for 5–7 days.

**Statistical analyses**

Statistical analyses and graphing were performed with GraphPad Prism 6.0 (http://www.graphpad.com). Statistical significance was determined by an unpaired two-tailed Student's t-test. P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Ms_rv2626c constitutively expresses the *M. tuberculosis* Rv2626c protein**

In this study, we generated two recombinant *M. smegmatis* strains, Ms_rv2626c and Ms_vec, to investigate the putative function of Rv2626c. Strain Ms_rv2626c was engineered to express a 6×His-tagged Rv2626c protein from a recombinant pMV361 vector. Strain Ms_vec harboured an empty vector. Since pMV361 contains the kanamycin resistance gene aph, both recombinant strains express the gene when cultured in M7H9 medium in the presence of kanamycin. However, only Ms_rv2626c had the ability to express the rv2626c gene, as detected by reverse transcription PCR (RT-PCR) (Fig. 1a).

Western blot analyses of whole-cell lysates (WCL) using the anti-6×His tag antibody detected two protein bands with molecular weights of approximately 16 kDa and 32 kDa (Fig. 1b), which is consistent with a previous observation that Hrp-1 migrated by SDS-PAGE as two major bands, corresponding to the monomer and dimer forms of the protein [31]. These results indicate that the Rv2626c protein from *M. tuberculosis* was successfully heterologously expressed in *M. smegmatis*.

**Rv2626c protein accelerates the growth rate and enhances the resistance of *M. smegmatis* to hypoxia**

The Rv2626c protein is similar to the *B. subtilis* YlbB protein, a homologue of IMP dehydrogenase [32]. It has been reported that IMP dehydrogenase is associated with cell proliferation [33]. The expression of the Rv2626c protein is upregulated during the transition of *M. tuberculosis* into persistent state,
suggesting that it might affect the proliferation of the bacterium. To assess whether expression of Rv2626c alters the growth of \textit{M. smegmatis} under aerobic conditions \textit{in vitro}, wild-type \textit{M. smegmatis}, Ms_rv2626c and Ms_vec were cultured in enriched M7H9 broth. When cultures were agitated, Ms_rv2626c exhibited a consistently increased growth rate compared to the wild-type parent or the Ms_vec strain (Fig. 2a). The wild-type parent and the Ms_vec strain exhibited no significant difference in growth (Fig. 2a). These results suggest that Rv2626c is able to accelerate the growth rate of \textit{M. smegmatis} under aerobic conditions \textit{in vitro}.

The rv2626c gene is a latency-associated gene from the dormancy regulon and may be involved in the response to hypoxic stress conditions. To validate this hypothesis, \textit{M. smegmatis} strains were cultured under hypoxic conditions as described for the Wayne model. When the methylene blue started to fade, Ms-rv2626c showed a higher c.f.u. value compared with the Ms_vec strain (Fig. 2b). At the decolorization time point (Ms_rv2626c and Ms_vec culture decolorization occurred approximately 30 and 36 h after inoculation, respectively), there were no significant differences in c.f.u. counts between Ms_rv2626c and Ms_vec (Fig. 2b). At 30 or 60 h after the methylene blue completely decoloured, the Ms_rv2626c strain showed a higher c.f.u. value compared with the Ms_vec strain (Fig. 2b), suggesting that \textit{M. smegmatis} harbouring the rv2626c gene acquired a better adaptability to hypoxia.

\textbf{Rv2626c contributes to the intracellular survival of \textit{M. smegmatis} in macrophages}

We assessed the intracellular survival of bacteria in resting and IFN-\gamma primed ANA-1 macrophages within 72 h. As

![Fig. 1. Ms_rv2626c constitutively expressing the \textit{M. tuberculosis} Rv2626c protein. (a) Detecting expression of the rv2626c and aph gene by reverse transcription (RT)-PCR. (b) Lysates were prepared from bacterial cells and subjected to Western blot analyses to detect 6×His-tagged Rv2626c protein. M, molecular weight standards.](image1)

![Fig. 2. Growth of \textit{M. smegmatis} strains under aerobic and anaerobic conditions, and intracellular survival. (a) Aerobic exponential cultures with a starting OD\textsubscript{600} of 0.02 and gentle stirring at 200 r.p.m.; the OD\textsubscript{600} was measured at various time points. Each analysis was performed in triplicate. (b) \textit{M. smegmatis} strains were cultured in sealed bottles and a head-to-space ratio of 0.5 was maintained. The cultures were stirred at 120 r.p.m. and plated onto 7H10 agar medium after appropriate dilution at various time points. D0, D30 or D60, 0, 30 or 60 h after the methylene blue completely decoloured. (c) Survival of recombinant \textit{M. smegmatis} strains after infection of resting or IFN-\gamma-primed ANA-1 macrophages at an m.o.i of 10:1. Aliquots of infected macrophages were lysed at indicated time points. Lysates were diluted and plated on M7H10 agar plates. The data represented here are the mean values ± standard deviation (SD) of three independent experiments. ns, non-significance; *** P<0.001 by Student’s two-tailed t-test.](image2)
shown in Fig. 2(c), both strains had a reduced survival rate at the tested time points. However, the Ms_rv2626c strain had a statistically significant higher intracellular viability than the Ms_vec strain at various time points ($P<0.01$).

**Enhanced intracellular survival is not correlated with increased resistance to hostile growth factors, except nitric oxide**

Bacilli within host cells generally suffer from nutritional deficiencies, hypoxia, redox stress and acidic pH. To understand the mechanism of Rv2626c-mediated enhancement of *M. smegmatis* survival in macrophages, we evaluated the resistance of the recombinant strains under several stress conditions, including acidified sodium nitrite (5 or $10\text{mM}$ at pH 5.4), malachite green ($1\text{mg}\text{l}^{-1}$), low pH (3.0 or 5.4), SDS (0.1 % w/v) and lysozyme ($2500\mu\text{g}\text{ml}^{-1}$). In tests assessing the susceptibility to reactive nitrogen intermediates (RNI), all strains were killed by NO in a time- and dose-dependent manner (Fig. 3a). However, the Ms_rv2626c strain treated with acidified sodium nitrite showed a higher viability than the Ms_vec. Furthermore, this significant difference was not due to the acidic pH (5.4) (Fig. 3b). For the other hostile factors tested, the Ms_rv2626c showed no increase in the resistance to acidic pH, lysozyme, malachite green or SDS (Fig. 3b–d). To evaluate whether the accumulation of Rv2626c alters sensitivity to drugs, the MICs to rifampicin, isoniazid, ofloxacin and ampicillin were determined by resazurin microplate assay and confirmed on solid medium. The results showed that the MIC of strain Ms_rv2626c for ofloxacin increased eightfold ($0.25$ vs $2\mu\text{g}\text{ml}^{-1}$), while the MICs for the other three drugs (rifampicin, isoniazid and ampicillin: $12.5$, $1$ and $125\mu\text{g}\text{ml}^{-1}$, respectively) had the same values as the control.

**Fig. 3.** Sensitivity of *M. smegmatis* strains to stress conditions. *M. smegmatis* strains were treated with (a) acidified nitrosative stress ($5$ or $10\text{mM}$ at pH 5.4), (b) acidic pH (3 or 5.4), (c) lysozyme ($2500\mu\text{g}\text{ml}^{-1}$), (d) malachite green ($1\text{mg}\text{l}^{-1}$) or (e) SDS (0.1 %) for various times. 100 µl samples were removed and serially diluted, and aliquots were plated onto 7H10 agar plates. The data represented here are the mean values ± standard deviation (SD) of three independent experiments. ns, non-significance; **, $P<0.01$ by Student’s two-tailed t-test.
Rv2626c promotes the release of pro-inflammatory cytokines from infected macrophages

To investigate the potential effect of Rv2626c on altering the immune response of infected macrophages, an in vitro assay using a macrophage infection model was utilized [34]. We found that Ms_rv2626c induced significantly higher levels of TNF-α, IL-1β and IL-6 in infected macrophages compared with Ms_vec (Fig. 4a-c), while similar results were obtained when ANA-1 cells were stimulated with recombinant His-tagged Rv2626 protein. The purity and molecular size of the recombinant protein were assessed by SDS-PAGE (Fig. S1, available with the online Supplementary Material). ANA-1 macrophages stimulated with recombinant Rv2626c protein (5 µg ml⁻¹) also secreted significantly higher levels of TNF-α, IL-1β and IL-6 compared with the control (Fig. 5a-c). Furthermore, heat denaturation abrogated the ability of rRv2626c to trigger cytokine secretion, while the heat-treated LPS maintained the ability to induce TNF-α, IL-1β and IL-6 secretion in macrophages (Fig. 5a-c), which indicates that cytokine secretion was induced by Rv2626c and not by LPS contamination. At 24 h post-infection, real-time PCR analyses showed that the expression of IL-1β, TNF-α and IL-6 mRNA in macrophages infected with Ms_rv2626c was increased compared with the expression in macrophages infected with Ms_vec (Fig. 4e), suggesting that rv2626c alters the expression profiles of these mRNAs in macrophages. It has been reported that rv2626c elicits the type-1 immune response, as manifested by IL-12, IFN-γ and IL-2 [10], but unfortunately we could not detect any of these proteins by ELISA. In addition, Ms rv2626c induced significantly higher levels of NO- and iNOS-related mRNA compared to Ms_vec (Fig. 4d, e), which is consistent with a previous observation that recombinant Rv2626c (rRv2626c) upregulates NO production and iNOS expression [10]. Collectively, our data suggest that Rv2626c promotes the release of pro-inflammatory cytokines from infected macrophages.

Rv2626c-induced cytokine release may involve the p38/ERK1/2/NF-κB signal pathway

It has been reported that the induction of iNOS expression and NO production by rRv2626c is mediated through the nuclear factor (NF)-κB-dependent pathway [10]. To clarify
Fig. 5. Rv2626c-induced cytokine release may involve the p38/ERK1/2/NF-κB signal pathway. To detect cytokine production by Rv2626c-treated ANA-1 macrophages, ANA-1 macrophages were stimulated with rRv2626c protein (5 µg ml⁻¹) or LPS (1 µg ml⁻¹) as a positive control for 24 h. The levels of IL-1β (a), IL-6 (b) and TNF-α (c) in culture supernatants were measured by ELISA. To examine the effect of endotoxin in the Rv2626c protein preparation, heat-treated LPS (H+LPS, 1 µg ml⁻¹) or rRv2626c protein (H+rRv2626c, 5 µg ml⁻¹) was incubated with macrophages for 24 h. The levels of IL-1β (a), IL-6 (b) and TNF-α (c) in culture supernatants were also measured by ELISA. ANA-1 cells were incubated for 1 h with or without an inhibitor of p38 (SB203580, 20 µM), nuclear factor (NF)-κB (Bay 11–7082, 10 µM) or extracellular signal-regulated kinases (ERK) 1/2 (U0126, 20 µM) before being stimulated with recombinant M. smegmatis strains or purified rRv2626c protein. Treatment with DMSO served as a control. After 1 h, the macrophages were infected with recombinant M. smegmatis strains (m.o.i.=10) or treated with rRv2626c (5 µg ml⁻¹). Culture
supernatants were harvested after 24 h of treatment and the concentrations of IL-1β, IL-6, TNF-α and NO were determined. (d), (e) and (f) were performed with recombinant Ms smegmatis strains; (g), (h) and (i) were performed with recombinant protein. The data represented here are the mean values ± standard deviation (SD) of three independent experiments. ***, P<0.01 by Student’s two-tailed t-test.

the functional roles of mitogen-activated protein kinases (MAPKs) and NF-κB in the activation of macrophages induced by Rv2626c, ANA-1 cells were treated with or without pharmacological inhibitors before being exposed to Ms smegmatis strains. Pharmacological inhibition data suggested that the inhibition of p38 and ERK1/2 reduced Ms smegmatis-induced secretion of the cytokines IL-1β, TNF-α and IL-6 from macrophages, and that secretion of these cytokines was almost completely abrogated by the NF-κB inhibitor (Fig. 5a–f). Specific signalling pathway inhibitors revealed that the ERK1/2, p38 and NF-κB pathways are involved in Ms smegmatis-induced TNF-α, IL-1β and IL-6 production in macrophages, and indicated that Rv2626c-induced secretion of cytokines may be dependent on those signalling pathways. To test this possibility, inhibitor-treated ANA-1 macrophages were stimulated with rRv2626c. rRv2626c-induced TNF-α, IL-1β and IL-6 were significantly inhibited in ANA-1 macrophages pre-treated with the specific NF-κB inhibitor (Bay 11–7082), ERK 1/2 inhibitor (U0126) or p38 inhibitor (SB203580) (Fig. 5g–i), suggesting that the NF-κB, ERK1/2 and p38 signal pathways are required for Rv2626c-induced TNF-α, IL-1β and IL-6 production by macrophages.

**M. smegmatis expressing Rv2626c leads to increased cell necrosis during infection**

To test the effect of Rv2626c expression in Ms smegmatis on macrophage viability, ANA-1 cells were infected with the recombinant Ms rv2626c or with the control strain. We found that both strains induced the release of considerable amounts of LDH, however, Ms rv2626c induced significantly higher levels of LDH than Ms vec at the 24 and 48 h time points (Fig. 6), indicating the propensity of macrophages to undergo necrosis in response to infection by Ms rv2626c, and that Rv2626c is partially responsible for this effect. Our results are consistent with previous observations that when the Rv2626c gene is deleted from the genome of M. tuberculosis, the bacterium displays significantly less necrosis in THP-1 cells, while, conversely, the overexpression of Rv2626c promotes host cell necrosis at early time points of infections, in contrast to the wild-type strain [12].

**Rv2626c enhances the viability of recombinant Ms rv2626c in mouse tissues**

A medium-dose i.p. mouse infection model was used to investigate the further impact of Rv2626c on the survival of Ms smegmatis in vivo [20]. The Ms rv2626c and Ms vec strains were intravenously injected into BALB/c mice, with 1x10⁷ c.f.u. of bacilli per mouse. When compared with Ms vec, we observed a significantly increased bacterial burden in the mouse liver (Fig. 7a), spleen (Fig. 7b) and lung (Fig. 7c) tissues infected with Ms rv2626c at various time points. Compared with the results of PE_PGRS 33 and PE3 expression in Ms smegmatis [20, 35], we put forward the same conclusion that the Ms rv2626c strain displayed enhanced infection and/or an increased persistence in mouse organs compared to the Ms vec strain.

**DISCUSSION**

*M. tuberculosis* is able to establish a long-term asymptomatic persistent infection in its host that is dependent on complex mechanisms of dormancy. Many investigations have indicated that a set of genes regulated by DosR/DosS participates in this process [36, 37]. The rv2626c gene of *M. tuberculosis*, encoding a conserved hypothetical protein, has one of the most strongly induced transcripts of the dormancy regulon. In the current study, we found that the expression of Rv2626c in *M. smegmatis* gave the bacteria some novel properties that may promote survival in hypoxic niches both *in vitro* and *in vivo* (e.g. hypoxic conditions *in vitro*, macrophages, mouse tissues) and under NO stress conditions, as well as eliciting stronger release of the pro-inflammatory cytokines and NO. Our results are consistent with the predicted functions of a protein that is associated with adaptation to hypoxia during infection. The Rv2626c protein may have several functions that allow *M. tuberculosis* to initiate a latent infection.
Rv2626c may be associated with cell proliferation and respiratory functions under hypoxic conditions

In this study, over-expression of Rv2626c significantly accelerated the growth rate of *M. smegmatis* under normal conditions and enhanced its tolerance to hypoxia. Using the TB database, we found that Rv2626c contains two CBS domains that could possibly be associated with the maintenance of redox balance and ATP homeostasis during hypoxia [13–15, 38]. During normal growth conditions, Rv2626c tends to be expressed under hypoxic conditions. Therefore, Rv2626c might be involved in respiratory functions and ATP production under hypoxic conditions, possibly via an effect on metabolism or at the level of ATP biosynthesis.

Rv2626c-induced cytokines and NO may mediate the complex pathogen–host relationship

Secreted proteins of *M. tuberculosis* have long been known to be a rich source of immunogens, such as the CFP-10/ESAT-6 pair and Ag85a, and have the ability to regulate the innate immune system of hosts as immunomodulators [10]. Rv2626c, as a secreted protein, was found to induce a T helper type 1 (Th-1)-mediated immune response (IFN-γ/iNOS induction) in a mouse model of latency [39] and in peripheral blood mononuclear cells from *M. tuberculosis*-infected patients [31, 40]. Consistent with the cytokine profiles reported elsewhere for macrophages infected with *M. smegmatis* [41, 42], ANA-1 macrophages infected with *M. smegmatis* also produced TNF-α, IL-6 and IL-1β, as well as NO. For IL-12, IFN-γ and IL-2, we could not detect any signals by ELISA, which may be due to their concentrations being lower than the kit can detect. In our study, Rv2626c triggered the secretion of pro-inflammatory cytokines (IL-1β, IL-6, NO and TNF-α) that are essential for cell recruitment, granuloma formation and maintenance [43, 44]. The pro-inflammatory responses elicited by Rv2626c might be significant in understanding the role of dormancy-related proteins in the maintenance of the latent state of tuberculosis.

During *M. tuberculosis* infection, pro-inflammatory cytokines such as TNF-α and IL-1β and IL-6, as well as NO, are essential for anti-mycobacterial activity [45–47]. Thus, it was a paradoxical phenomenon that *M. smegmatis* harbouring the rv2626c gene simultaneously elevated the pro-inflammatory cytokine levels and had increased survival. However, the same paradoxical phenomenon was observed in several other studies [22, 34, 42]. These results may be attributed to the pleiotropic effects of cytokines and NO. NO from exogenous sources or activated murine macrophages exhibits anti-mycobacterial properties, and can irreversibly damage bacteria [47]. However, NO also induces an *M. tuberculosis* dormancy programme by reversible inhibition of respiration that is expressed in *vivo* and adapts the organism for survival during extended periods of *in vitro* dormancy [29]. Although there is a great deal of evidence for protective roles for TNF-α or IL-6 [48–50], treatment of infected macrophages with neutralizing anti-TNF or anti-IL-6 antibodies reduced the growth rate of intracellular mycobacteria, and bacterial replication was augmented by the addition of TNF or IL-6 [51, 52]. From this result, it can be seen that Rv2626c has two roles: stimulation of the release of cytokines, and promotion of survival. Enhanced intracellular survival of mycobacteria may depend on the regulation of a variety of factors. However, the complex mechanisms credited with the greater ability of *M. tuberculosis* to survive in host cells remain unclear.

Rv2626c may be redundant and promote the survival of mycobacteria

In this study, we observed that, *M. smegmatis* harbouring the rv2626c gene gained significantly enhanced survival ability in macrophages and *in vivo*. According to recent
In this study, there were significant differences between M. _svec_ and M. _rv2626c_ for certain phenotypes, even though the differences were small, e.g., small differences in f.u. numbers and cytokine levels, which may bring into question their biological significance. However, our results still provide some valuable clues for further study of the precise function of Rv2626c. In summary, in the present study we investigated the expression of Rv2626c and its role in enhancing the survival of _M. smegmatis_ under different situations, as well as in promoting necrosis and augmenting the pro-inflammatory response of macrophages. This study revealed that Rv2626c may be involved in the metabolism under hypoxic conditions and complex pathogen–host interactions.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All the animal experiments were approved by and carried out under the guidelines of the Institutional Animal Ethics Committee of Sichuan University. Animals were sacrificed by cervical dislocation, and all efforts were made to minimize suffering.

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