Expression of Rv2031c-Rv2626c fusion protein in *Mycobacterium smegmatis* enhances bacillary survival and modulates innate immunity in macrophages

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Abstract. Dormancy-associated antigens encoded by the dormancy survival regulon (DosR) genes are required for survival of *Mycobacterium tuberculosis* (Mtb) in macrophages. However, mechanisms underlying survival of Mtb in macrophages remains to be elucidated. A recombinant *Mycobacterium smegmatis* strain (rMs) expressing a fusion protein of two dormancy-associated antigens Rv2031c and Rv2626c from Mtb was constructed in the present study. In an *in vitro* culture, growth rate of rMs was lower compared with Ms. A total of 24 h following infection of murine macrophages with rMs or Ms, percentage of viable cells decreased and the number of bacteria in viable cells increased compared with Ms, demonstrating that virulence and intracellular survival of rMs were enhanced. Compared with macrophages infected with Ms, necrosis of macrophages infected with rMs was increased, while apoptosis was inhibited. Macrophages infected with rMs secreted more interferon-γ and interleukin-6, but fewer nitric oxide and tumor necrosis factor-α, compared with macrophages infected with Ms. The present study demonstrated that the fusion protein composed of dormancy-associated antigens Rv2031c and Rv2626c in Ms serves a physiological function of a dormancy-associated antigen and modulates innate immunity of host macrophages, therefore favoring intracellular bacillary survival.

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Introduction

Tuberculosis (TB) is a worldwide public health concern caused by *Mycobacterium tuberculosis* (Mtb), and ~ a third of the world's population is latently infected with Mtb (1). The majority of infected people do not present symptoms immediately, but ~10% of those people develop an overt disease later in their lives. Latently infected individuals represent a reservoir of infection and potential reactivation of TB can be a source of transmission (2). Macrophages represent a primary target of infection and the most frequently infected cell type by Mtb in host individuals. The initial interaction between macrophages and Mtb determines the outcome of infection, but the mechanism underlying the interaction between macrophages and Mtb remains to be elucidated (3).

It has been demonstrated that during the latency period, Mtb remains in a dormant or non-replicating state, and the dormancy survival regulon (DosR), composed of 48 co-regulated genes, is necessary for survival of dormant Mtb (4). However, the role of dormancy-associated antigens in mediating interactions between Mtb and macrophages remains to be elucidated. The 16-kDa α-crystallin protein, (Rv2031c), also known as hspX, acr and Hsp16.3, is a predominant protein produced by Mtb, accounting for up to 25% of all proteins expressed during dormancy of Mtb (5). Rv2031c can be identified by mass spectrometry in culture filtrates, membrane protein fractions and whole cell lysates of Mtb (6). Rv2031c has been hypothesized to enhance long-term stability of proteins and cell structures, which in turn aids in maintaining long-term survival of Mtb (7). Rv2626c is a hypoxic response protein encoded by Mtb open reading frame *Rv2626c*. Rv2626c is also one of the highly expressed proteins by Mtb in hypoxic conditions and can be identified in culture filtrates and lysates of Mtb (8), but the role served by Rv2626c remains to be elucidated.

In the present study, a fusion protein of Rv2031c and Rv2626c was expressed in a non-pathogenic, fast growing *Mycobacterium semegmatis* (Ms), to describe the physiological function of the fusion protein in mycobacteria and to investigate its immuno-modulatory functions in macrophages.

Materials and methods

Strains of bacteria, media and growth conditions. The Ms strain MC^2 155 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Bacillus Calmette-Guérin (BCG) was obtained from Shaanxi Research Institute for Tuberculosis Control and Prevention (Shaanxi, China). Mycobacterial strains Ms mc^2155 and BCG were cultured in Middle brook 7H9 broth and 7H10 agar (Difco Laboratories, Detroit, MI, USA) containing albumin dextrose complex [5 g bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) 2 g glucose and 0.85 g NaCl/l], 0.5% volume/volume (v/v) glycerol and 0.05% Tween-80. *E. coli* DH5α (Takara Biotechnology Co., Ltd., Dalian, China) was cultured in Luria Bertani media (Takara Biotechnology Co., Ltd.). Both *E. coli* and mycobacteria were cultured at 37˚C in an incubator, with agitation. Hygromycin (Sigma-Aldrich; Merck KGaA) was added to certain treatment groups: 50 mg/ml to the *E. coli* culture and 15 mg/ml to the mycobacteria culture. All recombinant (r)Ms strains were cultured in the presence of 15 mg/ml hygromycin.

Construction of rMs strain expressing rv2031c-rv2626c fusion protein. In order to construct a rMs strain expressing Rv2031c-Rv2626 fusion protein, an expression vector was constructed by cloning Rv2031c and Rv2626 genes into the *E. coli-Mycobacterium* shuttle vector pDE22 (constructed in-house) (Fig. 1) (9). Primers were designed based on nucleotide sequences of Rv2031c and Rv2626c genes from the Mtb H37Rv strain. Rv2031 gene was amplified using the following primers: 5'-CGGGATCCATGGCCACCACCCTTC-3' (*Bam*HI site underlined; forward) and 5'-AGCGATATCGTTGGTGGA CCGG-3' (*Eco*RV site underlined; reverse). Rv2626c gene was amplified using the following primers: 5'-AGCGATATC*GGT GGCGGTAGCGGCGGTGGCTCCGGCGGTGGCAGCGGT GGCGGTAG*CACCACCGCACGC-3' (*Eco*RV site underlined; forward) and 5'-AGCAAGCTTCTAGCTGGCGAGGGC-3' (*Hin*dIII site underlined; reverse). A 48-base pair sequence encoding a hydrophobic linker (italics) was added in the linker sequence between the 3'end of *Rv2031c* and the 5'end of *Rv2626c* to ensure the correct folding of each protein. The following thermocycling conditions were used for the polymerase chain reaction (PCR): Following an initial denaturation at 95˚C for 1 min, 30 cycles of 94˚C for 45 sec, 65˚C for 45 sec, 72˚C for 50 sec; and a final extension at 72˚C for 5 min. Sequences of all resulting PCR products were validated by Sunny Biotechnology Co. (Westmont, IL, USA) and the correct sequences were identical to those reported by the GeneBank database. PCR products corresponding to each gene were cloned into the multiple cloning site region of the shuttle vector pDE22 using restriction endonucleases. The resulting recombinant plasmids were transfected into competent Ms cells by electroporation, and the transformed Ms were selected on solid 7H10 agar containing hygromycin (50 μg/ml; Sigma-Aldrich, Merck KGaA) for 3 days. Following selection, hygromycin-resistant colonies were transferred to fresh middlebrook 7H9 media with 15 mg/ml hygromycin. The optical density was measured at a wavelength of 600 nm (OD_{600nm}) and when a colony reached OD_{600nm} = 1.0, cells were incubated at 42˚C for 4 h.

A total of 2 ml of each cell culture was harvested by centrifuging at 8,000 x g for 20 min at room temperature. The supernatant was transferred into an Amicon ultrafiltration tube with a membrane NMWL of 10 kDa, centrifuged at 3,000 x g at 4˚C until approximately 10 μl fluid remained in the chamber, before adding 100 μl sterile-distilled water and centrifuged again at 3,000 x g at 4˚C until ~10 μl fluid remained in the chamber. After centrifugation, the upper chamber of the unltrfiltraion tube was transferred to a fresh mirofuge tube and centrifuged at 3,000 x g at 4˚C for 2 min, and the volume in the tube was determined and added to an equal volume of 2X SDS-PAGE sample buffer. The cell pellet was resuspended in 1 ml sterile-distilled water and re-centrifuged at 8,000 x g for 10 min at room temperature, then resuspended in 100 μl sterile-distilled water and sonicated on ice using 4 pulses for 15 sec on maximum output, then 100 μl 2X SDS-PAGE sample buffer was added. A total of 10 μl samples were loaded onto an 12% SDS-PAGE gel and proteins from gel were electrotransferred to a polyvinylidene difluoride membrane (pore size 0.2 μm; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 70 V for 2 h at 4˚C in Tris-Glycine transfer buffer composed of 25 mM Tris, 192 mM glycine and 20% methanol at pH 8.3.

For immunoblotting, non-specific binding sites were blocked with PBS containing 5% non-fat milk for 1 h at room temperature. Blocked membranes were incubated overnight at 4˚C in PBS with mouse anti-Rv2031c monoclonal antibody (cat. no. ab64786, dilution 1:500), mouse anti-Rv2626c monoclonal antibody (cat. no. ab64786, dilution 1:500) (both from Abcam, Cambridge, UK). Washed membranes were incubated 1 h at room temperature with IRDye 800CW anti-mouse antibody (1:5,000; LI-COR Bioscience, Lincoln, NE, USA), washed and immunodetection was performed using an ODYSSEY Infrared Imaging system (LI-COR Bioscience). Following screening, positive recombinant Ms strains were classified as rMs.

In vitro growth kinetics of rMs. To examine the growth pattern of rMs, rMs and Ms strains were cultured until late exponential phase, diluted to $OD_{600\ nm}$ = 0.2 and cultured in Middlebrook 7H9. Growth curves were generated by measuring alterations in $OD_{600\ nm}$ over time for 57 h.

Macrophage infection. RAW264.7 murine macrophages (provided by Dr. Shi CH, the Fourth Military Medical University, Xi'an, China) were cultured at 37˚C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 1% L-glutamin and antibiotics [60 mg/ml penicillin G sodium, 50 mg/ml streptomycin sulphate and 30 mg/ml gentamycin sulphate, purchased from Leagene Co., Beijing, China; www.leagene.bioon.com.cn). Cells were seeded in 6-well plates at a density of $0.5x10^5$ cells/well and used for infection 24 h later. Exponentially growing bacteria cultured in the presence of 15 mg/ml hygromycin were pelleted, washed and resuspended in DMEM (without antibiotics) to $OD_{600\ nm}$ = 1.0. Single cell suspensions of rMs and Ms strains were obtained by passing cultures ~5-6 times through 26 gauge needles. Bacillary viability was assessed at each step by colony-forming unit (CFU) counts. Equal numbers of each strain were used to infect macrophages at

a multiplicity of infection (MOI) =10:1, selected based on pilot infections (data not shown) with multiple MOIs that we performed with cell lines used in the present study. Following incubation with bacteria for 4 h, non-phagocytosed bacteria were washed off using PBS. Cells were washed with PBS and post-infection CFU counts were determined by lysing infected cells. Subsequently, complete DMEM containing gentamycin (Gibco; Thermo Fisher Scientific, Inc.) was added to eliminate extracellular bacteria. Infected cells were transferred to fresh DMEM and incubated for 24 h at 37˚C with 5% CO_2. Following the incubation, 20 μl MTT (Sigma-Aldrich; Merck KGaA) was added to each sample. Following 4 h of incubation with MTT, dimethyl sulfoxide was added and the samples were incubated at 37˚C for 10 min. Each sample was observed under an optical microscope and the absorbance was measured at a wavelength of 490 nm on a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). CFU counts were performed at 3, 6, 12 and 21 h post infection by lysing $1x10^3$ infected cells with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) followed by dilution plating on Middlebrook 7H10 agar, the results were expressed as $\log_{10}$ CFU/10^3 cells.

Apoptosis and necrosis of macrophages. RAW264.7 murine macrophages (10^5 cells) were left uninfected as controls or infected with Ms or rMs at 10:1 MOI for 24 h. Macrophages were subsequently removed from plates using accutase solution (Sigma-Aldrich; Merck KGaA), washed twice in ice-cold PBS and stained with propidium iodide (PE)-conjugated Annexin V and 7-aminoactinomycin D (7AAD), according to the manufacturer's protocol (BD Biosciences). Cells were fixed in PBS containing 5% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature and analyzed with a FACSCanto II cytometer and FACSDiva software (version 6.1.2; BD Biosciences). Apoptosis was expressed as the percentage of Annexin V-positive 7-AAD-negative cells, and necrosis was expressed as the percentage of Annexin V and 7-AAD-double positive cells.

Cytokine and nitrite assays. Levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in macrophage culture supernatants 24 h post infection were quantitated with a mouse IFN-γ ELISA development kit (cat. no. 3321-1H-6), mouse TNF-α development kit (cat. no. 3511-1H-6) and mouse IL-6 ELISA development kit (cat. no. 3361-1H-6) according to the manufacturer's instructions. All the kits were purchased from Mabtech AB, Stockholm, Sweden. Estimation of nitric oxide (NO) levels was performed using the Griess test. Equal volumes of cell culture supernatants were transferred in duplicate into 96-well culture plates and mixed with an equal volume of Griess reagent, composed of 1% weight/volume (w/v) sulphanilamide, 0.1% (w/v) napthyl-ethylenediamine hydrochloride and 2.5% (v/v) H_3PO_4. Following incubation at room temperature for 5 min, the absorbance was measured at a wavelength of 540 nm using an Ultra Microplate Reader (Omega Bio-Tek, Inc., Norcross, GA, USA). The concentration of nitrate was calculated using a $NaNO_2$ standard curve.

Statistical analysis. All experiments were performed in triplicate. Differences between groups were analyzed by one-way analysis of variance using SPSS software (version 15.0; SPSS, Inc., Chicago, IL, USA), followed by the Fisher-Tukey least significant difference post hoc test. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Figure 1. Schematic representation of Rv2031c and Rv2626c fusion construct generated in the *Escherichia coli-Mycobacterium* shuttle plasmid pDE22 under the control of the constitutive hsp60 promoter.

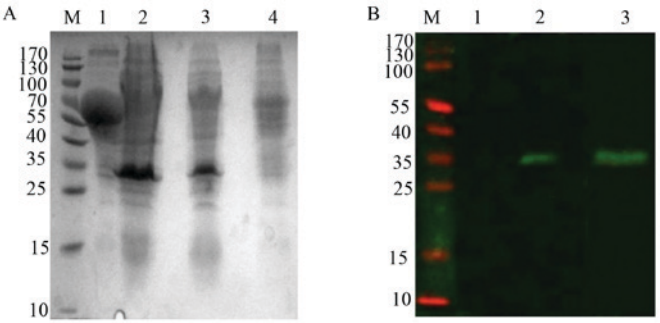

Figure 2. Expression of Rv2031c-Rv2626c fusion protein in rMs. (A) Western blot analysis of expression of Rv2031c and Rv2626c fusion protein in rMs induced by heat shock at 42˚C. Lane M, molecular weight of standard protein markers; lane 1, supernatant of rMs culture; lanes 2 and 3, cell lysates of rMs; and lane 4, the cell lysate of Ms. (B) Western blot analysis of the fusion protein by anti-Rv2031c mAb and anti-Rv2626c mAb. Lane M, pre-stained protein markers; lane 1, the cell lysate of Ms; lane 2, cell lysate of rMs stained with an anti-Rv2031 mAb; and lane 3, cell lysate of rMs stained with anti-Rv2626c mAb. mAb, monoclonal antibody; rMs, recombinant *Mycobacterium smegmatis*.

Results

Expression of Rv2031c-Rv2626c fusion protein in rMs. Total proteins from whole cell lysates of rMs and Ms strains were obtained following induction at 42˚C. Western blot analysis revealed that a specific expression band ~34 kDa, corresponding to the combined molecular weight of Rv2031c (16.3 kDa) and Rv2626c (16 kDa), was present in the cell lysate of rMs, and absent in Ms cells (Fig. 2A). The above results were further confirmed by western blot analysis with anti-Rv2031c and anti-Rv2626 monoclonal antibodies, which indicated that both Rv2031c and Rv2626c were correctly folded in the Rv2031c-Rv2626c fusion protein (Fig. 2B).

Intracellular and in vitro growth characteristics of rMs. In order to determine whether the expression of Rv2031c-Rv2626c fusion protein in rMs alters growth characteristics of the strain, growth rates of rMs and Ms *in vitro* cultures were identified by $OD_{600\ nm}$ measurement. When log-phase cultures of rMs and Ms were allowed to grow to saturation and were equivalently diluted, the duration of the growth lag phase of rMs was significantly prolonged in rMs compared with Ms (P<0.05). The final log-phase rates were not significantly different (Fig. 3A). To identify

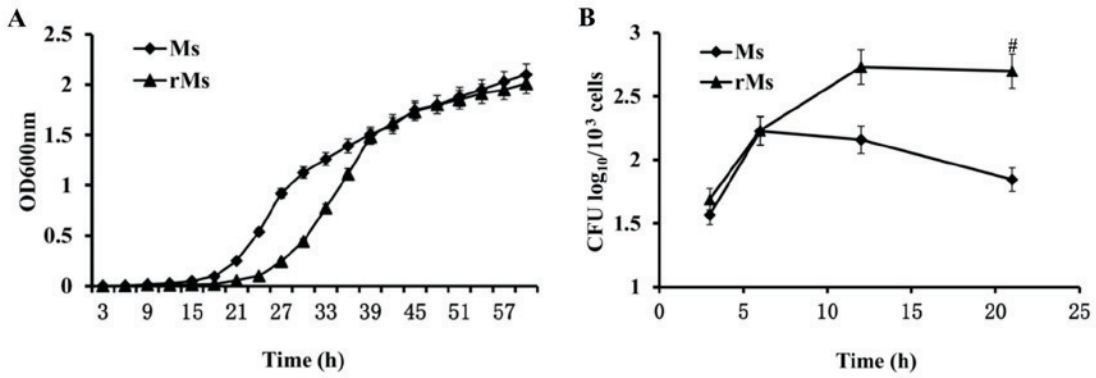

Figure 3. *In vitro* and *ex vivo* growth of rMs and Ms. (A) Growth of rMs and Ms was monitored in 3 h intervals by measuring optical density at a wavelength of 600 nm. (B) Growth of rMs and Ms in Raw264.7 murine macrophages. Data are presented as the mean ± standard deviation. #P<0.05 vs. the Ms group. rMs, recombinant *Mycobacterium smegmatis*.

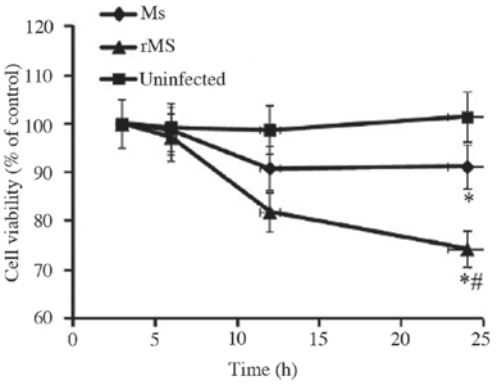

Figure 4. Viability of Raw264.7 murine macrophages analyzed by MTT. The experiment was repeated three times. *P<0.05 vs. the uninfected group; #P<0.05 vs. the Ms group. rMs, recombinant *Mycobacterium smegmatis*.

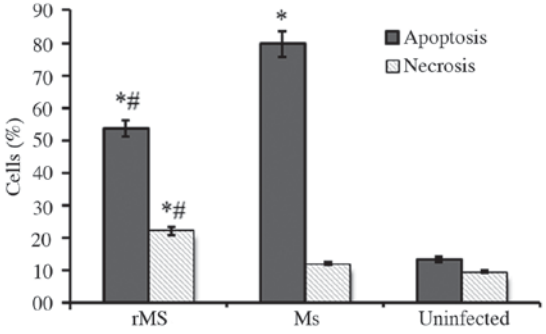

Figure 5. Apoptosis and necrosis of Raw264.7 murine macrophages infected with rMs or Ms. Percentage of apoptotic and necrotic cells was determined using a propidium iodide-conjugated Annexin V and 7-aminoactinomycin D staining and flow cytometry analysis 24 h following infection. Data are presented as the mean ± standard deviation. *P<0.05 vs. uninfected; #P<0.05 vs. Ms. rMs, recombinant *Mycobacterium smegmatis*.

intracellular growth characteristics of rMs and Ms, infectivity and intracellular survival ability of rMs and Ms in RAW264.7 murine macrophages were examined by CFU estimation of viable bacteria. The results demonstrated that similar numbers of cells (1.99±0.09 $\log_{10}$ CFU for rMs and 1.86±0.10 $\log_{10}$ CFU for Ms) were present 3 h following infection, suggesting that infectivity of rMs was unaffected by transfection. However, 21 h following infection, growth of rMs in macrophages was enhanced and the number of viable bacteria in 10^3 macrophage cells was equal to 2.70±0.14 lgCFU, which was significantly increased compared with 1.85±0.07 lgCFU in macrophages infected with Ms (P<0.05; Fig. 3B). The above results revealed a significant difference in survival ability inside macrophages between rMs and Ms, potentially associated with expression of Rv2031c-Rv2626c fusion protein in rMs.

Effect of rMs on viability of macrophages. To determine the effect of rMs on viability of macrophages, MTT analysis was performed at different time points following infection of RAW264.7 murine macrophages with rMs or Ms. A total of 24 h following infection, the viability of macrophages was equal to 78.8±3.9% in cells infected with rMs, 90.9±4.5% cells infected with Ms and 101.4±5.1% in uninfected control cells. Both rMs and Ms inhibited the viability of macrophages (P<0.05); however, the viability of macrophages infected with rMs demonstrated a significant decrease compared with Ms from 12 h onwards (P<0.05; Fig. 4). The above results indicate that rMs may be more virulent compared with Ms.

Apoptosis and necrosis of macrophages. RAW264.7 murine macrophages infected with rMs or Ms were stained with PE-conjugated Annexin V and 7AAD, and analyzed by flow cytometry to identified apoptotic and necrotic cells. A total of 24 h following infection, 50.6±3.2% macrophages infected with rMs were apoptotic and 20.7±2.2% were necrotic, while 80.2±4.6% Ms infected macrophages were apoptotic and 15.3±1.4% were necrotic. Compared with Ms, rMs significantly inhibited apoptosis and induced necrosis of infected macrophages (P<0.05; Fig. 5). The above results can be associated with expression of Rv2031c-Rv2626c fusion protein by rMS.

Modulatory effects of rMs on the innate immunity of macrophages. To identify factors contributing to the increased survival ability of macrophages infected with rMs, compared with Ms, levels of nitric oxide were measured by Griess assay. NO is a determinant of intracellular bacillary burden in host cells. Following infection, NO was down-regulated in macrophages infected with rMs, compared with macrophages infected with Ms (P<0.05; Fig. 6A). Levels of IFN-γ, IL-6 and

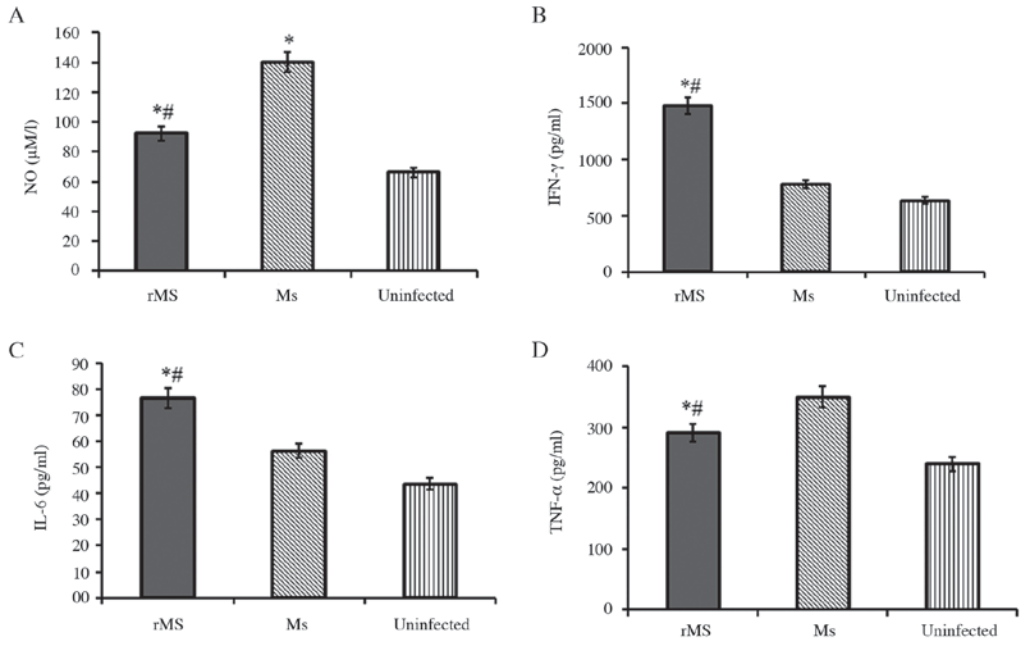

Figure 6. Innate immunity of Raw264.7 murine macrophages infected with rMs or Ms. Levels of (A) NO, (B) IFN-γ, (C) IL-6 and (D) TNF-α in culture supernatants of infected or uninfected macrophages 24 h after infection. Data are presented as the mean ± standard deviation. *P<0.05 vs. the uninfected group; #P<0.05 vs. the Ms group. rMs, recombinant *Mycobacterium smegmatis*. NO, nitric oxide; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

TNF-α in infected macrophages were determined by ELISA. The results demonstrated that secretion of IFN-γ and IL-6 in macrophages infected with rMs was significantly up-regulated (P<0.05), but levels of TNF-α were down-regulated (P<0.05), compared with macrophages infected with Ms. The above results demonstrate that expression of Rv2031c-Rv2626c fusion protein in rMs can modulate the innate immunity of macrophages infected with rMs to favor intracellular survival of rMs.

Discussion

It has been demonstrated that DosR regulon, composed of 48 co-regulated genes, is essential for the survival of Mtb in macrophages (10). Dormancy-associated antigens encoded by DosR genes in Mtb serve physiological and immuno-modulatory functions of the host immune system (11,12). In the present study, a fusion protein of dormancy-associated antigens Rv2031c and Rv2626c was expressed in a non-pathogenic strain of Ms. The results of the present study demonstrated that expression of the fusion protein Rv2031c-Rv2626c in rMs prolonged the duration of growth lag-phase of rMs *in vitro*. The aforementioned data are consistent with a previous report, in which overexpression of Rv2031c in Ms resulted in a significant lag in growth of Ms (5). In order to determine whether expression of Rv2031c-Rv2626c alters the intracellular survival ability of Ms, macrophages were infected with rMs or Ms. Compared with the Ms strain, infectivity of rMs was not affected by expression of the fusion protein Rv2031c-Rv2626c. Virulence and survival of rMs in macrophages were enhanced, the number of viable cells in macrophages infected with rMs was markedly decreased and the number of intracellular rMs bacteria increased. A previous study reported that a Mtb mutant, in which Rv2031c gene was replaced by a hygromycin resistance gene, was attenuated and demonstrated inhibited growth in a macrophage model (7); however, Hu *et al* (13) reported that increased numbers of CFU were observed in mice or macrophages that they were infected with an unmarked Rv2031c deletion mutant of Mtb when compared with the orginal Mtb strain.

It has been reported that virulent Mtb can inhibit apoptosis and trigger necrosis of host macrophages to evade innate immunity and delay the initiation of adaptive immunity (3). By contrast, attenuated Mtb and non-pathogenic mycobacteria induce apoptosis of macrophages, an innate defense mechanism that reduces bacterial viability (14,15). Therefore, in the present study, apoptosis and necrosis of infected macrophages were observed. The results of the present study demonstrated that, compared with Ms, apoptosis of macrophages infected with rMs was decreased, while necrosis was increased. NO production is an antimicrobial mechanism employed by macrophages (16). In the present study, compared with macrophages infected with Ms, macrophages infected with rMs demonstrated decreased NO levels in macrophages. Therefore, it can be hypothesized that inhibition of NO enhanced the survival of rMs in macrophages.

Macrophages eliminate invading Mtb directly and secrete cytokines to mediate host immune responses (17). The present study investigated secretion of IFN-γ, TNF-α and IL-6 from infected macrophages. The results of the present study demonstrated that levels of IFN-γ and IL-6 markedly increased compared with macrophages infected with Ms, while the levels of TNF-α decreased. TNF-α is an extrinsic mediator of apoptosis, which has been demonstrated to have a negative impact on the survival of mycobacteria within macrophages. More virulent strains appear to inhibit expression of TNF-α (18). It can be hypothesized that reduced apoptosis of macrophages

infected with rMs may be associated with inhibition of expression of TNF-α, but the underlying mechanism remains to be elucidated.

In conclusion, the present study demonstrated that expression of the fusion protein of dormancy-associated antigens Rv2031c and Rv2626c in Ms can serve a physiological function of a dormancy-associated antigen. The fusion protein also modulated the innate immunity of host macrophages, favoring intracellular bacillary survival. However, the mechanism underlying intracellular survival mediated by dormancy-associated antigens in Mtb, remain to be elucidated.

Acknowledgements

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