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Mechanisms of Pathogenesis

EspR promotes mycobacteria survival in macrophages by inhibiting MyD88 mediated inflammation and apoptosis

Chunyan Jin[1], Xiaoyu Wu[1], Chunsheng Dong, Fengge Li, Lingbo Fan, Sidong Xiong[**], Yuanshu Dong[*]

Jiangsu Key Laboratory of Infection and Immunity, Institute of Biology and Medical Sciences, Soochow University, Postal address: 199 Renai Road, SIP, Suzhou, Jiangsu, China

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ABSTRACT

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb), leading to about a million deaths each year. EspR is a DNA binding protein of Mtb which regulates expression of multiple genes and the activity of ESX-1 secretion system of the bacteria, with itself being secreted out as a substrate of ESX-1. We explored the function of secreted EspR in host cells by overexpressing the protein in murine macrophage cell line RAW264.7, infecting the cells with BCG which does not secrete EspR, and evaluating the antimicrobial responses of the cells. We found that EspR resulted in an increased intracellular bacteria load in macrophages. This is due to its inhibition on BCG induced expression of inflammatory cytokines and inducible nitric oxide synthase (iNOS), as well as host cell apoptosis. Mechanism study showed that EspR directly interacted with adaptor protein myeloid differentiation factor 88 (MyD88), suppressed MyD88 dependent Toll-like receptor (TLR) and IL-1R signal activation, thus reduced inflammatory responses and apoptosis in macrophages and promoted mycobacteria survival.

1. Introduction

Tuberculosis (TB) is a contagious chronic disease caused by *Mycobacterium tuberculosis* (Mtb). One third of population in the world is infected by Mtb, among which 1.3 million died in 2016, making TB the most deadly disease caused by a single infectious agent [40]. Although most TB patients can be cured by the first line antibiotics, the treatment lasts for 6–9 months, giving rise to poor patient compliance which not only results in treatment failure but also leads to expansion of drug-resistant strains [12,15,21,35]. In 2016, 600,000 new cases are drug-resistant [40] which can only be treated with second or third line drugs with a success rate of merely 54%. New drugs against TB are seriously needed, and this depends on a deep understanding of the interplays between Mtb and its host cells.

Mtb is an airborne pathogen invading human lung alveolus with inhalation, at where it is mostly phagocytized by pulmonary macrophages which act as both the host cells of Mtb as well as the cells eradicating the bacteria [10,25,37]. Various components of Mtb can be recognized by pattern recognition receptors (PRRs), especially Toll-like receptors (TLRs) of macrophages. For example, lipoproteins and cell wall components can be recognized by TLR2 [13,19,27,28,38], phosphatidyl inositol mannoside 6 (PIM6) by TLR4 [1], and Mtb genomic DNA by TLR9 [5]. Binding of TLRs activates their downstream NF-κB and MAPK pathways, which induce the expression of numerous antimicrobial genes, including iNOS for producing reactive nitrogen species (RNS) to kill Mtb, and inflammatory cytokines for activating macrophages and regulating the immune system. Activation of TLRs also induces host cell apoptosis [3,23,27,32,33], leading to arrest of the bacteria in apoptotic bodies which are phagocytized by standby macrophages, thus destroying the bacteria [3,9,22].

The importance of TLRs in defense against Mtb is evidenced by genetic studies. Polymorphisms on multiple TLRs show genetic association with human susceptibility to Mtb infection [2,7,24,34,41]. TLR2 mutant mice respond normally when infected by Mtb at low dosage, possibly due to redundant functions of TLRs. During high-dose infection, TLR2−/− mice produced less amount of pro-inflammatory cytokines and NO, had much higher bacteria CFU in their lungs and lower survival rate compared with the wild-type mice [14,30,39]. Reports on

[*] Corresponding author.
[**] Corresponding author.
E-mail addresses: 804465697@qq.com (C. Jin), 1810402622@qq.com (X. Wu), chunshengdong@suda.edu.cn (C. Dong), fengge.li@crownbio.com (F. Li), flb7369@126.com (L. Fan), sdxiongfd@126.com (S. Xiong), yuanshudong@126.com (Y. Dong).
[1] The authors contributed equally to this work.

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the function of TLR4 in defensing Mtb is inconsistent. TLR4 was found dispensable in initiating protective responses [30], or required to control chronic infection [1]. Myeloid differentiation factor 88 (MyD88) is a common adaptor for all TLRs except TLR3. Infection of MyD88 deficient mice with Mtb resulted in a dramatically decreased cytokine and NO production, a 2 log increase in bacteria burden, and a very short survival time of less than 4 weeks [18]. The high susceptibility of MyD88−/− mice to Mtb is not only due to their damaged TLR signaling, but also owing to their loss of IL-1R signal activation, which as well uses MyD88 as the adaptor protein and has been showed to play an essential role in the innate immunity against Mtb [17].

Mtb has developed strategies to interrupt the immunological reactions of macrophages during its long history of evolution. It secretes various virulence factors into host cells to suppress inflammatory responses, inhibit the fusion of phagosome and lysosome, and block apoptosis and autophagy of host cells to support its intracellular survival [20]. Many virulence factors are secreted out by Mtb protein secretion system ESAT-6 secretion system-1 (ESX-1), including crucial virulence factors ESAT-6 and CFP-10 which lead to Mtb phagosome escaping and intercellular spreading [36]. EspR (Rv3849) was reported to be a key regulator of the ESX-1 system [29]. It is a DNA binding protein functioning as both a transcription factor and a nucleoid-associated structural protein [8]. It regulates the expression of multiple genes including espACD operon which is necessary for ESX-1 activity [29]. EspR mutation totally blocks the protein secretion by ESX-1 system [29]. When infecting mice, Mycobacteria strain deficient of EspR grows poorly in lung and results in a ten times less bacterial burden than the wild type [29]. Crystal structure of EspR showed that its amino terminus is a helix-turn-helix DNA binding domain, and carboxyl terminus consists of hydrophobic protein-protein interaction domain [31]. Interestingly, Raghavan et al. reported that EspR itself is a substrate of ESX-1 system and is secreted out of bacteria when the system is activated [29]. Secretion of EspR lowers its intracellular concentration, leading to reduced expression of espACD and decreased activity of ESX-1 system, thus realizes a negative feedback regulation [29]. Blasco et al., however, reported that they could not detect secreted EspR [8].

Secreting transcription factors out of bacteria is an extremely rare mechanism to regulate gene transcription. We therefore suspect whether EspR secreted into macrophages, if any, has pathogenic functions against the host cells. To test this hypothesis, we first confirmed that EspR is a secreted protein of mycobacteria H37Rv. Then we overexpressed EspR protein in macrophage cell line RAW264.7 and infected the cells with BCG, a mycobacteria strain expressing but not secreting EspR, to study the effect of EspR on antimicrobial responses of host cells. We found that EspR expressed in macrophages resulted in elevated intracellular bacteria burden, reduced iNOS and pro-inflammatory cytokine expression, and decreased cell apoptosis level. Mechanism study showed that EspR inhibited NF-κB and MAPK signal activations induced by BCG, TLR2, TLR4, IL-1β but not TLR3, and the inhibition was abolished in cells deficient of MyD88, the common adaptor protein of TLR2/TLR4 and IL-1β, suggesting that EspR inhibits MyD88 mediated signal activation. Further exploration on EspR interaction proteins showed that EspR physically interacted with MyD88. These results indicated that by interacting with MyD88, EspR suppressed MyD88 dependent TLR and IL-1β signal activation, thereby promoted the intracellular survival of mycobacteria.

2. Materials and methods

2.1. Mice, cells and bacterial strains

BALB/c, C57BL/6, and MyD88 knockout mice were purchased from the Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in the animal breeding center of Soochow University. Animal experimental protocols used in this study followed the Guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and were approved by the Ethics Committee of Soochow University.

RAW264.7 and HEK293T cells (ATCC) was cultured with DMEM (HyClone) supplemented with 10% Fetal Bovine Serum (Gibco, Australia), 0.1 mg/ml streptomycin, 100 U/mL penicillin at 37 °C with 5% CO_2.

Murine bone marrow cells were isolated from femurs of 6–12 weeks old wild type or Myd88 knockout C57BL/6 mice, and cultured in RPMI1640 supplemented with 10% fetal bovine serum, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 10 mM glutamine with 5% CO_2 at 37 °C. BMDMs were obtained by inducing bone marrow cells with 50 ng/ml M-CSF for 7 days.

BCG was obtained from the Center for Disease Control of Suzhou. H37Rv was from Reference Lab of TB control center in Guangdong province. Both strains were grown in Middlebrook 7H9 broth (BD) supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD), 0.5% glycerol (sigma) and 0.05% Tween 80 (sigma), or on solid Middlebrook 7H11 medium (BD) supplemented with OADC. They were also cultured in Sauton's medium when examining secreted EspR in culture supernatant by Western Blot. All protocols were approved by the Institutional Biosafety Committee of Soochow University.

2.2. Plasmid construction

EspR coding sequence was PCR amplified using H37Rv genomic DNA as template and sub-cloned into prokaryotic expression vector pET28a (Novagen) down-stream of the His-tag to construct the pET28a-EspR plasmid. His-EspR expression was driven by T7 promoter, which was inducible by IPTG when transformed into *E. coli* BL21 cells. For eukaryotic expression, EspR coding sequence was cloned into pFLAG-CMV2 (Sigma) down-stream of FLAG-tag under CMV promoter to construct the plasmid pFLAG-EspR. It was also inserted into retroviral vector pMSCV-eGFP (Clontech) to create pMSCV-eGFP-EspR, in which FLAG-EspR expression was driven by CMV LTR and eGFP expression was directed by PGK promoter.

2.3. Purification of E. coli expressed EspR protein and preparation of EspR antisera

E. coli BL21 strain was transformed with pET28a-EspR, and the expression of EspR was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Beyotime, China) at 16 °C overnight. His-EspR protein was then purified using Ni-NTA His-binding resin (GE healthcare) and eluted with 300 mM imidazole (Fig. S1A). Protein concentration was determined using BCA Protein Assay Kit (Beyotime, China).

BALB/c female mice were immunized subcutaneously on weeks 0, 2, and 4 with 25 μg EspR protein emulsified with complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (Fig. S1B). Antisera against EspR were collected 6 weeks after the first immunization and tested by Western blot (Fig. S1C).

2.4. Generation of RAW264.7 cell line stably expressing EspR

HEK293T cells (4×10^6) were co-transfected with 10 μg each of pMSCV-eGFP-EspR and pCL-Ampho (Novus) plasmids to generate retrovirus containing EspR coding sequence. pCL-Ampho is an amphotropic retroviral packing vector targeting most mammalian cells including human cell lines. 72 h after transfection, the culture supernatant containing virus was collected and applied to RAW264.7 cells. 72 h after infection, virus infected cells showed GFP expression and were sorted out by BD FACS AriaTMIII sorter. The sorting was repeated 2 weeks later to obtain the RAW-EspR cell line with more than 98% GFP positive cells. Empty vector plasmid pMSCV-

eGFP was used in parallel to generate the RAW-vector control cell line.

2.5. Cytotoxicity assay

RAW-vector or RAW-EspR cells were seeded into 96 well plates at 5×10^3 cells/well and cultured for 6 h. 0, 24, and 48 h later, amount of life cells in each well were measured using Cell Counting Kit (CCK-8) (Beyotime, China) following manufacture's instruction.

2.6. CFU assay

RAW-vector or RAW-EspR cells were seeded at a density of 3×10^5 cells/well in 6-well plates and cultured for 12 h. The cells were then infected with BCG (MOI = 10) for 4 h, washed three times with PBS to remove bacteria in media, cultured in media containing 200 μg/ml of amikacin for 4 h and changed media into DMEM with 10% FBS. Cells were lysed at 0, 2, and 3 days after infection using water. Lysates were serially diluted, and 100 μl of each dilution was spread on 7H11 plates and cultured at 37°Cfor three weeks. Number of colonies on each plate was counted to obtain the CFUs.

2.7. Real-time PCR

RAW-vector or RAW-EspR cells were seeded at a density of 3×10^5 per well in 6-well plates and cultured for 12 h. The cells were then infected with BCG at MOI of 10 for 12 h. RNA was extracted using TRIzol reagent (Invitrogen), and reversely transcribed into cDNA with oligo-dT primers (Takara). Real-time PCR was performed using SYBR Premix Ex Taq (Takara) and LightCycler® 480 to measure the mRNA levels of IL-6, TNF-α, iNOS and GAPDH. Primers used in Real-time PCR analysis were listed in Table 1. Gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method.

2.8. ELISA

RAW-vector or RAW-EspR cells were seeded at a density of 3×10^5 per well in 6-well plates and cultured for 12 h. The cells were then infected with BCG at MOI of 10 for 24 h. Protein levels of TNF-α and IL-6 in cell culture supernatants were determined using ELISA kit (BD) according to manufacturer's instruction.

2.9. Western blot

RAW-vector or RAW-EspR cells were stimulated with BCG (MOI = 10), LPS (100 ng/ml) (Sigma), or Pam3CSK4 (1 μg/ml) (Invivogen) for indicated time and lysed in sample buffer (Beyotime, China). 10% or 12% SDS-PAGE were used to separate cell lysates and proteins were transferred to polyvinylidene fluoride membrane for blotting. p65, p-p65, JNK, p- JNK, Erk1/2, *p*-Erk1/2, p38, p-p38, MyD88, IRAK1, *p*-IRAK1, Caspase3, Caspase8, Caspase9, iNOS, tubulin, and GAPDH antibodies were purchased from Cell Signaling Technology. FLAG and GroEL antibodies were from Sigma. EspR antisera were produced in our lab. Secondary antibodies HRP-conjugated anti-mouse or anti-rabbit IgG were from Southern-Biotech.

Table 1
Primers for real time PCR.

Name	Sequence
Murine IL-6 F	ACAACCACGGCCTTCCCTACTT
Murine IL-6 R	CACGATTTCCCAGAGAACATGTG
Murine TNF-α F	AAGCCTGTAGCCCACGTCGTA
Murine TNF-α R	GGCACCACTAGTTGGTTGTCTTTG
Murine iNOS F	GAGCTCGGGTTGAAGTGGTATG
Murine iNOS R	GAAACTATGGAGCACAGCCACAT
Murine GAPDH F	GAAGGGCTCATGACCACAGT
Murine GAPDH R	GGATGCAGGGATGATGTTCT

2.10. Immunoprecipitation

4×10^6 HEK293T cells were transfected with 10 μg of indicated plasmids each. 24 h after transfection, cells were lysed with IP buffer (Beyotime, China), and the lysates were subjected to immunoprecipitation using anti-HA (Pierce) or anti-FLAG (Sigma) beads as indicated in the figures. Cell lysates and IP complexes were resolved by SDS-PAGE gel, and EspR and MyD88 signals were detected by indicated antibodies.

2.11. TUNEL assay

RAW-vector or RAW-EspR cells were seeded into 8-well plates and infected with BCG (MOI = 10) for 24 h. TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) according to manufacturer's instruction, and photographed using Nikon A1 confocal microscope (Nikon).

2.12. LDH assay

RAW-vector or RAW-EspR cells were seeded at a density of 3×10^5 per well in 6-well plates and cultured for 12 h. The cells were then infected with BCG at MOI of 10 for 0, 6, 12, and 24 h. Cell supernatants were subjected to LDH assay using LDH assay kit (Beyotime, China) according to the manufacturer's instruction.

2.13. Luciferase assay

RAW-vector or RAW-EspR cells were cultured in 6-well plate at 3×10^5 cells per well and transfected with NF-κB luciferase reporter plasmid (1.5 μg) plus internal control pRL-TK (500 ng). For BMDMs, pFLAG-EspR or pFLAG-CMV2 (1 μg) was also co-transfected. 24 h after transfection, cells were stimulated with LPS (100 ng/ml), Pam3CSK4 (1 μg/ml), poly IC (10 μg/ml), IL-1β (10 ng/ml) (R&D Systems), or BCG (MOI = 10) for 12 h and lysed by passive lysis buffer. Dual-Luciferase®Reporter Assay System (Promega) was used to measure luminescence from NF-κB-Luc, and the results were normalized by Renilla luminescence to give the relative luciferase activity. Cell culture media were subjected to ELISA to detect secreted IL-6 and TNF-α proteins in each sample.

2.14. Statistical analysis

The two-tailed unpaired *t*-test was used for statistical analysis. P value < 0.05 means statistically significant.

3. Results

3.1. EspR promoted Mtb survival in macrophages

As conflict reports exist on EspR secretion, we first tested whether we could detect EspR in Mtb culture filtrate. BCG was used as a negative control. The amino acid sequence of EspR is 100% identical in BCG and H37Rv. BCG, however, is deficient of ESX-1 system and could not be able to secrete EspR, according to Raghavan et al. [29]. H37Rv and BCG were grown in Sauton's medium till OD600 reached 0.8. Culture filtrate and bacterial pellet of H37Rv and BCG were examined by Western Blot for the existence of EspR. The results showed that EspR can be detected in the bacterial pellets of both H37Rv and BCG, but only in culture filtrate of H37Rv (Fig. 1A and Fig. S2). GroEL is an intracellular protein of mycobacteria and used as a cytosol protein marker here. It is undetectable in H37Rv filtrate, indicating that EspR in H37Rv filtrate was not from bacteria autolysis. The results showed that EspR was expressed in both H37Rv and BCG, but secreted out only by H37Rv, consistent

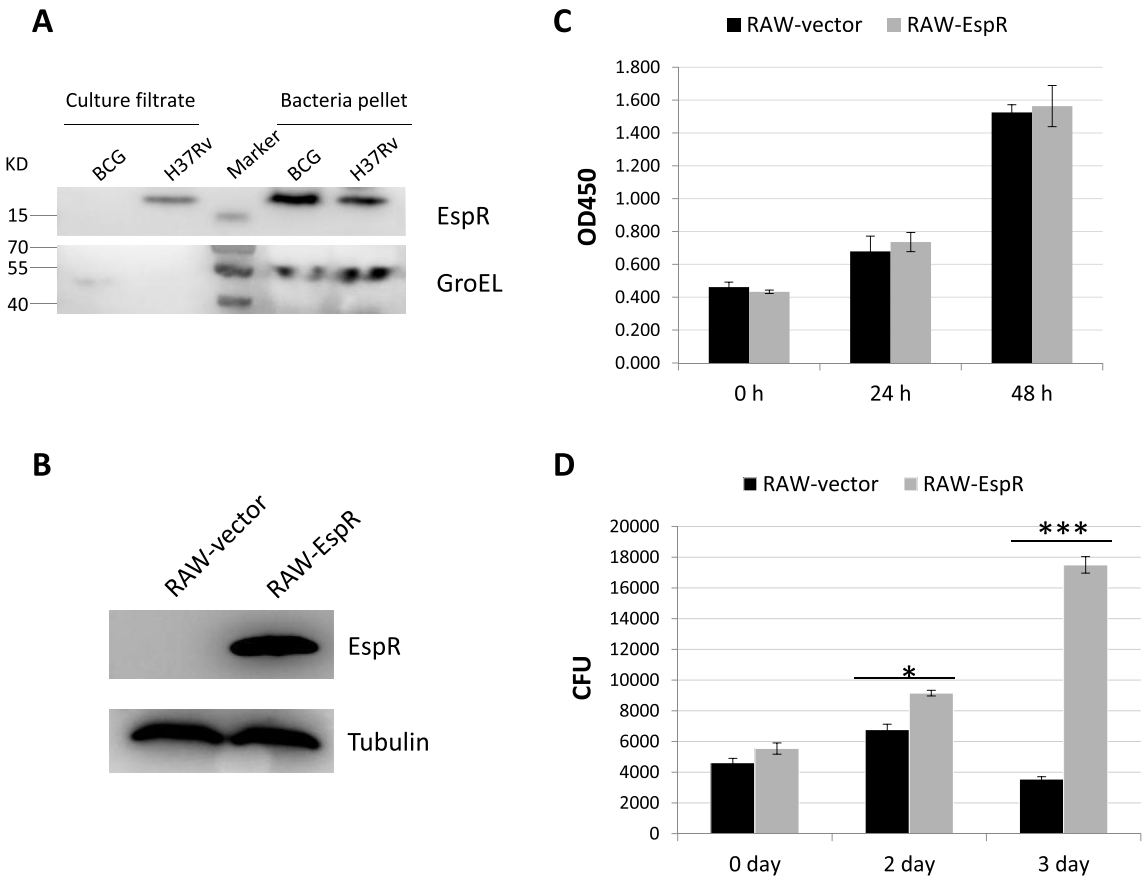

Fig. 1. EspR promoted Mtb survival in macrophages. (A) BCG and H37Rv were cultured in Sauton's medium till OD600 = 0.8. Bacterial pellets were collected from 1 mL of each sample and boiled in 200 μL sample buffer. 10 mL of culture supernatants from each sample were concentrated by millipore ultra-centrifugal filter (MWCO3KD) to 0.5 mL. EspR and GroEL protein levels in culture filtrates and bacteria pellets were determined by Western blot. **(B)** EspR expression in RAW-vector and RAW-EspR cells were measured by Western blot using antisera against EspR. **(C)** RAW-vector and RAW-EspR cells were seeded at 5×10^3 cells/well in 96 well plates. CCK-8 reagent was added at indicated time, and the amount of life cells in each well was determined by absorbance at 450 nm. Data shown are mean ± SD of three independent experiments. **(D)** RAW-vector and RAW-EspR cells were infected by BCG at MOI of 10 for 4 h. Cells were washed by PBS for 3 times and cultured in fresh media. Cells were lysed 0, 2, or 3 days after infection, and CFU of intracellular BCG was measured by spreading cell lysate on 7H11 plates, culture for 3 weeks and count the number of colonies. Data shown are mean ± SD of three independent experiments. *P < 0.05; ***P < 0.001.

with the report of Raghavan et al.

EspR plays important roles inside the bacteria. It is therefore not possible for us to use EspR deficient Mtb strain to identify its function in host cells only. To investigate whether secreted EspR interrupts the antimicrobial activities of macrophages, we decided to stably express EspR in murine macrophage cell line RAW264.7, infect the cells with BCG, and examine the immunological responses of the cells. BCG retains the function of EspR in bacteria without secreting it into host cells, therefore serves as a perfect stimulus to identify the effect of EspR in macrophages.

To construct RAW264.7 macrophage cell line stably expressing EspR, we PCR amplified the EspR coding sequence using H37Rv genomic DNA as a template, cloned it into the retroviral expression vector pMSCV-eGFP, and packaged it into retrovirus to transduce RAW264.7 cells. Western blot analysis showed that EspR was successfully expressed in the established cell line RAW-EspR but not the vector control cell line RAW-vector (Fig. 1B). To determine whether EspR has any cytotoxic effect in macrophages, we monitored the growth of RAW-vector and RAW-EspR cells for 48 h by CCK-8 life cell detecting reagent (Fig. 1C). We found that the cell numbers of the two cell lines are comparable at both 24 and 48 h after plating, suggesting that EspR does not have significant cytotoxic effect.

To investigate the function of EspR in host cells, we infected RAW-EspR and RAW-vector cells with BCG and measured the intracellular bacteria loads 2 and 3 days after infection. We found that the amount of survived BCG was higher in RAW-EspR cells at both time points (Fig. 1D). Especially, the bacterial burden in RAW-EspR cells was about 4 times of that in RAW-vector cells 72 h after infection, indicating that EspR interfered with the host cell bactericidal activities and supported mycobacteria survival.

3.2. EspR inhibited BCG induced expression of pro-inflammatory cytokines and iNOS

To determine the mechanism how EspR promoted mycobacteria survival, we examined multiple antimicrobial functions of RAW-EspR cells after BCG infection, including pro-inflammatory cytokines and iNOS expression, maturation of phagosome, cell apoptosis and autophagy. We found that EspR inhibited BCG induced pro-inflammatory cytokines and iNOS expression in macrophages. RAW-EspR and RAW-vector cells were infected with BCG at multiplicity of infection (MOI) of 10. Cells were harvested 12 h after infection to measure mRNA levels of TNF-α, IL-6, and iNOS by real time PCR, or 24 h after infection for protein levels by ELISA or Western blot. The results showed that EspR expression led to about 10 fold inhibition on BCG induced IL-6 mRNA and protein expressions (Fig. 2A and B). Inhibition on TNF-α expression was also observed at a less extent (Fig. 2C and D). In addition, we found that EspR reduced the expression of iNOS at both mRNA and protein levels (Fig. 2E and F). These results indicated that EspR had a potent inhibitory effect on BCG induced transcription of TNF-α, IL-6, and iNOS

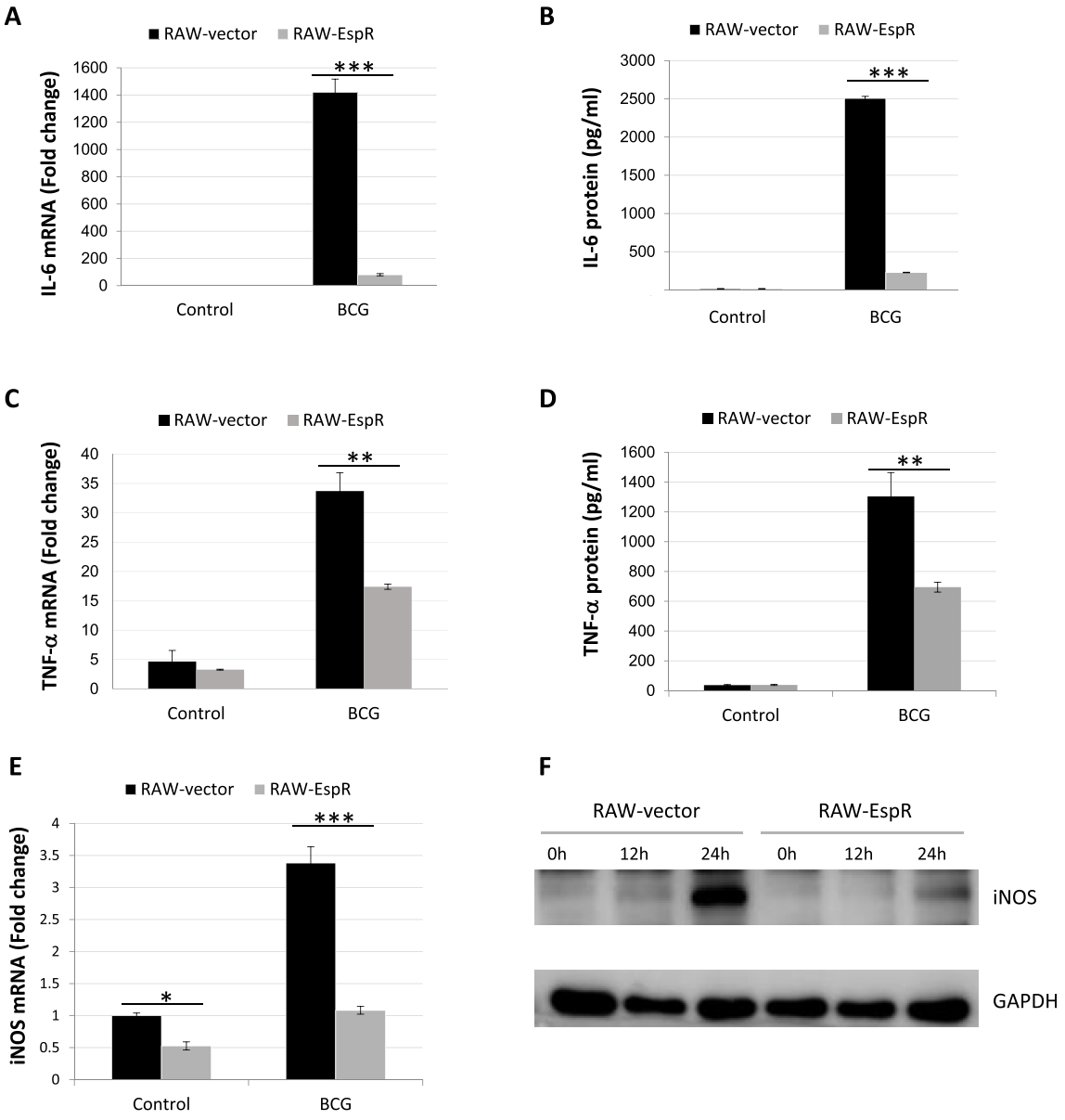

Fig. 2. EspR inhibited BCG induced expression of pro-inflammatory cytokines and iNOS. (A,C,E) RAW-vector and RAW-EspR cells were stimulated with BCG (MOI = 10) for 12 h and subject to RNA extraction. mRNA levels of TNF-α, IL-6, and iNOS were determined by reverse transcription followed by real-time PCR. Data shown are mean ± SD of three independent experiments. **(B, D)** RAW-vector and RAW-EspR cells were stimulated with BCG (MOI = 10) for 24 h. Levels of TNF-α and IL-6 protein in culture supernatants were assayed using ELISA kit. Data shown are mean ± SD of three independent experiments. **(F)** RAW-vector and RAW-EspR cells were stimulated with BCG (MOI = 10) for 12 or 24 h. Cells were lysed by sample buffer, and iNOS protein levels were analyzed by Western blot. *P < 0.05; **P < 0.01; ***P < 0.001.

genes, and subsequently suppressed their protein expression.

3.3. EspR inhibited BCG induced host cell apoptosis

In additional to hampering the expression of inflammatory mediators, we found that EspR also inhibited BCG induced cell apoptosis. RAW-EspR and RAW-vector cells were infected with BCG for 24 h, and apoptotic cells were determined by TUNEL assay. As shown in Fig. 3, the percentage of PE stained apoptotic cells was much lower in RAW-EspR than in RAW-vector, indicating that EspR inhibited BCG induced macrophage apoptosis (Fig. 3A and B). To further verify this effect of EspR, we examined the protein levels of active (cleaved) caspase 3 in BCG infected RAW-EspR and RAW-vector cells by Western blot. 24 h after infection, we found a significant amount of cleaved caspase 3 in RAW-vector cells, but a much lower level in RAW-EspR cells (Fig. 3C), indicating that EspR inhibited BCG induced cell apoptosis, consistent with the TUNEL results. To investigate whether EspR blocks cell apoptosis induced by extrinsic pathway or intrinsic pathway, represented by activation of caspase 8 or caspase 9, respectively, we checked the cleaved caspase 8 and caspase 9 levels in the two cell lines. The results showed that BCG induced caspase 8 activation was evidently repressed by EspR, while caspase 9 activation was not significantly changed (Fig. 3C), suggesting that EspR inhibited cell apoptosis induced by extrinsic pathway. Levels of cell necrosis in BCG infected RAW-vector and RAW-EspR cells were also measured by LDH assay, without finding significant difference between the two cell lines (Fig. S3).

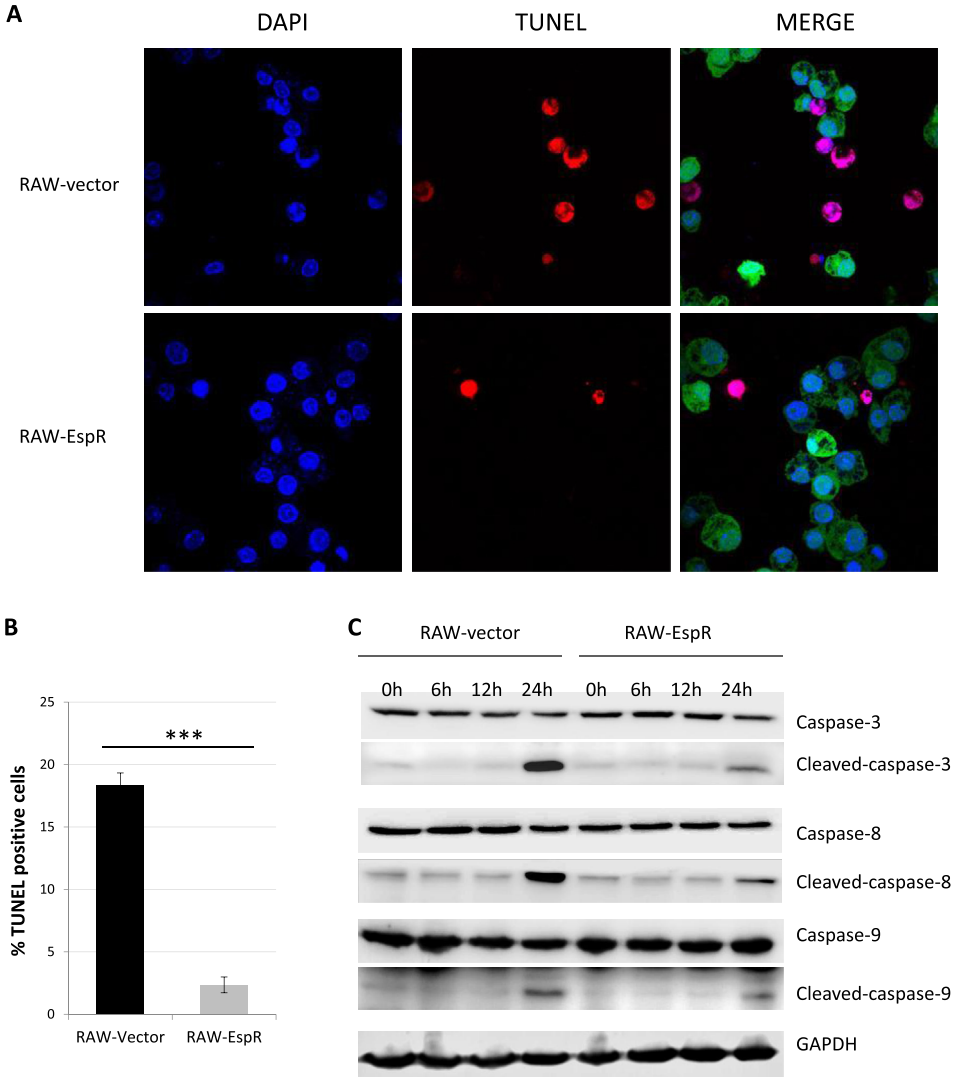

Fig. 3. EspR inhibited BCG induced host cell apoptosis. (A) RAW-vector and RAW-EspR cells were seeded into 8-well plates and infected with BCG (MOI = 10) for 24 h. TUNEL staining was performed using In Situ Cell Death Detection Kit and photographed using Nikon A1 confocal microscope. **(B)** is quantification of (A) by counting TUNEL positive cells and calculating its percentage in 10 random fields from two separate experiments for each cell line. Data represents mean ± SEM of 10 fields. **(C)** RAW-vector and RAW-EspR cells were stimulated by BCG (MOI = 10) for indicated time, and cells were lysed and subject to Western blot to detect the levels of total and active (cleaved) caspase 3, caspase 8, and caspase 9. $^{**}P < 0.01$.

3.4. EspR suppressed BCG and TLR2/TLR4 induced NF-κB and MAPK signal activation

BCG induces pro-inflammatory cytokine and iNOS expression in macrophages majorly by activating TLRs. TLRs signal through MyD88/TRIF/TRAM adaptor proteins and activate IRAK1 followed by TAK, which then phosphorylates NF-κB and MAPK that initiate the transcription of inflammatory cytokines and iNOS. To identify the mechanism how EspR inhibits iNOS and cytokine expression, we examined the effect of EspR on NF-κB and MAPK signal activation. RAW-EspR and RAW-vector cells were infected with BCG for 0–4 h, and the phosphorylation status of signal molecules in the two pathways were determined by Western blot (Fig. 4A). We found that EspR inhibited the activation of both pathways, evidenced by reduced p65, Erk, and JNK phosphorylation in RAW-EspR cells. As both NF-κB and MAPK activation were impeded by EspR, we checked their common upstream molecule IRAK1. Western blot results showed that EspR also repressed IRAK1 phosphorylation (Fig. 4A), indicating that EspR inhibited TLR signal activation at a very upstream position.

TLR2 and TLR4 play important roles in the defense against BCG in macrophages. We therefore checked the role of EspR on TLR2/TLR4 activation. RAW-vector and RAW-EspR were stimulated with the TLR2 agonist Pam3CSK4, and phosphorylation of TLR signal molecules were examined by Western blot. As shown in Fig. 4B, Pam3CSK4 stimulated p65, Erk, JNK, p38, and IRAK1 phosphorylation was all significantly reduced in RAW-EspR cells, indicating that EspR inhibited TLR2 signal activation (Fig. 4B). We also stimulated the cells with TLR4 agonist LPS and observed similar results (Fig. S4). These data indicate that EspR blocked BCG induced, TLR2 and TLR4 mediated NF-κB and MAPK activation, therefore inhibited the expression of inflammatory cytokines and iNOS.

3.5. EspR suppressed MyD88 dependent signal activation by directly interacting with MyD88

We have showed that EspR inhibited TLR2 and TLR4 signal activation at a point upstream of IRAK1 phosphorylation, indicating that EspR interrupts the functions of TLRs or their adaptor proteins. To identify the target of EspR, we transfected NF-κB-Luciferase reporter and pRL-TK internal control plasmids into RAW-vector and RAW-EspR cells, stimulated the cells with PamsCSK4 (TLR2 agonist), Poly IC (TLR3 agonist), LPS (TLR4 agonist), IL-1β, or BCG, and measured the NF-κB activities in these cells. IL-6 and TNF-α protein levels in the culture media were analyzed by ELISA in the same time. Signaling of TLR2 and IL-1β is totally dependent on adaptor protein MyD88; that of TLR4 is partially dependent on MyD88; and TLR3 signal is mediated by TRIF only. We found that all 5 stimuli generated a 7–10 fold induction of NF-κB activity in RAW-vector cells. The induced activity was inhibited in RAW-EspR cells stimulated by TLR2, TLR4, IL-1β, and BCG, but not TLR3 (Fig. 5A). Results of IL-6 and TNF-α protein assay were consistent

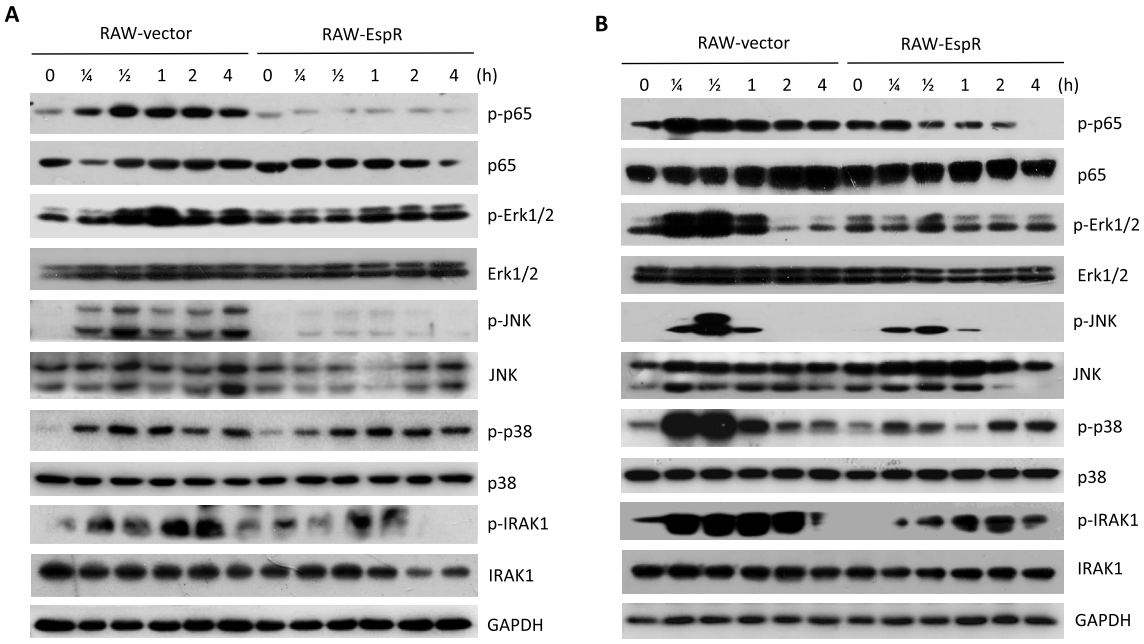

Fig. 4. EspR suppressed BCG and TLR2 induced NF-κB and MAPK signal activation. (A) RAW-vector and RAW-EspR cells were stimulated by BCG (MOI = 10) for indicated time. Cells were lysed and subject to Western blot to determine the protein levels of total and phosphorylated p65, Erk1/2, JNK, p38, and IRAK1. **(B)** RAW-vector and RAW-EspR cells were stimulated by Pam3CSK4 (1 μg/ml) for indicated time. Cells were lysed and subject to Western blot to determine the protein levels of total and phosphorylated p65, Erk1/2, JNK, p38, and IRAK1.

with that of luciferase activity assay (Fig. 5B and C). The results suggested that EspR blocked the function of MyD88 instead of TLR, as TLR3 signal pathway, the only one not requiring MyD88 among the five, was not suppressed by EspR, while that of IL-1β, involving MyD88 but not TLR, was significantly inhibited.

To confirm that EspR targets MyD88, we tested the inhibitory effect of EspR on NF-κB activation using MyD88 deficient BMDM. Wild type and MyD88−/− BMDM were transfected with NF-κB-Luc reporter and pRL-TK internal control plasmids, plus pFLAG-CMV2 or pFLAG-EspR. The cells were stimulated with Pam3CSK4, IL-1β, or BCG, and NF-κB luciferase activities were measured (Fig. 5D and E). We found that in wild type BMDM, the 3 stimuli induced 5–9 folds of NF-κB activity, which were inhibited by EspR by 50–80% (Fig. 5D). In MyD88−/− BMDM, Pam3CSK4 and IL-1β could not bring a significant induction of NF-κB activity due to absence of MyD88, indicating that MyD88 dependent pathways were totally blocked (Fig. 5E). BCG induced a mild 3 fold NF-κB activity in MyD88−/− BMDM without EspR expression, probably through PRR signal pathways not dependent on MyD88. This moderate induction, however, was not affected by EspR, indicating that EspR only inhibit MyD88 mediated NF-κB activation (Fig. 5E).

Having identified MyD88 as the target of EspR, we wonder if EspR blocks the function of MyD88 by directly interacting with it. To verify this hypothesis, we did co-immunoprecipitations (co-IPs) between EspR and MyD88. HEK293T cells were transiently transfected with pFLAG-EspR plus HA-vector or HA-MyD88 encoding murine MyD88. Cells were lysed 24 h after transfection and immunoprecipitated using anti-HA beads. As shown in Fig. 5F, EspR co-IP with HA-MyD88 but not the HA beads control, suggesting a direct interaction between EspR and MyD88. We also did reverse IP by transfecting HEK293T cells with HA-MyD88 plus pFLAG-CMV2 or pFLAG-EspR, and immunoprecipitating the lysates with anti-FLAG beads (Fig. 5G). We found MyD88 co-IP with FLAG-EspR but not the FLAG beads control, consistent with the results of Fig. 5F. The data demonstrated that EspR interacted with MyD88 directly, suggesting that EspR disrupted the function of MyD88 by physically interacting with the protein.

4. Discussion

Mtb secreted various virulence factors into host cells to block their antimicrobial functions. Most of these virulence factors do not have critical functions inside the bacteria. EspR, however, showed essential functions both in bacteria and in host cells. In bacteria it is a DNA binding protein, regulating multiple gene expression and the activity of ESX-1 system; In macrophage, we showed in this study that EspR physically interacted with MyD88 and inhibited signals mediated by this key adaptor protein. This results in reduced expression of pro-inflammatory cytokines and iNOS, as well as suppressed apoptosis induced by Mtb infection. EspR thus interrupted the anti-bacteria activities of macrophages and improved the intracellular survival of Mtb. To our knowledge this is the first report on Mtb virulence factor holding crucial functions inside both bacteria and host cells.

In macrophages TLRs play a significant role in stimulating antimicrobial responses. With ligand binding, TLRs recruit adaptor proteins MyD88/TRIF/TRAM which mediates phosphorylation of IRAKs and recruitment of TRAFs. TAK is then activated by TRAF complex and stimulates the downstream NF-κB and MAPK pathway, inducing transcriptional activation of numerous anti-bacteria genes including inflammatory cytokines and iNOS. Among TLRs, TLR2 and TLR9 signaling is totally dependent on MyD88. TLR4 uses both MyD88 and TRIF, while TLR3 employs only TRIF. Our results showed that EspR inhibited NF-κB activation induced by TLR2 and TLR4 agonists. TLR3 mediated NF-κB activity was not affected by EspR, suggesting that EspR targets MyD88 mediated signal activation. This is confirmed by the fact that EspR lost its inhibitory effect in MyD88−/− BMDM. Further more, we found that EspR interacted with MyD88 physically. All together, the results demonstrated that EspR inhibited MyD88 dependent TLR activation in macrophages.

Activation of TLRs can also induce cell apoptosis. MyD88 can directly recruit FADD and mediates cell apoptosis through extrinsic pathway [3]. It has been reported that Mtb lipoprotein LpqH is capable of inducing macrophage apoptosis through activating TLR2 (32). Apoptosis is a strategy of macrophages to clear Mtb. It has been showed that attenuated strains of mycobacteria are more likely to induce cell

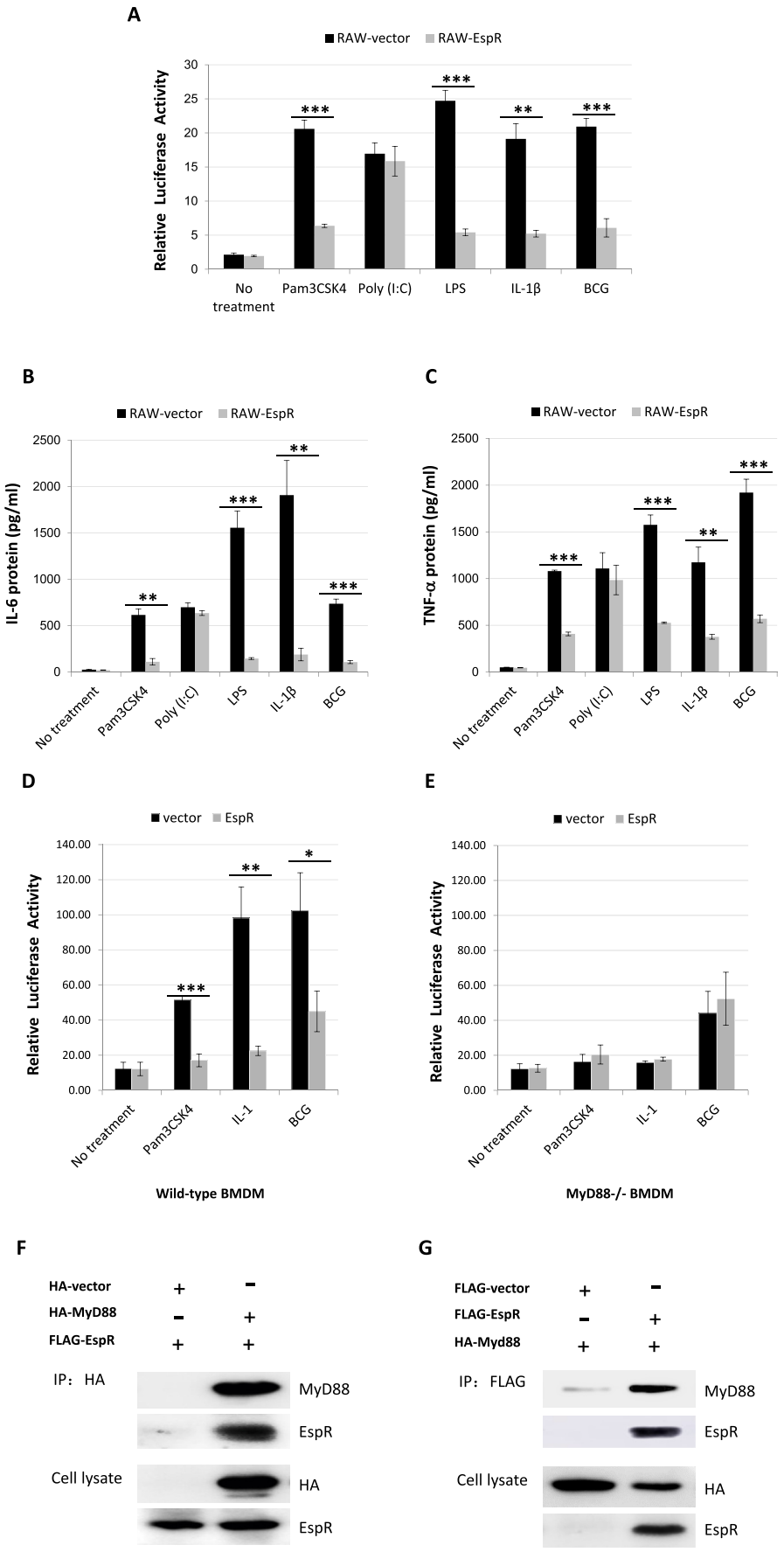

Fig. 5. EspR suppressed MyD88 mediated signal activation by directly interacting with MyD88. (A) RAW-vector and RAW-EspR cells were transfected with NF-κB luciferase reporter plasmid (1.5 μg) plus pRL-TK (500 ng). 24 h after transfection, cells were treated with indicated stimuli for 12 h. Cells were lysed, and luminescence from NF-κB-Luc was measured and normalized by Renilla luminescence using Dual-Luciferase®Reporter Assay System. Data shown are mean ± SD of three independent experiments. **(B–C)** Culture media of samples in (A) were analyzed for IL-6 **(B)** and TNF-α **(C)** protein levels by ELISA. Data shown are mean ± SD of three independent experiments. **(D–E)** BMDM cells from wild type **(D)** or MyD88 −/− **(E)** mice were transfected with NF-κB luciferase reporter plasmid (1.5 μg), pRL-TK (500 ng), and pFLAG-CMV2 or pFLAG-EspR (1 μg). 24 h after transfection, cells were treated with indicated stimuli for 12 h. Cells were lysed, and luminescence from NF-κB-Luc was measured and normalized by Renilla luminescence using Dual-Luciferase®Reporter Assay System. Data shown are mean ± SD of three independent experiments. **(F)** HEK293T cells were transfected with the indicated plasmids. 24 h after transfection, cells were lysed by IP buffer, and lysates were incubated with anti-HA resin at 4 °C overnight. IP complexes and cell lysates were resolved by Western blot, and protein levels of EspR and HA-MyD88 were determined by indicated antibodies. **(G)** HEK293T cells were transfected with the indicated plasmids. 24 h after transfection, cells were lysed by IP buffer, and lysates were incubated with anti-FLAG resin at 4 °C overnight. IP complexes and cell lysates were resolved by Western blot, and protein levels of EspR and MyD88 were determined by indicated antibodies.

apoptosis, while virulent strains can suppress apoptosis and induce cell necrosis, a cell death mode favoring Mtb survival and spreading [22]. We showed in this study that EspR could inhibit BCG induced macrophage apoptosis, evidenced by reduced TUNEL positive cells and suppressed caspase 3 activation in EspR expressing macrophages. Western blot analysis also indicated that EspR blocked extrinsic apoptotic pathway, implying that EspR could inhibit TLRs induced apoptosis by interrupting MyD88 signaling. Cell necrosis was not significantly changed by EspR, implying that virulent strains of Mycobacteria might use different factors to manipulate host cell apoptosis and necrosis.

MyD88 mediates signals not only from TLRs, but also from IL-1R. IL-1R1 deficient mice showed a very high susceptibility to Mtb infection at a level close to that of MyD88 deficient mice [17], indicating that IL-1R mediated signal contributes significantly to MyD88 dependent innate immune responses against Mtb. Our study showed that EspR inhibited IL-1β induced NF-κB activation by about 70%, indicating that EspR also blocked MyD88 mediated IL-1R signal pathway. EspR therefore can potently support the survival of Mtb in macrophages by suppressing antimicrobial activities from both TLRs and IL-1R pathways.

Due to the critical role of MyD88 dependent signal pathways in macrophage antimicrobial responses, it is not surprising that bacteria develop strategies to suppress these pathways. TlpA protein expressed by *Salmonella enterica* suppresses TLR4 and IL-1 receptor mediated transactivation of NF-κB [26]; *Staphylococcus aureus* TIR domain protein TirS interferes with signaling through TLR2 and inhibits NF-κB and/or MAPK pathways [4]; Tcps identified in *Escherichia coli* and Brucella melitensis impede TLR signaling by direct binding to MyD88 [11]. Accumulating evidences showed that MyD88 dependent signal pathways are targeted by various strains of bacteria. Interestingly, Rahman et al. reported that only virulent strain H37Rv, but not avirulent BCG, can disrupt the TLR2-MyD88 pathway in macrophage and translocate from phagosome into cytosol [42], suggesting the existence of virulent strain specific factors that block TLR/MyD88 signaling in host cells, which may include EspR.

EspR is not the only factor in Mtb that represses TLR pathway. Secreted protein tyrosine phosphatase MptpB inhibits TLR induced NF-κB and MAPK activation [16]; Putative sulfotransferase Stf1 was shown to inhibit TLR2 induced pro-inflammatory responses by interacting with IRAK1 and disrupt the binding between MyD88 and IRAK1 [6]. Here we showed that EspR suppressed MyD88 dependent pathways by interacting with MyD88 and blocked inflammatory responses and apoptosis of macrophages. These reports suggested that multiple virulence factors from Mtb could work on the same pathway, at different points, by interacting with different host proteins, to repress these critical signals to a low level so as to improve the survival of Mtb in host cells.

5. Conclusion

In this study, we investigated the functions of the Mtb secreted DNA binding protein EspR in host cells. We found that EspR improved intracellular bacteria survival in macrophages by inhibiting mycobacteria induced inflammatory cytokine expressions and host cell apoptosis. By interacting with adaptor protein MyD88, EspR suppressed TLR and IL-1R mediated signal activation, thus blocked the downstream inflammatory responses and apoptosis in macrophages.

Conflicts of interest

None.

Author contributions

Yuanshu Dong and Sidong Xiong designed the study. Chunyan Jin and Xiaoyu Wu performed the experiments mostly and contributed equally. Chunsheng Dong, Fengge Li and Lingbo Fan helped to complete the experiments partly. Yuanshu Dong wrote the manuscript. Sidong Xiong and Yuanshu Dong authorized the final version of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.03.010.

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