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ORIGINAL ARTICLE

Mycobacterium tuberculosis EspB protein suppresses interferon-γ-induced autophagy in murine macrophages

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Abstract *Background: Mycobacterium tuberculosis* (Mtb) persists within immature phagosomes by preventing their maturation into phagolysosomes. Although the early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B (EspB) of Mtb is strongly linked to immunogenicity and virulence of this organism, its mechanism of action remains largely unclear. This study aimed to investigate EspB effects on autophagy in murine ANA-1 macrophage cells.

Methods: EspB gene was amplified by polymerase chain reaction from Mtb H37Rv genomic DNA to express recombinant EspB protein. Levels of autophagic markers, including Microtubule-associated protein 1 light chain 3 beta (LC3B-I and -II), phosphorylated signal transducer and activator of transcription (STAT)1 and total STAT1 in ANA-1 cells treated with EspB proteins were assessed by Western blotting. In addition, autophagic vacuoles were detected by fluorescence microscopy. Finally, *IFN-γR1* expression was evaluated by semiquantitative reverse transcriptase polymerase chain reaction and flow cytometry.

Results: EspB gene was expressed in Escherichia coli cells to yield a soluble N-terminal glutatione S-transferase tag fusion protein used in subsequent experiments. Preincubation with EspB significantly suppressed autophagosome formation and LC3B expression induced by interferon (IFN)-γ stimulation, in a dose-dependent manner. These results were confirmed by the reduced incorporation of monodansylcadaverine, a marker for the acidic compartment of autolysosomes, after treatment with EspB. Interestingly, we found that IFN-γ receptor 1 mRNA and protein levels were decreased in EspB-stimulated ANA-1 cells in comparison with untreated cells. Finally, EspB protein also inhibited IFN-γ-activated STAT1 phosphorylation, thereby downmodulating macrophage responsiveness to IFN-γ.

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Conclusion: EspB inhibits autophagosome formation in murine macrophages, at least in part by downregulating IFN-γ receptor 1 expression. Overall, EspB should be considered a relevant factor in the pathogenesis of mycobacterial infections in humans.

Introduction

Autophagy is a major homeostatic cellular mechanism, responsible for the removal of damaged organelles and elimination of intracellular pathogens.[1–4] *Mycobacterium tuberculosis* (Mtb) is a facultative intracellular pathogen, characterized by its intracellular survival in host macrophages.[5] It has been shown that Mtb persists within immature phagosomes by preventing their maturation into phagolysosomes.[6] However, induction of autophagy by interferon (IFN)-γ can overcome mycobacterial inhibition of phagosome maturation and is therefore critical for Mtb eradication from murine and human macrophages.[7] Interestingly, **Mtb** downregulates autophagy, the main feature enabling persistence of this microorganism in the host cellular environment.[8] However, the mechanisms by which **Mtb** reduces autophagy as well as its implications for host cell viability remain to be elucidated.

Although the early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion apparatus is critical to Mtb virulence,[9] its mechanisms of action are not fully understood. ESX-1 secretion-associated protein B (EspB), a 48-kDa protein encoded by the *Rv3881c* gene of Mtb H37Rv, constitutes a novel substrate of the ESX-1 secretion system and plays an important role in Mtb virulence.[10] Indeed, EspB is required for cytolysis, bacterial spreading, and ESAT-6 secretion.[11,12] More importantly, EspB is critical to inhibition of phagosome maturation and EspB deletion results in acidification of the bacterial phagosome.[13] Despite its implication in host–pathogen interactions during Mtb infection, the precise role of EspB in innate immune regulation remains to be determined.

In this work, we aimed to investigate further the role of EspB in host immune responses. We found that the EspB protein pretreatment significantly suppressed autophagosome formation and LC3B expression after interferon (IFN)-γ stimulation in ANA-1 macrophage cells. In addition, EspB treatment resulted in downmodulation of IFN-γ receptor 1 (R1) expression on macrophage cell surface, and inhibition of IFN-γ-induced autophagy by suppressing IFN-γR1 signal transducer and activator of transcription (STAT)1 phosphorylation. These data suggest that EspB inhibits autophagosome formation in murine macrophages, effects due, at least in part, to downmodulation of IFN-γR1 expression.

Methods

Cloning, expression, and purification of EspB protein

The gene encoding EspB was amplified by polymerase chain reaction (PCR) from **Mtb** H37Rv genomic DNA (AL123456.3),

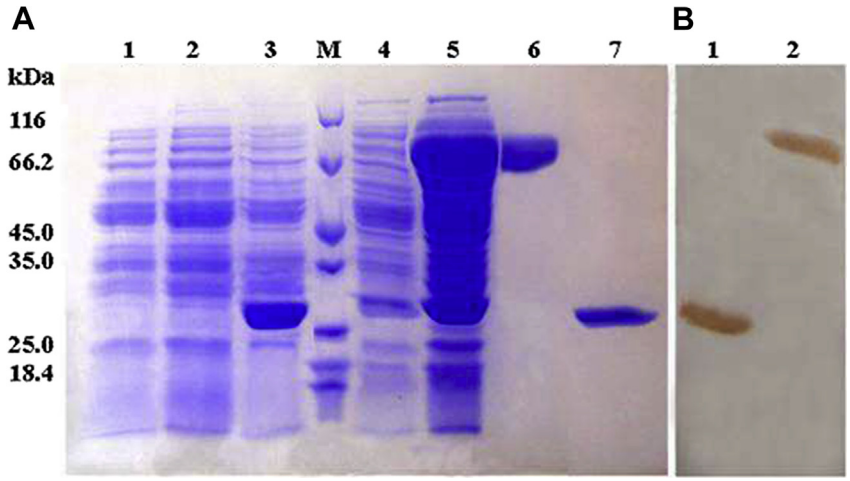

Figure 1. Detection of EspB protein and EspB–GST fusion expressed in *Escherichia coli*. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on 12% sodium dodecyl sulfate polyacrylamide gel and stained with Coomassie brilliant blue. M, molecular mass marker; Lanes: 1, *E. coli* BL21 control; 2, BL21 containing plasmid pGEX-4T-1 prior to induction; 3, BL21 containing pGEX-4T-1 plasmid after induction; 4, bacterial culture before induction of the fusion protein in BL21; 5, bacterial culture after induction of the fusion protein in BL21; 6, pGEX–EspB fusion protein purified on a GST column; 7, purified GST fusion protein. (B) Immunoblot analysis was performed with mouse anti-GST tag monoclonal antibodies. *E. coli* harboring pGEX-4T-1-EspB was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 5 hours at 30°C. Lanes 1, 26-kDa GST fusion partner protein; and Lane 2, 74-kDa pGEX–EspB fusion protein. EspB = The early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B; GST = glutathione S-transferase.

using the following primers: EspB forward P1, 5′-TT GAATTC ATG ACGCAGTCGCAGACCGT-3′; and EspB reverse P2, 5′-GCCTCGAGTCACTTCGACTCCTTACTGT-3′ (*Eco*RI and *Xho*I sites, respectively, are underlined). Restriction endonucleases and *Taq* DNA polymerase were purchased from MBI Fermentas (Shanghai, China). The PCR product was cloned into the expression vector pGEX-4T-1 (Zihan, Tianjing, China). After the clone integrity was confirmed by DNA sequencing, the recombinant vector was transformed into *Escherichia coli* BL21 (DE3) for expression. The EspB protein was purified using Ni sepharose high-performance purification columns (GE Healthcare, USA) according to standard procedures. The purified fusion protein underwent cleavage with thrombin to remove the glutathione S-transferase (GST) tag, which was separated using a GST-affinity column (GE Healthcare, USA). After this purification step, the EspB protein was subjected to an endotoxin-removing gel (Detoxi-Gel; Pierce, Rockford, IL, USA) according to manufacturer's instructions, for removal of *E. coli* lipopolysaccharide contamination. The purified endotoxin-free EspB was lyophilized and stored at −70°C until use. The protein concentration was assessed using the BCA Protein Assay Kit (Pierce).

Cell culture

ANA-1 cells (Fanke, Shanghai, China) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified environment containing 5% CO_2.

Cell lysis and Western blot analysis

Approximately 10^7 cells were collected, washed twice with ice-cold phosphate buffered saline (PBS), lysed in cell lysis RIPA Buffer (Sigma—Aldrich, St. Louis, MO, USA) for 20

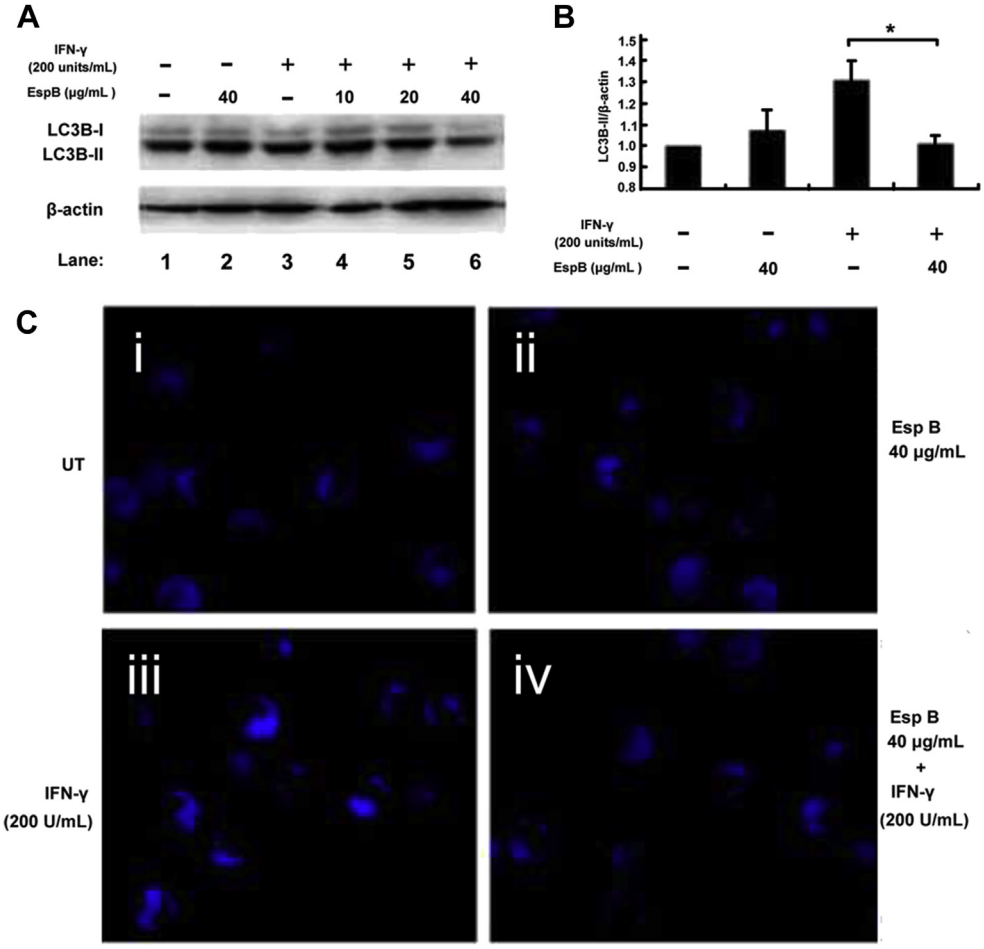

Figure 2. EspB suppressed IFN)-γ-induced autophagy. (A) ANA-1 cells were treated with EspB at the indicated concentrations for 6 hours, followed by 16 hours challenge with IFN-γ (200 U/mL). Cell lysates were assessed by Western blotting using anti-LC3B or anti-β-actin antibodies. (B) LC3B-II band intensities were normalized to β-actin. The ratio of LC3B-II to β-actin in each sample was compared with that obtained for cells without IFN-γ treatment. The data represent mean ± standard deviation from three independent experiments. (C) ANA-1 cells were fixed and stained with monodansylcadaverine (original magnification, 200×) after (1) no treatment (UT); (2) pretreatment with EspB (40 μg/mL); (3) treatment with IFN-γ (200 U/mL) for 16 hours; and (4) pretreatment with EspB (40 μg/mL) and treatment with IFN-γ (200 U/mL) for 16 hours. *$p < 0.05$. EspB = The early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B; IFN = interferon; LC3B = Microtubule-associated protein 1 light chain 3 beta.

minutes on ice, and subjected to sonication for 20 seconds. Cell lysates were centrifuged at 12,000× *g* for 20 minutes at 4°C and the resulting supernatants were collected. Protein concentrations were determined as described above. Equal amounts of protein were separated by 15% or 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking in Tris-buffered saline—Tween-20 containing 5% nonfat milk, membranes were incubated overnight at 4°C with primary antibodies against Microtubule-associated protein 1 light chain 3 beta (LC3B-I and -II) (Cell Signaling Technology, Danvers, MA, USA), phosphorylated STAT1 (Tyr701), and total STAT1 (Signalway Antibody, College Park, MD, USA). Afterwards, membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase (ZSGB-Bio, Beijing, China) for 1 hour at room temperature. The blots were probed using the ImageQuant ECL system (Sigma) and quantified by laser densitometry.

Fluorescence microscopy analysis

For detection of autophagic vacuoles, cells were seeded on sterile coverslips and treated with 40 μg/mL EspB protein for 6 hours, followed by 16 hours challenge with 200 U/mL mouse recombinant IFN-γ (Peprotech, Rocky Hill, NJ, USA). Then, cells were incubated with 50 μM monodansylcadaverine (Sigma—Aldrich) in PBS at 37°C for 1 hour. Finally, coverslips were washed four times with PBS, immediately sealed, and images were acquired using a fluorescence microscope manufactured by Carl Zeiss (Jena, Germany).

Semiquantitative reverse transcriptase PCR for assessment of IFN-γR1 expression

RNA was isolated from ANA-1 cells using the total RNA extraction kit (Bio Flux, USA). Total RNA (1 μg) was added to a 50-μL reaction mixture of the Quant One-Step RT-PCR kit (Tiangen Biotech, Beijing, China). Primers used for reverse transcriptase PCR were: IFN-γR1 forward, 5′-GTGTATTC GGGTTCCTGGACTGATT-3′; IFN-γR1 reverse, 5′-CGAATGTG-TAACAGGTGCTCCCGTC-3′; β-actin forward, 5′-GAGACCTT-CAACACCCCAGC-3′; and β-actin reverse, 5′-ATGTCACGCAC GATTTCCC -3′. The PCR products were analyzed using the Quantity One analysis software (BioRad, Hercules, CA, USA).

Flow cytometry assays

For flow-cytometry analysis, ANA-1 cells (treated and untreated) were incubated with biotin anti-mouse CD119 (IFN-γR1 chain) antibodies (Biolegend, San Diego, CA, USA) followed by streptavidin—phycoerythrin (Biolegend). Afterwards, the stained cells were washed and resuspended in PBS at 10^6 cells/300 μL. Finally, the samples were analyzed by flow cytometry on a FACSCalibur (Becton—Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Data are presented as mean ± standard deviation of triplicate observations. Student *t* test was used to analyze the data. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

Purification of recombinant EspB expressed in *E. coli*

The *EspB* gene was expressed in *E. coli* cells harboring pGEX-4T-1, to yield a soluble N-terminal GST-tag fusion protein which was purified by affinity chromatography. Western blot data clearly revealed high expression levels of a 74-kDa fusion protein, which was equivalent to the predicted molecular weight (Fig. 1). The endotoxin content of the purified EspB protein devoid of the GST tag was <0.05 endotoxin units per microgram of protein.

EspB suppresses IFN-γ-induced autophagy

We measured LC3B protein expression in ANA-1 cells treated with EspB prior to IFN-γ treatment and found that

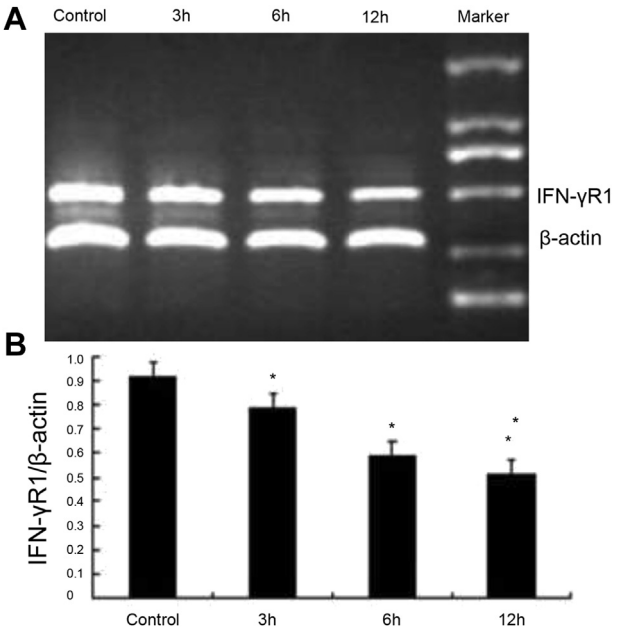

Figure 3. Effects of EspB protein on expression of IFN-γR1 mRNA in ANA-1cells. ANA-1 cells were treated with 40 μg/mL EspB and total RNA was extracted at the indicated time points. Untreated ANA-1 cells were used as controls. Expression of mRNA was assayed by semiquantitative RT-PCR. Percent inhibition was calculated by densitometry analysis and normalization to β-actin mRNA levels. (A) Representative gel image of semiquantitative RT-PCR depicting the variation of IFN-γR1 mRNA expression at various time points. (B) Histogram showing the normalized data for IFN-γR1 (IFN-γR1/β-actin) in treated and untreated ANA-1 cells. Data represent mean ± standard deviation from three independent experiments with similar results. *$p < 0.05$. EspB = The early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B; IFN-γR1 = interferon-γ receptor 1; RT-PCR = reverse transcriptase polymerase chain reaction.

EspB alone did not affect the expression level of LC3B (Fig. 2A, lanes 1 and 2; Fig. 2B, lanes 1 and 2). With EspB pretreatment for 6 hours followed by IFN-γ treatment, the IFN-γ-induced enhancement of LC3B protein expression was reduced in a dose-dependent manner (Fig. 2A, lanes 3—6) as quantified by laser densitometry (Fig. 2B).

To confirm that autophagosome formation was inhibited by EspB, we assessed the incorporation of monodansylcadaverine, a marker for the acidic compartment of autolysosomes.[14] As shown in Fig. 2C, EspB alone did not affect ANA-1 cell autophagic vacuoles. Upon treatment with 200 U/mL IFN-γ, an increased production of autophagic vacuoles in ANA-1 cells was observed (Fig. 2C). By contrast, 40 μg/mL EspB pretreatment markedly reduced the IFN-γ-induced accumulation of vacuoles (Fig. 2C). Taken together, these results demonstrate that EspB inhibited IFN-γ-induced autophagosome formation in ANA-1 cells (Fig. 2).

EspB protein decreases IFN-γR1expression in macrophage cells

To assess whether the expression of IFN-γR1 was modulated by EspB, ANA-1 cells were stimulated for 6 hours with EspB protein. Semiquantitative reverse transcriptase PCR showed decreased mRNA levels of IFN-γR1 in EspB-stimulated ANA-1 cells in comparison with untreated cells (Fig. 3). The IFN-γR1 proteins on the cell surface were labeled with fluorescent antibody. Flow cytometry showed that the mean fluorescence intensity on the cell surface was significantly lower in EspB-stimulated cells compared with untreated cells ($p < 0.05$; Fig. 4), indicating decreased surface IFN-γR1 level.

EspB protein suppresses IFN-γ-induced STAT1 phosphorylation

As shown in Fig. 5, western blot analysis of STAT1 and its phosphorylated form showed that IFN-γ treatment significantly increased phosphorylation of STAT1 in ANA-1 cells. This enhancement of STAT1 phosphorylation by IFN-γ was suppressed by EspB protein.

Autophagy is a process that controls the quality and quantity of intracellular biomass in eukaryotic cells by targeting autodigestion of cytoplasmic components that range in complexity and size from individual proteins to whole organelles.[15] During autophagy, parts of the cytoplasm and intracellular organelles are enwrapped in

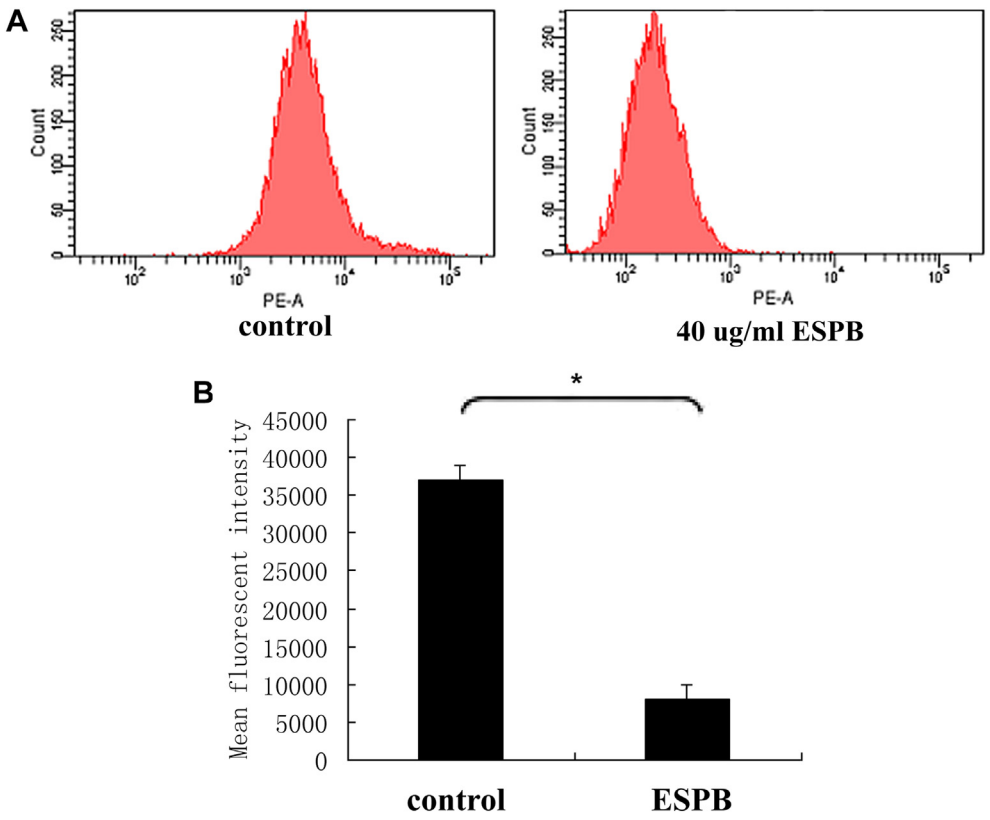

Figure 4. Effects of EspB on IFN-γR1 expression in ANA-1cells. ANA-1 cells were stimulated with the EspB protein (40 μg/mL) for 6 hours. After treatment, the cells were washed and stained for IFN-γR1. Untreated cells were used as controls. (A) Representative histogram plots showing the surface expression of IFN-γR1 in untreated controls and EspB-treated ANA-1 cells. (B) Quantitation of the cumulative mean fluorescent intensity of IFN-γR1 surface expression in untreated and treated ANA-1 cells. Data represent mean ± standard deviation from three independent experiments with similar results. *$p < 0.05$. EspB = The early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B; IFN-γR1 = interferon-γ receptor 1.

double-membrane vesicles called autophagosomes, which are ultimately delivered to lysosomes for bulk degradation.[16] Autophagy is a crucial weapon for the host cell in fighting against bacterial and viral invasion. However, it is now evident that highly evolved intracellular pathogens including Mtb have specialized anti-autophagy adaptations that allow them to prevent, block, or elude autophagic elimination.[7] Despite extensive studies, the specific bacterial factors that undermine host autophagy remain largely unknown. A limited number of Mtb components, including lipoprotein LpqH[17] and Eis protein,[18] have been suggested to contribute to this process.

Mtb is known to evade successfully the host immune system by various cellular mechanisms. The ability of Mtb to block macrophage responses to IFN-γ is an important trait developed by the bacteria to evade cell-mediated immunity. Mtb-infected human macrophages are desensitized to activation by IFN-γ.[19–21] The antimicrobial properties of IFN-γ result in part from its ability to stimulate autophagy in host cells.[8,22,23] We hypothesized that the Mtb attenuation of cytokine responsiveness of host cells also alters the IFN-γ-dependent activation of autophagy pathways. Indeed, the present data demonstrated that EspB inhibits IFN-γ-induced autophagy; a possible mechanism by which the bacteria evade the host immune system.

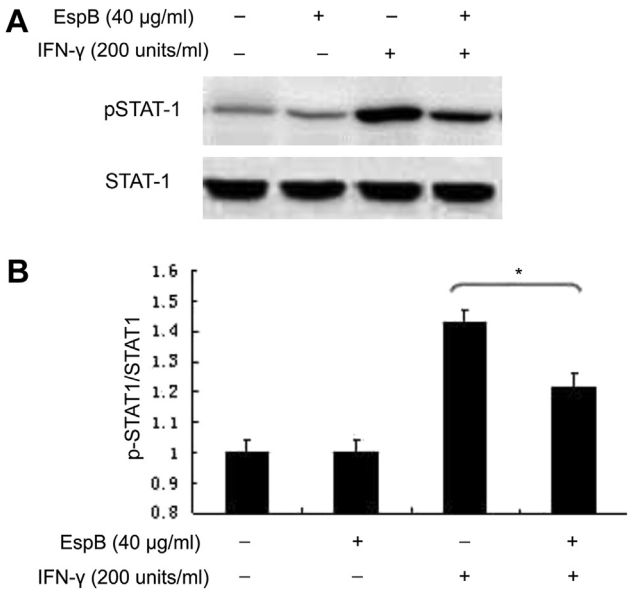

Figure 5. EspB protein suppressed IFN-γ-induced STAT1 phosphorylation. (A) ANA-1 cells were treated with EspB (40 μg/mL) for 6 hours, followed by IFN-γ (200 U/mL) incubation for 1 hour. Cell lysates were examined by Western blotting using anti-pSTAT1 or anti-total STAT1 antibodies. (B) The intensity values obtained for pSTAT1 were normalized to total STAT1. The ratio of pSTAT1/STAT1 in each sample was compared to that of untreated cells. Data represent mean $\pm$ standard deviation from three independent experiments. *$p < 0.05$. EspB = The early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion-associated protein B; IFN-γ = interferon-γ; pSTAT = phosphorylated signal transducer and activator of transcription.

The autophagy process involves formation and maturation of autophagosomes, and is mediated by the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3), a mammalian homolog of yeast autophagy related gene 8 (Atg8). It has been shown that LC3B cleavage into LC3B-I and subsequently LC3B-II plays a critical role in autophagosome formation.[24] This is observed in Western blot assays where two bands are usually detected by anti-LC3B antibodies: LC3B-I (18 kDa) and LC3B-II (16 kDa). Interestingly, it has been shown that LC3B-II amounts correlate well with autophagosome numbers, allowing the use of LC3 conversion in monitoring autophagy.[25]

The data presented here showed that treatment of ANA-1 cells with EspB alone did not alter LC3B-II production. However, reduced LC3B-II protein expression was observed in EspB-pretreated murine ANA-1 cells triggered by subsequent treatment with IFN-γ. These findings suggest that EspB suppresses the IFN-γ-induced autophagy, an effect that possibly correlates with IFN-γ signaling.

Classical IFN-γ-induced cellular effects involve activation of the Janus kinase STAT1-regulated signaling events. The binding of IFN-γ to its cognate receptor induces the assembly of an active receptor complex and subsequent transphosphorylation of the receptor-associated Janus tyrosine kinases Jak1 and Jak2. This is followed by recruitment of the cytosolic STAT1 protein to the active complex. Following translocation into the nucleus, the dimerized STAT1 induces transcription of cellular genes to mediate the initiation of various antimicrobial processes, including cytokine expression and autophagy.[26–29] Li et al[30] confirmed the crucial role of STAT1 activation in IFN-γ-induced autophagy.

The EspB protein downregulates IFN-γR1 and inhibits IFN-γ-activated STAT1 phosphorylation, thereby downmodulating macrophage responsiveness to IFN-γ. The present characterization of the mycobacterial effector EspB as a modulator of autophagy presents a previously unknown paradigm in the understanding of host–pathogen interactions in mycobacterial infection. However, because EspB is only one of many molecules that mediate Mtb immune modulation, the autophagy-dysregulating effect of EspB could be masked by an entire set of mycobacterial components in the context of the whole bacillus. For instance, Mtb is known to modulate macrophage function by affecting the host pathway that is dependent on nitric oxide synthase 2, which generates toxic reactive nitrogen intermediates.[31] Future studies using EspB mutant bacteria as well as *in vivo* experiments are needed to assess further the effect of EspB.

In conclusion, we demonstrated here that the EspB protein inhibits IFN-γ-induced autophagy in mouse macrophages. This inhibitory effect is due in part to the downregulation of IFN-γR1 and results in the inhibition of IFN-γ-induced STAT1 phosphorylation. The ability of purified EspB to inhibit macrophage function is a relevant component in the pathogenesis of such chronic mycobacterial infections in humans.

Conflicts of interest

All the authors declare they have no conflict of interests.

Acknowledgments

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