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Mycobacterium tuberculosis ESAT6 induces IFN-β gene expression in Macrophages via TLRs-mediated signaling

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ABSTRACT

Mycobacterium tuberculosis is a highly virulent bacterium that causes tuberculosis. It infects about one third of the world's population. Type I interferons (IFNs) play a detrimental role in host defense against *M. tuberculosis* infection. Proteins secreted by *M. tuberculosis* through ESX-1 secretion system contribute to type I IFNs production. However, the precise mechanism by which 6-kDa early secretory antigen target (ESAT6), one of ESX-1-mediated secretory proteins, induces type I IFNs production in host cells is currently unclear. Therefore, the objective of the present study was to determine the underlying molecular mechanism regulating ESAT6-mediated gene expression of IFN-β in macrophages. Recombinant ESAT6 produced from *E. coli* expression system induced IFN-β gene expression in various types of macrophages such as mouse bone marrow-derived macrophages (BMDMs), peritoneal macrophages, and MH-S cells (murine alveolar macrophage cell line). Deficiency of TLR4 and TRIF absolutely abrogated ESAT6-induced IFN-β gene expression. TLR2 and MyD88 were partially involved in IFN-β gene expression in response to low dose of ESAT6. Another recombinant ESAT6 produced from baculovirus system also upregulated IFN-β gene expression via TLR4-dependent pathway. Polymyxin B (PMB) treatment impaired LPS-induced IFN-β expression. However, IFN-β expression induced by ESAT6 was not influenced by PMB. This suggests that ESAT6-mediated IFN-β expression is not due to LPS contamination. Treatment with ESAT6 resulted in activation of TBK1 and IRF3 in macrophages. Such activation was abolished in TLR4- and TRIF-deficient cells. Moreover, inhibition of IRF3 and TBK1 suppressed IFN-β gene expression in response to ESAT6. Our results suggest that ESAT6 might contribute to virulence of *M. tuberculosis* by regulating type I IFNs production through TLR4-TRIF signaling pathway.

1. Introduction

Mycobacterium tuberculosis is the etiologic agent of tuberculosis. It has been estimated that one-third of the world's population is infected by *M. tuberculosis* [1]. Region of difference 1 (RD1) is considered as the pathogenic gene region of *M. tuberculosis*. It is absent in avirulent *M. bovis* BCG but present in various virulent strains [2,3]. Depletion of RD1 locus reduces *in vivo* replication of *M. tuberculosis* in lungs and spleens, thus improving survival and lung pathology in mice [4]. Moreover, complementation with RD1 markedly increases virulence, persistence, and dissemination of BCG strain in mice [5]. RD1 locus is approximately 9.5 kb in length and contains nine genes including Rv3875 (6-kDa early secretory antigen target, ESAT6) and Rv3874 (10-kDa culture filtrate protein, CFP10) [6]. *M. tuberculosis* has five secretion systems (ESAT6 system-1 (ESX-1) to ESX-5) to export many of their virulence factors [7,8]. One of these systems, ESX-1 is encoded by genes in RD1 locus and involved in the secretion of virulence proteins ESAT6 and CFP10 [9,10].

Type I interferons (IFNs) are originally known to be potent antiviral factors. However, recent studies have revealed that IFNs are involved in pathological severity caused by various bacterial infections [11,12]. During *M. tuberculosis* infection, type I IFNs seem to have detrimental effects on the host. Manca *et al.* have demonstrated that intranasal administration of purified IFN-α/β can lead to increased bacterial burden in lungs and reduced survival in mice [13]. Moreover, type I IFNR-deficient mice have displayed reduced growth of *M. tuberculosis* in spleens of infected mice [14]. Although several microbial and host factors have been suggested to be able to induce type I IFNs production

Abbreviations: RD1, Region of difference 1; ESAT6, 6-kDa early secretory antigen target; ESX-1, ESAT6 system-1; CFP10, 10-kDa culture filtrate protein; Type I IFNs, Type I interferons; TBK1, TANK-binding kinase; IRF, Interferon regulatory factor; BMDMs, Bone marrow-derived macrophages; PMB, Polymyxin B

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in response to *M. tuberculosis*, the precise underlying mechanism by which *M. tuberculosis* induces the production of IFNs remains unclear. Stanley *et al*. have shown that depletion of genes coding ESX-1 secretion system as well as ESAT6 (*esxA*) impairs IFN-β gene expression both *in vitro* (macrophages) and *in vivo* (spleens and lungs) [14]. They have suggested that TLRs signaling and Rip2 are not required for *M. tuberculosis*-induced IFN-β expression in macrophages, whereas TANK-binding kinase 1 (TBK1) is critical [14]. On the contrary, Pandey et al. have shown that Nod2-Rip2 pathway contributes to IFN-β gene expression in macrophages in response to *M. tuberculosis* in interferon regulatory factor 5 (IRF5)-dependent manner [15]. Although studies using mutant strains have shown that ESX-1 secretion system and its secretory protein ESAT6 are essential for the production of type I IFNs in response to *M. tuberculosis*, the molecular mechanism by which ESAT6 induces such production in host cells remains unclear. Therefore, the objective of the present study was to determine the underlying molecular mechanism regulating ESAT6-mediated gene expression of IFN-β in macrophages. Our results showed that TLR2/4-mediated signaling regulated IFN-β gene expression in macrophages in response to recombinant ESAT6 in a dose-dependent manner. In addition, both IRF3 and TBK1 were involved in gene expression in ESAT6-treated macrophages.

2. Materials and methods

2.1. Animals

Wild type (WT), TLR2-, TLR4-, and MyD88- deficient mice with C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). TRIF-deficient mice were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan). TLR2/4 double-deficient mice were generated in our lab by crossing mice deficient of single TLR2 and TLR4 and intercrossing F1 generations. Animal studies were performed using protocols approved by the Institution Animal Care and the Use Committee of Chonnam National University (Approval No. CNU IACUC-YB-2015-32).

2.2. Reagents

Poly (I:C) as a synthetic analog of dsRNA and LPS from *Escherichia coli* O111:B4 were purchased from InvivoGen (San Diego, CA, USA). For inhibitor assay, Polymyxin B sulfate (PMB; Sigma-Aldrich, St. Louis, MO, USA) as inhibitor of LPS and BX795 (InvivoGen) as inhibitor of TBK1 were used.

2.3. Preparation of recombinant ESAT6

Recombinant ESAT6 antigen was expressed as a His-tagged protein in *E. coli* BL21 and purified by Ni-NTA affinity chromatography as described previously [16]. In addition, recombinant ESAT6 protein produced from baculovirus system (NBP1-99045) was commercially available (Novus Biologicals, Littleton, CO, USA).

2.4. Cell culture

Bone-marrow-derived macrophages (BMDMs) were isolated and differentiated as described previously [17]. Briefly, BMDMs were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY, USA) containing 30% L929 cell culture supernatant, 10% fetal bovine serum (FBS, Corning costar, Corning NY, USA), 1% penicillin/streptomycin (P/S), 1% MEM Non-Essential Amino Acids (MEM NEAA), 1% sodium pyruvate, and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37 °C for 6 days. Fresh medium was added 3 days later and cells were cultured for an additional 2 days and used in this study. Additionally, to differentiate BMDMs into M1 and M2 phenotypes, cells were stimulated with combination of LPS (100 ng/ml) and IFN-γ (20 ng/ml) (BioLegend, San Diego, CA, USA) and 50 ng/ml of IL-4 (R & D Systems, Minneapolis, MN, USA) for 2 days, respectively. The M1 or M2 phenotype was confirmed by gene expression of iNOS and CD206, representative markers of M1 and M2, respectively. For preparation of peritoneal macrophages, mice were i.p. injected with 2 ml of 4% thioglycollate broth (Sigma-Aldrich). Four days later, peritoneal lavage was performed twice with 5 ml of cold PBS. After lysing contaminated blood with RBC lysis buffer (eBioscience, San Diego, CA, USA), the cells were cultured in DMEM (Welgene, Gyeongsan, Gyeongbuk, Korea) containing 10% FBS and 1% P/S in a 5% CO_2 incubator at 37 °C. Murine alveolar macrophage cell line MH-S (American Type Culture Collection, Manassas, VA) was also cultured in RPMI 1640 medium (Welgene) containing 10% FBS, 0.05 mM 2-mercaptoethanol (Sigma-Aldrich), and 1% P/S in a 5% CO_2 incubator at 37 °C.

2.5. Real-time quantitative PCR (qPCR)

For real-time quantitative PCR, BMDMs or MH-S cells were seeded into 6-well plates at a density of 2×10^6 cells/well and incubated overnight. To determine the expression of IFN-β, BMDMs and MH-S cells were treated with ESAT6 (1 μg/ml) for 2 and 4 h. Total RNA was isolated from cells using easy-BLUE™ Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea) and cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix (TOYOBO Bio-Technology, Osaka, Japan) according to the manufacturer's instructions. Target gene expression was quantified using QGreen™2X SybrGreen qPCR Master Mix (Cellsafe, Suwon, Korea). PCR primers used for real-time quantitative PCR amplification were as follows: IFN-β (forward 5′-ATGAACTC CACCAGCAGACAG-3′, reverse 5′-ACCACCATCCAGGCGTAGC-3′); iNOS (forward 5′-GAGATTGGAGTTCGAGACTTCTGTG-3′, reverse 5′-TGGC TAGTGCTTCAGACTTC-3′); CD206 (forward 5′-GTGGAGTGATGG AACCCCAG-3′, reverse 5′-CTGTCCGCCCAGTATCCATC-3′); GAPDH (forward 5′-CGACTTCAACAGCAACTCCCACTCTTCC3′, reverse, 5′-TGGGTGGTCCAGGGTTTCTTACTCCTT-3′). qPCR data were normalized using GAPDH as an endogenous control. Real-time PCR was performed in a Rotor-Gene Q real-time PCR system (Qiagen, Hilden, Germany) using a two-step protocol of 95 °C for 10 seconds followed by 40 cycles of 58 °C for 45 seconds.

2.6. Immunoblotting

For immunoblotting, BMDMs or MH-S cells were seeded into 6-well plates at a density of 2×10^6 cells/well and incubated overnight. These cells were treated with ESAT6 (1 μg/ml) or Poly (I:C) (100 μg/ml). Cells were lysed at indicated time point in a buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, and 0.02% NaN_3 supplemented with protease inhibitor (complete, Mini, EDTA-free, Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich), and 2 mM dithiothreitol. To detect target protein, lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were probed with primary antibodies against regular and phosphorylated forms of IFR3 and TBK1 (Cell signaling Technology, Beverly, MA, USA). A primary antibody against β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used to verify equal loading of protein samples. After immunoblotting with relevant secondary antibodies (Santa Cruz Biotechnology), proteins were detected using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA).

2.7. siRNA transfection

For targeted gene silencing of IRF3, synthetic duplex RNA oligonucleotides were transfected into MH-S cells. On-Target plus smart pool short interfering RNA (siRNA) IRF3 was purchased from Dharmacon. On-target plus non-targeting siRNA (Dharmacon, Lafayette, CO) was