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Early secreted antigenic target of 6-kDa of *Mycobacterium tuberculosis* promotes caspase-9/caspase-3-mediated apoptosis in macrophages

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Abstract
Mycobacterium tuberculosis (Mtb) is an intracellular pathogen that triggers several survival mechanisms against the host immune system. Many studies show that the diverse components of Mtb can modulate apoptosis in various types of cells differently. So far, apoptosis induced by ESAT-6, an early secreted antigenic target of 6-kDa of Mtb, has been studied but the details of molecular mechanism and signaling pathway remain incompletely defined. This study investigated the role of recombinant ESAT-6 in inducing apoptosis in primary bone marrow-derived macrophages (BMDMs) of mice using Annexin V/PI assay with FACS analysis and Western blotting technique. It has been found that ESAT-6-induced apoptosis in BMDMs in a dose- and time-dependent pattern. Apoptosis induced by ESAT-6 was mainly via the intrinsic pathway with elevated protein levels of cleaved caspase-9 and -3. Furthermore, ESAT-6 also induced Bim activation during this process. Interestingly, this event was TLR2-dependent since the effect of ESAT-6 on apoptosis vanished in BMDM from mice with TLR2 deficiency. Furthermore, ROS generation and MAPKs phosphorylation induced by ESAT-6 were also involved in caspase-9 and caspase-3 activation. Taken together, these data suggest that ESAT-6-mediated apoptosis is involved in ROS-MAPKs signaling and further activating the intrinsic pathway, which provides new insights into the basic physiology of macrophage death in tuberculosis.

Keywords Macrophage · ESAT-6 · TLR2 · ROS · MAPK · Apoptosis

Introduction

Tuberculosis (TB) is a chronic inflammatory disease caused by *Mycobacterium tuberculosis* (Mtb). It is distributed world widely with high rate of morbidity and mortality [1]. Interestingly, about one-third of the world's population has a contagion affected with Mtb but only less than 10% of them suffer from TB. Usually people with a hypofunction of immunity have a higher risk of falling ill from TB. Therefore, the immune response of the host critically influences the progression of Mtb infection. Understanding the interaction between Mtb and host immune response is the key point for controlling TB.

From infection to apoptosis, many signaling are involved in target cells. Apoptosis is a very important response of host defense mechanism against pathogen, which involves multiple constituents and highly coordinated signaling. Mtb can proactively trigger the apoptosis in phagocytic cells, which can benefit the dissemination of Mtb. Macrophage-mediated innate immune response functions are a first line of host defense against Mtb including secreting various cytokines to mediate the inflammatory response [2, 3], and further inducing different types of cell death to control the bacterial growth [4]. Many studies have showed the virulent Mtb strain exposed as a much stronger apoptosis inducer compared with the attenuated Mtb strain [5–7] which indicates apoptosis can benefit the dissemination of Mtb. Up till now, two classical signal pathways have been reported for the induction of apoptosis in Mtb-infected macrophages. One is the extrinsic pathway. Macrophage-apoptosis induced by Mtb depends on the induction of TNF-α cytokine that is

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mediated by the executioner caspases-3 and -7 [8]. Meanwhile TNF-α also mediates the activation of the kinases p38, ASK1and c-Abl, which identifies the early components of the extrinsic apoptotic pathway [9]. Mtb-infection-induced apoptosis in macrophages could mediate ROS-endoplasmic reticulum stress response leading to calreticulin (CRT) exposure on the macrophage plasma membrane, which further forms CRT/CXCR1/TNFR1 complex activating caspases-8-dependent apoptosis [10]. The other pathway is the intrinsic pathway. Infection of macrophages with the attenuated H37Ra or the virulent H37Rv could upregulate Bax expression and cytochrome c release from the mitochondrial intermembrane space [11]. One more study shows *M. fortuitum*, a natural fish TB pathogen, could mediate apoptosis in fish macrophage via calpain-caspase-12-caspase-9 signaling pathway [12], which indicates Mtb may activate intrinsic apoptotic pathway. As known, BH-3-only proteins of Bim has been implicated in the apoptosis signaling in lymphocytes [13]. A study also reported that Mtb-induced apoptosis in J774 cells via Bim activation [14]. Therefore, different apoptosis inducers activate their own signaling pathways during host response since many components of Mtb can impact innate immune response signaling.

Toll-like receptors' (TLRs') family is crucial to bridging the innate and adaptive immunity. To date, 10 TLRs have been identified in humans and 12 found in mice. Each type of TLR is responsible for distinguishing different types of ligands and evoking various innate immune responses. Many studies have shown that the established pathogen-associated molecular patterns (PAMPs) from Mtb activate immune cells through the plasma membrane receptors. For example, some Mtb components, such as MPT83, Rv1016c, MTBRa, Rv2660c and ESAT-6, bind to TLR2, and some other Mtb components, such as 60-/65-HSPs, 3-/4-acylated lipomannan, 50S ribosomal protein, bind to TLR4 on the surface of cells [15, 16], whereas TLR8 and TLR9 are also important recognition receptors in endosomes that can bind to Mtb components such as DNA and related mimic Mtb CpG motif molecules. TLR9-deficient mice are susceptible to Mtb infection than wild-type [17]. TLR8 plays an important role in susceptibility to pulmonary tuberculosis in different populations [18].

Caspase-1 activation depends on live intracellular Mtb that expresses a functional ESX-1 system [19]. Inhibition of caspase-1 can strongly inhibit Mtb-induced apoptosis [20]. ESAT-6, secreted from ESX-1 system of Mtb as virulence factor, has been described as a pro-apoptotic factor [21]. Some observations indicate that ESAT-6 not only upregulates expression of caspase-3, -5, -7 and -8 in THP-1 cell [22] but also causes endoplasmic reticulum stress increasing caspase-12 and -3 activation in A549 cell [23], downregulates BCL-2 and increases caspase-3 expression in macrophage [24]. These findings could infer that ESAT-6 plays an important role in Mtb-induced apoptosis. Despite the observations mentioned above, the precise molecular mechanisms of ESAT-6-modulating macrophage apoptosis signaling pathway remain unclear. This study investigated ESAT-6-induced apoptosis in BMDM from mice and the interplay between ROS-mediated MAPKs phosphorylation and the activation of caspases signaling pathways.

Materials and methods

Experimental animal and cell culture

C57BL/6 mice (male wild type, 6–8 weeks old) were purchased from the Central Animal Facility of Tongji University (Shanghai, China). The isolation and cultures were performed as described in this lab previously [25]. Briefly, BMDMs were isolated by flushing the bone marrow from the femur and tibia of the hind legs of mice. The extracted cells were incubated for 6–7 days in RPMI 1640 containing 25% L929 mouse fibroblasts conditioned media and 20% fetal bovine serum allowing cells to differentiate. Then the cells were collected for further studies as described in each experiment. This project involving mice study has been approved by the Institutional Ethics Committee of Animal Experimentation of Tongji University. All protocols complied with the National Institutes of Health Guide for the care and use of laboratory animals, and conformed to directive 2010/63/EU and NIH guidelines.

Drugs and chemicals

Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit was obtained from BD Company (Franklin Lakes, NJ, USA). Commercial standard ESAT-6 was purchased from ProSpec-Tany Technogene Ltd. (Ness Ziona, Israel). EtEraser Endotoxins Removal Kit was obtained from Houshiji Corporation (Ximan, China). TUNEL apoptosis assay kit and ROS detection kit were obtained from KeyGen (Nanjing, China). Anti-GADPH mouse monoclonal antibody was purchased from Multi Sciences (Hangzhou, China). Bid and Bim antibodies were purchased from Abcam (Cambridge, UK). All other primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All secondary antibodies were obtained from Jackson (West Grove, PA, USA). Caspase-9 inhibitor Z-LEHD-FMK, ERK inhibitor PD098059, p38 inhibitor SB203580 and JNK inhibitor SP600125 were purchased from Selleck (Huston, TX, USA). ROS inhibitor dimethyhhiourea (DMUT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). General laboratory chemicals were obtained from Sangon (Shanghai, China), including nitrilotriacetic acid (Ni-NTA) columns. Reagents

for cell culture were obtained from Gibco (Waltham, MA, USA).

Expression and purification of ESAT-6

The constructed plasmid pET21a (+)-ESAT-6 was generated in our Lab as described previously [25]. The ESAT-6 gene was constructed in the expression plasmid pET21a and tagged with 6×His at the C terminus. *Escherichia coli* BL21 cells carrying the recombinant plasmid were induced with isopropyl-*β*-D-thiogalactopyranoside (IPTG), and His-tagged recombinant ESAT-6 was purified with nitrilotriacetic acid (Ni-NTA) columns (Sangon, Shanghai, China). ESAT-6 protein was concentrated through Amicon® Ultra-15 column (Millipore, Billerica, MA, USA). The purity of the recombinant ESAT-6 protein was analyzed by performing SDS-PAGE, followed by Coomassie staining. The biological function of recombinant ESAT-6 was tested and compared with commercial standard ESAT-6 (ProSpec, Rehovot, Israel) after removing contaminated endotoxins with the Kit. The purified recombinant ESAT-6 was stored − 80 °C for further experiments.

ROS Detection

The levels of intracellular ROS were measured with ROS detection kit. The ROS-specific cell-permeable fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used. Cells were treated with 1 μg/ml commercial standard ESAT-6 or purified ESAT-6 for 6 h before probing with 10 mM DCFH-DA for 20 min. The positive fluorescent signals were measured by Flow Cytometer (FACS Calibur, BD Biosciences).

Detection of apoptotic cell

Apoptotic cell was detected with Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit after cells treatment. According to the manufacturer's instruction, stained cells were assayed by flow cytometry (FACS Calibur, BD Biosciences) after fixation with FACS fixed buffer (FACS buffer containing 1% paraformaldehyde). The positive cells were calculated and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). For TUNEL assay, the frequency of apoptosis was evaluated using terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) method according to the manufacturer's protocol. Briefly, the cells are cultured in 96-well plates and fixed with 4% paraformaldehyde phosphate buffer saline for 20 min, then permeabilized by 0.3% Triton X-100 for 10 min, followed by TUNEL for 1 h at 37 °C and DAPI staining for 5 min. The Cy3-labeled TUNEL-positive cells were stimulated at 550 nm excitation and 570 nm radiation under a fluorescence microscope. The proportions of apoptotic cells were calculated by counting stained cells.

Western blot analysis

Total proteins were extracted from cell lysates (KeyGen, Nanjing, China). To detect target protein, lysates were resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvindylidene fluoride (PVDF) membranes (Millipore, USA). These membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) buffer with nonfat milk (5% w/v) for 1 h at room temperature and then probed with primary antibodies for an additional 18 h at 4 °C. After washing with TBS-T, the immunoblots were incubated with relevant secondary antibodies for 60 min. The protein levels were detected using Clarity Western ECL Substrate (Millipore, Billerica, MA, USA). Bands were quantified by grey levels using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All experiments were performed for at least three times. The data were expressed as mean ± SEM. Calculation analysis was performed using GraphPad Prism software (San Diego, CA, USA). The unpaired two-tailed *t* test was applied for statistical analysis (EXCEL software). Differences were considered statistically significant at p value < 0.05.

Results

ESAT-6 induces apoptosis via caspase 9/3 signaling pathway

Compared to attenuated Mtb strain, virulent Mtb is a stronger apoptosis inducer inducing macrophage apoptosis whereas attenuated Mtb is tend less to elicit regulated cell death of host cells [5, 22]. ESAT-6 is a virulent secreted protein from virulent Mtb. To clarify whether ESAT-6 is involved in this event, the effect of ESAT-6-induced BMDM apoptosis was investigated. First, recombinant ESAT-6 protein was prepared from constructed *E. coli* and purified. The purity of the ESAT-6 preparation was evaluated using SDS-PAGE. As shown in Fig. 1a, a bright band was observed on the gel with the molecular weight of about 12 kDa, a recombinant ESAT-6 with histidine tag. The biological activity of the recombinant ESAT-6 was determined after LPS has been removed. The generation levels of ROS in BMDM induced by the recombinant ESAT-6 was the same as commercial standard ESAT-6's (STD) (Fig. 1b), which indicates that recombinant ESAT-6 is in good quality for the

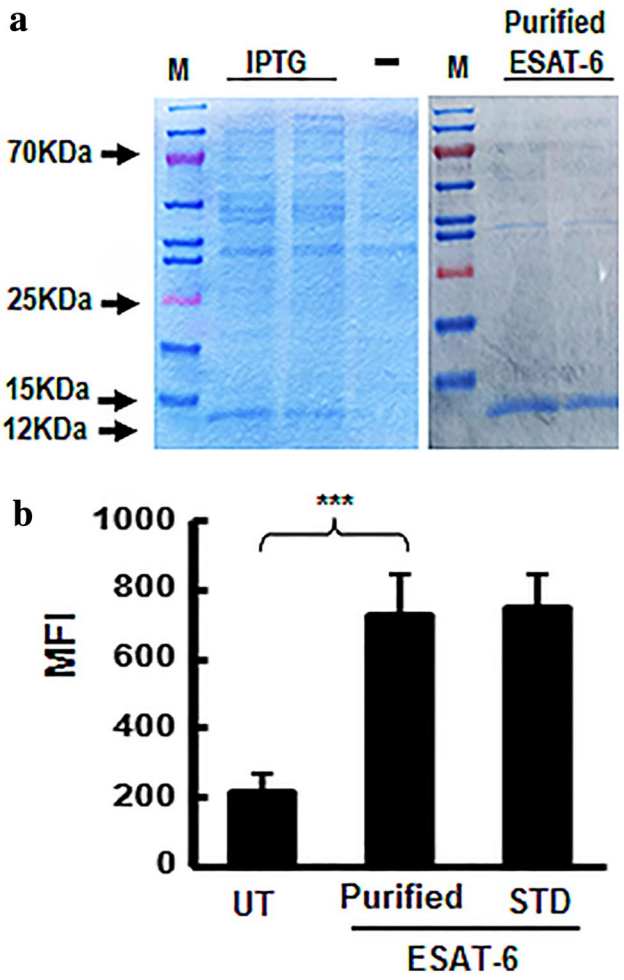


Fig. 1 Recombined ESAT-6 is purified and the biological activity was detected with ROS production. **a** Recombinant ESAT-6 protein was purified from *E. coli* containing ESAT-6 gene after IPTG induction. The cells were induced with or without IPTG inducer for 4 h before lysis. The cell lysates and purified recombinant ESAT-6 protein were analyzed by 15% SDS-PAGE. The gel was visualized after staining with Coomassie Blue followed by de-staining. **b** The biological activity of purified recombinant ESAT-6 was tested for ROS production. Cells were treated with 1 μg/ml of commercial standard ESAT-6 (STD) or purified recombinant ESAT-6 for 6 h. The levels of intracellular ROS induction were measured by flow cytometry after probing with DCFH-DA. Data are represented as mean ± SEM of three independent experiments. *** indicates $p < 0.001$ compared with the untreated group

study although there may be very low level of LPS existed with no biological effect.

Next, to investigate ESAT-6-induced apoptosis in dose- and time-response curve, BMDMs were stained with Annexin V/PI after the cells were stimulated with ESAT-6 in a various concentration for 24 h. As shown in Fig. 2a, the apoptotic cells were obviously increased when BMDMs were stimulated with ESAT-6 of concentration greater than 1 μg/ml condition. The time course demonstrates that the apoptotic cells were significantly increased after 12 h of stimulation with 1 μg/ml ESAT-6, and reached a maximal value after 36 h of stimulation (Fig. 2b). Furthermore, apoptotic-related molecules including cleaved caspase-9, -8 and -3 were measured. As shown in Fig. 2c, the levels of cleaved caspase-9 and -3 proteins were markedly increased after 6 h of stimulation and reached a peak value after 24 h. However, cleaved caspase-8 did not exhibit significant changes in the time period indicated. Interestingly, in the presence of the specific caspase-9 inhibitor Z-LEHD-FMK, the apoptotic cells dramatically dropped (Fig. 2d). To further confirm the specific caspase-9 inhibitor effect, cells were stained with TUNEL/DAPI and observed under inverted fluorescence microscope. The number of apoptotic cells was obviously reduced in the presence of specific caspase-9 inhibitor (Fig. 2e). It has been reported that casapase-8 can activate caspase-3 by directing cleavage caspase-3 or inducing the cleavage of Bid into tBid, which subsequently activate casapase-9 and caspase-3 cascades [26]. To exclude caspase-8 activation-induced apoptosis, ESAT-6-activated Bid was measured. As shown in Fig. 3a, there was no significant change in the protein levels of Bid in the time period indicated, which implies that caspase-8 was not be disturbed by ESAT-6 during this study. Collectively, these results provided an evidence that ESAT-6 has the ability to induce cell apoptosis by activating caspase-9 and caspase-3.

In addition, BH3-only members are essential initiators of apoptosis that are activated in response to many cytotoxic stimuli. It has been reported that only virulence Mtb can activate Bim, one of BH3-only members, and induces pro-apoptosis capacity but not BCG [14]. To identify whether ESAT-6 alone could activate Bim, a further study was carried out. BMDMs were stimulated with 1 μg/ml ESAT-6 for the time period indicated. Not surprisingly, the three main isoforms, BimEL, BimL and BimS, were visibly augmented after 24 h stimulation (Fig. 3b), indicating ESAT-6 could trigger the pro-apoptotic Bim cascade that leads to cell death.

TLR2 was required for ESAT-6-triggered apoptosis in macrophage

Recognition of mycobacterial components by Toll-like receptors (TLRs) is a key step in initiating innate immune responses upon mycobacterial infection. Previous studies have shown that ESAT-6 can directly bind TLR2 [27] and activate downstream signaling pathways to generate ROS in macrophages and produce TNF-α, MCP-1 [25, 28], but not IL-6 [29]. To identify whether TLR2 is responsible for ESAT-6-induced apoptosis in macrophage, TLR2-/- mice were used for this study. BMDMs were isolated from wild-type (WT) and TLR2-/- mice, respectively, and treated with or without ESAT-6 for 24 h. Interestingly, the apoptotic cells

induced by ESAT-6 completely vanished in TLR2-/- group compared with WT group (Fig. 4). These results indicated that initial signal for ESAT-6-induced macrophage apoptosis is mainly via TLR2 transduction, as the similar machinery for inducing ROS generation and TNF-α and MCP-1 production by ESAT-6 [25].

ESAT-6-induced activation of MAPK signaling partly activates intrinsic apoptotic signaling pathway

The MAPK signaling pathway regulates cell proliferation and apoptotic signaling [30]. MAPK activation has been observed in virulent Mtb-infected macrophages but not in BCG-infected cells [14]. Our previous study reported that ESAT-6 can significantly increase the activation of MAPKs to produce cytokine TNF-α and MCP-1 secretion [25]. To determine whether this signaling pathway induced by ESA-6 is associated with the apoptosis, the levels of phosphorylated MAPK-associated proteins were assessed after cells were stimulated with 1 μg/ml ESAT-6. The results showed that ESAT-6 stimulation could significantly increase the phosphorylation levels of p-ERK, p-JNK and p-p38 MAPKs (Fig. 5a). To further clarify whether ESAT-6-induced changes in MAPKs proteins activation are involved in apoptosis, the status of the cleaved caspase-9 and -3 were further measured in the presence of MAPK inhibitors. As shown in Fig. 5b, the ERK inhibitor PD98059 (20 μM), JNK inhibitor SP600125 (10 μM) and p38 inhibitor SB203508 (10 μM) could significantly mitigate the elevated levels of cleaved caspase-9 and -3 proteins induced by ESAT-6. These data indicate that ESAT-6 regulates the activation of apoptotic-related proteins partly through MAPK signaling pathway.

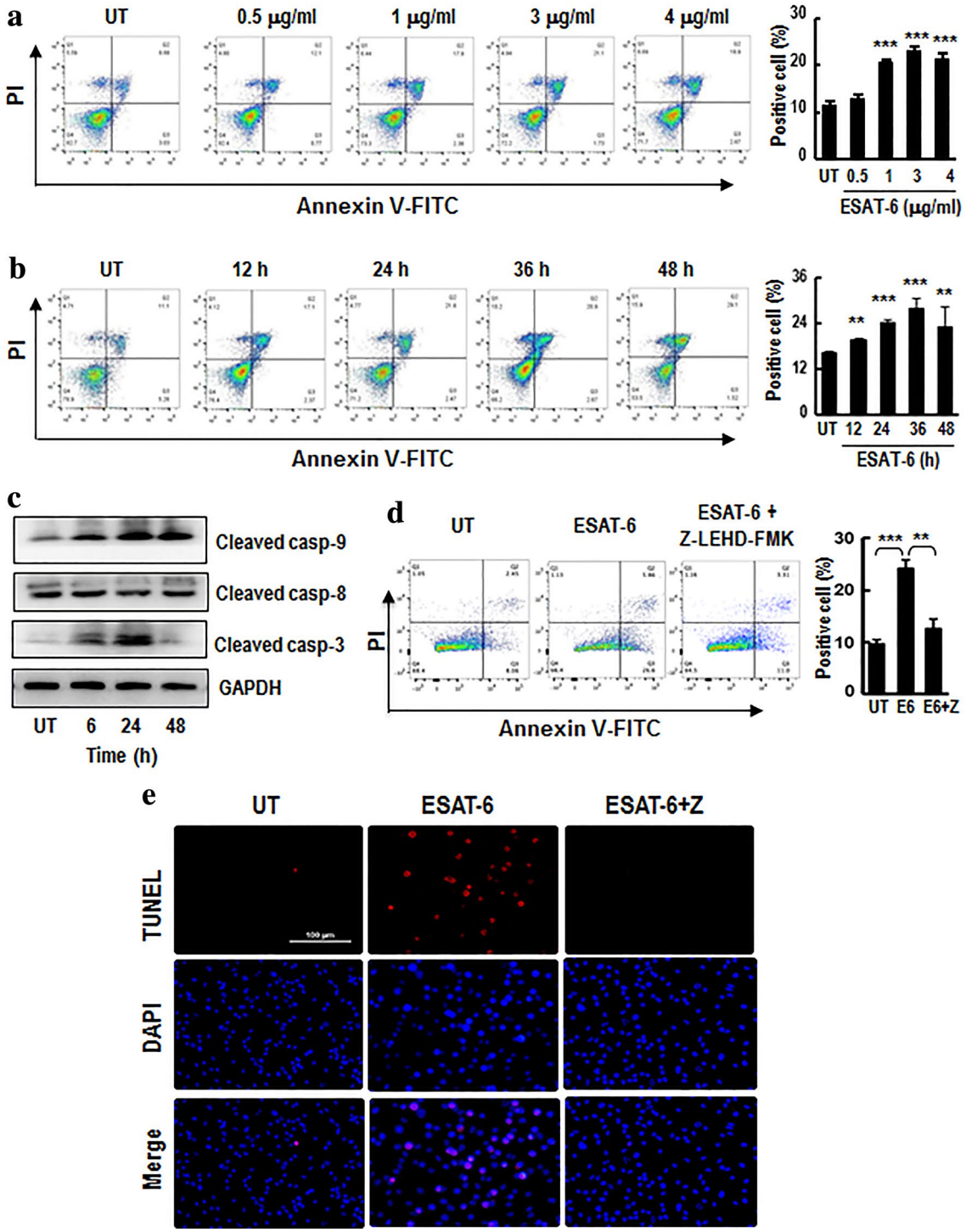
Critical roles of ROS-MAPKs signaling pathways in ESAT-6-induced caspase-dependent apoptosis

The production of ROS and the oxidative stress reaction are essential for apoptosis [31]. Interestingly, it has been reported tuberculin-purified protein derivative (PPD)/TLR2/ROS signaling is responsible for the induction of proinflammatory responses during Mtb infection [32], and moreover ESAT-6 can initiate TLR2-dependent signal transduction pathways which will generate many biological effects [25, 27]. To further explore the effect of ROS on ESAT-6-induced apoptosis, ROS antioxidants DMTU was used to suppress ROS production in BMDMs. Cells were treated with 30 mM DMTU for 60 min before treating with 1 μg/ml ESAT-6 for a further 24 h. A significant inhibition of ESAT-6-induced apoptosis was observed (Fig. 6a). In addition, cleaved caspase-9 and -3 were remarkably decreased in the presence of DMTU (Fig. 6b). It has been reported that ROS are possible upstream signaling molecules for the activation of the cellular kinases ERK, JNK and p38MAPK [33]. The interactions between ROS generation and MAPKs phosphorylation in ESAT-6-treated BMDMs were unclear during ESAT-6-induced apoptosis. To address this issue, the effect of ROS inhibitor DMTU on ESAT-6-induced phosphorylation of ERK, JNK, and p38 MAPK was evaluated. Cells were treated with 15 mM or 30 mM DMTU for 60 min before treating with 1 μg/ml ESAT-6 for a further 30 min. It is shown that MAPK activation could be significantly interrupted by ROS inhibitor DMTU in a dose-dependent manner (Fig. 6c). Taken together, these data suggest that the inhibition of ESAT-6-induced ROS generation could impact phosphorylation of ERK, JNK, and p38MAPK and further downregulate ESAT-6-induced cell apoptosis.

Discussion

Macrophage is the primary target cells during Mtb infection in lung. After phagocytosing the Mtb, macrophages express several antimicrobial mechanisms to limit the growth of the intracellular infection. Among these innate defense mechanisms, apoptosis has been linked to the killing of intracellular Mtb and limiting Mtb replication [34, 35]. Interestingly, a clinical study showed significantly elevated apoptotic cells in bronchoalveolar lavage (BAL) from patients with reactive pulmonary TB than that of normal controls [36]. Recently, many studies attempted to interpret evaluated apoptosis in the macrophage after virulent Mtb strains infection [21, 37, 38], but the role of ESAT-6 in pro-apoptotic cell formation and the signaling pathways remain to be clarified. This study has demonstrated that ESAT-6 significantly induced apoptosis in BMDMs in dose- and time-dependent patterns. ESAT-6-induced activated caspase-9 and -3 were associated with ROS generation and MAPKs phosphorylation via TLR2 receptor that responds to ESAT-6 stimulation on the cell surface of BMDM. These findings suggest that ESAT-6-mediated apoptosis is involved in ROS-MAPKs signaling and plays a significant role in TB pathogenesis.

Numerous studies have shown that treatment of macrophages with Mtb can lead to increasing rates of apoptosis. However, more research is needed to identify the Mtb components that are responsible for evoking the apoptotic response or the apoptotic mechanisms. Using a purified recombinant ESAT-6 protein preparation, results from this study shows that ESAT-6-mediated apoptosis mainly via activation of caspase-9 and -3, not caspase-8. The similar response pattern was also reported in human lung type II epithelial cell (A549) [23] but not in THP-1 cells [22]. Interestingly, ESAT-6 mediates apoptosis in A549 cells through ER stress resulting in increasing the activation of caspase-12 and -3 signaling pathway [23]. *M. fortuitum*, as a natural fish TB pathogen, could mediate apoptosis in fish macrophage via calpain-caspase-12-caspase-9 signaling pathway [12].



Surprisingly, one research group has reported that ESAT-6 not only elevated level of caspase-8 and -3 mRNA expression, but also increased expression of anti-apoptotic genes (bcl-2 and the apoptosis inhibitory protein 3) in THP-1 cells [22]. Although it is not yet clear which of caspase-9 or caspase-8 apoptotic pathway plays a critical role in regulating apoptosis, data from this study suggest that caspase-9 and caspase-3 are required for the induction of BMDMs apoptosis during ESAT-6 stimulation since the activation of caspase-8 and Bit was not observed. The reason for this discrepancy could be due to the different types of cells used, and probably a consequence of interactions that exist between the diversity of immune cells. Several studies have identified Bim as a crucial mediator of cytokine-induced

◂**Fig. 2** Recombinant ESAT-6 induces BMDM apoptosis in a dose- and time-dependent manner via mainly caspase-9 signaling pathway. **a** The dose–response curves of EAST-6-induced apoptosis were detected by Annexin V/PI assay after treating BMDMs with a concentration range of ESAT-6 in the experiments for 24 h. **b** The time–response curves of EAST-6-induced apoptosis were detected by Annexin V/PI assay after BMDMs were stimulated with 1 μg/ml ESAT-6 for a time period indicated. Cells were stained with Annexin V/PI and analyzed by flow cytometry. A representative experiment is shown in the left panels. Data in the graphs (right panels) are represented as mean ± SEM of three independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively, compared with the untreated group. **c** The protein levels of cleaved caspase-9, -8, -3 were analyzed by Western blotting after cells were treated with 1 μg/ml ESAT-6 for a time period indicated. GAPDH served as internal control. These blots were obtained from one of three independent experiments, respectively. **d** Cells were treated with caspase-9 inhibitor Z-LEHD-FMK (20 μM) for 60 min before treatment with 1 μg/ml ESAT-6 for a further 24 h. The apoptotic cells were detected by Annexin V/PI assay with flow cytometry. A representative experiment is shown in the left panels. Data in the graphs (right panels) are represented as mean ± SEM of three independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$ respectively, compared with the untreated group. **e** Above treated cells were also double stained with TUNEL (red) and DAPI (blue). A representative group image of stained cells was observed under inverted fluorescence microscope. Bar = 100 μm. Similar results were observed in three separate experiments

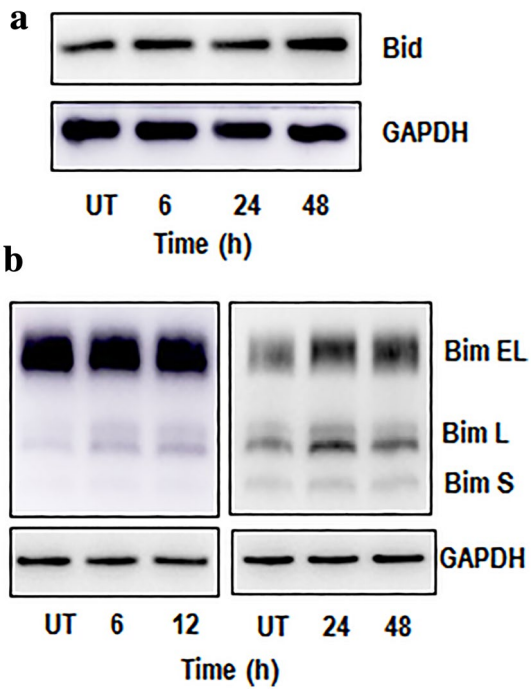


Fig. 3 ESAT-6 does not induce activations of Bid but activate Bim in macrophages. **a** The cells were treated with 1 μg/ml ESAT-6 for a time period indicated. Total cell lysates were prepared and the protein levels of Bid were analyzed by Western blotting. GAPDH served as internal control. These blots were obtained from one of three independent experiments, respectively. **b** The cells were treated with 1 μg/ml ESAT-6 for a time period indicated. Total cell lysates were prepared and the protein levels of three Bim isoforms were analyzed by Western blotting. GAPDH served as internal control. These blots were obtained from one of three independent experiments, respectively

apoptosis [14, 39]. It has been reported that upregulation of Bim was observed only in Mtb-infected macrophages but not in BCG-infected cells [14] indicating ESAT-6 may play a vital role in this process. This study has further confirmed that ESAT-6 can activate Bim protein-promoting cell undergoing pro-apoptosis.

TLRs are a family of membrane receptors for PAMP detection and are involved in the innate immune responses. They can be mainly divided into two groups according to their subcellular localization and evoke different innate immune response. An increasing body of evidence has showed that the principal TLRs for Mtb are TLR2 and TLR4 expressed on the plasma membrane, and TLR8 and TLR9 expressed in endosomes. For example, some components of Mtb have been shown to elicit the production of a broad range of immune response in a TLR2-dependent manner, such as MPT83, Rv2660c protein, MTBRa and ESAT-6 [17]. TLR2 gene polymorphisms or mutation may also increase the risk of susceptibility to Mtb. It has been reported that the extreme C terminus of ESAT-6 can directly bind to a distinct groove in the ECD of TLR2 [27]. Our previous studies and other reports have showed that ESAT-6 not only induces secretion of MCP-1 and TNF-α in macrophage via TLR2-dependent pathway but also induces secretion of IL-6 and TGF-β in DCs through TLR2-My88-dependent signaling [25, 40]. However, a group reported a different observation that ESAT-6-induced IL-6 production in macrophage may not require TLR2 [29], which drops a hint that ESAT-6 could directly or indirectly activate multiple signaling pathways to produce various cytokines in macrophages. Obviously, more studies are required to address this association. Nevertheless, this study has indicated that TLR2 is an important trigger for ESAT-6-induced apoptosis in macrophage.

In intra-macrophage bacterial infections, ROS generation seems to play a significant role in the host defense mechanism against bacteria. The roles of intracellular ROS in mediating inflammatory responses to Mtb infection are not well understood. Our previous studies along with other reports have demonstrated that the components of Mtb ESAT-6 and PPD elicit inflammatory responses in monocytes/macrophages via TLR2 and ROS-mediated activation of MAPK signaling pathways [25, 32]. It has been reported that apoptosis induced by Mtb correlates with the presence of a functional ESX-1 system and secretion of ESAT-6, which suggests ESAT-6 may play a pro-apoptotic role under physiological conditions [21]. As known, ROS

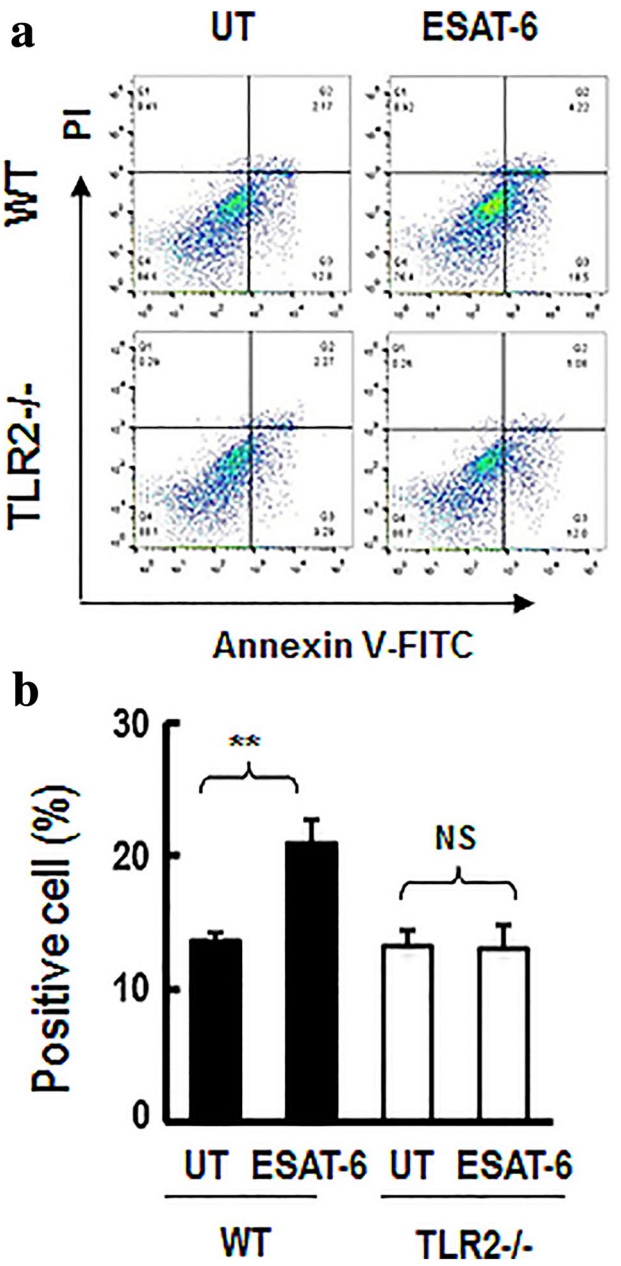


Fig. 4 TLR2 is required for ESAT-6 trigger apoptosis in macrophage. BMDMs from wild-type (WT) or TLR2-/- mice were challenged with 1 μg/ml ESAT-6 for 24 h before analyzing cell apoptosis. **a** One of the three representative experiments is shown after cells were stained with Annexin V/PI assay and analyzed by flow cytometry. **b** Data in the graphs are represented as mean ± SEM of the three independent experiments. ** indicates $p < 0.01$ compared with the untreated group

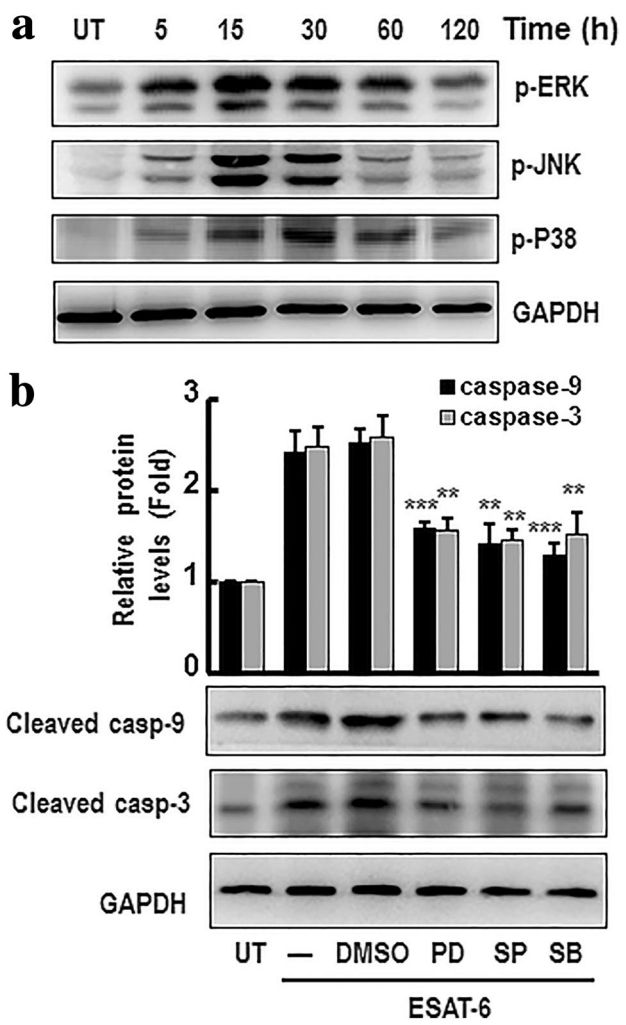


Fig. 5 The activation of MAPK signaling pathway takes part in ESAT-6-induced apoptosis. **a** BMDMs were stimulated with 1 μg/ml ESAT-6 for a time period indicated and the protein levels of phosphorylated ERK, JNK and P38 were detected by Western blotting. GAPDH served as internal protein control. These blots were obtained from one of three independent experiments, respectively. **b** BMDMs were pretreated with ERK inhibitor PD98059 (20 μM), JNK inhibitor SP600125 (10 μM) or p38 inhibitor SB203508(10 μM), respectively for 60 min before treatment with 1 μg/ml ESAT-6 for a further 24 h. The protein levels of cleaved caspase-9/-3 in the cells were analyzed by Western blotting and semi-quantified by Image J software. A typical group of blots is shown and similar results were obtained in three separate experiments. DMSO served as vehicle control. GAPDH served as the internal control. Each relative value is normalized to the level of GAPDH in the same sample. Data represented are the means ± SEM of three independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively, compared with the DMSO control group

serves as an important second messenger to control a wide range of physiological and pathological processes. It has been reported that elevated ROS activates PKC and MAPK signaling pathways to drive NF-κB and AP-1 pathways and ultimately alter target gene expression [41, 42]. Interestingly, it has been reported that activation of p38MAPK in Mtb-infected macrophages not only upregulates Bims activation but also increases pro-apoptotic cell and impedes cell necrosis [14, 43]. Not surprisingly, this study showed ESAT-6-stimulated cells could increase MAPKs phosphorylation and enhance cleaved caspase-9/-3 protein levels. The further study has demonstrated that ESAT-6 causes

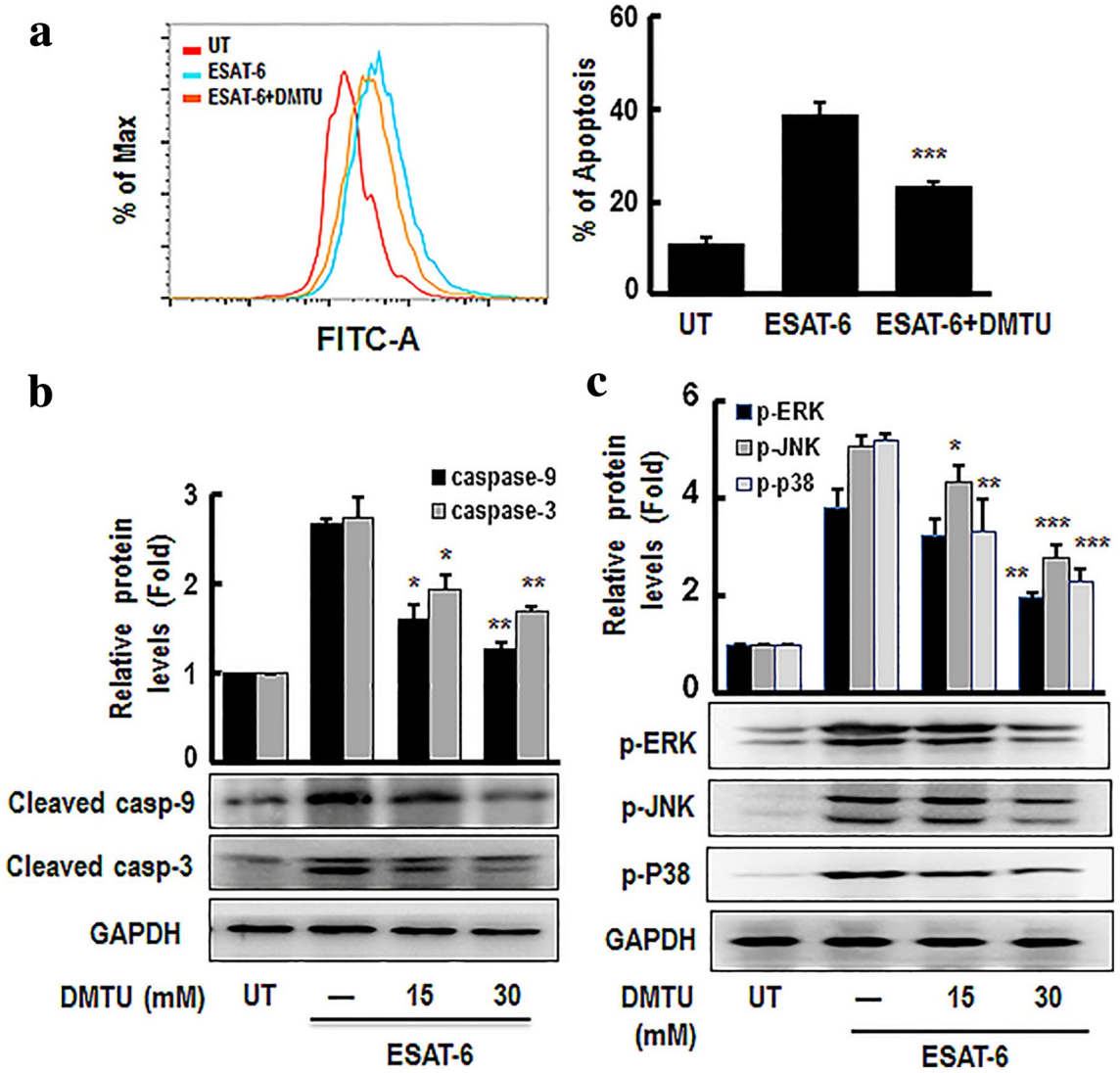


Fig. 6 ESAT-6-mediated apoptosis is partly involved in ROS-MAPKs signaling. **a** BMDMs were pretreated with or without ROS inhibitor DMTU (30 mM) for 60 min before treatment with 1 μg/ml ESAT-6 for a further 24 h. Apoptotic cells were detected by Annexin V-FITC/PI and observed by FACS. A typical group of FITC distribution curve is shown (left panels) and similar results were obtained in three separate experiments. The percentage of apoptotic cells were calculated in the graphs (right panels). Data are expressed as means ± SEM from three separate experiments. *** indicates $p < 0.001$ compared with ESAT-6 treated group. **b** BMDMs were pretreated with or without ROS inhibitor DMTU (15 or 30 mM) for 60 min before treatment with 1 μg/ml ESAT-6 for a further 24 h. The protein levels of cleaved caspase-9, -3 were analyzed by Western blotting and semi-quantified by Image J software. A typical group of blots is shown and similar results were obtained in three separate experiments. GAPDH served as internal control. Data represented are the means ± SEM of three independent experiments. * and ** indicate $p < 0.05$, and $p < 0.01$, respectively, compared with ESAT-6 treated group. **c**. BMDMs were pretreated with or without ROS inhibitor DMTU (15 or 30 mM) for 60 min before treatment with 1 μg/ml ESAT-6 for a further 30 min. The protein levels of phosphorylated ERK, JNK and p38 in the cells were analyzed by Western blotting and semi-quantified by Image J software. A typical group of blots is shown and similar results were obtained in three separate experiments. GAPDH served as internal control. Data represented are the means ± SEM of three independent experiments. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with ESAT-6 treated group

elevated pro-apoptotic cell via increasing intracellular ROS, which has been confirmed by the addition of DMTU, a ROS scavenger, along with ESAT-6 leading to a significant reduction in ERK/JNK/p38 MAPKs phosphorylation and cleaved capase-9/-3 formation. The association of ROS production with MAPKs-capase-9/-3 activation indicates an intracellular signaling pathway for ESAT-6-inducted apoptosis in macrophage. To fully understand ESAT-6-induced signaling pathway during this process, more studies are needed

to explore how ESAT-6 induces NADPH oxidase activation and its possible signaling pathway.

In conclusion, the result presented in this study confirms that ESAT-6-promoted apoptosis could activate intrinsic pathway in macrophages by rapidly targeting the ROS-MAPKs signaling that is dependent on ESAT-6 band with TLR2. This study provides a basis for a new avenue to explore various immunomodulatory mechanisms of ESAT-6 in host innate immunity against mycobacterial infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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