Cellular Immunology 335 (2019) 41–50

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Regulation of host cell pyroptosis and cytokines production by *Mycobacterium tuberculosis* effector PPE60 requires LUBAC mediated NF-κB signaling

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ARTICLE INFO

Keywords:
Mycobacterium tuberculosis
PE/PPE
Pyroptosis
LUBAC
PPARγ

ABSTRACT

Tuberculosis, caused by *Mycobacterium tuberculosis* infection, remains a global public health threat. The success of *M. tuberculosis* largely contributes to its manipulation of host cell fate. The role of *M. tuberculosis* PE/PPE family effectors in the host destiny was intensively explored. In this study, the role of PPE60 (Rv3478) was characterized by using *Rv3478* recombinant *M. smegmatis*. PPE60 can promote host cell pyroptosis via caspases/NLRP3/gasdermin. The production of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12p40 and TNF-α was altered by PPE60. We found that LUBAC was involved in PPE60-elicited NF-κB signaling by using Linear Ubiquitin Chain Assembly Complex (LUBAC)–specific inhibitor gliotoxin. The PPE60 recombinant *M. smegmatis* survival rate within macrophages is increased, as well as elevated resistance to stresses such as low pH, surface stresses and antibiotics exposure. For a first time it is firstly reported that *M. tuberculosis* effector PPE60 can modulate the host cell fate via LUBAC-mediated NF-κB signaling.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* infection remains a major public health concern claimed millions lives annually [1]. *M. tuberculosis* capability to manipulate host cell fate during the interaction is crucial to the grave endemic of TB. Many *M. tuberculosis* components are engaged in host-pathogen interaction [2]. The PE/PPE family, named after the conserved proline-glutamate (PE) and proline-proline-glutamate (PPE) motifs at N-terminus, accounts for around 10% *M. tuberculosis* genome encoding capacity. PE/PPE family contains 69 members of PPE proteins classified into three subfamilies PPE_SVP, PPE_PPW and PPE_MPTR, and 100 members of PE proteins classified into the PE and PE_PGRS subfamily [3,4]. The presence of repeat motifs and conserved structure suggest a role in *M. tuberculosis* antigen variation [5].

Cell fate determination is fundamental to biomedicine. Pathogen infection can alter the host fate, such as pyroptosis, apoptosis, necrosis or autophagy. Many Caspases family proteins are involved in pyroptosis, such as Caspase-1, Caspase-3, Caspase-4/5/11 [6,7]. Some PE/PPE proteins can modulate the fate of host cells and the production of TNF-α or other pro-inflammatory cytokines. PE_PGRS33 triggered apoptosis in Jurkat T cells [8]; PE11 and PE_PGRS17 altered the cytokines expression and induced macrophage cell death [9,10], PE9 and PE10 could induce macrophage apoptosis through engaging TLR4 [11]. Moreover, Rv3478, Rv2875 and Rv1886 decreased the burden of *M. tuberculosis* in the lungs of infected mice and induced stronger host immune response, [12,13] which is consistent with the reported features of PE/PPE proteins [14]. Most of *M. tuberculosis* PE/PPE family proteins are up-regulated during pathogen growth within mouse and human macrophages [15]. PPE26 regulates the host inflammatory response via Toll-like receptors [16–20].

Many pathogens can manipulate host immune response by secreted effectors. Enteropathogenic *Escherichia coli* intimin receptor could be translocated into host cell and suppresses the production of inflammatory cytokines [21]. *Shigella* effector protein IpaH9.8 targets the NEMO/IKKγ and inhibits NF-κB-mediated inflammatory response [22]. LUBAC, a recently discovered ubiquitin ligase consisting of three subunits including HOIP, HOIL-1L and SHARPIN, can alter the NF-κB

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https://doi.org/10.1016/j.cellimm.2018.10.009
Received 3 August 2018; Received in revised form 13 October 2018; Accepted 30 October 2018
Available online 30 October 2018

signaling to modulate host cell fate [23–26], as well as cancer prognosis [27,28]. PPE60 protein encoded by *Rv3478*, localized on the mycobacterial cell wall, altered the host inflammatory response. PPE60 was previously shown to promote the maturation of dendritic cells through TLR2 [29]. However, the role of PPE60 in the microbe-host interaction and the underlying mechanism of action remain elusive. We found that PPE60 promoted the expression of pro-inflammatory cytokines and altered the host cell fate through the LUBAC mediated NF-κB signaling.

2. Materials and methods

2.1. Reagents and media

The antibiotics used in this study includeampicillin, kanamycin, gentamicin, and penicillin–streptomycin, and were purchased from Sigma Company. The RPMI164 medium used in cell culture experiment was purchased from Corning/Costar and BD-Falcon, and FBS was purchased from Hyclone. The NF- κB pathway inhibitor TPCK, the JNK pathway inhibitor Sp600125 and the P38 pathway inhibitor SB202190 are purchased from Sigma Company. The LUBAC inhibitor gliotoxin was purchased from Solarbio. Myc-tag, Caspase-3, Caspase-7 and β-actin antibody were purchased from Proteintech. Catalog number: 66004-I-Ig, 19677-I-AP, 27I55-I-AP and 20536-I-AP.

2.2. Construction of recombinant M. smegmatis

We have successfully cloned the *Rv3478* gene (Protein and DNA sequences Obtained from https://mycobrowser.epfl.ch/) by using the *M. tuberculosis* H37Rv genomic DNA (Our Laboratory saved) and specific primers (Table 1). The PCR product was directly ligated to plasmid pNIT. The recombinant plasmid Rv3478-pNIT was cloned into *Escherichia coli* DH5α and cultured at 37 °C. The plasmid Rv3478-pNIT and pNIT were electroporated into *M. smegmatis* mc^2 155. The bacterial clone PCR was used to verify the positive bacteria. Then, the recombinant bacteria Ms_PPE60 and Ms_pNIT were cultured in 7H9 medium containing 0.05% (v/v) Tween 80 and 100 μg/mL kanamycin. All plasmids and strains were obtained from our laboratory stock.

Table 1
The primers used in this study.

Primer Sequence (5′–3′)
PPE60-F GAATTCGTGGTGGATTTCGGGGCGTTAC
PPE60-R ATGCATGCAAACCGGTCGTGGTATCC
IL-1β-F ATGCCTTCAGCAGAGT
IL-1β-R CTGGGTCTTGGTTCTC
IL-6-F GCCTTCGGTCCAGTTGCC TTCT
IL-6-R TGCCAGTGCC TC TTTGCTGCTTT
IL-12p40-F GGACCAGAGCAGTGAGGTCTT
IL-12p40-R CTCCTTGTTGTCCCCTCTGA
TNF-α-F GGCGGTGCTTGTTCCT
TNF-α-R GCTACAGGCTTGTCACTCG
Caspase-1-F GAAGGTACAATAAATGGCTTAC
Caspase-1-R GAAGGTACAATAAATGGCTTAC
Caspase-4-F TCACAGGGATGAAGGAGC
Caspase-4-R TGGCGTTGAAGAGCAGAA
Gasdermin D-F ATGAGGTGCCTCCACAACTTCC
Gasdermin D-R CCAGTTCCTTGGAGATGGTCTC
Caspase-3-F TGG AATTGA TGC GTG ATG TT
Caspase-3-R GTC GGC ATA CTG TTT CAG CA
NLRP3-F GATCTTCGCTGCGATCAACA
NLRP3-R GGGATTCGAAACACGTGCATTA
HOIP-F CTTTCTTCCCCTAATCCTGCAAG
HOIP-R CCCAAGGCTCATTTAGCATGG
HOIL-1L-F AAACACCAAGCACTCTCAGC
HOIL-1L-R ACTGTGCAGTCTCCTTCCTG
SHARPIN-F GTGGCCCTTCCCTCACAAGTCCGAC
SHARPIN-R CCCAACCCTGGTGACTGTCCCAGCA
PPARγ-F CTTCACTGATACACTGTCTGC
PPARγ-R CTTGTGAATGGAATGTCTTCG
β-actin-F CGGCTCCGGCATGTGCAA
β-actin-R ATG TCACGCACGATTTCC

2.3. Subcellular fractionation of PPE60 in M. smegmatis

The total ultrasonic cell lysate was centrifuged at 3000*g*, 4 °C for 5 min to remove intact cells and cell debris. The supernatant was ultracentrifuged, 27,000*g*, 4 °C for 45 min, to separate the cell wall/membrane and cytoplasm. Western blotting was used to monitor the location of target protein and native GroEL2 protein as control.

2.4. The viability of recombinant bacteria upon stresses

Recombinant *M. smegmatis* Ms_PPE60 and Ms_pNIT were cultured in 7H9 medium containing 20 μg/mL kanamycin, 0.05% (v/v) Tween 80 and 1% glucose, cultured to OD_{600} = 0.6–0.8. Then 28 mM caprolactam was added for 16 h induction. The recombinant bacteria were treated with 0.05% SDS for 1, 2, 3 and 4 h, then CFU was detected on the 7H9 agar medium, and the results were observed after 2–3 days. Ms_PPE60 and Ms_pNIT were incubated in 7H9 culture to OD_{600} = 0.8, then centrifuged to collect bacteria. 7H9 medium (pH = 3) was used to suspend bacteria and adjusted OD_{600} to equal, and CFU was detected at 0, 3, 6, and 9 h. 100 μL bacteria was mixed with 0.7% water agar, spotted on 7H9 agar plate containing 28 mM caprolactam. 10 μL H_2O_2 (0.5%, 1% and 2%) was added to different regions of the plate, and the size of the inhibition zone was measured three days later. To compare the survival of Ms_PPE60 and Ms_pNIT upon diamide exposure. The recombinant bacteria were induced and spotted on 7H9 agar plates containing different concentrations diamide and 28 mM caprolactam by 10-fold serial dilution. CFU was counted after 2–3 days.

2.5. Intracellular survival analysis

THP-1 cell was a gift from Chongqing Medical University and cultured in RPMI1640 medium containing 10% (v/v) FBS + 100 U/mL penicillin + streptomycin + 2 mM glutamine. The culture environment was 37 °C, 5% CO_2. 100 μg/mL phorbol 12-myristate13-acetate (PMA, Sigma) was added when the number of cells was up to 1×10^6. Then the cells were seeded at 1×10^6 cells per well in 12-well plates. The cells become adherent macrophage after 48 h. The recombinant bacteria were induced by 28 mM caprolactam for 16 h. Before infection, cells were rinsed three times with 1 × PBS and infected at MOI = 10 (bacteria-to-THP-1 cells ratio). After 4 h, the planktonic and adsorbant bacteria were removed with 1 × PBS. Fresh medium was added containing 250 nM IVN (Sigma) and 200 μg/mL hygromycin B (Roche, USA). At the time point of 6 h, 24 h, 48 h and 72 h, the cells were lysed by 0.025% SDS, and the lysate was spotted on the 7H9 agar plates with tenfold serial dilution. The bacteria were counted after 3–5 days culture.

2.6. Cytokines analysis

After 24 h or 48 h THP-1 cells were infected by recombinant bacteria Ms_PPE60 and Ms_pNIT. Cells were lysed by RNA lysate (TIANGEN) for RNA extraction by using RNA Extraction Kit (Promega), then reversed transcribed into cDNA by using following parameters: 37 °C/15 min → 85 °C/5 s. The mRNA levels of cytokines were detected by RT-PCR using specific primers (Table 1).

2.7. Cell signaling pathway inhibition

All three inhibitors used in this study, namely TPCK (NF-κB), SB202190 (P38 MAPK) and Sp600125 (JNK), were purchased from Sigma Company. TPCK (tosyl-phenylalanine chloromethyl-ketone) is a serine protease inhibitor blocking the degradation of IκBα and the activation of NF-κB [30]. SB202190 (a pyridinium imidazole) competes

with ATP to inhibit p38 MAP kinase activity and p38 MAPK phosphorylation [31,32]. Sp600125, a selective inhibitor of JNK-1, JNK-2 and JNK-3, can inhibit c-Jun phosphorylation in a dose-dependent manner [33]. The working concentration is 30 mM, 10 mM and 20 mM, respectively. The cells were treated with inhibitors for 1 h before infection by recombinant bacteria. The control cells were treated with 0.1% DMSO. 1 × PBS rinsed cells were transferred to fresh medium. Infected cells were harvested at 24 h and 48 h, lysed, mRNA was extracted, and the transcription level of cytokines was detected by RT-PCR.

2.8. Cell pyroptosis analysis

Cell pyroptosis detection kit was purchased from the BestBio. Before detecting, cells were washed with 1 × PBS three times, 400 μL 1 × binding Buffer and 5 μL Annexin V-FITC were added, gently mixed and incubated for 15 min at 2–8 °C, without light. Then 10 μL PI was added, incubated for 5 min. Detection was made by flow cytometry or fluorescence microscopy within 1 h.

2.9. Mitochondrion stability detection

ELISA kit was purchased from MBBIOLOGY. 24 h after infection, cells were centrifuged at 4000g, the supernatant was collected. The the protein level of Mfn2 was detected according to the instructions provided by the ELISA kit.

2.10. Statistical analysis

Student's *t*-test was used for the analysis of significant difference. In this study GraphPad Prism 7 was used to analyze the differences between the experimental group and the control group. The *P* value less than 0.05 was considered significant, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. All experiments were performed three times.

3. Results

3.1. Ms_PPE60 up-regulates the production of pro-inflammatory cytokines by macrophages and promotes the pyroptosis of macrophages.

3.1.1. Rv3478 heterologous expressed in M. smegmatis

M. smegmatis is a well-recognized surrogate of virulent mycobacteria due to its fast growing and availability of genetic tools. To characterize PPE60 (Rv3478), recombinant strain Ms_PPE60 and Ms_pNIT (vector control) were constructed. Ms_PPE60 recombinant and Ms_pNIT were grown to OD_{600} = 0.8. To induce the expression of *Rv3478*, caprolactam was added (final concentration 28 mM). Total protein lysate was analyzed by Western blotting. The PPE60 protein was present in Ms_PPE60, while absent in Ms_pNIT control strain (Fig. 1). The Ms_PPE60 recombinant strain can be used for further characterization.

3.1.2. Ms_PPE60 promotes host cell death

Some PE/PPE proteins are well documented cell fate manipulators.

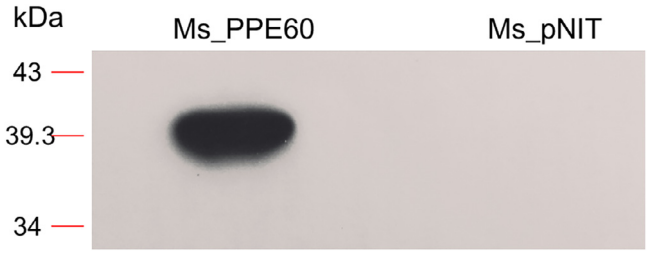

Fig. 1. Recombinant bacteria expressing PPE60 protein. Mouse anti-Myc antibody was used to detect PPE60 protein by Western blotting.

Ms_PPE60 infection promoted host cell death. To test whether and how PPE60 effects in host cell death, THP-1 macrophages were infected with Ms_PPE60 and Ms_pNIT and after 24 h, the infected host cells were treated with FITC-Annexin V and Propidium iodide (PI). Ms_PPE60 infected cells showed higher death rate at 24 h compared to the Ms_pNIT infected cells (Fig. 2). Quantative analysis with flow cytometry showed that the death level of Ms_PPE60 infected cells was 78.5%, while that of Ms_pNIT infected cells was only 45.4%. These evidences showed that PPE60 plays a role in macrophages death.

3.1.3. Ms_PPE60-promoted cell death does not engage mitochondrion fragmentation

Mitochondrial fusion protein 2 (Mfn2) is mitochondria's outer membrane protein and essential for its integrity. Which is closely related with cell apoptosis. Stress cues triggered JNK signaling which mediates the degradation of mitochondrial rupture related Mfn2 [34], a protein with anti-apoptotic activity [35,36]. To test whether mitochondria integrity was involved in Ms_PPE60 promoted THP-1 cells death, we quantified the expression level of Mfn2 gene in cells infected by Ms_PPE60 or Ms_pNIT, Sp600125 treated or not. As shown in Fig. 3, Mfn2 expression did not show discernible difference. This precluded a role of mitochondria integrity in Ms_PPE60 promoted cell death. In contrast, the mRNA levels of important pyroptosis molecules such as Caspase-1/4, NLRP3 and gasdermin D (Fig. 2e-h) were significantly increased, suggesting Ms_PPE60 promotes cell pyroptosis instead of apoptosis.

3.1.4. Ms_PPE60 alters the production of cytokines

Host cell pyroptosis usually accompanies with changed profile of pro-inflammatory cytokines, while the cell apoptosis elicits much less prominent inflammatory response. To test whether Ms_PPE60-promoted host cell death has characteristic cytokines profile change, PMA-differentiated THP-1 macrophages were infected with Ms_pNIT and Ms_PPE60, and the transcription levels of cytokines were detected at different intervals (24 h and 48 h). The expression levels of pro-inflammatory cytokines IL-1β, IL-6 and IL-12p40 at 24 h and 48 h post infection were significantly up-regulated by Ms_PPE60 (Fig. 4a-d).

Peroxisome proliferator-activated receptor-gamma (PPARγ), a nuclear hormone receptor, can competitively bind NF-κB p65/p50 subunit to inhibit the function of macrophages/monocytes as well as the expression of pro-inflammatory cytokines. We used RT-PCR to test the effect of Ms_PPE60 on the transcription of PPARγ. The data showed that PPARγ transcription was significantly inhibited in Ms_PPE60-infected cells. TPCK is NF-κB-specific inhibitor. Upon TPCK exposure, NF-κB activity was inhibited and the production of pro-inflammatory cytokines was decreased, together with increased PPARγ (Fig. 4e). The data showed that Ms_PPE60-infected cells elicited much broader response, consistent with the expectation that Ms_PPE60 can manipulate host immune response. Taken together, we demonstrated that Ms_PPE60 promotes cell death via pyroptosis.

3.2. Ms_PPE60 modifies the production of pro-inflammatory cytokines through Linear Ubiquitin Chain Assembly complex (LUBAC) mediated NF-κB signaling

3.2.1. Ms_PPE60 up-regulates the production of selected cytokines via NF-κB signaling

To define the signaling pathway underlying PPE60-altered cytokines profile, we choose inhibitors specific to NF-κB, JNK and p38 MAPK signaling pathway to pre-treat PMA-differentiated macrophage THP-1 for 1 h and then infected by Ms_PPE60 or Ms_pNIT for 24 h. Total RNA was collected from cell lysate. Transcription levels of IL-12p40 and TNF-α were detected by RT-PCR. TPCK (NF-κB signaling pathway specific inhibitor), SB202190 (inhibitor of p38 MAPK) and Sp600125 (inhibitor of JNK signaling) were used to treat the cells. As shown in Fig. 5b, the transcription level of IL-12p40 is dramatically reduced in

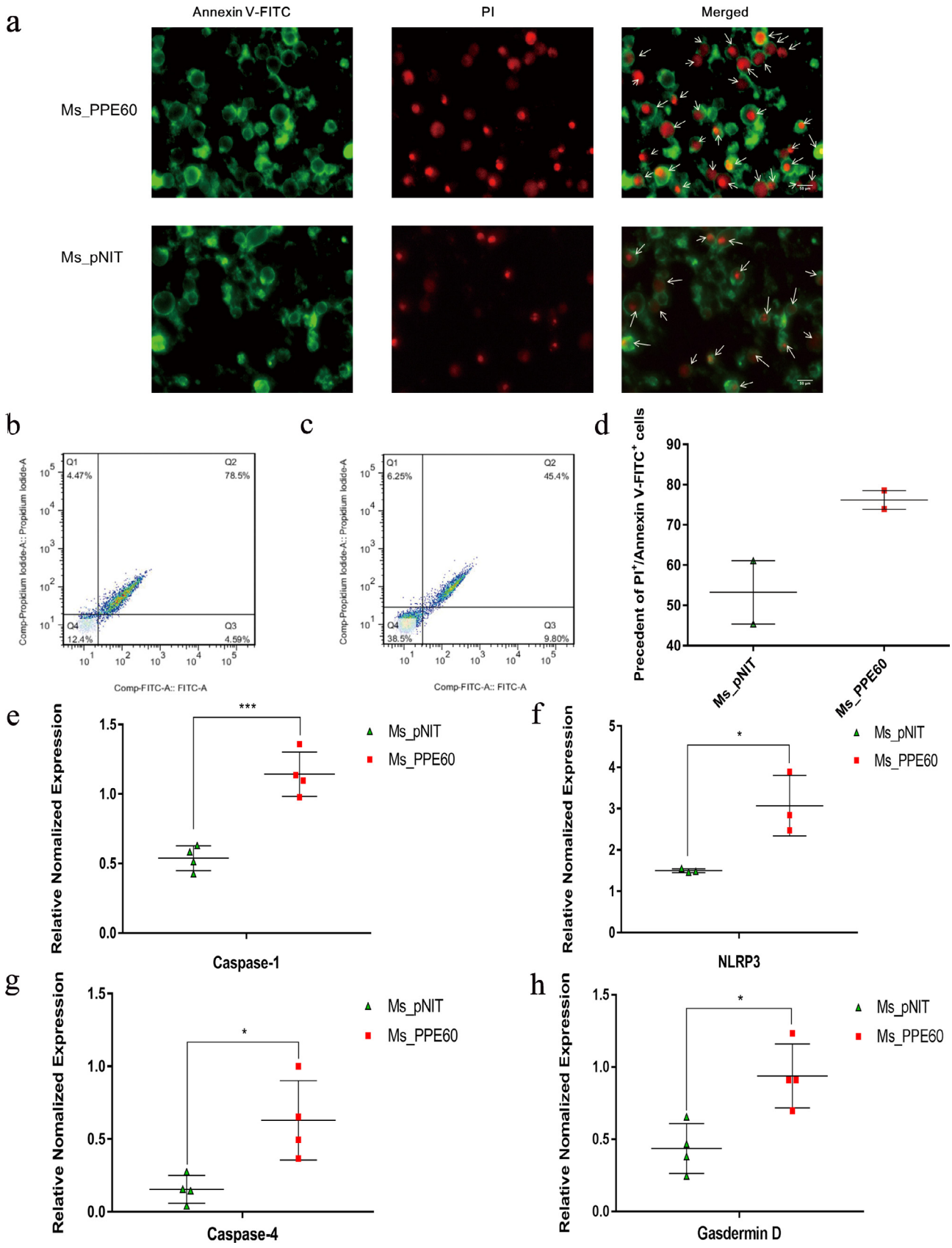

Fig. 2. PPE60 promoted the pyroptosis of THP-1 macrophages. THP-1 macrophages were incubated with Ms_PPE60 and Ms_pNIT for 24 h, the cells were washed and subjected to Annexin V/PI stain. The results were visualized by fluorescence microscopy and flow cytometry. Early apoptotic populations (Annexin V^+/PI^-, green color) or pyroptosis populations (PI^+/Annexin V^+, yellow color) were compared. (a-d) cells were collected and the level of pyroptosis was detected by fluorescence microscope and flow cytometry. (e-h) the mRNA levels of Caspase-1, Caspase-4, NLRP3 and Gasdermin D.

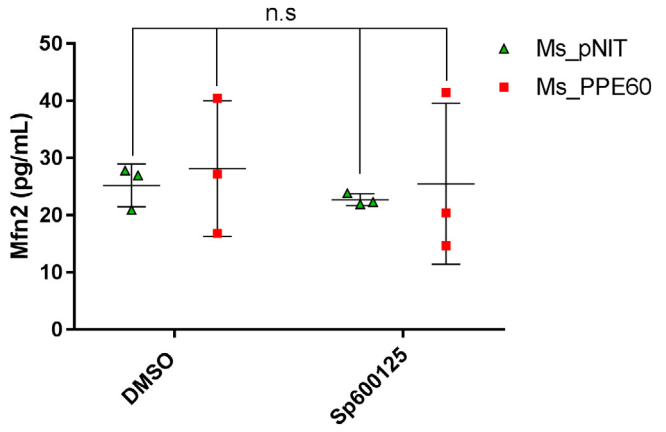

Fig. 3. PPE60 failed to change the level of Mfn2. Sp600125 was used to treat cell to block JNK. 24 h post infection, the infected cells and supernatant were collected. ELISA kit was used to detect the protein level of Mfn2 (n = 3).

TPCK treated cells; Upon SB202190 treatment, the transcription of IL-12p40 was slightly reduced, while Sp600125 treatment had no effect on the transcription of IL-12p40. While in the DMSO treated control group, Ms_PPE60 could induce higher level of TNF-α, and the inhibitory effect of the three inhibitors on TNF-α transcription was more prominent (Fig. 5a, b). These results indicated that Ms_PPE60 can up-regulate the transcription of cytokines via NF-κB, P38 MAPK and JNK axis, among which NF-κB predominates.

3.2.2. Ms_PPE60-elicited cytokines profile change depends on LUBAC

NF-κB signaling can be activated by multiple pathways. LUBAC-mediated NF-κB signaling activation is a recent finding. The ubiquitin ligase LUBAC catalyzes the linear ubiquitination of NEMO, which is an adaptor of the IκB kinases (IKKs) and regulates NF-κB signaling by degrading IκBα, thereby activating NF-κB. To test whether LUBAC is involved in Ms_PPE60-elicited NF-κB activity change, the cells were treated 1 h with 1 uM gliotoxin prior to infection. The levels of TNF-α and IL-12p40 were detected at 24 h post infection. Gliotoxin is a selective inhibitor of LUBAC binding with HOIP [37], thereby blocking the ubiquitin ligase activation of LUBAC. As shown in Fig. 5d-e, the levels of TNF-α and IL-12p40 significantly lowered in gliotoxin-treated cells, consistent with NF-κB inhibitor TPCK treated cells. The data showed that LUBAC and NF-κB signaling overlapped. One possibility is that the ubiquitin ligase LUBAC in some way can directly regulate the activity of NF-κB, thereby reducing the expression of pro-inflammatory factors. However, Ms_PPE60 failed to alter the transcriptional levels of the three subunits of LUBAC in macrophages (Fig. 5c). Further studies of post-transcriptional regulation are required.

3.3. PPE60 confers recombinant M. smegmatis intracellular survival advantage via elevated stress resistance

3.3.1. PPE60 enhances intracellular survival of M. smegmatis

PE/PPE proteins are well-documented mycobacteria virulence factors and intracellular survival potentiators [38,39]. To test whether Ms_PPE60 survival was enhanced in macrophage, the CFUs of Ms_PPE60 and Ms_pNIT collected from THP-1 macrophages infected with either recombinant were calculated. Ms_PPE60 CFU survival was significantly higher than Ms_pNIT (Fig. 6a). In vitro growth rate between Ms_PPE60 and Ms_pNIT was compared to preclude the effect of possible difference. As shown in Fig. 6b, both strains showed similar growth kinetics, thereby precluding the bias of microbes' biomass.

3.3.2. PPE60 elevates the M. smegmatis stresses resistance capacity

We measured the growth of recombinant Ms_pNIT and Ms_PPE60 upon exposure to different stresses which mimic the intracellular milieu of macrophage, such as low pH, surfactant stress, oxidative stress to test the higher survival rate of Ms_PPE60 is related to stress resistance capacity. The cell wall location of PPE60 (Fig. 7a) supports its role in stress resistance. Ms_PPE60 growth was more robust than Ms_pNIT under pH = 3, and Ms_PPE60 survival rate was significant higher than Ms_pNIT 9 h post acid stress (pH = 3) (Fig. 7b). Upon exposed to 0.05%, 1%, 2% and 4% SDS, Ms_PPE60 was more tolerant than

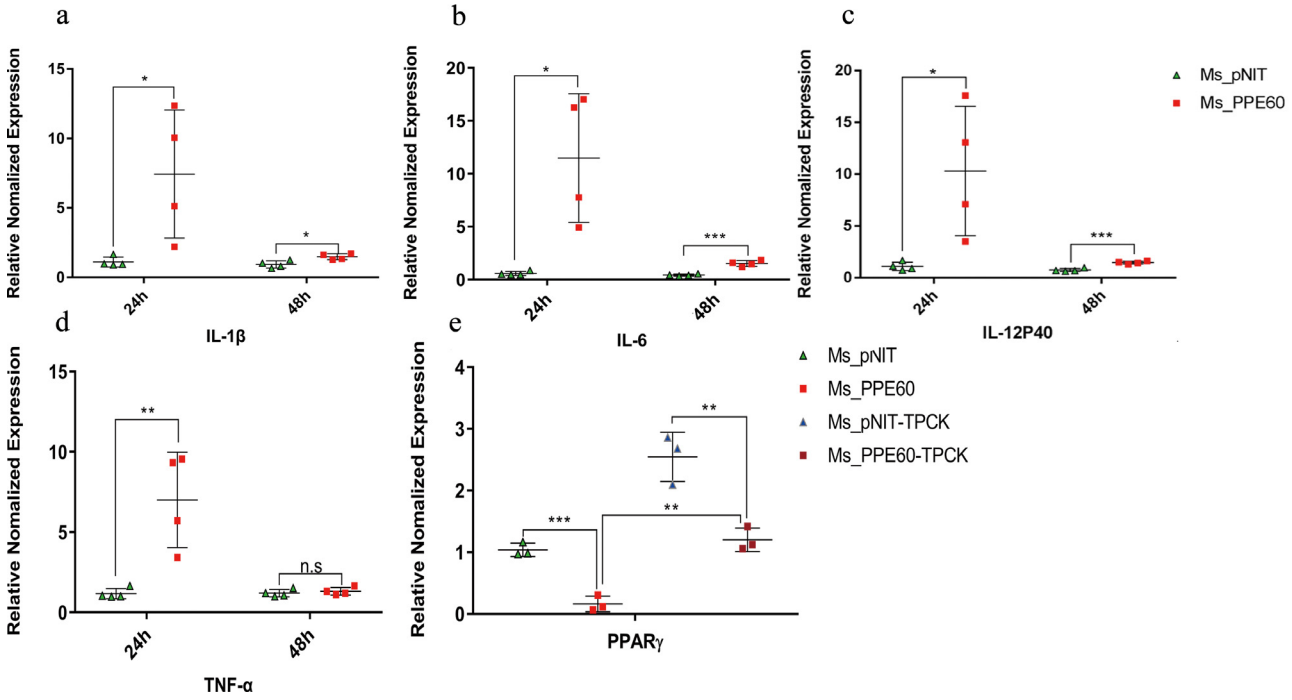

Fig. 4. PPE60 up-regulated the expression of pro-inflammatory cytokines and down-regulated the production of PPARγ. RT-PCR was used to quantify the mRNA level of (IL-1β (a), IL-6 (b), IL-12p40 (c), TNF-α (d) n = 4), PPARγ (e) (n = 3) at 24, and 48 h post infection ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, determined by Student's *t* test).

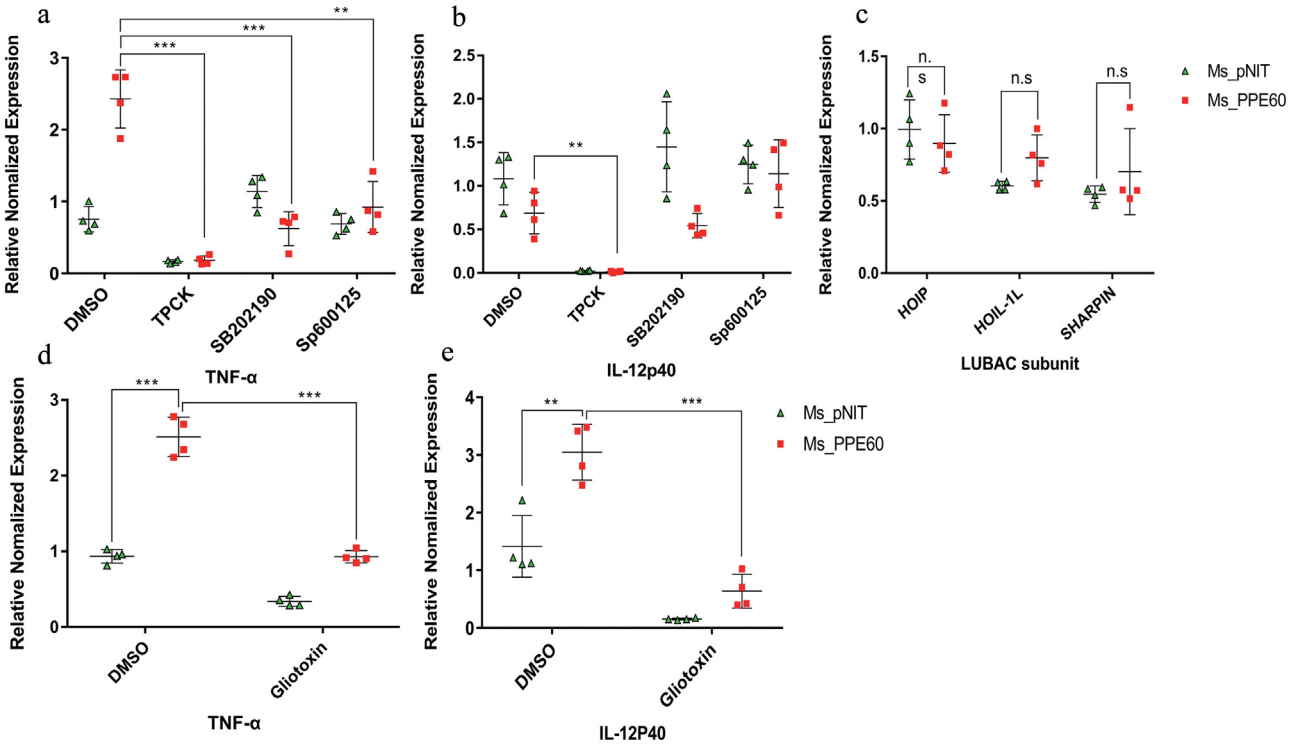

Fig. 5. Signaling underlying Ms_PPE60 stimulated pro-inflammatory response in THP-1 macrophages. THP-1 macrophages were treated with specific inhibitors of JNK (Sp600125), p38MAPK (SB202190), NF-κB (TPCK) and LUBAC (Gliotoxin) for 1 h before infected with recombinant bacteria, a and b: TNF-α and IL-12p40 mRNA was detected by RT-PCR, c: changes in the transcriptional level of LUBAC subunits; d and e: mRNA detection of IL-12p40 and TNF-α after treatment with gliotoxin for 1 h (n = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, determined by Student's *t* test).

Ms_pNIT (Fig. 7c and d). For H_2O_2 (0.5%, 1% and 2%) oxidative stress, Ms_PPE60 also was more resistant than Ms_pNIT (Fig. 7e and f). For diamide (0 mM, 2 mM and 5 mM) treatment, Ms_PPE60 and Ms_pNIT showed comparable growth under 0 mM and 2 mM diamide. For 5 mM diamide, Ms_PPE60 was more resistant than Ms_pNIT (Fig. 7g). Taken together, PPE60 confers recombinant bacteria resistance against stresses mimic macrophage intracellular milieu.

3.3.3. PPE60 increases the survival of M. smegmatis upon multiple antibiotics exposure

Bacteria cell wall is crucial for defense against antibiotics, especially for *M. tuberculosis* [40]. To test whether cell wall-located PPE60 can confer more antibiotics resistance, we compared the CFUs of Ms_PPE60 and Ms_pNIT upon exposure to Isoniazid (4 μg/mL), Vancomycin (10 μg/mL), Rifampicin (4 μg/mL) and Capreomycin (1.25 μg/mL). The data showed that Ms_PPE60 was more resistant against Vancomycin, Isoniazid and Rifampcin than the Ms_pNIT; no significant difference was spotted upon Capreomycin exposure (Fig. 7h). This is consistent with the subcellular location of PPE60.

4. Discussion

M. tuberculosis has evolved multiple tactics to thrive and persist within host [41]. PE/PPE family molecules are prominent virulence and persistence effectors in the genome of *M. tuberculosis* H37Rv [4,42]. The host cell fate during *M. tuberculosis* infection is critical for the prognosis. Pyroptosis of host cells can facilitate scavenge of intracellular bacteria, such as *Salmonella*, *Bacillus*, *Listeria*, *Shigella* and *Legionella* [43–45]. However, how *M. tuberculosis* manipulates host cell pyroptosis remains elusive.

In this study, Ms_PPE60 significantly promoted the pyroptosis of macrophages. Ms_PPE60 induced cell pyroptosis in Caspases/NLRP3/

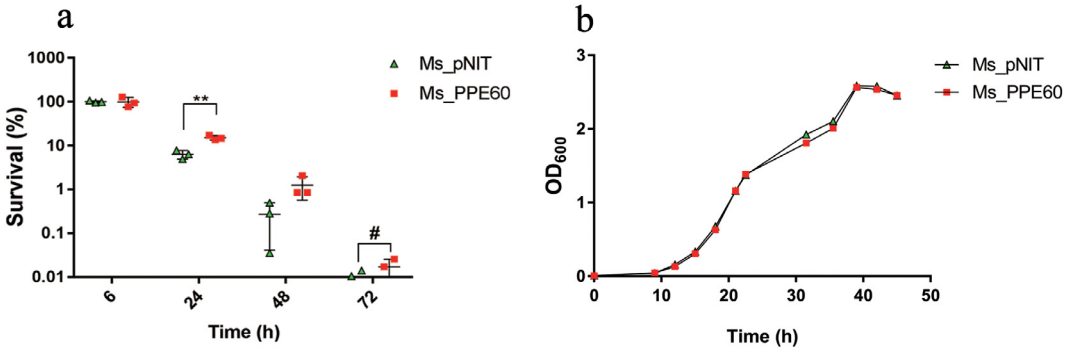

Fig. 6. The survival of the Ms_PPE60 and Ms_pNIT recombinant *M. smegmatis* in macrophages (a) and Ms_PPE60 and Ms_pNIT showed similar growth (b). (a) Cell lysates containing the live bacteria were diluted gradually and then plated on 7H9 agar plates to determine, "#" means cannot be detected (n = 3). (b) The growth rate of Ms_PPE60 and Ms_pNIT at 37 °C in 7H9 liquid medium and all data were the results of three independent experiments (n = 3, $^{**}p < 0.01$, determined by Student's *t* test).

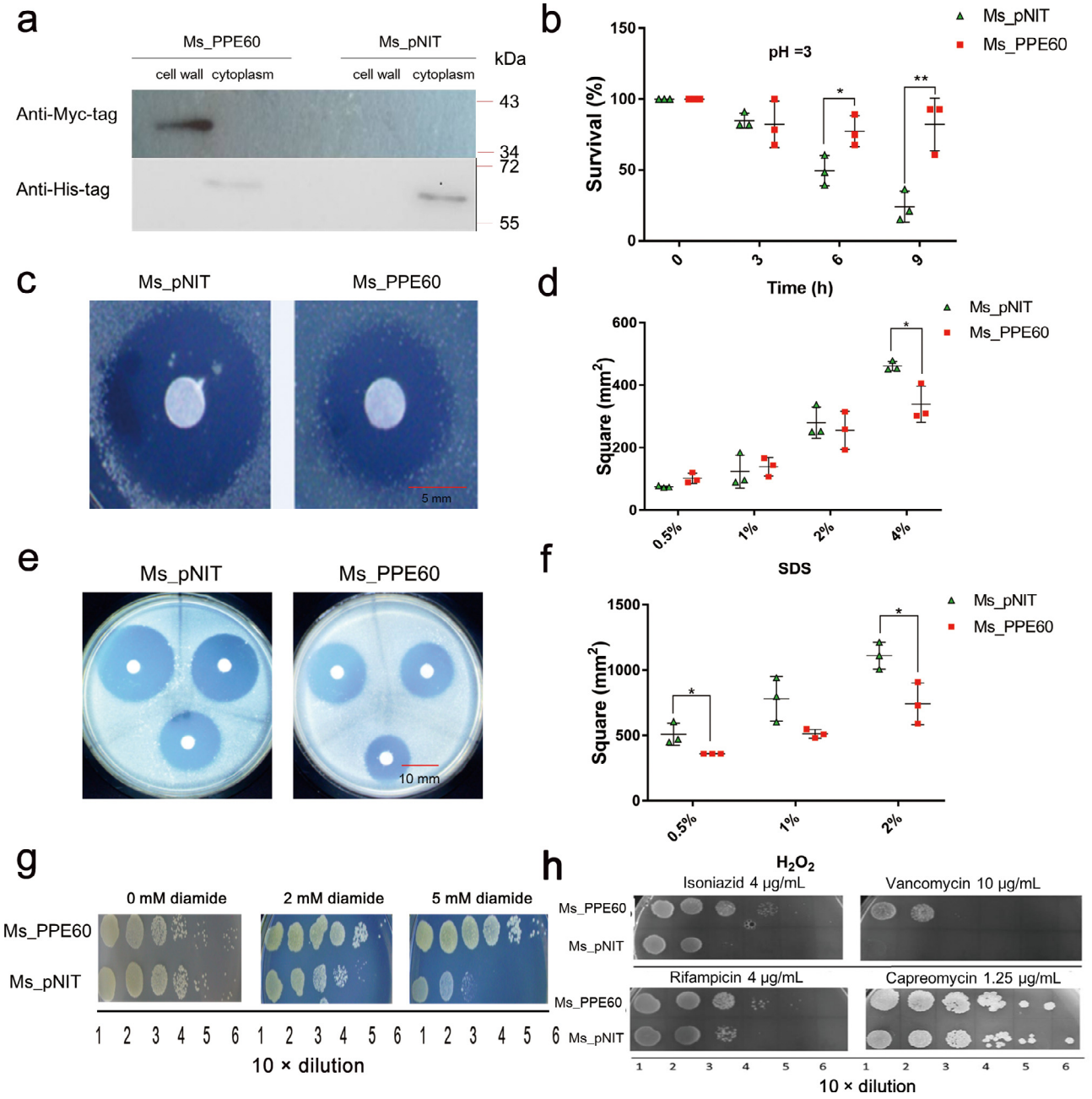

Fig. 7. Subcellular localization of PPE60 and survival of Ms_PPE60 and Ms_pNIT under stress conditions and antibiotics treatment. a: Subcellular localization revealed that PPE60 was located in the cell wall of bacteria; (b-g): PPE60 increases the survival of *M. smegmatis* in low pH, SDS, H_2O_2 and diamide stress (n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$, determined by Student's *t* test), h: Isoniazid, vancomycin, rifampicin, and capreomycin.

gasdermins dependent manners. As previously mentioned, caspases arecrucial for cell growth and differentiation, as well as cell pyroptosis. Apoptosis was once indistinguishable from pyroptosis. However, there are profound differences between them. No inflammatory reaction was observed in the process of apoptosis, the cell crinkle, and the membrane evaginate, but cell maintains a complete membrane structure which preventing PI from staining the nucleus. Pyroptosis is a normal cell fate during infections. It is more rapid than apoptosis, accompanied by robust inflammatory response, cell expansion and rupture and releasinginflammatory factors and bacteria. Pyroptosis is mediated by Caspase-1/4/5/11 and NLRPs, while apoptosis is mediated by Caspase-3. Caspases cannot determine the cell death manner. Caspase-3 also cut Gasdermin E and cause pyroptosis [7]. Elevated Gasdermins during infection and digestion by caspases results in pyroptosis. The cell death-facilitated spread of bacteria [46] might underlie the observed higher survival rate of recombinant mycobacteria in this study. Mitochondria is important for cell apoptosis [34]. Using the marker protein of mitochondrial fission Mfn2, we found that Ms_PPE60 failed to alter the integrity of mitochondria. These evidences indicate that Ms_PPE60 promoted host cell death via pyroptosis instead of apoptosis.

M. tuberculosis infection usually altered the cytokines profile of macrophages [47], such as IL-1β, IL-6 and TNF-α, which can initiate the early immune response, activating T cells and B cells [48]. IL-12p40 is important for activating the protective Th1 immune response and producing of interferon – γ [49]. Most of PE/PPE proteins such as PPE18 [50], PPE26 [17], PPE37 [51], PPE38 [52] and PPE57 can alter the macrophages cytokines profile [53]. PPE60 (*Rv3478*) can elicit strong protective immune response [12]. Which is consistent with our result that Ms_PPE60 up-regulated (5–8 folds) the levels of IL-1β, IL-6, IL-12p40 and TNF-α (24 h and 48 h post infection). PPARγ can

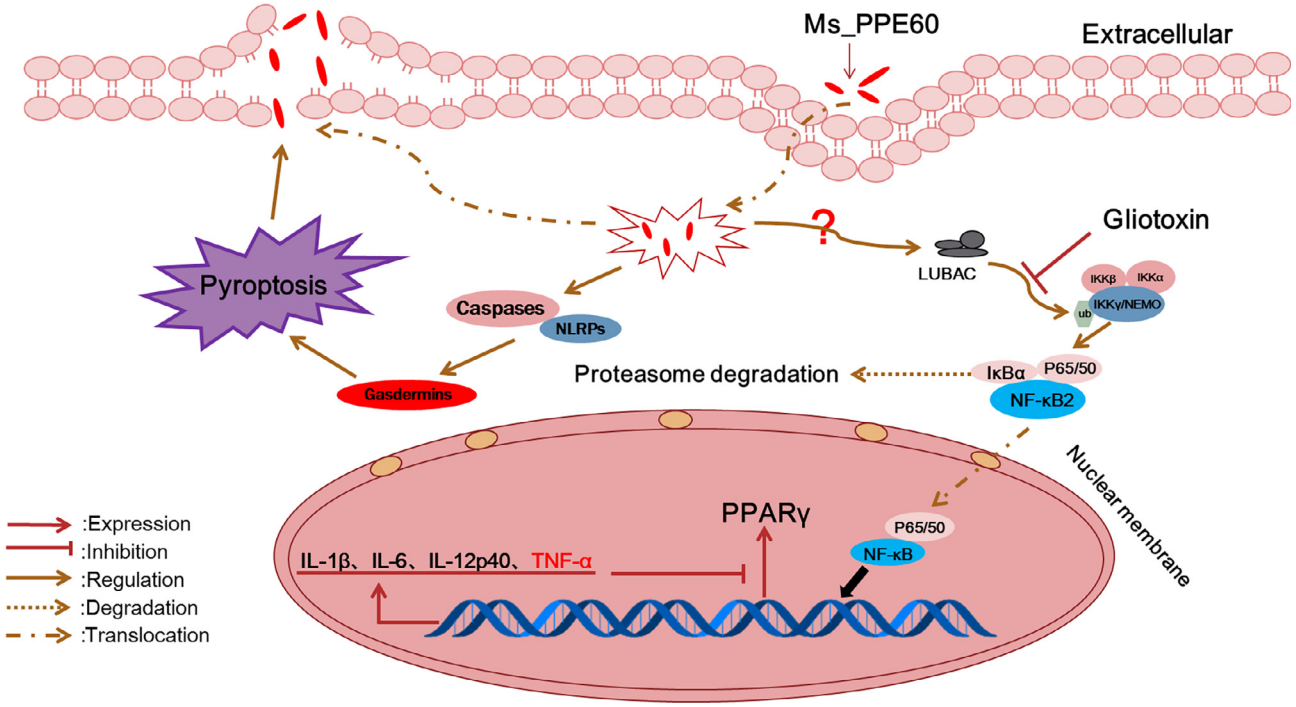

Fig. 8. Model depicting PPE60 interaction with macrophage. PPE60 promoted expressions of pro-inflammatory cytokines IL-1β, IL-6, IL-12p40 and TNF-α through LUBAC mediated NF-κB signaling and cell pyroptosis. Ms_PPE60 phagocytosed by macrophage activated NF-κB signaling, which is mediated by LUBAC, thereby increasing the production of IL-1β, IL-6, IL-12p40 and TNF-α. Ms_PPE60 inhibited the transcription of PPARγ and promotes cell pyroptosis, cell membrane rupture, and facilitates the spread of bacteria.

moderate the NF-κB signaling and inhibit the immune response of macrophages [54], representing an ideal drug target for the treatment of many autoimmune diseases, such as Alzheimer's disease and rheumatoid arthritis [55,56]. PPARγ agonists effectively reduced the incidence of these diseases. The PPARγ level is significantly decreased in Ms_PPE60 infected macrophages. Upon inhibition of NF-κB, the production of pro-inflammatory cytokines was decreased rapidly, while the production of PPARγ was significantly increased. We found that Ms_PPE60-activated NF-κB signaling can repress the transcription of PPARγ, thereby dramatically restricting the anti-inflammatory effect of PPARγ.

Ms_PPE60-promoted production of IL-12p40 and TNF-α depended on NF-κB, p38 MAPK and JNK, among which NF-κB predominates. A quite unexpected finding is that LUBAC is involved in Ms_PPE60-elicited activation of NF-κB. Ubiquitin ligase LUBAC, when activated, linearly concatenates ubiquitin to the NF-κB regulatory protein NEMO, thereby activating IKK complex. This culminates in IκBα degradation by proteasome and inducing- the NF-κB signaling. LUBAC plays an important role in cell physiology, immune response, cell fate and tumor [57–59]. In this study, we found that Ms_PPE60-elicited NF-κB activation depend on LUBAC. However, the mechanism details of Ms_PPE60-elicited LUBAC activation remain to be elucidated.

M. tuberculosis cell wall is important for its survival and persistence [60–62]. Many PE/PPE proteins are annotated as "cell wall and cell processes" [63]. In this study, we confirmed that PPE60 is a cell wall associated protein with a role in cell wall integrity, permeability and resistance against host macrophage bactericidal factors [64]. Interestingly, PPE60 significantly enhances the viability of recombinant mycobacterium Ms_PPE60 in macrophage. Macrophages have multiple bactericidal means, such as ROS, NO, H_2O_2, SDS, low pH and diamide stress which can mimic intracellular milieu similar to those encountered upon mycobacteria infection. Results showed that the viability of Ms_PPE60 under these stresses is significantly higher than that of Ms_pNIT. Meanwhile, Ms_PPE60 became more resistant to selected antibiotics, consistent with expectation.

5. Conclusion

In summary, in this study we report that *M. tuberculosis* PPE60 plays a role in *Mycobacterium* survival, and this capability is associated with manipulation of LUBAC, NF-κB signaling and host cell pyroptosis (see Fig. 8). How PPE60 affects the LUBAC activity remains to be determined.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by National Natural Science Foundation [grant number 81871182, 81371851, 81071316, 81271882, 81301394], National Key Research and Development Program (2016YFC0502304), Open Fund of Shanghai Key Laboratory of Tuberculosis (2017-001) the Fundamental Research Funds for the Central Universities [grant numbers XDJK2016E093, XDJK2012D011, XDJK2012D007, XDJK2013D003], The Chongqing Municipal Committee of Education for postgraduates innovation program [grant numbers CYS16073].

Author contribution statement

Zhen Gong designed, performed all experiments, analyzed the data and wrote article; Hui Li, Zhongmei Kuang, Chunyan Li, Md Kaisar Ali, Fujing Huang, Jiang Li and Sai Ren conducted part of the experiments, Ping Li, Qiming Li and Xue Huang wrote the manuscript; Jianping Xie designed the experiments, wrote the manuscript. All authors read and approved the manuscript.

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