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The endoplasmic reticulum stress-mediated unfolded protein response protects against infection of goat endometrial epithelial cells by *Trueperella pyogenes* via autophagy

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ABSTRACT

Trueperella pyogenes is an important bacterial pathogen of a wide range of domestic and wild animals. Autophagy plays a key role in eliminating *T. pyogenes* in a process that is dependent on mechanistic target of rapamycin (mTOR). The endoplasmic reticulum (ER) stress response also is critical for autophagy regulation. However, the relationship between ER stress and *T. pyogenes* is uncharacterized and the intracellular survival mechanisms of *T. pyogenes* have not been investigated adequately. In this study, we show that *T. pyogenes* invades goat endometrial epithelial cells (gEECs). Meanwhile, we observed that GRP78 was upregulated significantly, and that unfolded protein response (UPR) also were activated after infection. Additionally, treatment with activators and inhibitors of ER stress downregulated and upregulated, respectively, intracellular survival of *T. pyogenes*. Blocking the three arms of the UPR pathway separately enhanced *T. pyogenes* survival and inflammatory reaction to different levels. We also show that LC3-labeled autophagosomes formed around the invading *T. pyogenes* and that autolysosome-like vesicles were visible in gEECs using transmission electron microscopy. Moreover, tunicamycin did not inhibit the intracellular survival of *T. pyogenes* under conditions in which autophagy was blocked. Finally, severe challenge with *T. pyogenes* induced host cell apoptosis which also may indicate a role for ER stress in the infection response. In summary, we demonstrate here that ER stress and UPR are novel modulators of autophagy that inhibit *T. pyogenes* intracellular survival in gEECs, which has the potential to be developed as an effective therapeutic target in *T. pyogenes* infectious disease.

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Introduction

Trueperella pyogenes is a Gram-positive, opportunistic pathogenic bacterium [1] which has been isolated from the endometrium, stomach, udder, and skin of numerous livestock and wild animals [2–5]. The bacterium is a major uterine pathogen during postpartum of beef and dairy cattle [6] and induces severe pathogenicity in the bovine uterus [7]. Infection by *T. pyogenes* is a source of considerable economic loss and animal distress. *T. pyogenes* previously was not considered a human pathogen. However, the bacterium was isolated recently from the human urinary tract [8]. Therefore, investigation of the mechanisms by which *T. pyogenes* causes infection and the development of effective therapeutic strategies are important and urgent priorities.

The process of invasion, survival and escape by bacterial pathogens relies both on bacterial virulence factors and on modulation of eukaryotic signal transduction pathways [9]. Virulence factors such as pyolysin (PLO), collagen-binding protein (CpbA), neuraminidases (NanP and NanH) and fimbriae (FimA, FimC and FimE) act in concert during the invasion of host cells by *T. pyogenes* [10]. The FimA virulence factor also is associated significantly with clinical endometritis in dairy cows [11]. *T. pyogenes* is considered an extracellular pathogen that adheres to cells and induces endometrium columnar epithelium injury before further invasion of endometrial stromal cells [10]. Invasion and intracellular survival experiments within HeLa epithelial cells and J774A.1 macrophages demonstrated that *T. pyogenes* may persist for up to 72 hours with a decreasing survival during this period [1]. However, the mechanisms by which *T. pyogenes* invades and is eliminated by epithelial cells are unclear. Host responsiveness to *T. pyogenes* infection has been of particular interest recently which has revealed

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that mechanistic target of rapamycin (mTOR)-mediated autophagy plays an important role in protecting mice from *T. pyogenes* challenge [12]. Autophagy is an evolutionarily-conserved intracellular degradative process in eukaryotic cells and is essential for cell homeostasis, during development, and in the response to diverse infectious pathogens [13,14]. Autophagy flux is initiated in the autophagosome which comprises membrane vesicles that form around damaged organelles, proteins and invading pathogens. Autophagosomes are delivered to lysosomes to form autolysosomes in which cargo is degraded by lysosomal proteases [14]. Different types of autophagy, including mitophagy, reticulophagy, pexophagy and xenophagy, are classified by the cargo that is sequestered [15,16]. Xenophagy targets invading pathogens such as group A *Streptococcus* [17], *Listeria monocytogenes* [9], *Shigella flexneri* and *Salmonella enterica* Typhimurium [18]. Thus, xenophagy is considered an important component of innate immunity [15]. In the process of infection, autophagy is induced by *T. pyogenes* PLO in endometrial stromal cells [19]. Moreover, induction of amino acid starvation pathways, which trigger autophagy immediately, is mediated by pore-forming toxin listeriolysin in *Listeria*-infected cells [9].

Starvation previously was considered the sole inducer of autophagy. Autophagy is negatively controlled by the metabolic checkpoint kinase mTOR [20], whereas the endoplasmic (ER) reticulum stress-mediated unfolded protein response (UPR) is involved in activating autophagy [21]. ER is the key organelle for synthesis and secretion of proteins in eukaryotic cells [22]. Cellular threats, such as pathogen infection, nutrition deprivation and hypoxia, induce an imbalance in ER homeostasis which is followed by activation of UPR which either restores homeostasis, thereby saving the cells, or activates cell death signals [23]. The ER transmembrane proteins inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) serve as ER stress transducers via spliced X-box binding protein 1 (XBP1s), the cytosolic DNA-binding portion of ATF6 and the phosphorylated α-subunit of eukaryotic translation initiation factor 2 (p-eIF2α), respectively [22]. Thapsigargin, which is a chemical inducer of ER stress, induces transcription factor EB (TFEB) expression [24]. XBP1s enhances transcription of the gene for TFEB by interacting with its promoter region to further regulate autophagy [25]. Small interfering RNA analysis of the PERK pathway also revealed that UPR is an important mediator of hypoxia-induced autophagy [26,27]. The importance of the ER stress and UPR in protecting against infection by diverse pathogenic bacteria has reported widely. For example, UPR improved the survival rate of *Caenorhabditis elegans* and mammalian cells which are exposed to Cry5B PFT which is a member of the pore-forming Cry toxin family [28], and UPR signaling is involved in defense against the early steps of *Campylobacter jejuni* invasion [29].

The aim of the present study was to investigate intracellular survival mechanisms of *T. pyogenes* in goat endometrial epithelial cells (gEECs). We show that *T. pyogenes* infection triggered the rapid induction of ER stress and UPR which are consistent with a rapid decrease in the levels of intracellular bacteria. *T. pyogenes* infection also induced LC3-labeled autophagosome and autolysosome-like vesicles in the cytoplasm of gEECs. We further demonstrate that ER stress and the three arms of the UPR inhibit *T. pyogenes* intracellular survival by autophagy which was confirmed by knockdown of the gene for autophagy related 5 (ATG5) protein. We also show that severe infection with *T. pyogenes* triggered the apoptosis signaling pathway which was accompanied by significant up-regulation of CHOP which is a key apoptosis-related protein in UPR. The results reveal the critical role played by ER stress-mediated UPR and autophagy in the host response to *T. pyogenes* infection, and demonstrate how gEECs control the autophagy and apoptosis processes as well as the inflammatory reaction by ER stress to defend and eliminate invasion by *T. pyogenes*.

Materials and methods

Cell lines and bacterial strains

The strain of *T. pyogenes* used in this study was isolated from a goat abscess by syringe sampling and was grown on Brain Heart Infusion (BHI; BD Difco) agar plates supplemented with 5% fetal bovine serum (FBS; Gibco) at 37°C and 5% CO_2 in a humidified incubator, or in BHI broth containing 5% FBS at 37°C. The identification of the *T. pyogenes* strain was based on colony characteristics on plates, sequence alignment of 16S rRNA, and hemolysis as described previously [30]. Goat endometrial epithelial cells (gEECs), which were immortalized by transfection with human telomerase reverse transcriptase (hTERT), are stored in the host laboratory [31] and were cultured in Dulbecco's modified Eagle medium:nutrient mixture F-12 (DMEM/F12; Gibco) supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C. When the cells grew to 90% confluence in six-well cell culture plates (Corning), *T. pyogenes* was inoculated at a multiple of infection (MOI) of 50 or 100. After adsorption for one hour, cells were washed three times with phosphate buffer solution (PBS) and the

culture medium was replaced with DMEM/F12 containing gentamicin (50 μg/ml).

Antibodies and reagents

Anti-GRP78 (Cat# ab21685, RRID: AB_2119834), anti-ATF6 (Cat# ab83504, RRID: AB_2058904), anti-p-IRE1 (Cat# ab124945, RRID: AB_11001365), anti-XBP1 (Cat# ab220783), anti-eIF2α (Cat# ab26197, RRID: AB_2096478), anti-p-eIF2α (Cat# ab32157, RRID: AB_732117), anti-LC3B (Cat# ab192890, RRID: AB_2827794) and anti-Caspase-3 (Cat# ab13847, RRID: AB_443014) antibodies were purchased from Abcam. Anti-CHOP (Cat# 2895, RRID: AB_2089254) antibody was from Cell Signaling Technology, and anti-LC3B (Cat# L7543, RRID: AB_796155) antibody, 4-phenylbutyric acid (4-PBA), tunicamycin (Tm), cholesterol, L-α-phosphatidylcholine, L-glutamine, polybrene, and puromycin were obtained from Sigma. Chemically Defined Lipids and Opti-MEM medium were purchased from Gibco. TurboFect was from Thermo Fisher Scientific and anti-β-actin antibody was obtained from Tianjin Sanjian Biotech. HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from Zhongshan Golden Bridge Biotechnology, and donkey anti-rabbit IgG (H + L) secondary antibody and Alexa Fluor 594 were from Invitrogen. 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was from Beyotime Biotechnology.

Immunofluorescence microscopy

For immunofluorescence analyses, cells were fixed with paraformaldehyde (PFA; 4%) for 20 minutes and permeabilized with Triton X-100 (0.1%) for 15 minutes at 4°C. Cells were washed three times using PBS and blocked with skim milk (10%) in Tris-buffered saline containing Tween 20 (TBST; 0.5%) for one hour and incubated with the indicated primary and Alexa Fluor-conjugated secondary antibodies diluted in the blocking buffer for eight hours at 4°C followed by one hour at room temperature. Nuclei and *T. pyogenes* were stained using DAPI before imaging by confocal microscopy (Nikon A1R-si).

Lentivirus packaging

The construction of cell lines sh-ATF6, sh-XBP1, sh-eIF2α and sh-N using the lentivirus package technology was described in detail elsewhere [32]. Briefly, lentiviral vectors containing short hairpin RNA (shRNA) sequences that target the required genes and a scrambled shRNA lentiviral vector were constructed, and then were co-transfected using TurboFect into HEK 293 T cells with packaging vectors that encoded Gag-Pol, Rev, Tat, and G-protein. The medium was replaced 12 hours later with advanced DMEM containing FBS (2%), cholesterol (0.01 mmol/L), L-α-phosphatidylcholine (0.01 mmol/L), L-glutamine (4 mmol/L) and Chemically Defined Lipids (1:1000 dilution) and the culture was continued for 48 hours. Supernatant containing the lentivirus was collected to infect gEECs with polybrene (8 μg/ml) for 12 hours. The cells were selected with puromycin (5 mg/mL) in complete culture medium. The efficiency of infection was observed by fluorescence microscopy and was analyzed further by Western blot. Short hairpin RNA sequences are listed in supplement Table S1.

Small interference RNA

The small interference RNA (siRNA) targeting ATG5 (si-ATG5) and the negative control siRNA (si-N) were designed by GenePharm (Shanghai). The gEECs were cultured in a six-well cell culture plate. When the gEECs reached 70% confluence, the siRNA oligo (50 nM final concentration) was used to transfect the cells with TurboFect transfection reagent supplemented with Opti-MEM. Cells were cultured in the normal cell culture medium mentioned above for 48 hours and then were subjected to the processing outlined in the preceding section. Small interference sequences are listed in supplement Table S2.

Western blotting

Whole-cell protein extraction from gEECs using the KGP2100 Kit (KeyGEN Biotech) was performed according to the manufacturer's protocol. The protein concentrations of the cell lysates were determined using a bicinchoninic acid assay (KeyGEN Biotech). The total cellular protein was degraded with 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer by boiling in water for 10 minutes. Samples containing an equal quantity of total cellular protein were resolved by 12% SDS-PAGE and the separated proteins were transferred onto PVDF membranes. The membranes were blocked with nonfat milk (5%) in TBST (0.5%) for one to two hours and were then incubated with the indicated primary antibodies diluted in TBST overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were administered and incubated for one hour at room temperature. Finally, the protein bands were

visualized with a gel imaging system (Tanon Biotech) and quantified with ImageJ.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from cultured cells using RNAiso Plus (TaKaRa) and the same amounts of total RNA were used to synthesize cDNAs by reverse-transcription using the Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology Co., Ltd.). The qPCR reactions were performed with a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc.) using SYBR® Premix Ex Taq II (TaKaRa) with a 20 µl reaction system. The melting peaks were determined by melting curve analysis to ensure product specificity, and the amplification efficiencies of all specific primers were determined by standard curves which suggested efficiencies of no less than 90%. The expression of each gene was determined with three technical replicates and biological duplicates were quantified by the $2^{-\Delta\Delta Ct}$ method. Results are reported relative to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in supplement Table S3.

Transmission Electron Microscopy (TEM)

For TEM analyses, cells were pre-fixed in PFA (2%) and glutaraldehyde (2.5%) for 4 hours at 4°C before fixation with osmic acid for 4 hours at room temperature. Fixed cells were dehydrated using 30%, 50%, 70%, 80%, and 90% ethanol followed by anhydrous ethanol for 8 minutes intervals. Cells were subsequently embedded in Epon812 for 48 hours, and 70 nm ultra-thin sections were obtained with a UC7 ultramicrotome (Leica Microsystems). Ultra-thin cell sections were stained with uranyl acetate and lead citrate. Finally, stained cell sections were analyzed using a Tecnai G2 Spirit Bio-Twin transmission electron microscope (FEI).

Flow cytometry

After infection with *T. pyogenes* for the times indicated in the figure legends, cells were collected and stained according to the protocol with the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN Biotech). In brief, cells were digested with trypsin without EDTA and collected by centrifugation at 2000 rpm for 5 min twice in PBS. Cells were resuspended in binding buffer, followed by staining with annexin V-FITC and propidium iodide for 15 minutes at room temperature. 10,000 cells were analyzed within an hour using flow cytometry (BD FACSAria).

Statistical analysis

All experiments were performed at least three times. Data are presented as means ± standard errors of the mean (SEM). Unpaired t-test was used in two-group comparison, One-way ANOVA or Two-way ANOVA followed by Dunnett's or Tukey's test were used for post hoc multiple comparisons in three or more group. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc.) with $P < 0.05$ considered as statistically significant.

Results

T. pyogenes intracellular survival in goat endometrial epithelial cells is inhibited during the early infection period

Epithelial cells, similarly to immune cells, are endowed with the capacity to control diverse microbial invaders through intrinsic defense mechanisms that synergize with the immune system to confer whole-body protection. To investigate whether *T. pyogenes* was capable of infecting gEECs and to probe the intracellular survival of this pathogen, cell lines were infected as outlined in Materials and Methods. *T. pyogenes* survived in the cytosol during the first two hours of infection (Figure 1a) although colony-forming units (CFU) decreased quickly post-infection (Figure 1b). The data reveal that *T. pyogenes* did not replicate in gEECs. These results suggest that antimicrobial activity, which remains poorly understood in gEECs, counteracted infection by *T. pyogenes*.

T. pyogenes infection induces endoplasmic reticulum stress and unfolded protein responses

Host cells activate a series of adaptive stress responses in reaction to threats from multiple environmental factors and to reestablish homeostasis. ER stress plays an important role in combatting bacterial and viral intracellular survival. Biomarker proteins of ER stress and UPR were analyzed by Western blot to investigate whether these processes participate in protecting against *T. pyogenes* in gEECs. Strikingly, *T. pyogenes* infection resulted in sustained upregulation of GRP78, a central regulator of ER homeostasis, which suggests the activation of ER stress during infection (Figure 2a). In parallel, the three arms of UPR were activated to different levels: ER membrane-embedded sensors ATF6

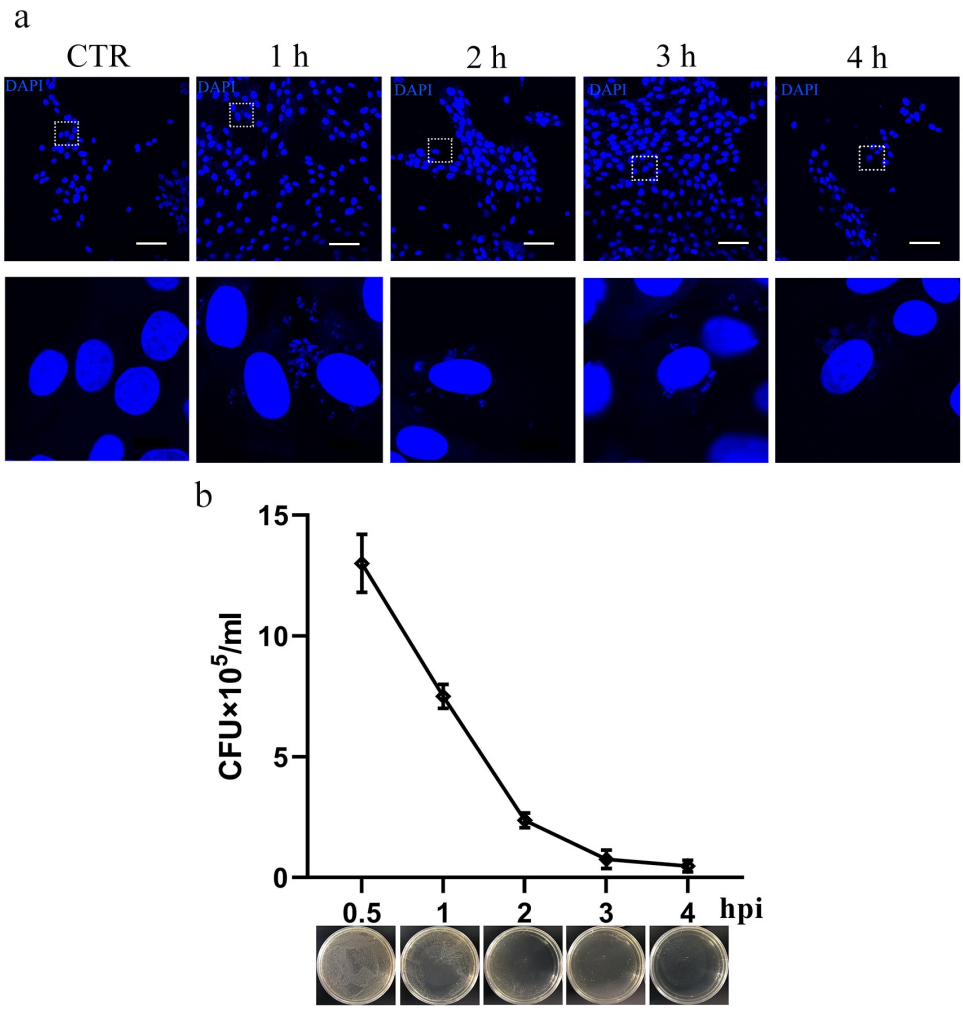

Figure 1. Survival of *T. pyogenes* in host cells. a. *T. pyogenes* can invade the cytosol of gEECs. gEECs were uninfected (CTR) or infected with *T. pyogenes* for one to four hours and were analyzed by immunofluorescence with DAPI to visualize both the nucleus and bacterial cells. Scale bar, 100 µm. b. gEECs were infected with *T. pyogenes* (MOI = 50) for 30 minutes to four hours. Cells were lysed with Triton X-100 (0.1%) and the lysate was inoculated into BHI medium, serially diluted, and plated on BHI agar. Colonies were counted after incubation at 37°C for 48 hours. The data are representative of three independent experiments (n = 3) .

(Figure 2b) and IRE1 (Figure 2c) were activated significantly, as were spliced XBP1 (for IRE1; Figure 2d), phosphorylated eIF2α and ATF4 (for PERK; Figure 2e and f). As our results suggest that gEECs respond to *T. pyogenes* infection by activating ER stress and UPR, we therefore reasoned that these signaling pathways might influence *T. pyogenes* intracellular survival which was investigated further.

Key role of endoplasmic reticulum stress and unfolded protein responses in regulating intracellular survival of *T. pyogenes*

4-PBA is a chemical molecular chaperone that alleviates ER stress, whereas the antibiotic Tm induces ER stress. 4-PBA and Tm were used initially to investigate whether ER stress influences the survival of *T. pyogenes* in gEECs. Pretreatment of gEECs with 4-PBA to inhibit ER stress significantly increased the CFU of *T. pyogenes* one to four hours post-infection, whereas the converse result was obtained when ER stress was activated by Tm (Figure 3a). UPR signaling pathways may be triggered selectively to address the imbalance caused by disruption of ER homeostasis resulting from ER stress. As described in the preceding section, the three arms of UPR were activated during *T. pyogenes* infection that suggests that these pathways may act together to protect against infection. Therefore, shRNA sequences that targeted *XBP1* (sh-XBP1-1, −2 and −3), *eIF2α* (sh-eIF2α-1, −2 and −3), or *ATF6* (sh-ATF6-1, −2 and −3) and a non-targeting shRNA (sh-N) were designed, and stable knockdown

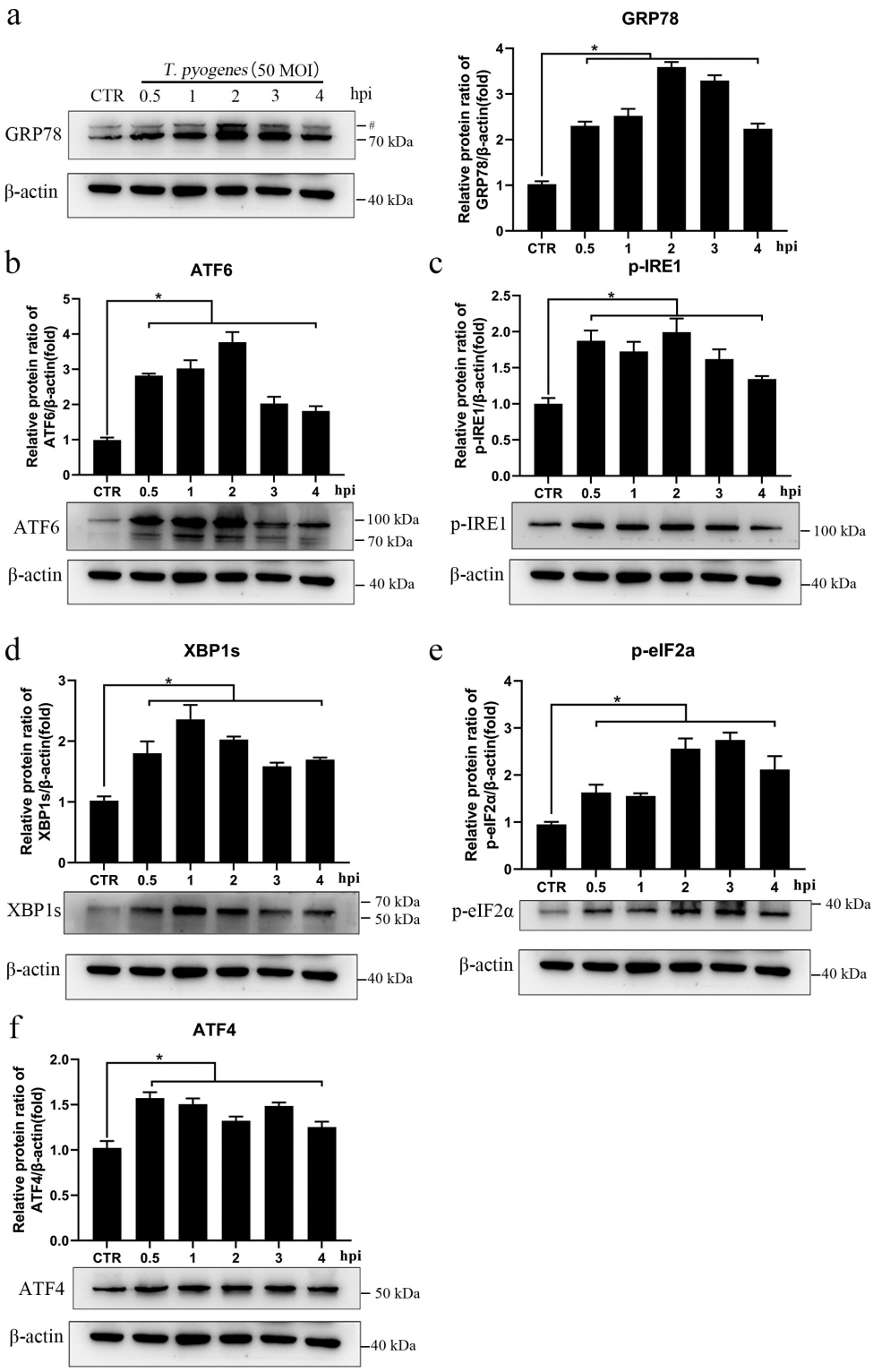

Figure 2. *T. pyogenes* induces ER stress and UPR. gEECs were uninfected (CTR) or infected with *T. pyogenes* (MOI = 50) for 30 minutes to four hours. Cells were lysed for Western blotting with antibodies against GRP78 (a), ATF6 (b), p-IRE1 (c), XBP1s (d), p-eIF2α (e), and ATF4 (f). The loading control was β-actin. #, nonspecific band. Target bands were quantified by ImageJ software relative to the corresponding loading control. The data are representative of three independent experiments (n = 3). One-way ANOVA was performed, and data were expressed as the mean ± SEM, *P < 0.05.

cell lines were constructed by recombinant lentivirus infection. The efficiencies of infection and interference were examined by observing GFP tags (Figure 3b) and by measuring target protein expression, respectively

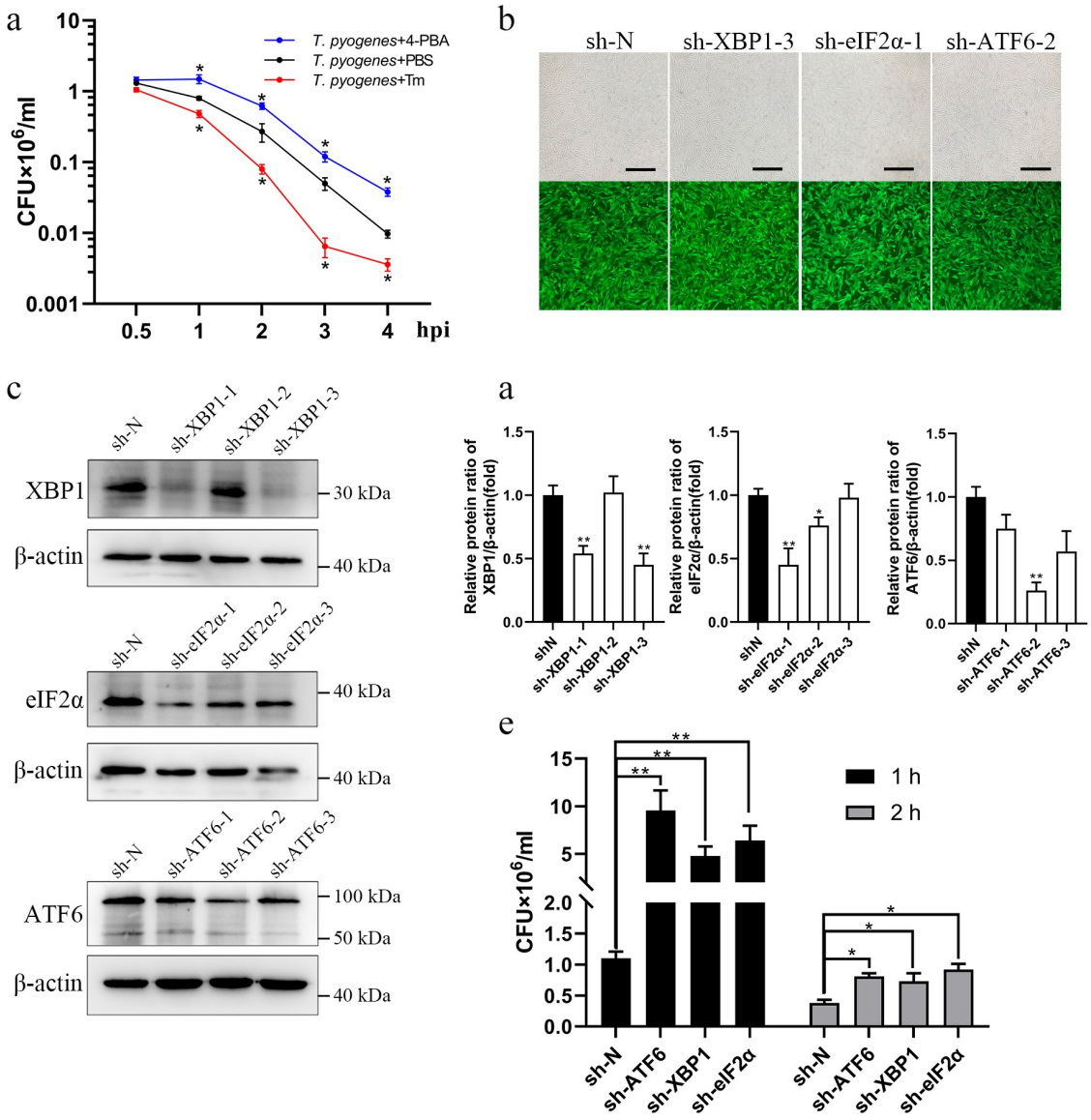

Figure 3. *T. pyogenes* intracellular survival is regulated by ER stress and UPR. a. gEECs were preincubated with 4-PBA (0.5 μM), Tm (0.5 μM) or PBS, and were then infected with *T. pyogenes* (MOI = 50) for 30 minutes to four hours. CFU were counted as outlined in Materials and Methods. b. Efficiency of lentivirus infection was examined by observing GFP tags after puromycin selection. Scale bar, 100 μm. c. Western blotting to analyze protein expression of XBP1, eIF2α and ATF6 in the constructed cell lines. d. Target bands were quantified by ImageJ software relative to the corresponding β-actin loading control to analyze interference efficiency. e. Cell lines of sh-N, sh-XBP1, sh-eIF2α and sh-ATF6 were infected with *T. pyogenes* (MOI = 50) for one or two hours. CFU were counted as outlined in the Figure 1 legend. The data are representative of three independent experiments ($n = 3$). In A, D and E, two-way ANOVA (a, e), or one-way ANOVA (d) were performed, and data were expressed as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

(Figure 3c). As shown in Figure 3d, target proteins were inhibited to different levels. Cell lines that harbored sh-XBP1-3, sh-eIF2α-1 and sh-ATF6-2 which exerted the highest knockdown efficiencies of the corresponding target genes were used for subsequent experiments. The CFU of *T. pyogenes* increased significantly in each stable knockdown cell line compared to sh-N cell lines at one and 2 hours after infection (Figure 3e). However, the CFU at 2 hours decreased significantly in all three cell lines compared to an hour post-infection (Figure 3e). These results suggest that ER stress mediated-UPR partially influences *T. pyogenes* survival.

It has been reported recently that autophagy regulated intracellular survival of *T. pyogenes* in RAW264.7 cell lines in a process that is dependent on the mTOR signaling pathway [12]. The survival of *T. pyogenes* in gEECs was invesigated here. LC3-positive puncta in vesicles with internalized bacteria were observed during *T. pyogenes* infection of gEECs (Figure 4a) and the

levels of the specific autophagy marker LC3-II were increased significantly (Figure 4b). Analogously, autolysosome-like vesicles were observed by TEM analyses of *T. pyogenes* infected cells (Figure 4c). Furthermore, small interference RNA (siRNA) revealed that *ATG5*, a protein that determines autophagosome elongation, was involved partly in this effect (Figure 4d). The decrease in CFU elicited by Tm in the si-N group is in agreement with the results described above (Figure 3a), but there was no significant difference in CFU in the si-ATG5 group (Figure 4e). Moreover, ER stress and UPR were more

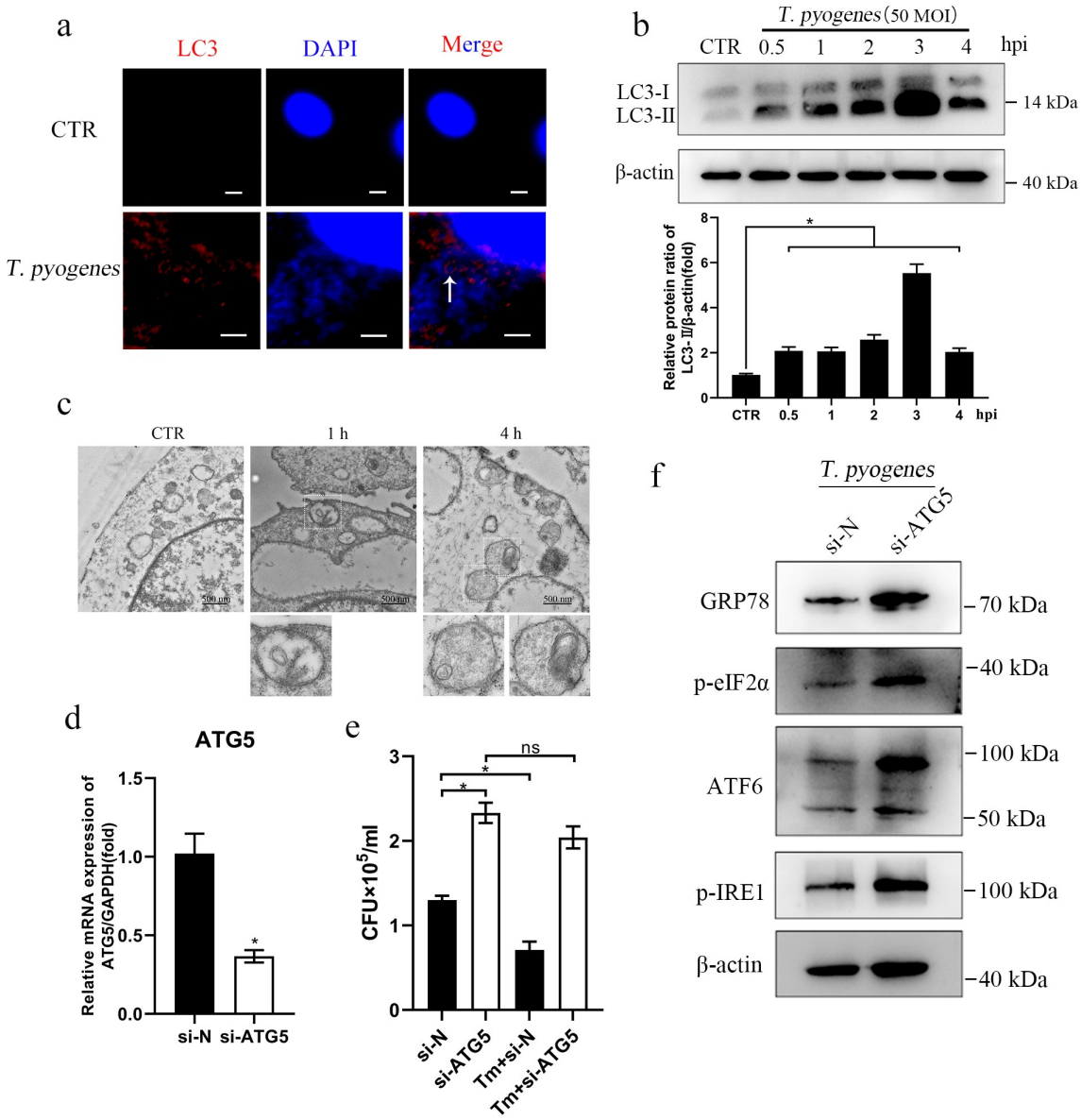

Figure 4. Knockdown of ATG5 blocks the process by which ER stress inhibits intracellular survival of *T. pyogenes*. a. gEECs were uninfected (CTR) or were infected with *T. pyogenes* for two hours and were analyzed by immunofluorescence with anti-LC3 antibody. The nucleus and bacterial cells were visualized wtih DAPI. Scale bar, 10 μm (CTR) and 5 μm (*T. pyogenes*). b. gEECs were uninfected (CTR) or were infected with *T. pyogenes* for 30 minutes to four hours and LC3-II protein expression was analyzed by Western blotting. c. gEECs were uninfected (CTR) or infected with *T. pyogenes* for one or four hours and cells were fixed and processed for transmission electron microscopy analysis. Scale bars are shown in the images. D-F. gEECs were transfected with small interference RNA (siRNA), including si-N and si-ATG5, for the following assays. d. Interference efficiency was detected by RT-qPCR and the relative ATG5 mRNA transcript levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. e. gEECs were preincubated with Tm (0.5 μM) or PBS and were then infected with *T. pyogenes* (MOI = 50) for two hours. CFU were counted as outlined in the Figure 1 legend. f. gEECs were infected with *T. pyogenes* (MOI = 50) for two hours and ER stress and UPR protein expression were analyzed by Western blotting. The data are representative of three independent experiments ($n = 3$). In B, D and E, one-way ANOVA (b), unpaired t-test (d) or two-way ANOVA (e) were performed, and data were expressed as the mean ± SEM, $^*P < 0.05$.

pronounced when autophagy flux was blocked by si-ATG5 (figure 4f). Our results overall suggest that ER stress-mediated UPR signaling pathways influence *T. pyogenes* intracellular survival in gEECs in a process that is dependent on autophagy.

Severe infection by *T. pyogenes* triggers apoptosis

We found in the preceding experiments that inhibition of autophagy flux induced severe ER stress and increased the intracellular survival of *T. pyogenes*. ER stress acts as a pro-survival mechanism under certain physiological conditions. However, apoptosis signal pathways, such as upregulation of CHOP, are triggered when ER stress is severe or prolonged. We adjusted the MOI to 100 to investigate whether severe infection with *T. pyogenes* triggers ER stress-mediated apoptosis signal pathway in gEECs. We observed that both mRNA concentrations and protein levels of CHOP were upregulated significantly by the increased MOI (Figure 5a and b). Cleaved Caspase 3, which is a key executioner in apoptosis, also was activated in gEECs during severe infection by *T. pyogenes* (Figure 5c). In addition, flow cytometry studies showed that *T. pyogenes* induced significant apoptosis in gEECs (Figure 5d and e). These results demonstrate that severe infection with *T. pyogenes* induces apoptosis in gEECs which may reflect ER stress-mediated UPR.

Unfolded protein responses inhibit the endometrial inflammatory response induced by *T. pyogenes*

The endometrium serves as the first barrier of the uterus and is vital for protecting against infection by diverse pathogenic bacteria. Therefore, the effect of *T. pyogenes* infection on the expression of inflammatory cytokines was examined. The inflammatory cytokines IL-1β, IL-6 and TNF-α and the proinflammatory gene COX-2 were expressed in gEECs in a time-dependent manner during the early stages of *T. pyogenes* infection (Figure 6a-6d). NF-κB p65, the key transcriptional regulator of the innate immune response, also was upregulated significantly by *T. pyogenes* infection (Figure 6e and f). We demonstrated in preceding experiments that ER stress mediated-UPR signal pathways influence the intracellular survival of *T. pyogenes* and that host cells activate the innate immune response to protect against infection (Figures 2–5). Thus, the interplay of ER stress mediated-UPR regulation of *T. pyogenes* and the inflammatory reaction was explored further. Immunoblotting experiments showed that *T. pyogenes* induced a significant increase of NF-κB p65 compared to the sh-N group when the UPR signal pathways that involve XBP1, eIF2α, or ATF6 were blocked in gEECs (Figure 7a-d). Similarly, the expression of proinflammatory cytokines IL-1β, IL-6, TNF-α and COX-2 was upregulated significantly (Figure 7d-f). Thus, knockdowns of key genes in each UPR signal pathway made gEECs more susceptible to *T. pyogenes* infection. These results further corroborate a critical role for ER stress mediated-UPR in protecting host cells against *T. pyogenes* by modulating the inflammatory reaction.

Discussion

Infection by pathogenic bacteria seriously impairs both animal-breeding efficiency and the quality of meat and milk in livestock farming. There is a clear correlation between *T. pyogenes* infection and endometritis, uterine and ovarian dysfunction, and dam infertility [33], but the precise infection mechanisms that are involved and the host cell response to this opportunistic pathogen remain unclear. Here, we demonstrated that wild-type *T. pyogenes* isolated from a sick goat invaded gEECs, but that the invasive bacteria decreased quickly post-infection. We noted that mTOR-mediated autophagy is a potent functional regulator of immune defense responses during *T. pyogenes* infection [12]. In addition, upstream regulators involved in activating autophagy flux, such as the mTORC1 complex and the ER stress signal pathway, are necessary for defense against infection [34]. Although ER stress is a critical adaptive cell response during adverse environmental conditions, the process also is utilized by pathogenic bacteria during replication in host cells [35]. The activation of the ER stress and UPR by cholesterol-dependent cytolysins (CDCs) also has been reported [28]. Hence, the relevance of exploring the role of ER stress in protecting against *T. pyogenes* in gEECs was clear. Our results suggest that ER stress and UPR were activated by gEECs in response to *T. pyogenes* infection, and that inhibition of ER stress elicited a significant increase in *T. pyogenes* intracellular persistence whereas activation of ER stress decreased the bacterial load. There are similarities in intracellular survival during early infection between *T. pyogenes* and *Brucella* spp., although VirB T4SS effectors of *Brucella* promote bacterial replication by forming Brucella-containing vacuoles in the following period [36]. Another vacuolar pathogen, *S. enterica* Typhimurium, delivers the SopD2 effector, a type III secretion protein with GAP activity toward Rab32, to counter host defense mechanisms [37]. *T. pyogenes* appears not to have evolved an equivalent "armed force" to counteract cell autonomous defense mechanisms during the early invasion period.

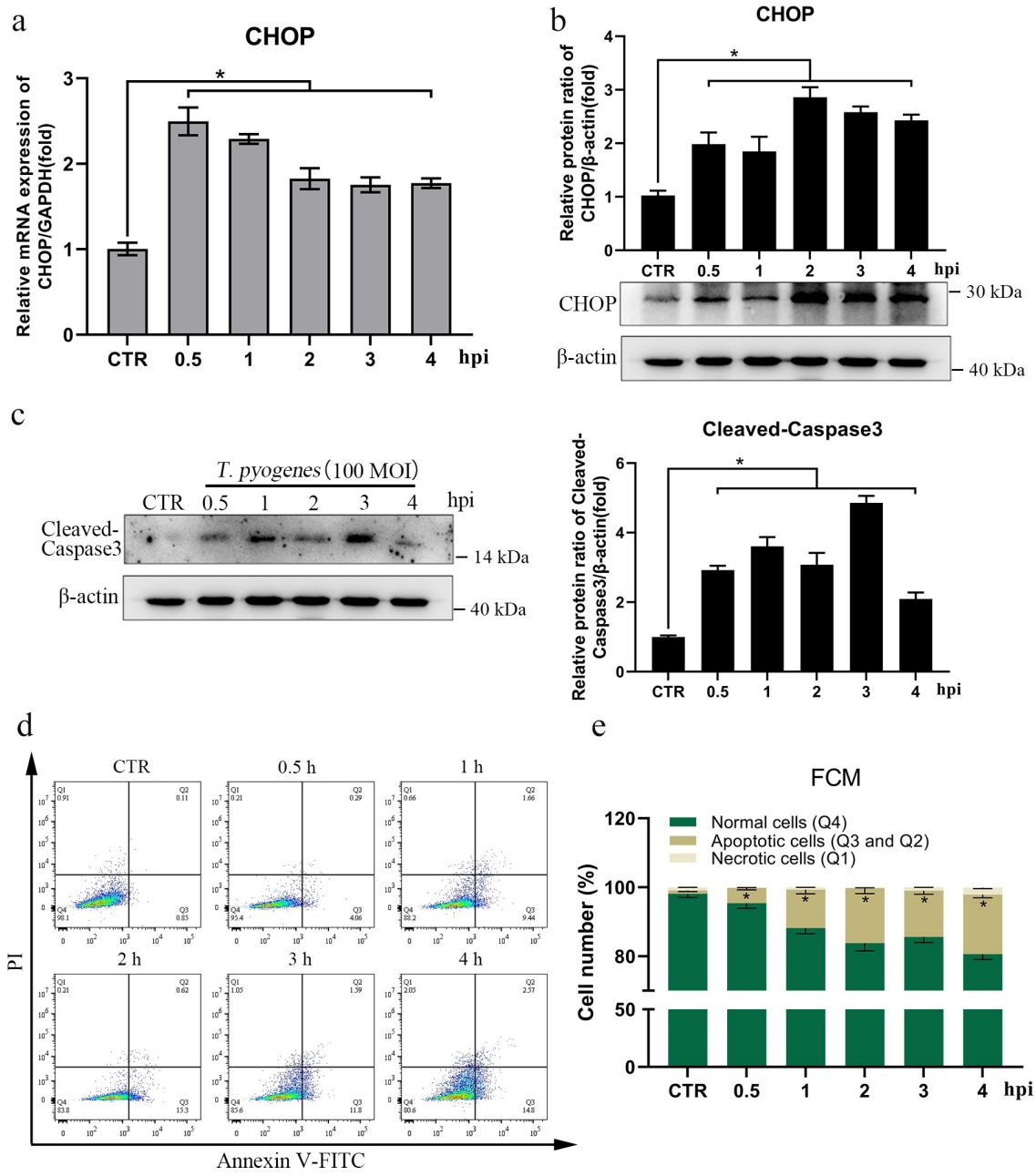

Figure 5. Apoptosis was induced in gEECs by severe infection with *T. pyogenes*. gEECs were uninfected (CTR) or were infected with *T. pyogenes* (MOI = 100) for 30 minutes to four hours. a. Relative mRNA transcript of CHOP was detected by RT-qPCR and was analyzed by the $2^{-\Delta\Delta Ct}$ method. b, c. CHOP and cleaved-Caspase3 protein expression was analyzed by Western blotting. d, e. Cells were analyzed by flow cytometry after stained with annexin V and propidium iodide (PI). One-way ANOVA was performed, and data were expressed as the mean ± SEM, *P < 0.05. The data are representative of three independent experiments (n = 3) .

LC3, a widely used marker for autophagy, was upregulated significantly during *T. pyogenes* infection of gEECs and the invading bacteria were surrounded by LC3 puncta observed by fluorescence microscopy. However, as autophagy is a dynamic and complex process, complementary concurrent methods should be used to assess accurately the status of autophagic activity [38]. TEM was performed on gEECs infected with *T. pyogenes* to find direct evidence that elimination of intracellular *T. pyogenes* involves autophagy as with group A *Streptococcus* which is enveloped by autophagosome-like compartments and killed after fusion with lysosomes [17]. Autolysosome-like vesicles were apparent in these ultrastructure observations. However, *T. pyogenes* enclosed by a phagophore or double-membrane structure (autophagosome) was not captured in the TEM studies. Nevertheless, the possibility that *T. pyogenes* enters into these structures cannot be

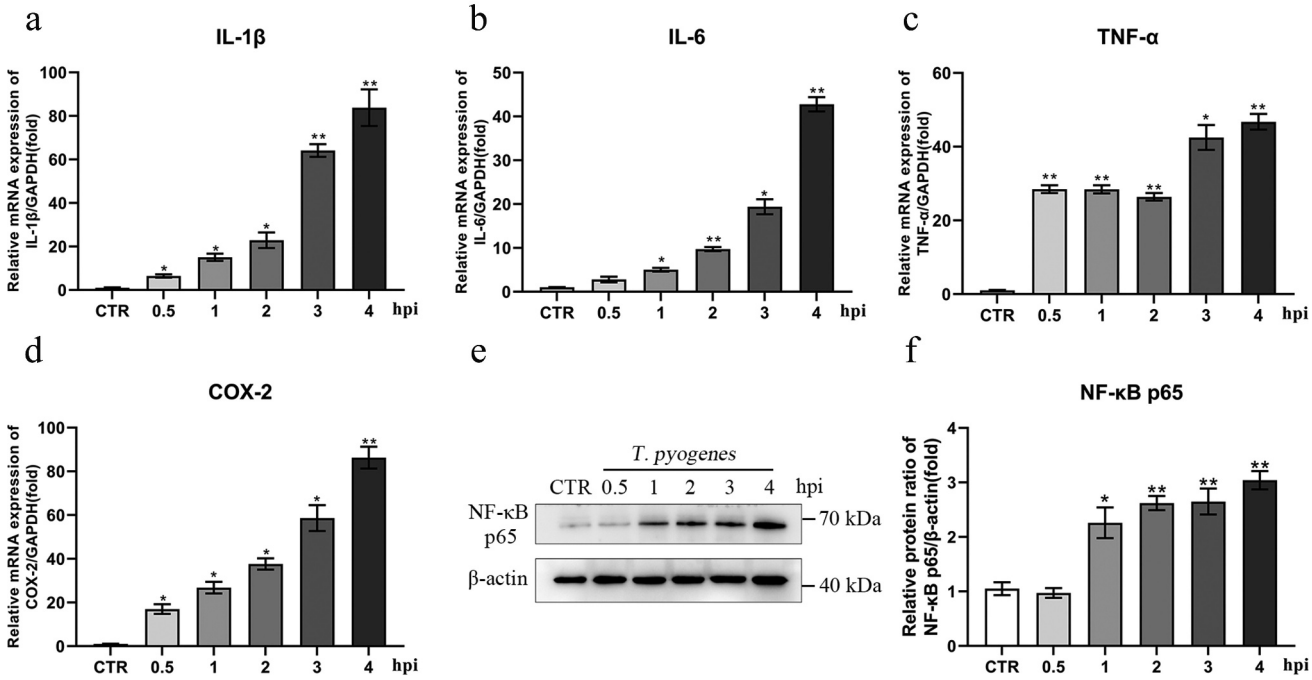

Figure 6. *T. pyogenes* induces an inflammatory reaction in gEECs. gEECs were uninfected (CTR) or were infected with *T. pyogenes* (MOI = 50) for 30 minutes to four hours. a-d. Relative mRNA transcripts of IL-1β, IL-6, TNF-α and COX-2 were detected by RT-qPCR and were analyzed the by $2^{-\Delta\Delta Ct}$ method. e. NF-κB p65 protein expression was analyzed by Western blotting. f. Target bands were quantified by ImageJ software relative to β-actin. One-way ANOVA was performed, and data were expressed as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$. The data are representative of three independent experiments ($n = 3$) .

excluded entirely as autophagy flux is a dynamic process in which cargo or pathogens within autophagosomes may be short-lived [38]. When autophagy flux was blocked by knockdown of ATG5, a primary protein in autophagosome formation, intracellular survival of *T. pyogenes* was enhanced which accords with previous studies [12]. The capacity of the Tm antibiotic to inhibit intracellular survival of *T. pyogenes* was reduced markedly in the knockdown cells in which *T. pyogenes* also induced severe ER stress. A possible explanation of these results is that autophagy served as the downstream effector of ER stress to eliminate *T. pyogenes* and that the bacterial burden was increased by deficient autophagy in the ATG5 knockdown cells which resulted in gEECs undergoing a severe stress response. Combined with results of immunofluorescence and immunoblotting, we suggest that autophagy plays a vital role in defending host cells against *T. pyogenes*.

It is well established that apoptosis is triggered when homeostasis cannot be reestablished by UPR [22]. The apoptosis effector Caspase3 and the UPR downstream pro-apoptotic transcription factor CHOP both were activated during *T. pyogenes* infection of gEECs. These observations, combined with flow cytometry data, demonstrated that severe challenge with *T. pyogenes* induced cellular apoptosis. This response may protect the entire organism from the threat posed by *T. pyogenes* infection by depriving the bacterium of tolerable survival conditions. Considering the predominantly cytoprotective role of autophagy under stress conditions, such as during pathogen infection, it seems plausible that autophagy and apoptosis are antagonistic events. Nevertheless, the mechanisms by which autophagy and apoptosis crosstalk are still not defined clearly. Autophagy is a self-protective process that shields cells from death by targeting invading pathogens, such as *T. pyogenes*, damaged organelles, and other defective cellular components. Although *T. pyogenes* induced ER stress that autophagy was unable to solve, cells undergo apoptosis as a rational and active decision to sacrifice specific cells for the greater benefit of the organism. Apoptosis is key not only in the self-defense process but also as part of the immune system that is crucial to organismal health [39]. Inflammation is an element of the immune system response that prevents, limits and repairs damage by invading pathogens. However, a persistent or severe inflammatory response is associated with tissue dysfunction and pathology [40]. In this study, the UPR signaling pathway inhibited inflammatory cytokine expression that was induced by *T. pyogenes* infection which may contribute to

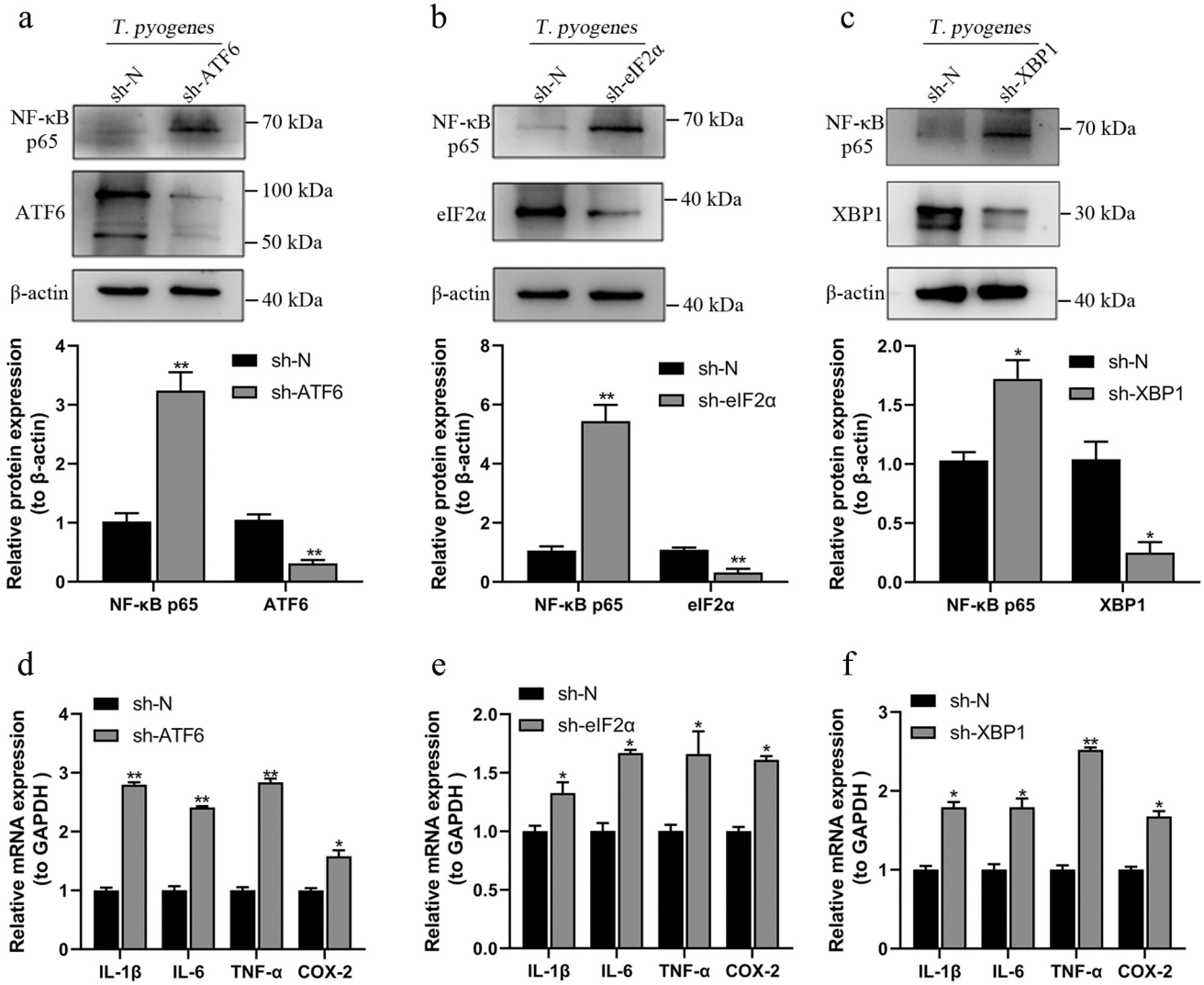

Figure 7. *T. pyogenes* induced an inflammatory reaction in gEECs regulated by ER stress and UPR. Cell lines of sh-N, sh-ATF6, sh-eIF2α and sh-XBP1 were infected with *T. pyogenes* (MOI = 50) for two hours. a-c. NF-κB p65 and the corresponding target proteins of the relevant cell lines were detected by Western blotting. Target bands were quantified by ImageJ software relative to β-actin. d-f. Relative mRNA transcripts of IL-1β, IL-6, TNF-α and COX-2 were detected by RT-qPCR in sh-ATF6 (d), sh-eIF2α (e) and sh-XBP1 (f) cell lines compared to sh-N. The $2^{-\Delta\Delta Ct}$ method was used to analyze mRNA expression relative to GAPDH. Unpaired t-test was performed. *$P < 0.05$; **$P < 0.01$. The data are representative of three independent experiments ($n = 3$) .

uterine homeostasis and fertilization in the following estrus cycle.

The current study raises numerous questions about the mechanism of *T. pyogenes* infection and the host cell response to this infection. For example, different virulence and survival mechanisms exist in different species of the same genus, such as *S. enterica* Typhimurium and *S. enterica* Typhi [41]. In this case, the intracellular survival of both *S. enterica* Typhi and *S. enterica* Typhimurium is restricted by the host Rab-family GTPase Rab32. Expression of SopD2, which is a Rab32 GTPase-activating protein, and GtgE, which is a protease that is specific for Rab32, counteracts this restriction for *S. enterica* Typhimurium. In contrast, the gene for GtgE is absent and *sopD2* is a pseudo-gene in *S. enterica* Typhi [37]. Variations in virulence also are pervasive in different strains of *T. pyogenes* and, among virulence factors, only PLO is expressed in all strains that have been tested [42]. Therefore, the presence of other potential mechanisms that promote *T. pyogenes* infection remains to be explored further. In this study, the influence of the primary virulence factor PLO, which can induce cell death and cytolysis by *T. pyogenes*, was discounted. According to the work of Preta et al. [19], the cytotoxic effects of PLO are dose-dependent which coincides with the results of our previous study [43]. Cell viability is not affected significantly by PLO at the sublytic concentrations (not more than 0.1 μg/ml or 50 HU). In the

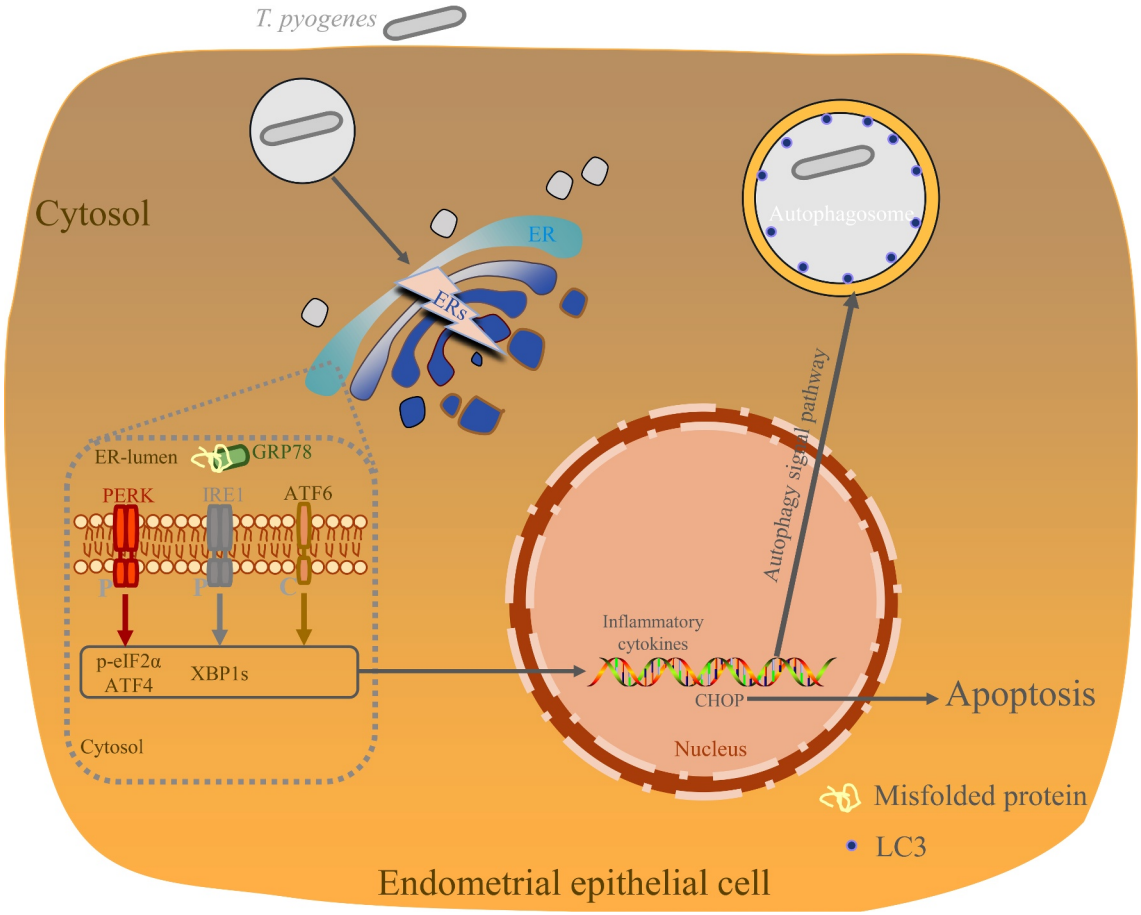

Figure 8. Schematic model delineating the pathway of the responsiveness of cells to *T. pyogenes*. *T. pyogenes* infection induces the ER stress mediated-UPR signaling pathway. Autophagy and apoptosis are triggered to counteract the invading bacteria.

infection mode (50 MOI) used here, cell viability did not decrease significantly which affirmed that the PLO concentration did not reach the threshold that induces significant cell death. Meanwhile, autophagy was activated by PLO at a concentration that does not affect cell viability [19]. Thus, PLO is the main virulence factor of tissue purulent and hemorrhagic lesions caused by *T. pyogenes* on the one hand, but on the other hand PLO also may act as a trigger to activate host clearance mechanism such as xenophagy, which has a critical role in the process of *T. pyogenes* infection.

In summary, we demonstrated that the ER stress mediated-UPR signaling pathway participates in regulating intracellular survival of *T. pyogenes* as well as in the inflammatory response elicited by this pathogen. Blocking the pathway made host cells more susceptible to *T. pyogenes* infection. Moreover, autophagy serves as a critical mechanism that may interfere with the ability of the ER stress response to inhibit *T. pyogenes* intracellular survival (Figure 8). Our study provides signposts for the prevention of *T. pyogenes* infection and for treatment of *T. pyogenes* and related diseases, and also provides important insights into the interplay between *T. pyogenes* and critical host defense systems.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethical statement

The experimental procedures were approved under the control of the Guidelines for Animal Experiments by the Committee for the Ethics on Animal Care and Experiments of Northwest A&F University and performed under the control of the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Author contributions

Maozhen Qi and Yaping Jin designed the experiments. Maozhen Qi, Qingran Jiang, Siwei Yang and Chenxi Zhang carried out the experiments and collected data. Maozhen Qi wrote this manuscript. Jianguo Liu, Wei Liu, Pengfei Lin, Huatao Chen, Dong Zhou, Keqiong Tang, Aihua Wang checked and revised the manuscript.

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References

[1] Jost BH, Billington SJ. *Arcanobacterium pyogenes*: molecular pathogenesis of an animal opportunist. Antonie Van Leeuwenhoek. 2005;88(2):87–102.
[2] Narayanan S, Nagaraja TG, Wallace N, et al. Biochemical and ribotypic comparison of Actinomyces pyogenes and A pyogenes-like organisms from liver abscesses, ruminal wall, and ruminal contents of cattle. Am J Vet Res. 1998;59(3):271–276.
[3] Jost BH, Post KW, Songer JG, et al. Isolation of *Arcanobacterium pyogenes* from the porcine gastric mucosa. Vet Res Commun. 2002;26(6):419–425.
[4] Ashrafi Tamai I, Mohammadzadeh A, Zahraei Salehi T, et al. Genomic characterisation, detection of genes encoding virulence factors and evaluation of antibiotic resistance of *Trueperella pyogenes* isolated from cattle with clinical metritis. Antonie Van Leeuwenhoek. 2018;111(12):2441–2453.
[5] van Engelen E, Dijkstra T, Meertens NM, et al. Bacterial flora associated with udder cleft dermatitis in Dutch dairy cows. J Dairy Sci. 2020;104(1):728–735.
[6] Lewis GS. Uterine health and disorders. J Dairy Sci. 1997;80(5):984–994.
[7] Amos MR, Healey GD, Goldstone RJ, et al. Differential endometrial cell sensitivity to a cholesterol-dependent cytolysin links *Trueperella pyogenes* to uterine disease in cattle. Biol Reprod. 2014;90(3):54.
[8] Semaan A, Tayeh GA, Chebel JA, et al. *Arcanobacterium pyogenes* and encrusted pyelitis. Future Sci OA. 2019;6 (1):Fso430.
[9] Tattoli I, Sorbara MT, Yang C, et al. *Listeria* phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures. Embo J. 2013;32 (23):3066–3078.
[10] Rzewuska M, Kwiecień E, Chrobak-Chmiel D, et al. Pathogenicity and Virulence of *Trueperella pyogenes*: a review. Int J Mol Sci. 2019;20(11):2737.
[11] Bicalho ML, Machado VS, Oikonomou G, et al. Association between virulence factors of *Escherichia coli, Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. Vet Microbiol. 2012;157(1–2):125–131.
[12] Huang T, Cui K, Song X, et al. MTOR involved in bacterial elimination against *Trueperella pyogenes* infection based on mice model by transcriptome and biochemical analysis. Vet Microbiol. 2019;235:199–208.
[13] Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132(1):27–42.
[14] Mizushima N, Levine B, Cuervo AM, et al. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069–1075.
[15] van der Vaart A, Mari M, Reggiori F. A picky eater: exploring the mechanisms of selective autophagy in human pathologies. Traffic. 2008;9(3):281–289.
[16] Lamb CA, Dooley HC, Tooze SA. Endocytosis and autophagy: shared machinery for degradation. Bioessays. 2013;35(1):34–45.
[17] Nakagawa I, Amano A, Mizushima N, et al. Autophagy defends cells against invading Group A streptococcus. Science. 2004;306(5698):1037–1040.
[18] Tattoli I, Sorbara MT, Vuckovic D, et al. Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. Cell Host Microbe. 2012;11(6):563–575.
[19] Preta G, Lotti V, Cronin JG, et al. Protective role of the dynamin inhibitor Dynasore against the cholesterol-dependent cytolysin of *Trueperella pyogenes*. Faseb J. 2015;29(4):1516–1528.
[20] Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell. 2010;40(2):310–322.
[21] Deegan S, Saveljeva S, Gorman AM, et al. Stress-induced self-cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. Cell Mol Life Sci. 2013;70(14):2425–2441.
[22] Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007;8(7):519–529.
[23] Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends Biochem Sci. 2015;40(3):141–148.
[24] Martina JA, Diab HI, Brady OA, et al. TFEB and TFE3 are novel components of the integrated stress response. Embo J. 2016;35(5):479–495.
[25] Zhang Z, Qian Q, Li M, et al. The unfolded protein response regulates hepatic autophagy by sXBP1-mediated activation of TFEB. Autophagy. 2021; 171:1841–1855.
[26] Rzymski T, Milani M, Pike L, et al. Regulation of autophagy by ATF4 in response to severe hypoxia. Oncogene. 2010;29(31):4424–4435.
[27] Rouschop KM, van Den Beucken T, Dubois L, et al. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J Clin Invest. 2010;120(1):127–141.
[28] Bischof LJ, Kao CY, Los FC, et al. Activation of the unfolded protein response is required for defenses against bacterial pore-forming toxin in vivo. PLoS Pathog. 2008;4(10):e1000176.
[29] Tentaku A, Shimohata T, Hatayama S, et al. Host cellular unfolded protein response signaling regulates *Campylobacter jejuni* invasion. PLoS One. 2018;13(10): e0205865.
[30] Sheldon IM, Noakes DE, Rycroft AN, et al. Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction. 2002;123:837–845.

[31] Zhang YY, Wang AH, Wu QX, et al. Establishment and characteristics of immortal goat endometrial epithelial cells and stromal cells with hTERT. J Anim Vet Adv. 2010;9(21):2738–2747.
[32] Chen F, Lin PF, Li X, et al. Construction and expression of lentiviral vectors encoding recombinant mouse CREBZF in NIH 3T3 cells. Plasmid. 2014;76:24–31.
[33] Miller AN, Williams EJ, Sibley K, et al. The effects of *Arcanobacterium pyogenes* on endometrial function in vitro, and on uterine and ovarian function in vivo. Theriogenology. 2007;68(7):972–980.
[34] Lee WS, Yoo WH, Chae HJ. ER stress and autophagy. Curr Mol Med. 2015;15(8):735–745.
[35] Zhu E, Chen W, Qin Y, et al. Classical swine fever virus infection induces endoplasmic reticulum stress-mediated autophagy to sustain viral replication in vivo and in vitro. Front Microbiol. 2019;10:2545.
[36] Celli J, Salcedo SP, Gorvel JP. *Brucella* coopts the small GTPase Sar1 for intracellular replication. Proc Natl Acad Sci U S A. 2005;102(5):1673–1678.
[37] Spanò S, Gao X, Hannemann S, et al. Targets a host rab-family GTPase defense pathway with a GAP. Cell Host Microbe. 2016;19(2):216–226.
[38] Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell. 2010;140(3):313–326.
[39] Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: disease message and therapeutic target potentials. Biosci Rep. 2019;39(1):BSR20180992.
[40] Okin D, Medzhitov R. Evolution of inflammatory diseases. Curr Biol. 2012;22(17):R733–740.
[41] Spanò S, Galán JE. A Rab32-dependent pathway contributes to salmonella typhi host restriction. Science. 2012;338(6109):960–963.
[42] Risseti RM, Zastempowska E, Twarużek M, et al. Virulence markers associated with *Trueperella pyogenes* infections in livestock and companion animals. Lett Appl Microbiol. 2017;65(2):125–132.
[43] Qi M, Liu J, Jiang Q, et al. *Trueperella pyogenes* pyolysin inhibits lipopolysaccharide-induced inflammatory response in endometrium stromal cells via autophagy- and ATF6-dependent mechanism. Braz J Microbiol. 2021;52(2):939–952.