

Estradiol inhibits autophagy of *Mycobacterium tuberculosis*-infected 16HBE cells and controls the proliferation of intracellular *Mycobacterium tuberculosis*

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Abstract. Tracheobronchial tuberculosis (TBTB) is most common in young, middle-aged females. Despite adequate anti-tuberculosis therapy, >90% of patients develop tracheobronchial stenosis, which has a high rate of resulting in disability. The present study aimed to explore the effect of estradiol on the development of TBTB. Estrogen receptor (ER) expression in granulomatous tissue was assessed via immunofluorescence. In order to determine whether estrogen affected the proliferation of intracellular *Mycobacterium tuberculosis* (Mtb), 16HBE cells were infected with Mtb *in vitro*, followed by estradiol treatment. Intracellular Mtb was quantified via colony counting. The effect of estradiol on autophagy of infected 16HBE cells was determined via western blotting and transmission electron microscopy. Necrosis assays of infected 16HBE cells were analyzed using propidium iodide staining and assessing lactate dehydrogenase (LDH) release. To determine how estradiol affects autophagy, infected 16HBE cells were treated with ER-specific and non-specific modulators. Reactive oxygen species (ROS) levels were analyzed via flow cytometry. Additionally, the protein expression levels of autophagy-associated proteins were determined via western blotting. Mtb could enter human lobar bronchial goblet cells and ciliated cells in patients with TBTB. The results also demonstrated that ER α was expressed in granulomatous tissue from patients with TBTB. Administration of 10^{-6} M estradiol reduced the number of intracellular Mtb colony-forming units *in vitro* in the 16HBE human bronchial epithelial cell line at day 3 after infection. Furthermore, cells treated with estradiol and infected with Mtb released less LDH at 72 h

and exhibited reduced necrosis levels at 24 h compared with the untreated cells. In addition, autophagy of infected 16HBE cells was inhibited by estradiol. Estradiol and the specific ER α agonist had similar effects on autophagy in infected 16HBE cells. Additionally, treatment with the ER α antagonist abolished the inhibition of autophagy by estradiol in infected 16HBE cells. Compared with the untreated infected 16HBE cells, the ROS levels in the infected 16HBE cells treated with estradiol and the ER α agonist significantly decreased. The levels of phosphorylated (p)-mTOR and p-AKT notably increased in estradiol- and ER α agonist-treated infected 16HBE cells. In summary, estradiol may serve a key role in the development of TBTB through binding to ER α .

Introduction

Tuberculosis (TB) imposes an important health burden worldwide. The 2020 Global World Health Organization TB report stated that an estimated 10 million people fell ill with TB in 2019 globally (1). Tracheobronchial TB (TBTB) is defined as *Mycobacterium tuberculosis* (Mtb) infection of the tracheobronchial tree. Although TB affects all age groups, the highest burden is in adult men. Indeed, adult men accounted for 56% of all cases in 2019, compared with 32% in adult women and 12% in children (1). However, previous studies have reported that TBTB is most common in young and middle-aged females (2-4). However, the reasons for this are unclear. Estradiol levels are significantly augmented in patients with TB compared with healthy controls (5,6). In addition to its effects on sexual differentiation and reproduction, estrogen may regulate autophagy by affecting the production of reactive oxygen species (ROS) (7,8). In certain cases, estrogen serves a role in promoting autophagy (9), but on other occasions, when cellular autophagy is stimulated by hypoxia or lipopolysaccharides, estrogen shows a restrictive effect on the expression of genes associated with autophagy (10,11). Previous studies have reported that ER modulators suppress intracellular Mtb growth by enhancing autophagy in infected macrophages (7). Therefore, we hypothesized that estrogen may serve an important role in the occurrence and development of TBTB.

Epithelial cells are known to contribute to the immune responses in lungs and can sense intra- and extracellular

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pathogens (12). *In vitro* studies have demonstrated that primary human airway epithelial cells and the type-II alveolar cell line A549 are capable of internalizing Mtb, although at a slower rate than macrophages (13,14). Bacteria have been shown to replicate extensively inside A549 cells. The replicated bacteria may then escape from the cell after bacteria-induced apoptosis or necrosis, which facilitates systemic dissemination (15,16). Autophagy also protects type-II alveolar epithelial cells, which are non-phagocytic cells, from Mtb infection and is not conducive to the spread of Mtb inside the cells (17). However, a previous study found that blocking autophagy using 3-methyladenine is advantageous to the infected A549 cells and inhibits bacterial replication and results in a significant decrease in bacterial viability compared with the untreated infected A549 cells (18). Therefore, it may be hypothesized that estradiol could serve a key role in the development of TBTB by affecting the intracellular Mtb proliferation and the autophagy of Mtb-infected bronchial epithelial cells.

To address this question, granulomatous tissue from patients with TBTB was collected to determine the expression of estrogen receptor (ER). Mtb is already known to be capable of infecting alveolar epithelial cells. However, whether Mtb could infect bronchial epithelial cells, including ciliated cells, goblet cells, basal cells and secretory cells, remains to be elucidated. Therefore, whether Mtb could infect above bronchial epithelial cells was determined. Additionally, a model that mimics the bronchial epithelial cell-Mtb interaction was employed using the human bronchial epithelial cell line 16HBE infected with Mtb and treating the 16HBE-infected cells with estradiol at different concentrations *in vitro*.

Materials and methods

Patients and tissue collection. Three lobar bronchial granulomatous tissues of 1 male and 2 female patients (age, 21-31 years) with lobar bronchial TBTB (the etiology test of these tissues was positive for Mtb) were obtained between March and June 2021 from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). For inclusion in the study patients needed to be confirmed to have TBTB and have positive etiology test of biopsy tissue for Mtb. Patients who was unable to tolerate bronchoscopy were excluded. Specimens for immunofluorescence analysis were fixed with 4% formaldehyde at 4°C for 24 h and embedded in paraffin wax. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (approval no. 20188502; Chongqing, China). Written informed consent was obtained from all patients.

Bacterial culture. The Mtb H37Rv and *Bacillus Calmette-Guerin* (BCG) strains were donated by Beijing Chest Hospital, Capital Medical University. For *in vitro* cell infection, frozen Mtb (strain H37Rv) or BCG was inoculated on Löwenstein-Jensen culture medium (Baso Diagnostic, Inc.) at 37°C until colony formation. Subsequently the cultures were transferred to bottles containing 60 ml Middlebrook 7H9 (BD Biosciences) culture medium supplemented with 10% Middlebrook albumin dextrose catalase (BD Biosciences) and 0.1 mM calcium chloride (Beijing Solarbio Science & Technology Co., Ltd.). Cultures were incubated in an orbital

shaker at 5 x g at 37°C for 7-12 days until they reached McFarland turbidity standard no. 5 (Shenzhen Kangtai Biological Products). Bacterial concentrations were then estimated according to the McFarland turbidity standard (19).

Cell preparation. The human bronchial epithelial cell line 16HBE (Shanghai Fuheng Biotechnology, Co., Ltd.) was grown in complete RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (SORFA Life Science; Beijing Shuohua Biotech Co., Ltd.) at 37°C in 5% CO₂.

Infection with Mtb and stimulation of cells with estradiol. Estradiol (Sigma-Aldrich; Merck KGaA) was diluted in DMSO (concentration 10⁻² M) and stored in the refrigerator at -80°C. Before the experiment, estradiol was diluted to 10⁻⁴ M and 10⁻⁶ M using PBS. Then 20 µl estradiol at 10⁻⁴ M and 10⁻⁶ M were added to cells containing 2ml medium to ensure final concentrations of estradiol were 10⁻⁶ M (E2) and 10⁻⁸ M (E1). Bacteria growing on the 7H9 broth were centrifuged at 400 x g at room temperature for 10 min and resuspended in PBS. Bacterial concentrations were then estimated according to the McFarland turbidity standard (19). To evaluate whether different concentrations of estradiol affect the growth of intracellular Mtb, 16HBE cells were infected with Mtb at a multiplicity of infection (MOI) of 10:1 at 37°C in 5% CO₂ for 24 h in the absence of estradiol and cells were then washed four times with PBS to remove extracellular bacilli. Infected 16HBE cells were incubated at 37°C in 5% CO₂ for 1 h (day 0), 24 h (day 1) and 72 h (day 3) in the presence or absence of estradiol. Mtb-infected cells were lysed with 1% SDS followed by 20% BSA (Sigma-Aldrich; Merck KGaA), serially diluted 10-10⁴ times in Middlebrook 7H9 medium, and plated in triplicate on Löwenstein-Jensen culture medium at 37°C for 4-6 weeks. The number of colony-forming units (CFUs) were manually quantified.

Immunofluorescence (IF). According to the methods of Hui *et al* (20), 5-mm-thick sections were deparaffinized in xylene followed by rehydration using an ethanol gradient. Sections were subjected to antigen retrieval using 1X citric acid antigen repair solution (Wuhan Boster Biological Technology, Ltd.), which was heated with high fire for 8 min and medium fire for 8 min. Membrane disruption was performed using 0.03% Triton X-100 for 30 min at room temperature. The sections were then blocked with 5% BSA for 20 min at room temperature. Mouse anti-human p63 (1:200; cat. no. NBP3-07430; Novus Biologicals, Ltd.), mouse anti-human secretoglobin 1A member 1 (SCGB1A1; 1:500; cat. no. H00007356-M01; Novus Biologicals, Ltd.), mouse anti-human mucin 5AC (MUC5AC; 1:200; cat. no. ab3649; Abcam), mouse anti-human forkhead box J1 (FOXJ1; 1:500; cat. no. 14-9965-82; Thermo Fisher Scientific, Inc.) or rabbit anti-Mtb antibody (1:100; cat. no. ab905; Abcam) was added overnight at 4°C. FITC-labeled goat anti-mouse IgG secondary antibody (1:200; cat. no. E031210; EarthOx; Beijing Canlife Technology Co.) was added and smears were incubated at 37°C for 50 min. Cy3-labeled goat anti-rabbit IgG secondary antibody (1:200; cat. no. E031620; EarthOx; Beijing Canlife Technology Co.) was simultaneously added and smears were incubated at 37°C

for 50 min. Sections and 16HBE cells on coverslips were fixed, permeabilized and blocked with 5% BSA at 37°C for 20 min. Mouse anti-human ER α (1:500; cat. no. NBP2-61764; Novus Biologicals, Ltd.) and mouse anti-human ER β (1:500; cat. no. NBP2-44366; Novus Biologicals, Ltd.) monoclonal antibodies were added and incubated overnight at 4°C. Cy3-labeled goat anti-mouse IgG secondary antibody (1:200; cat. no. E031610-02; EarthOx; Beijing Canlife Technology Co.) was added and incubated at 37°C for 50 min. DAPI solution was added and incubated at room temperature for 5 min. An appropriate amount of anti-fluorescence quenching medium was dropped on the tissue, which was then observed under a fluorescence microscope (Olympus Corporation).

Transmission electron microscopy (TEM). According to the methods of Hui *et al* (20), for transmission electron microscopy examination, 16HBE cells were infected with Mtb at a MOI of 10:1 for 24 h in the absence of estradiol and cells were then washed four times with PBS to remove extracellular bacilli. Infected 16HBE cells were then treated with different concentrations of estradiol at 37°C for 24 h and were centrifuged at 410 x g at room temperature for 5 min. Cells were fixed in 1% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7.0) at 4°C for 24 h, postfixed in 2% osmium tetroxide at room temperature for 24 h, dehydrated with increasing concentrations of ethanol and gradually infiltrated with Epon resin (Ted Pella, Inc.) at room temperature for 2 h. Thin sections (60 nm) were contrasted with 90% uranyl acetate at 37°C for 30 min and 90% lead citrate at 45°C for 10 min. Finally, thin sections were observed using a transmission electron microscope JEOL JEM-1400PLUS (JEOL, Ltd.), which was equipped with DigitalMicrograph 3.9 (Gatan, Inc.) image management software.

Necrosis assays. 16HBE cells at 80-90% confluence in 24-well dishes were infected with Mtb at MOI=10:1. The infected cells were designated as Mtb cells and divided into the following five groups: i) Untreated infection control group (Mtb group); and infected cells treated with ii) 3-Methyladenine (3-MA; 10 mM; Sigma-Aldrich; Merck KGaA); iii) rapamycin (RAPA; 100 nM; Sigma-Aldrich; Merck KGaA); or estradiol at iv) E1; and v) E2. Uninfected and untreated cells were used as negative controls (PBS group). Uninfected cells treated with 3-MA, RAPA or estradiol at E1 and E2 were used as the treatment control. After 24 h, the cells were assessed for necrosis by incubation with 0.5 ml PBS and propidium iodide (PI) (Shanghai Biyuntian Biotechnology, Co., Ltd.) at 37°C for 20 min, followed by observation them under a fluorescence microscope (Olympus Corporation) according to the manufacturer's instructions. Cells were harvested at 6, 24 and 72 h to measure the level of released lactate dehydrogenase (LDH) in the medium using the LDH-Cytotoxicity Assay Kit II (cat. no. C0017; Shanghai Biyuntian Biotechnology, Co., Ltd.). The LDH reaction results were determined by reading the absorbance at 490 nm using a microplate reader (iMark; Bio-Rad Laboratories, Inc.).

Western blot analysis. Infected 16HBE cells (2.75×10^6 cells) were incubated with ER α -specific agonist [4, 4, 4, 4-(4-propyl-[1H]-pyrazole-1, 3, 5-triyl) trisphenol (PPT);

10^{-6} M] or ER β -specific agonist [diarylpropionitrile (DPN); 10^{-6} M] at 37°C in 5% CO₂ for 24 h. Infected 16HBE cells were pre-treated with ER α -specific antagonist (AZD9496; 10^{-6} M) or nonspecific ER modulator [Bazedoxifene (BAZ); 0.5 μ M] at 37°C in 5% CO₂ for 1 h and co-incubated with E2 at 37°C in 5% CO₂ for 24 h. ER agonists and antagonists were purchased from Tocris Bioscience and Sigma-Aldrich; Merck KGaA. Stock solutions of PPT, DPN, AZD9496 and BAZ were prepared using absolute ethyl alcohol and serially diluted using PBS to the aforementioned concentrations. Additionally, 3-MA (10 mM) and RAPA (100 nM) were used as negative and positive controls. After 24 h, 16HBE cells were washed with PBS and cells were lysed in RIPA lysis buffer (Cell Signaling Technology, Inc.). The protein concentration in the lysates was measured with a BCA protein kit (Beyotime Institute of Biotechnology). The protein samples were boiled for 10 min. Total protein (25 μ g total protein/lane) was separated via SDS-PAGE on 4-20% gels (Nanjing ACE Biological Technology Co., Ltd.), then transferred onto a PVDF membrane (MilliporeSigma). The membrane was blocked with protein-free rapid blocking buffer (cat. no. PS108P; Epizyme, Inc.) for 1 h at room temperature, then incubated with antibodies against β -actin (1:1,000; cat. no. ab8227; Abcam), LC3B (1:1,000; cat. no. ab192890; Abcam), beclin1 (1:1,000; cat. no. ab207612; Abcam), P62 (1:500; cat. no. ab207305; Abcam), p-AKT (1:500; cat. no. AF0016; Affinity Biosciences), AKT (1:500; cat. no. AF6263; Affinity Biosciences), p-mTOR (1:500; cat. no. AF3308; Affinity Biosciences) and mTOR (1:500; cat. no. AF6308; Affinity Biosciences) at 4°C for 12 h. After washing for 30 min using TBS with 0.1% Tween-20, the membrane was incubated for 2 h at room temperature with either goat anti-mouse, HRP-linked antibody (1:3,000; cat. no. 7076; Cell Signaling Technology, Inc.) or goat anti-rabbit biotinylated antibody (1:3,000; cat. no. 14708; Cell Signaling Technology, Inc.) secondary antibodies. The protein band was visualized using an ECL detection solution (Thermo Fisher Scientific, Inc.). The digital images of protein bands were acquired using an ImageQuant LAS 4000 system (Cytiva).

Measurement of ROS. ROS can be detected using a fluorescence microplate reader, laser scanning confocal microscope and flow cytometry. Due to the infectivity of Mtb, the biosafety level of the laboratory where the above three detection methods were located did not meet the requirements. Only the biosafety level of the laboratory where flow cytometry was located met the requirements of BCG experimental biosafety. Therefore, ROS levels were determined using flow cytometry using BCG instead of Mtb infected 16HBE. BCG-infected 16HBE cells (2.75×10^6) were incubated with PPT or DPN in the absence of estradiol at 37°C in 5% CO₂ for 24 h. Infected 16HBE cells (2.75×10^6) were pre-treated with AZD9496 or BAZ at 37°C in 5% CO₂ for 1 h and co-incubated with estradiol at 37°C in 5% CO₂ for 24 h. The cells were incubated with 2,7-dichlorodihydrofluorescein diacetate (10 μ M; Shanghai Biyuntian Biotechnology, Co., Ltd.) for 15 min at 37°C in the dark. Fluorescence intensity was measured using flow cytometer FACS Aria II (BD Biosciences) and analyzed using FlowJo software (version 6.1; Tree Star, Inc.) according to the manufacturer's protocol.

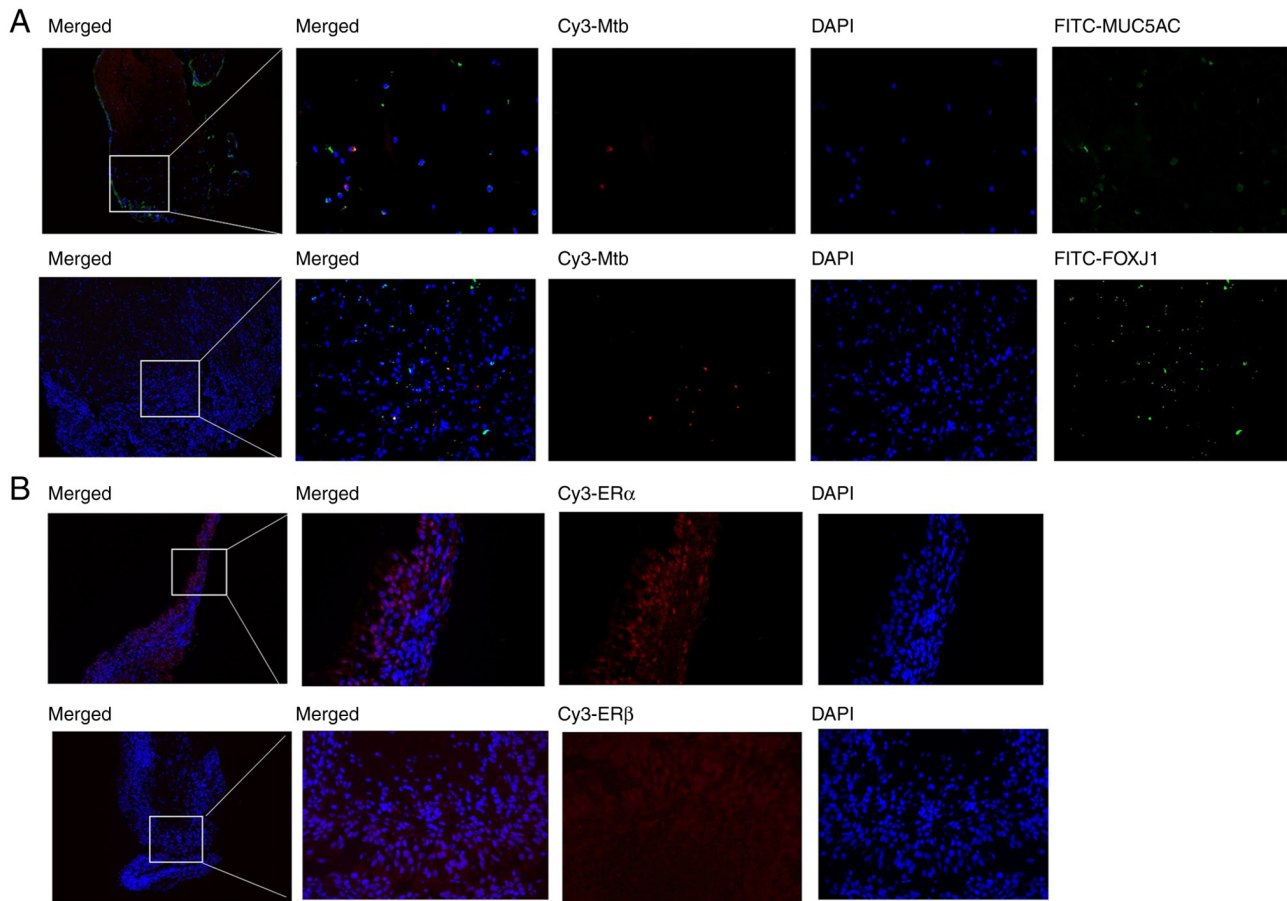


Figure 1. Mtb-infected epithelial cells and the expression of ER α and ER β in granulomatous tissues of patients with TBTB. (A) Mtb-positive epithelial cells were analyzed using double-labeled immunofluorescence. Mtb was observable in goblet cells and ciliated cells. (B) Only ER α was strongly expressed in tissue samples from patients with TBTB. MUC5AC is commonly used to identify goblet cells. FOXJ1 is a marker of ciliated cells. Magnification, x100 and x400 for the low and high-magnification panels, respectively. Mtb, *Mycobacterium tuberculosis*; TBTB, tracheobronchial tuberculosis; ER, estrogen receptor; MUC5AC, mucin 5AC; FOXJ1, forkhead box J1.

Statistical analysis. With the exception of the TEM experiment, each *in vitro* experiment was performed independently in triplicate. After the emergence of COVID-19, due to the infectivity of Mtb, TEM laboratory (Biosafety Level II Laboratory) was not allowed to conduct Mtb related tests, but we repeated the test twice with BCG instead of Mtb, the results were consistent with those of Mtb. The data are presented as the mean \pm SEM. Statistical comparisons were performed using GraphPad Prism 8 (GraphPad Software, Inc.). One-way ANOVA (followed by Dunnett's post hoc test) was used to analyze the differences in ROS levels. Two-way ANOVA (followed by Tukey's post hoc test) was used to analyze the difference in LDH release and CFU between the groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ER α is strongly expressed in granulomatous tissue of patients with TBTB. It was known from previous studies that Mtb could enter alveolar type II epithelial cells (21,22). To gain a better understanding in whether Mtb could enter human bronchial epithelial cells, a double-label IF analysis was conducted using an antibody against Mtb combined with antibodies against each of the following markers: p63 (data not

shown), SCGB1A1 (data not shown), MUC5AC and FOXJ1, commonly used to identify basal cells, secretory club cells, goblet cells and ciliated cells, respectively. The IF analysis revealed that Mtb could enter human lobar bronchial goblet cells and ciliated cells in patients with TBTB (Fig. 1A). The IF analysis also revealed that there were no Mtb infected basal cells (p63-marked) or Mtb infected secretory club cells (SCGB1A1-marked) in granulomatous tissue (data not shown). Clipping normal bronchial epithelial cells from TBTB patients is likely to spread Mtb, which was not approved by the ethics committee. Therefore, only collected granulomatous tissue was used, which is mainly Langerhans cells. Consequently the number of epithelial cells was very few and it was hard to find Mtb-infected epithelial cells. Therefore, only a small number of infected bronchial cells were observed. As aforementioned, estrogen acts mainly by binding to ER. Therefore, the present study aimed to elucidate whether ER was expressed in TBTB tissues and found that only ER α was expressed in granulomatous tissues in patients with TBTB. More specifically, ER α was expressed in lobar bronchial epithelial cells (Fig. 1B).

Estradiol decreases CFUs in 16HBE cells as the time elapses. In order to study the role of estradiol in Mtb-infected bronchial epithelial cells *in vitro*, a model that mimics the bronchial

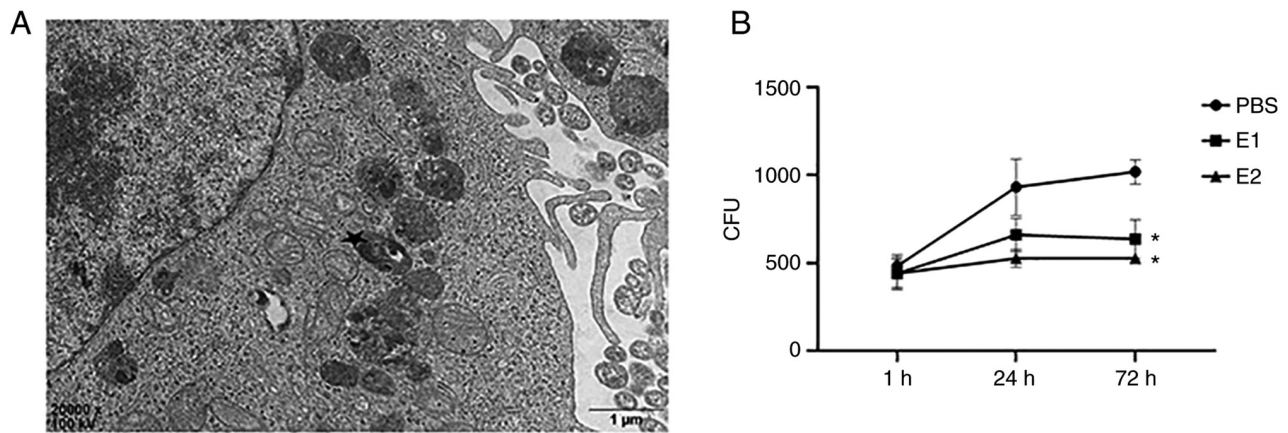


Figure 2. Estradiol decreases the number of CFUs in 16HBE cells over time. (A) Mtb (black star) could enter 16HBE cells, as confirmed by transmission electron microscopy. Magnification, x20,000. (B) The number of CFUs in all groups did not differ at day 0. 16HBE cells exposed to Mtb alone (PBS group) showed an increase in the number of CFUs from day 0 to day 3. By contrast, the difference was not significant in the number of CFUs over time in estradiol-treated 16HBE cells. Compared with PBS group, the number of CFUs in E1 and E2 groups significantly decreased at 72 h * $P < 0.05$ vs. PBS. Mtb, *Mycobacterium tuberculosis*; E1, 10^{-8} M estradiol; E2, 10^{-6} M estradiol; CFU, colony-forming unit.

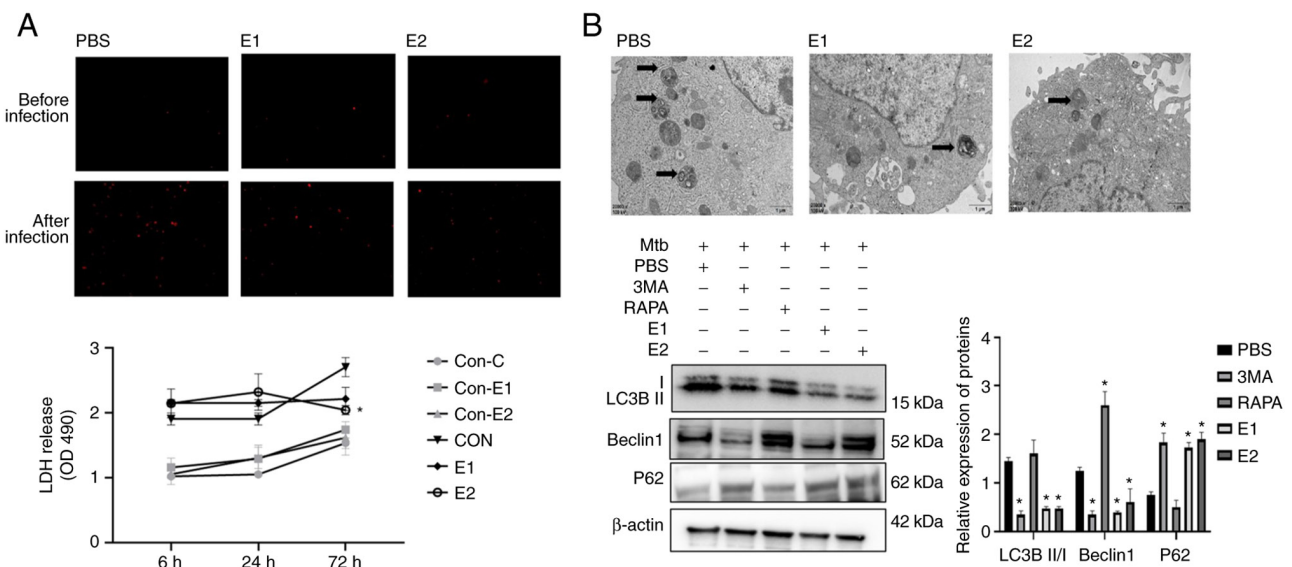


Figure 3. Estradiol inhibits the growth of intracellular Mtb and necrosis of the infected host cells by inhibiting autophagy. (A) Cells treated with estradiol and infected with Mtb released less LDH at 72 h and displayed decreased necrosis levels at 24 h compared with the untreated cells. Magnification, x100. Compared with the con-PBS group, estradiol treatment of uninfected 16HBE for 72 h did not affect LDH release. However, compared with the PBS group, estradiol treatment of infected 16HBE cells for 72 h significantly reduced LDH release, especially at 10^{-6} M. * $P < 0.05$ vs. con-PBS. (B) Compared with PBS group, Mtb-infected 16HBE cells in the E1 and E2 groups had markedly fewer intracellular autophagolysosomes and the protein expression levels of LC3B II/I and beclin1 significantly decreased, whereas the protein expression levels of P62 were significantly increased. Arrows indicate autophagic lysosomes. Magnification, x20,000. Mtb, *Mycobacterium tuberculosis*; RAPA, 100 nM rapamycin; 3-MA, 10 mM 3-methyladenine; E1, 10^{-8} M estradiol; E2, 10^{-6} M estradiol; Con, uninfected 16HBE cells; LDH, lactate dehydrogenase; OD, optical density.

epithelial cell-Mtb interaction was established by treating with estradiol the 16HBE cells infected with Mtb. Mtb was internalized into 16HBE cells, as indicated by TEM (Fig. 2A). To further confirm whether estradiol affects the intracellular Mtb growth, infected 16HBE cells were treated with estradiol at E1 and E2. The number of CFUs did not differ between the groups at day 0. 16HBE cells exposed to Mtb alone (PBS group) showed an increase in the number of CFUs from day 0 to day 3. However, the differences were not significant as the time elapsed in estradiol treated 16HBE cells from day 0 to day 3 (Fig. 2B). Compared with the PBS group, the number of CFUs in E1 and E2 groups was significantly decreased at 72 h.

Estradiol impacts the proliferation of intracellular Mtb and the survival of the infected host cells by inhibiting autophagy. Fine *et al* (18) showed that the level of infected cell necrosis and autophagy determines the proliferation of intracellular Mtb. Therefore, the effect of estradiol on necrosis and autophagy of infected cells was examined. LDH release experiments and PI staining were conducted using the same infected monolayers to determine whether bacterial viability was associated with cell survival (Fig. 3A). Cells treated with estradiol and infected with Mtb released less LDH at 72 h and exhibited decreased necrosis at 24 h compared with the untreated cells. Compared with the PBS group, Mtb-infected 16HBE cells

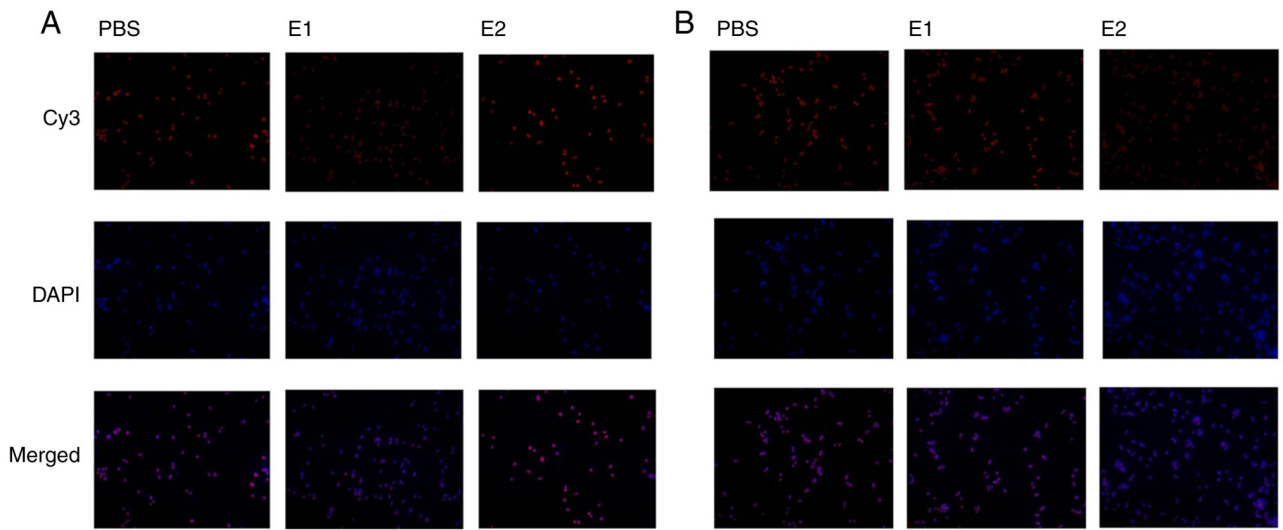


Figure 4. ER α and ER β are expressed in 16HBE cells. 16HBE cells were treated with E1 or E2 for 24 h and then ER expression was examined by immunofluorescence. 16HBE cells strongly expressed ER α and ER β . (A) Compared with the PBS group, the fluorescence intensity of ER α in the E1 group was markedly decreased, while it did not significantly change in the E2 group. (B) Compared with PBS group, the fluorescence intensity of ER β exhibited no significant change in the E1 group, while the fluorescence intensity of ER β was markedly decreased in the E2 group. Magnification, $\times 100$. E1, 10^{-6} M estradiol; E2, 10^{-8} M estradiol.

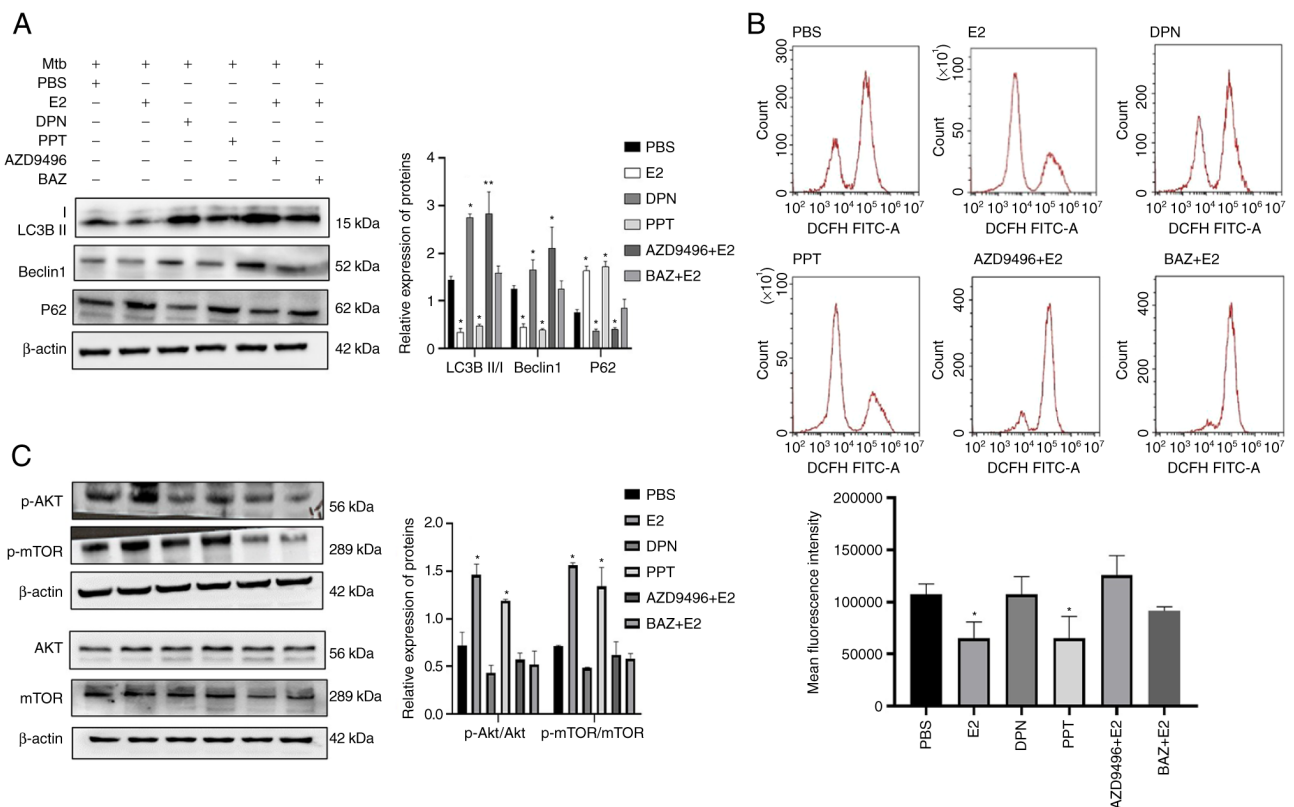


Figure 5. Inhibition of autophagy by estradiol promotes ROS production and phosphorylation of AKT/mTOR by binding to ER α . (A) E2 inhibited autophagy by binding to ER α . Infected 16HBE cells were incubated with PPT or DPN for 24 h in the absence of estradiol. Moreover, infected 16HBE cells were pre-treated with AZD9496 or BAZ for 1 h and co-incubated with estradiol for 24 h. (B) ROS levels in infected 16HBE cells treated with ER agonist or antagonist for 24 h were detected using the DCFH-diacetate probe ($10 \mu\text{M}$). Mean fluorescence intensity of ROS was analyzed by flow cytometry. (C) Protein levels of p-AKT, total AKT, p-AMPK, total AMPK, p-mTOR and total mTOR in Mtb-infected 16HBE cells were analyzed using western blotting. * $P < 0.05$ and ** $P < 0.01$ vs. PBS. E2, 10^{-6} M estradiol; PPT, 4, 4, 4, 4(4-propyl-[1H]-pyrazole-1, 3, 5-triyl) trisphenol or ER α -specific agonist (10^{-6} M); DPN, diarylpropionitrile or ER β -specific agonist (10^{-6} M); AZD9496, ER α -specific antagonist (10^{-6} M); BZA, Bazedoxifene or nonspecific ER modulator ($0.5 \mu\text{M}$); Mtb, *Mycobacterium tuberculosis*; p, phosphorylated; DCFH, 2,7-dichlorodihydrofluorescein.

in the E1 and E2 groups had markedly fewer intracellular autophagolysosomes. Furthermore, compared with the PBS

group the protein expression levels of LC3B II/I and beclin1 significantly decreased, whereas P62 protein expression were

significantly increased in the E1 and E2 groups. Autophagy of 16HBE cells infected with Mtb was inhibited following treatment with estradiol (Fig. 3B).

Estradiol-inhibited autophagy is associated with ROS production and phosphorylation of AKT/mTOR by binding to ER α . The aforementioned results showed that autophagy of Mtb-infected 16HBE cells was inhibited by estradiol. To examine whether estradiol inhibited autophagy by binding to ER α and/or ER β , further experiments were conducted to determine whether 16HBE cells expressed ER. Both ER α and ER β were strongly expressed in 16HBE cells. Compared with the PBS group, the fluorescence intensity of ER α in E1 group was markedly decreased, whereas it was significantly changed in the E2 group (Fig. 4A). Compared with the PBS group, fluorescence intensity of ER β had no significant change in the E1 group. However, the fluorescence intensity of ER β was markedly decreased in the 1 E2 group (Fig. 4B). Subsequently, Mtb-infected 16HBE cells were treated with ER agonists or antagonists. Compared with the PBS group, the protein expression levels of LC3B II/I and beclin1 significantly increased and the protein expression levels of p62 significantly decreased in the DPN group. The protein expression levels of LC3B II/I and beclin1 significantly decreased, whereas the protein expression levels of P62 significantly increased in the E2 and PPT group compared with the PBS group. Furthermore, these results showed that autophagy of Mtb-infected 16HBE cells was inhibited by PPT (a specific ER α agonist), which was similar to the effect of E2. Moreover, increased expression levels of LC3B II/I and beclin1 and decreased protein expression level of P62 by estradiol was ameliorated when Mtb-infected 16HBE cells were treated with both estradiol and AZD9496 (a special ER α antagonist) (Fig. 5A). The protein expression levels of LC3B II/I, beclin1 and p62 in the BAZ + E2 group were similar to those observed in the PBS group. ROS levels in Mtb-infected 16HBE cells treated with estradiol and PPT were similar and significantly lower than that in Mtb-infected 16HBE cells treated with PBS. When Mtb-infected 16HBE cells were treated with both E2 and AZD9496, the ROS levels were similar compared with that in the Mtb-infected 16HBE cells treated with PBS. The ROS levels in Mtb-infected 16HBE cells treated with DPN or BAZ + E2 were not significantly different compared with the PBS group (Fig. 5B).

Phosphorylation of AKT/mTOR is a key signaling step for estradiol-associated autophagy (23,24). To understand the signaling pathway involved in the estradiol-mediated inhibition of autophagy, the phosphorylation levels of mTOR and AKT were measured. Similar total protein amounts of AKT and mTOR among all groups were observed. By contrast, the levels of p-mTOR and p-AKT were notably increased in E2 and PPT-treated Mtb-infected 16HBE cells compared with that in the other groups (Fig. 5C).

Discussion

It is well known that women have stronger immunity to infection than men (25). People in all age groups can be affected by TB, although the highest burden is in adult men (1). However, previous studies have demonstrated that TBTB is most common in young and middle-aged female (2-4). Therefore,

it may be hypothesized that estrogen is involved in the pathogenesis of TBTB.

Previous studies have demonstrated that Mtb could enter alveolar and interstitial macrophages, type-II pneumocytes, endothelial cells and fibroblasts (17,18,21,22). In the present study, it was demonstrated that Mtb could enter goblet cells and ciliated cells. There have been numerous studies on the expression of ER in the lung and gonad (26,27), few studies on the airway (28,29) and none in patients with pulmonary TB and TBTB. Previous studies have demonstrated that ERs were expressed in human airway mast cells (28) and human airway smooth muscle cells (29). In the present study, it was found that only ER α was expressed in TBTB tissues and that ER β was not expressed in TBTB tissues. Therefore, it was hypothesized that estradiol, through binding to ER α , may serve a role in the pathogenesis of TBTB.

Several studies have shown that autophagy of non-phagocytic cells infected with Mtb is closely related to cell necrosis and dissemination of intracellular Mtb and autophagy protects type II alveolar epithelial cells from Mtb infection (14,17,18). Behar *et al* (30) found that Mtb infection caused different levels of death in human macrophages and alveolar epithelial cells. With respect to non-phagocytic cells, Mtb infection mainly causes necrosis in Mtb-infected cells (31). Inhibition of the autophagy pathway using 3-MA has been indicated to improve host cell viability and decreased numbers of viable intracellular Mtb (18). The present study also suggested that estradiol controlled the growth of intracellular Mtb by inhibiting the autophagy of infected cells. The regulation of autophagy in Mtb-infected macrophages is a complex process. ROS have been reported to induce autophagy (32) through inhibition of the AKT and mTOR signaling pathway (33). Jin *et al* (34) suggested that estradiol alleviates intervertebral disc degeneration through modulating the antioxidant enzymes and inhibiting autophagy in a menopausal rat model. Cook *et al* (35) showed that knockdown of ER α induces autophagy and promotes ROS-induced breast cancer cell death. Another study also found that BAZ inhibits the intracellular Mtb growth in macrophages by increasing autophagy, associated with ROS production and phosphorylation of mTOR and AKT signaling (7). A previous study on osteoarthritis suggested that estradiol can protect chondrocytes against mitophagy by activating the PI3K/AKT signaling pathway (36). Lambert *et al* (37) showed that when ER α -deficient peritoneal macrophages were exposed to *Mycobacterium avium* *in vitro*, the bacterial load decreased significantly compared with wild-type mouse macrophages. ER β -deficient peritoneal macrophages, on the other hand, did not differ from wild-type peritoneal macrophages in the bacterial load. The results of these studies are consistent with the findings of the present study in that estradiol inhibits autophagy primarily by binding to ER α rather than ER β , which resulted in reduced production of ROS and affected the AKT pathway. However, the current data have not addressed the exact signaling pathway from ROS to autophagy during estradiol treatment. BCG is known as *Mycobacterium bovis*, which is a subtype of Mtb. BCG and Mtb not only have several similar antigens, but also can induce macrophage necrosis by enhancing the accumulation of ROS (7,17,38,39). In addition, BCG could induce upregulation of ROS in A549 cells (40). Thus, the effect of estrogen and its

receptor modulator on ROS was measured using BCG-infected 16HBE cells. The number of necrotic cells among different groups was not further compared by flow cytometry and only fluorescence observation after PI staining was carried out.

In summary, the present study demonstrated that estradiol may serve a key role in the development of TB/TB through binding to ER α and affect the growth of Mtb in bronchial epithelial cells. Previous studies have shown that ER modulators (tamoxifen and BAZ) have anti-TB effects *in vitro* (7,41-43). Rey *et al* showed (44) that endocrine changes, including an increase in estradiol, in patients with TB would favor a reduction in protective cell-mediated immunity and an exacerbation of inflammation, leading to perpetuation of the lung injury. A cross-sectional study found that low serum estradiol levels are related to *Mycobacterium avium* complex lung disease (45). Exogenous estradiol supplementation was found to be beneficial in controlling lung disease in a *Mycobacterium avium* lung infection animal model (46). In another animal study, exogenous administration of 2-methoxyestradiol (an endogenous metabolite of estradiol) accelerated disease progression in the early stage of Mtb infection by inhibiting hypoxia-inducible factor 1 α . In the late stage, Mtb load was reduced by inducing apoptosis of Mtb-infected macrophages in BALB/c mice (47). Therefore, estrogen or ER modulators may be promising for the treatment of TB/TB, but further animal experiments are needed to explore the value of estrogen and its receptor modulators in the treatment of TB/TB.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SLG contributed to the conception and design of the study and manuscript preparation. YLG conducted experiments, analyzed the data and prepared the manuscript. QFH assisted in the experiments, analyzed the experimental data and drafted parts of the manuscript. AML and LG contributed to the design of the study and drafted parts of the manuscript. AML and LG confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (approval no. 20188502; Chongqing, China). Written informed

consent was obtained from all the patients whose tissues were used in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. World Health Organisation. Global tuberculosis report 2020. <https://www.who.int/publications/i/item/9789240013131>. Accessed July 3, 2021.
2. Lee JH, Park SS, Lee DH, Shin DH, Yang SC and Yoo BM: Endobronchial Tuberculosis: Clinical and bronchoscopic features in 121 cases. *Chest* 102: 990-994, 1992.
3. Jung SS, Park HS, Kim JO and Kim SY: Incidence and clinical predictors of endobronchial tuberculosis in patients with pulmonary tuberculosis. *Respirology* 20: 488-495, 2015.
4. Xue Q, Wang N, Xue X and Wang J: Endobronchial tuberculosis: An overview. *Eur J Clin Microbiol Infect Dis* 30: 1039-1044, 2011.
5. Bottasso O, Bay ML, Besedovsky H and del Rey A: The immuno-endocrine component in the pathogenesis of tuberculosis. *Scand J Immunol* 66: 166-175, 2007.
6. Fernández R, Díaz A, D'Attilio L, Bongiovanni B, Santucci N, Bertola D, Besedovsky H, Del Rey A, Bay ML and Bottasso O: An adverse immune-endocrine profile in patients with tuberculosis and type 2 diabetes. *Tuberculosis (Edinb)* 101: 95-101, 2016.
7. Ouyang Q, Zhang K, Lin D, Feng CG, Cai Y and Chen X: Bazedoxifene Suppresses Intracellular *Mycobacterium tuberculosis* growth by enhancing autophagy. *mSphere* 5: e00124-20, 2020.
8. Xiang J, Liu X, Ren J, Chen K, Wang HL, Miao YY and Qi MM: How does estrogen work on autophagy? *Autophagy* 15: 197-211, 2019.
9. Totta P, Busonero C, Leone S, Marino M and Acconcia F: Dynamin II is required for 17 β -estradiol signaling and autophagy-based ER α degradation. *Sci Rep* 6: 23727, 2016.
10. Lin CW, Chen B, Huang KL, Dai YS and Teng HL: Inhibition of autophagy by estradiol promotes locomotor recovery after spinal cord injury in rats. *Neurosci Bull* 32: 137-144, 2016.
11. Wang F, Xiao J, Shen Y, Yao F and Chen Y: Estrogen protects cardiomyocytes against lipopolysaccharide by inhibiting autophagy. *Mol Med Rep* 10: 1509-1512, 2014.
12. Invernizzi R, Lloyd CM and Molyneaux PL: Respiratory microbiome and epithelial interactions shape immunity in the lungs. *Immunology* 160: 171-182, 2020.
13. Reuschl AK, Edwards MR, Parker R, Connell DW, Hoang L, Halliday A, Jarvis H, Siddiqui N, Wright C, Bremang S, *et al*: Innate activation of human primary epithelial cells broadens the host response to *Mycobacterium tuberculosis* in the airways. *PLoS Pathog* 13: e1006577, 2017.
14. Scordo JM, Knoell DL and Torrelles JB: Alveolar epithelial cells in *Mycobacterium tuberculosis* infection: Active players or innocent bystanders? *J Innate Immun* 8: 3-14, 2016.
15. Birkness KA, Deslauriers M, Bartlett JH, White EH, King CH and Quinn FD: An in vitro tissue culture bilayer model to examine early events in *Mycobacterium tuberculosis* infection. *Infect Immun* 67: 653-658, 1999.
16. Mehta PK, King CH, White EH, Murtagh JJ Jr and Quinn FD: Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. *Infect Immun* 64: 2673-2679, 1996.
17. Guo XG, Ji TX, Xia Y and Ma YY: Autophagy protects type II alveolar epithelial cells from *Mycobacterium tuberculosis* infection. *Biochem Biophys Res Commun* 432: 308-313, 2013.
18. Fine KL, Metcalfe MG, White E, Virji M, Karls RK and Quinn FD: Involvement of the autophagy pathway in trafficking of *Mycobacterium tuberculosis* bacilli through cultured human type II epithelial cells. *Cell Microbiol* 14: 1402-1414, 2012.
19. Walker SV, Wolke M, Plum G, Weber RE, Werner G and Hamprich A: Failure of Vitek2 to reliably detect vanB-mediated vancomycin resistance in *Enterococcus faecium*. *J Antimicrob Chemother* 76: 1698-1702, 2021.

20. Hui KPY, Ching RHH, Chan SKH, Nicholls JM, Sachs N, Clevers H, Peiris JSM and Chan MCW: Tropism, replication competence, and innate immune responses of influenza virus: An analysis of human airway organoids and ex-vivo bronchus cultures. *Lancet Respir Med* 6: 846-854, 2018.
21. Hernández-Pando R, Jeyanathan M, Mengistu G, Aguilar D, Orozco H, Harboe M, Rook GA and Bjune G: Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. *Lancet* 356: 2133-2138, 2000.
22. Rodrigues TS, Alvarez ARP, Gembre AF, Forni MFPAD, de Melo BMS, Alves Filho JCF, Câmara NOS and Bonato VLD: *Mycobacterium tuberculosis*-infected alveolar epithelial cells modulate dendritic cell function through the HIF-1 α -NOS2 axis. *J Leukoc Biol* 108: 1225-1238, 2020.
23. Zhang L, Zhao Y and Guo L: 17 β -estradiol protects INS-1 insulinoma cells from mitophagy via G protein-coupled estrogen receptors and the PI3K/Akt signaling pathway. *Int J Mol Med* 41: 2839-2846, 2018.
24. Xue JF, Shi ZM, Zou J and Li XL: Inhibition of PI3K/AKT/mTOR signaling pathway promotes autophagy of articular chondrocytes and attenuates inflammatory response in rats with osteoarthritis. *Biomed Pharmacother* 89: 1252-1261, 2017.
25. Moulton VR: Sex hormones in acquired immunity and autoimmune disease. *Front Immunol* 9: 2279, 2018.
26. Carey MA, Card JW, Voltz JW, Germolec DR, Korach KS and Zeldin DC: The impact of sex and sex hormones on lung physiology and disease: Lessons from animal studies. *Am J Physiol Lung Cell Mol Physiol* 293: L272-L278, 2007.
27. Tang ZR, Zhang R, Lian ZX, Deng SL and Yu K: Estrogen-Receptor expression and function in female reproductive disease. *Cells* 8: 1123, 2019.
28. Zhao XJ, McKerr G, Dong Z, Higgins CA, Carson J, Yang ZQ and Hannigan BM: Expression of oestrogen and progesterone receptors by mast cells alone, but not lymphocytes, macrophages or other immune cells in human upper airways. *Thorax* 56: 205-211, 2001.
29. Bhallamudi S, Connell J, Pabelick CM, Prakash YS and Sathish V: Estrogen receptors differentially regulate intracellular calcium handling in human nonasthmatic and asthmatic airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 318: L112-L124, 2020.
30. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, Gan HX, Divangahi M and Remold HG: Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. *Mucosal Immunol* 4: 279-287, 2011.
31. Lam A, Prabhu R, Gross CM, Riesenberger LA, Singh V and Aggarwal S: Role of apoptosis and autophagy in tuberculosis. *Am J Physiol Lung Cell Mol Physiol* 313: L218-L229, 2017.
32. Shin DM, Jeon BY, Lee HM, Jin HS, Yuk JM, Song CH, Lee SH, Lee ZW, Cho SN, Kim JM, *et al*: *Mycobacterium tuberculosis* regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathog* 6: e1001230, 2010.
33. Bolisetty S and Jaimes EA: Mitochondria and reactive oxygen species: Physiology and pathophysiology. *Int J Mol Sci* 14: 6306-6344, 2013.
34. Jin LY, Lv ZD, Wang K, Qian L, Song XX, Li XF and Shen HX: Estradiol alleviates intervertebral disc degeneration through modulating the antioxidant enzymes and inhibiting autophagy in the model of menopause rats. *Oxid Med Cell Longev* 2018: 7890291, 2018.
35. Cook KL, Clarke PA, Parmar J, Hu R, Schwartz-Roberts JL, Abu-Asab M, Warri A, Baumann WT and Clarke R: Knockdown of estrogen receptor- α induces autophagy and inhibits antiestrogen-mediated unfolded protein response activation, promoting ROS-induced breast cancer cell death. *FASEB J* 28: 3891-3905, 2014.
36. Fan DX, Yang XH, Li YN and Guo L: 17 β -Estradiol on the expression of G-Protein coupled estrogen receptor (GPER/GPR30) mitophagy, and the PI3K/Akt signaling pathway in ATDC5 chondrocytes in vitro. *Med Sci Monit* 24: 1936-1947, 2018.
37. Lambert KC, Curran EM, Judy BM, Lubahn DB and Estes DM: Estrogen receptor-alpha deficiency promotes increased TNF-alpha secretion and bacterial killing by murine macrophages in response to microbial stimuli in vitro. *J Leukoc Biol* 75: 1166-1172, 2004.
38. Wu X, Deng G, Li M, Li Y, Ma C, Wang Y and Liu X: Wnt/ β -Catenin signaling reduces *Bacillus Calmette-Guérin*-induced macrophage necrosis through a ROS-mediated PARP/AIF-dependent pathway. *BMC Immunol* 16: 16, 2015.
39. Shah G, Zielonka J, Chen F, Zhang G, Cao Y, Kalyanaraman B and See W: H₂O₂ generation by *bacillus Calmette-Guérin* induces the cellular oxidative stress response required for *bacillus Calmette-Guérin* direct effects on urothelial carcinoma biology. *J Urol* 192: 1238-1248, 2014.
40. Méndez-Samperio P, Pérez A and Torres L: Role of reactive oxygen species (ROS) in *Mycobacterium bovis* *Bacillus Calmette-Guérin* mediated up-regulation of the human cathelicidin LL-37 in A549 cells. *Microb Pathog* 47: 252-257, 2009.
41. Chen FC, Liao YC, Huang JM, Lin CH, Chen YY, Dou HY and Hsiung CA: Pros and cons of the tuberculosis drug use approach-an empirical analysis. *PLoS One* 9: e100829, 2014.
42. Abdmouleh F, El Arbi M, Saad HB, Jellali K, Ketata E, Amara IB, Pigeon P, Hassen HB, Top S, Jaouen G, *et al*: Antimicrobial, antitumor and side effects assessment of a newly synthesized tamoxifen analog. *Curr Top Med Chem* 20: 2281-2288, 2020.
43. Jang WS, Kim S, Podder B, Jyoti MA, Nam KW, Lee BE and Song HY: Anti-*Mycobacterial* activity of tamoxifen against drug-resistant and intra-macrophage *Mycobacterium tuberculosis*. *J Microbiol Biotechnol* 25: 946-950, 2015.
44. Rey AD, Mahuad CV, Bozza VV, Bogue C, Farroni MA, Bay ML, Bottasso OA and Besedovsky HO: Endocrine and cytokine responses in humans with pulmonary tuberculosis. *Brain Behav Immun* 21: 171-179, 2017.
45. Uwamino Y, Nishimura T, Sato Y, Tamizu E, Asakura T, Uno S, Mori M, Fujiwara H, Ishii M, Kawabe H, *et al*: Low serum estradiol levels are related to *Mycobacterium avium* complex lung disease: A cross-sectional study. *BMC Infect Dis* 19: 1055, 2019.
46. Tsuyuguchi K, Suzuki K, Matsumoto H, Tanaka E, Amitani R and Kuze F: Effect of oestrogen on *Mycobacterium avium* complex pulmonary infection in mice. *Clin Exp Immunol* 123: 428-434, 2001.
47. Baay-Guzman GJ, Duran-Padilla MA, Rangel-Santiago J, Tirado-Rodriguez B, Antonio-Andres G, Barrios-Payan J, Mata-Espinosa D, Klunder-Klunder M, Vega MI, Hernandez-Pando R and Huerta-Yepez S: Dual role of hypoxia-inducible factor 1 alpha in experimental pulmonary tuberculosis: Its implication as a new therapeutic target. *Future Microbiol* 13: 785-798, 2018.



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