Establishment of a lipopolysaccharide-induced inflammation model of human fetal colon cells

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Research Article

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Abstract

AIM: To culture a human fetal colon (FHC) cell line *in vitro* and establish an FHC cell inflammation model that meets the requirements for high expression of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).

Methods: FHC cells were cultured with various concentrations of *Escherichia coli* lipopolysaccharide (LPS) in appropriate media for 0.5, 1, 2, 4, 8, 16 and 24 h to stimulate an inflammatory reaction. The viability of FHC cells was detected by a Cell Counting Kit-8 (CCK-8) assay. The transcriptional levels of IL-6 and tumor necrosis factor-α and the protein expression changes in FHC cells were detected by PCR and ELISA, respectively. Appropriate stimulation conditions were selected (i.e., LPS concentration and treatment time), based on changes in cell survival rate, and IL-6 and TNF-α expression levels.

Results: An LPS concentration higher than 100 μg/mL or a treatment time longer than 24 h resulted in morphological changes and decreased cell survival. By contrast, expression levels of IL-6 and TNF-α significantly increased within 24 h when LPS concentration lower than 100 μg/mL and peaked at 2 h, whilst maintaining cell morphology and viability in FHC cells.

Conclusion: The treatment of FHC cells with 100 μg/mL LPS within 24h was optimal. was optimal in terms of stimulating IL-6 and TNF-α expression.

Introduction

Inflammatory bowel disease (IBD) is a chronic, non-specific inflammatory state of the gastrointestinal tract. IBD mainly includes ulcerative colitis and Crohn's disease[1]. The incidence of IBD has increased rapidly in recent years[2]. It has been reported that abnormal immune responses caused by heredity and environmental factors are major risk factors for the development of IBD[3]. In patients with IBD, chronic intestinal inflammation is a major risk factor for the development of gastrointestinal cancer, including colon cancer, small intestinal adenocarcinoma, intestinal lymphoma, anal cancer, and bile duct cancer[4], hence IBD interventions can prevent the occurrence of gastrointestinal cancer[5]. At present, despite the use of various protocols such as immunomodulators, thiopurine agents, and anti-TNF monoclonal antibodies in the treatment of IBD, the therapeutic outcomes are still not satisfactory[5]. Interleukin-6 (IL-6), a member of the pro-inflammatory cytokine family, can induce the expression of a variety of proteins related to acute inflammation[6] and its increased expression is related to the pathogenesis of many diseases, such as chronic inflammatory diseases, autoimmune diseases, and tumor development[7]. Tumor necrosis factor-α (TNF-α) is a cytokine that has pleiotropic effects on multiple cell types. It has been identified as a major regulator of inflammatory responses and is thought to be involved in the pathogenesis of certain inflammatory and autoimmune diseases[8]. A large number of studies reported that IL-6[9-11] and TNF-α[12, 13] participated in the occurrence and development of IBD.

The stimulation of an inflammatory response by lipopolysaccharide (LPS) and the feeding of mice with dextran sodium sulfate (DSS) are currently common cell and animal models for IBD research. The animal DSS model is well-established, with clearly defined stimulation conditions. However, for the cell inflammation model, a wide range of different stimulation conditions have been used among studies, along with a variety of cell types, including NCM460[14, 15], HT29[16, 17], and Caco2[16] cells. Only a few studies have reported the use of human fetal colon (FHC) cells. Among them, some researchers used proinflammatory cytokines to establish an FHC cell inflammation model[18], and others used DSS[19]. Only a few studies have reported the establishment of cell inflammation models using LPS. In our study, we induced an inflammatory response in FHC cells using LPS, and cell viability was determined by the CCK-8 method. The transcriptional levels of IL-6 and TNF-α and the protein expression changes in FHC cells were detected by PCR and ELISA, respectively. Based on these findings, we selected appropriate stimulation conditions (i.e., LPS concentration and treatment time), to establish a reliable inflammation model with high expression of IL-6 and TNF-α in FHC cells.

Materials And Methods

Cell culture and preparation of LPS

FHC cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were incubated at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco #C11995500BT, Beijing, China) containing fetal bovine serum (10%; Natocor #NTC-HK009, Argentina), streptomycin (0.1 mg/mL), and penicillin (100 units/mL) (STR identification of FHCs see Supplemental Figures 1).

LPS (#L2880, Sigma-Aldrich, St. Louis, MO, USA) was suspended in DMEM to a final concentration of 1 mg/mL.

CCK-8 assay

FHC cells were digested, centrifuged, and resuspended, then inoculated into each well (5000 cells/well) of a 96-well plate. The plate was incubated for 1 h at 37 °C in 5% CO_2. Following cell adhesion, LPS was added at different concentrations for different incubation times, then 10 μL of CCK-8 (BOSTER #AR1160, Wuhan, China) solution was added into each well and incubated for 1 h (37 °C, 5% CO_2). A microtiter plate reader (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the absorbance of the plate at 450 nm, and the OD_{450} value was recorded.

Real-time PCR and primers

Total RNA was extracted from FHC cells using TRIzol (Takara #9109, Dalian, China) according to the manufacturer's protocol. Total RNA was reverse transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara #RR047A). Quantitative PCR was performed in triplicate using 2.5 to 10 ng of cDNA and TB Green® Premix Ex Taq™ II (Takara #RR820A) in a total volume of 10 μL on a CFX Connect™ Thermal Cycler (BIO-RAD, BR005222, Singapore). The relative quantities (D cycle threshold values) were

obtained by normalization to the GAPDH gene, depending on the experiment. The thermal cycle: initial denaturation at 95˚C for 30 sec, denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec for a total of 40 cycles, and then at 65˚C for 5 sec and at 95˚C for 5 sec. The following primers were used: IL-6 F primer 5'- CACTGGTCTTTTGGAGTTTGAG -3', R primer 5'- GGACTTTTGTACTCATCTGCAC -3'; TNF-α F primer 5'- TGGCGTGGAGCTGAGAGATAACC -3', R primer 5'- CGATGCGGCTGATGGTGTGG -3'.

ELISA

The cell supernatant was centrifuged at 3000 rpm at room temperature for 30 min. Then, the supernatant was transferred to a fresh tube and the IL-6 and TNF-α levels were measured using a Human IL-6 (Jiubang #QZ-20469, Fujian, China) ELISA kit and a Human TNF-α ELISA kit (Jiubang #QZ-20789) according to the manufacturer's instructions.

Statistical analysis

All data are shown as the mean ± standard error of the mean and each experiment was performed in triplicate in this study. Statistical analyses were performed using GraphPad Prism 8 software for analysis of variance with $P < 0.05$ considered statistically significant.

Results

Effects of different concentrations of LPS on the morphology and viability of FHC cells

To explore the effect of LPS concentration on the viability and morphology of FHC cells, we treated FHC cells with different concentrations of LPS for 24 h. The results showed that when the LPS concentration was less than 31.25 μg/mL, the number of viable cells and the cellular morphology were consistent with those in the control group (Fig. 1A–F). When the concentration reached 156.25 μg/mL or higher, cell viability decreased and cell morphology changed significantly, with the cells becoming rounder and smaller (Fig. 1G and H). The results of the CCK8 assay confirmed that when the concentration of LPS reached 156.25 μg/mL, cell viability was significantly decreased (Fig. 2A).

To further explore the effects of LPS concentration and stimulation time on FHC cells, we cultured cells under different conditions, i.e., different LPS concentrations and different stimulation times. The results of the CCK-8 assay revealed that the viability of FHC cells was significantly decreased with longer incubation times of 36 and 48 h and viability was further decreased with increased LPS concentration (Fig. 2D and E). Higher levels of viability were detected when FHC cells were cultured for 12 and 24 h with an LPS concentration of 100 μg/mL or less, when the concentration of LPS exceeded 100 μg/mL, the viability of FHC cells was significantly decreased (Fig. 2B and C). This suggested that LPS concentrations greater than 100 μg/mL and incubation times greater than 24 h decrease the viability of FHC cells, which was consistent with the results in Figure 1.

Effects of LPS on the mRNA expression of IL-6 and TNF-α

We next investigated the transcriptional effects of different LPS concentrations on FHC cell-associated inflammatory factors over a range of treatment times that did not affect the cell state. We cultured FHC cells with different concentrations of LPS for different times. PCR analysis revealed that the mRNA expression levels of IL-6 and TNF-α increased with the increase in LPS concentration over 24 h of culture, this increase was not significant at LPS concentrations of 16 μg/mL or lower, but when the LPS concentration was 64 μg/mL or higher, the mRNA expression levels of IL-6 and TNF-α were significantly increased (Fig. 3A–G, Fig. 4A–G). As we previously found that the viability of FHC cells begins to decrease significantly at LPS concentrations above 100 μg/mL, we added 100 μg/mL LPS alone and incubated for different times. The results revealed that at an LPS concentration of 100 μg/mL, the mRNA expression levels of IL-6 and TNF-α significantly increased (>3-4 times, respectively) within 24 h, with the expression levels peaking at 1 h after LPS stimulation (Fig. 3H, Fig. 4H).

Effect of LPS on the expression of IL-6 and TNF-α

We also determined the expression levels of IL-6 and TNF-α in the supernatants of FHC cells, cultured under the conditions mentioned above, by ELISA. The results of the ELISA suggested similar findings (Fig. 3I–O, Fig. 4I–O). Interestingly, when the concentration of LPS was 100 μg/ml, expression levels of IL-6 and TNF-α significantly increased within 24 h and peaked at 2 h (>2-4 times, Fig. 3P, Fig. 4P).

Discussion

LPS is a key cytotoxic factor causing inflammation[20], and it can be applied to induce and establish a variety of injury models[21, 22]. To date, few studies have reported the use of LPS to stimulate inflammation in FHC cells. Furthermore, the LPS concentration used in previous studies was much lower than the concentration recommended in the current study[23-26]. A low LPS concentration may result in insignificant changes in the expression levels of inflammatory factors related to the inflammatory model, thus leading to inconclusive data. This may explain the poor application of this type of inflammatory model. Other common cell types used in inflammation models to date include a range of cancer cells, such as HT29 and Caco2. The only human normal colon cells that have been used for modeling are NCM460 and FHC cells. Of these two cell types, only FHC cells are available in the ATCC, so FHC cells were used to establish the cell inflammation model.

In this study, we first determined the optimal LPS concentration and incubation time for good cell growth without significant cell damage. We found that at an LPS concentration of 100 μg/mL or less and a culture time of no more than 24 h, cell morphology was maintained and the number of viable cells remained high (i.e., inhibition rate < 10%). It was preliminarily determined that an LPS concentration of no more than 100 μg/mL was safe for modeling.

Inflammatory factors IL-6 and TNF-α were selected as markers to measure the success of the inflammatory model. Our results showed that when the concentration of LPS was 100 μg/mL and the treatment time was within 24 h, the mRNA and protein expression levels of IL-6 and TNF-α were

significantly higher than those of the control group. However, the difference in mRNA expression was more pronounced than the corresponding difference in protein expression, which we propose may be related to mutual regulation of these inflammatory factors at the protein level. We also found that the peak of IL-6 and TNF-α expression in LPS-stimulated FHC cells was delayed by 1 h at the protein level relative to the mRNA level, which we propose may be related to the time required for protein translation and post-transcriptional regulation.

As a cellular model of inflammation, here, we only assessed changes in inflammatory factors IL-6 and TNF-α, but clearly other inflammatory factors such as IL-17, IL-10, and IL-1 also play a role. However, as classical markers of cellular inflammation, significant increases in IL-6 and TNF-α are sufficient evidence for the successful establishment of a cellular inflammation model.

In conclusion, we have successfully established a cell inflammation model by inducing FHC cells with LPS and determined that stimulation of FHC cells with an LPS concentration of 100 μg/mL within 2 h could establish a reliable inflammation model in FHC cells of high expression of IL-6 and TNF-α, also, the expression levels of IL-6 and TNF-α peak at 2h, which is of great reference value for the experiment of acute inflammation requiring high expression of IL-6 and TNF-α.

Declarations

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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

LL and ZM designed the study and performed the experiments, KY and YS collected the data, KY and SL analyzed the data, KY and CL prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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Figures

Figure 1

Viability of FHC cells cultured with different concentrations of LPS for 24 h

(A–H) The images observed under the microscope after FHC cells were cultured with LPS at concentrations of 0, 0.05, 0.25, 1.25, 6.25, 31.25, 156.25, and 781.25 μg/mL for 24 h. Magnification, 1:40.

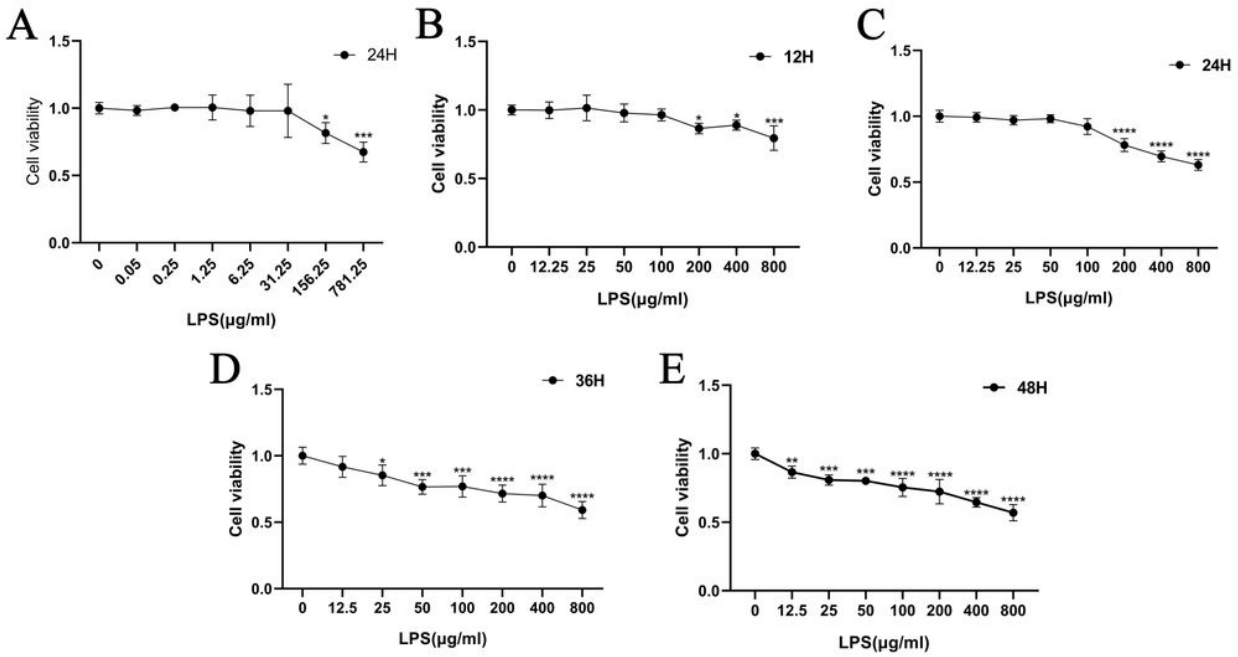

Figure 2

Detection of viability using a CCK8 assay after FHC cells were cultured with different concentrations of LPS for different times

(A) The CCK-8 assay results after FHC cells were cultured with LPS at concentrations of 0, 0.05, 0.25, 1.25, 6.25, 31.25, 156.25, and 781.25 μg/mL for 24 h. (B-E) The CCK-8 assay results after FHC cells were cultured with LPS at concentrations of 0, 12.25, 25, 50, 100, 200, 400, and 800 μg/mL for 24 h. *P<0.05, **P<0.01, ***P<0.001, ****P <0.0001.

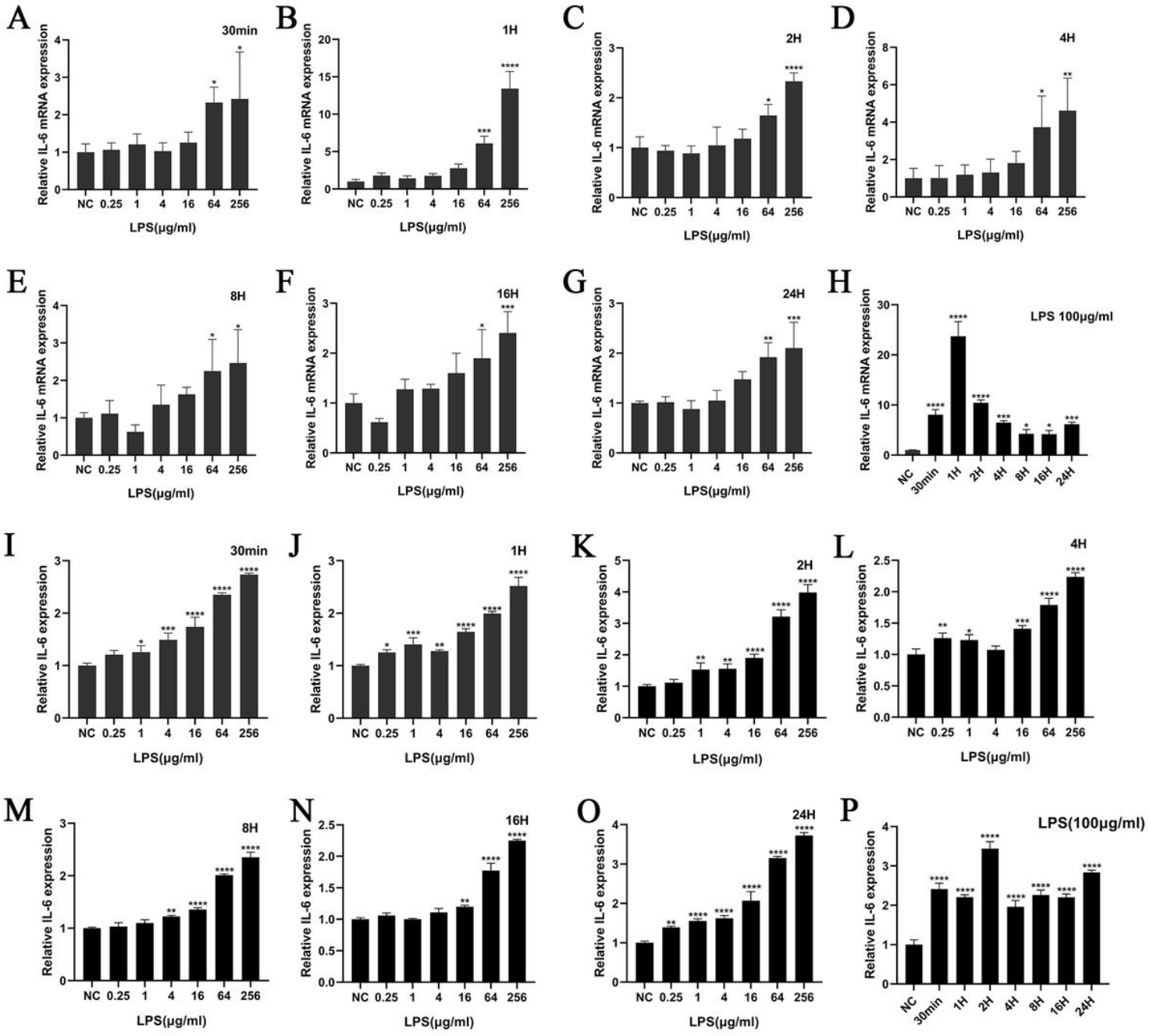

Figure 3

Expression of IL-6 in FHC cells cultured with different concentrations of LPS for different time durations

FHCs were incubated at LPS concentrations of 0, 0.25, 1, 4, 16, 64, 100, and 256 μg/mL for 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h, respectively, as shown in the figure. (A–H) Expression level of IL-6 was determined by RT-qPCR. (I–P) Expression level of IL-6 was determined by ELISA. *P < 0.05, **P < 0.01 *** means P<0.001 ****P < 0.0001.

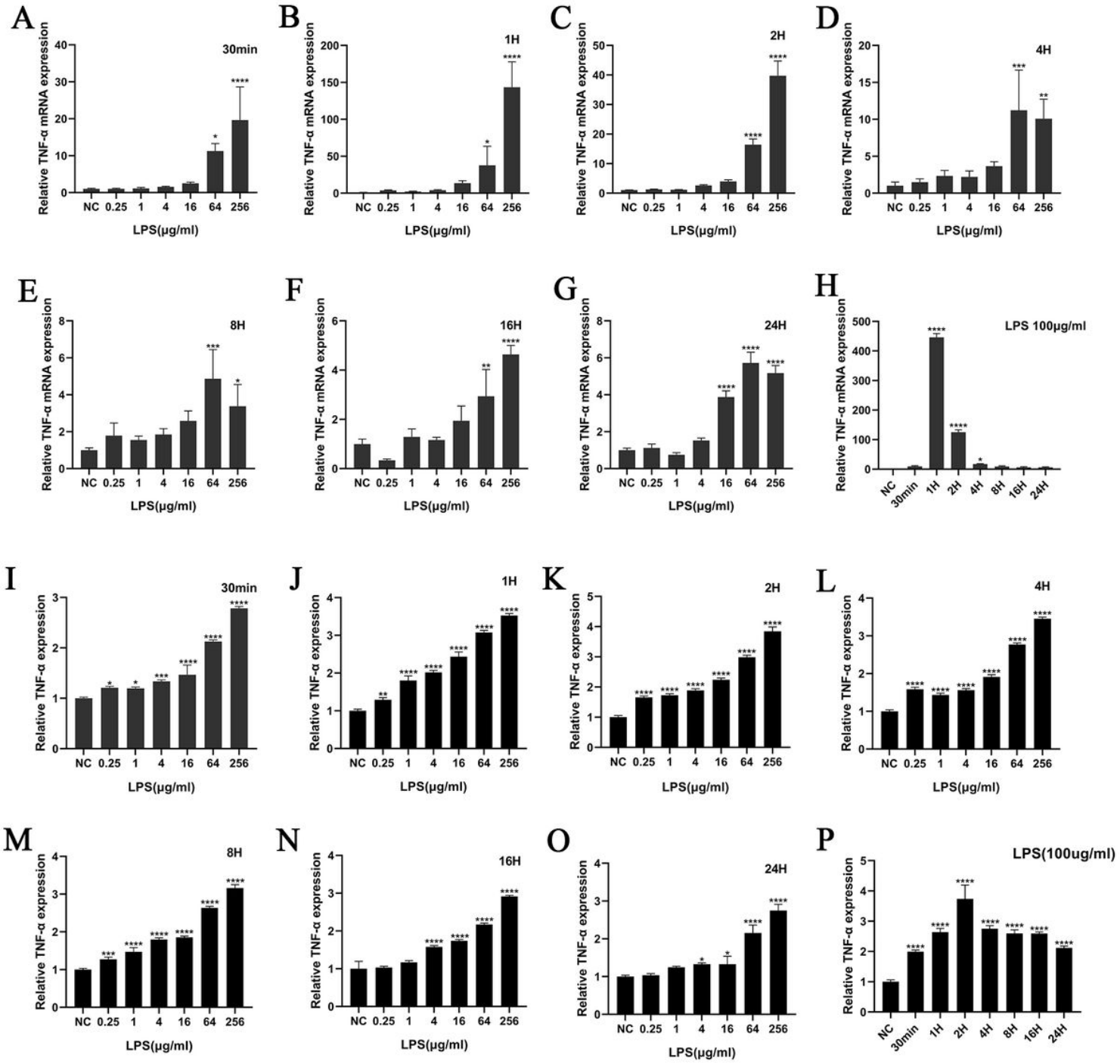

Figure 4

Expression of TNF-α in FHC cells cultured with different concentrations of LPS for different time

FHCs were incubated at LPS concentrations of 0, 0.25, 1, 4, 16, 64, 100, and 256 μg/mL for 30 min and 1, 2, 4, 8, 16, and 24 h, respectively, as shown in the figure. (A–H) Expression level of TNF-α was determined by RT-qPCR. (I–P) Expression level of TNF-α was determined by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Supplementary Files

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- SupplementaryFigure1.jpg