Nicotine promotes chronic obstructive pulmonary disease via inducing pyroptosis activation in bronchial epithelial cells

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Abstract. Nicotine is one of the primary components in cigarettes, which is responsible for addiction. Numerous studies have investigated the effects of nicotine on pulmonary disease. The health of epithelial cells is important in the development of chronic obstructive pulmonary disease (COPD). Accumulating evidence has suggested that epithelial cell death may initiate or contribute to the progression of a number of lung diseases via airway remodeling. Pyroptosis is a unique form of inflammatory cell death mediated by the activation of caspase-1 and the NOD-like receptor protein-3 (NLRP3) inflammasome. The present study aimed to evaluate whether pyroptosis of epithelial cells was involved in the progression of COPD. The normal human bronchial epithelial cell line 16HBE was treated with 0.1 or 1 μM nicotine. Then the proliferation ability of 16HBE cells was detected by CCK-8. Cell death was detected by flow cytometry analysis and TUNEL assay. Subsequently, the levels of pro-caspase 1, caspase 1, IL-1β, IL-18, NLRP3, ASC and cleaved GSDMD were examined by western blotting. It was revealed that nicotine treatment significantly induced cell death and suppressed proliferation of 16HBE cells. Furthermore, nicotine exposure increased the expression levels of caspase-1, IL-1β, IL-18, NLRP3, apoptosis-associated speck-like protein and gasdermin D in 16HBE cells. Therefore, the present study concluded that nicotine treatment induced pyroptosis in 16HBE cells, which may be associated with the progression of COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is responsible for >1,400,000 annual mortalities worldwide, and is characterized by persistent airflow limitation and limited therapeutic options (1). There are currently 64 million patients with COPD and 3 million cases of COPD-associated mortality every year estimated by the WHO. COPD is predicted to be the third leading cause of mortality in the world by 2030 (2). Annually, COPD treatment costs ≤€141.4 billion in Europe and ~$50 billion in America (3,4). Emphysema is the major pathological diagnostic factor associated with COPD, which affects the distal space of the terminal bronchioles, and is associated with an irregular inflammatory response and oxidative imbalance of the lungs to toxic particles or gases (1,5). COPD mainly manifests by incomplete reversible airflow limitation, which significantly decreases the quality of life and exercise endurance of patients (6). Therefore, it is important to determine the underlying mechanism and identify effective preventative measures of COPD to further improve the survival rate of patients with COPD.

Overwhelming evidence has suggested that cigarette smoking is the leading cause of COPD worldwide (5). Between 80 and 90% of patients with COPD are smokers, and 10-15% of smokers will develop COPD (5). It has been shown that there are >4,500 chemical compounds in the gas mixture generated by cigarette smoking, most of which are toxic, and 72 compounds are carcinogenic substances (7,8). Nicotine, the principal addictive component in cigarette smoking, is considered to be the main factor driving the pathogenesis and progression of pulmonary disease (9,10). Substantial evidence has supported the promoting effect of nicotine on COPD on a long-term basis (11) and smoking cessation is the top priority in the treatment of COPD (12); however, the underlying mechanisms remain largely unknown.

Inflammation and cell death are the two critical pathological mechanisms of COPD (13,14). Enhanced cell death can be observed during the destruction of lung tissue in both humans and mice (14,15). Previously, cell death was typically ascribed to apoptosis and necrosis; however, several other forms of cell death have been identified recently, such as pyroptosis (16).

Pyroptosis is a unique form of inflammatory cell death, which is mediated by the activation of caspase-1 and the NOD-like receptor protein-3 (NLRP3) inflammasome (17). The NLRP3 inflammasome is a multiprotein intracellular innate immune sensor mainly composed of NLRP3, apoptosis-associated speck-like protein (ASC) and pro-caspase-1 (18). Caspase-1 activation is responsible for the maturation of pro-IL-18 and pro-IL-1β (19), whereas NLRP3 activation induces the cleavage of gasdermin D (GSDMD), which rapidly creates cell membrane rupture and leads to intracellular protein release, resulting in an inflammatory reaction (20). Non-infectious and infectious stimuli can both trigger pyroptosis (21). It has been reported that cigarette smoke can induce the expression of caspase-1 and its downstream target molecules, IL-1β and IL-18, in the bronchoalveolar lavage, serum and sputum of patients with COPD (22,23). Furthermore, inflammasome activators have been shown to be increased in the airway of patients with COPD, including extracellular ATP, ROS and damage-associated molecular patterns (24). These previous findings indicate a critical role for pyroptosis in COPD progression.

Bronchial epithelial cells are the first anatomical barrier exposed to noxious gases and particles of cigarette smoke, which can initiate airway remodeling in COPD (25). Apoptosis of epithelial cells serves a crucial role in the pathogenesis of COPD via airway remodeling (26,27). Numerous factors associated with COPD, including cigarette smoke, have the potential to cause apoptosis of epithelial cells (28,29). Nevertheless, to the best of our knowledge, it remains to be fully elucidated as to whether pyroptosis is involved in epithelial cell death upon nicotine exposure.

The present study aimed to investigate whether epithelial cells undergo pyroptosis in COPD progression. These findings may improve understanding of the underlying mechanisms of COPD and provide valuable information for the diagnosis and treatment of COPD.

Materials and methods

Cell culture. The normal human bronchial epithelial cell line 16HBE is a common cell line used to study COPD pathogenesis *in vitro* (30). 16HBE cells (American Type Culture Collection) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were grown in 75-cm^2 flasks at 37˚C in a humidified atmosphere containing 5% CO_2 and were passaged 1:3 using 0.25% trypsin when they reached 80-90% confluence. Nicotine was purchased from Nacalai Tesque, Inc. 16HBE cells were treated with 0.1 or 1 μM nicotine for 48 h at 37˚C, as previously described (31).

Cell proliferation assay. The proliferation of 16HBE cells was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Inc.). 16HBE cells were seeded in 96-well plates ($1.0x10^4$ cells/well), and were exposed to 0, 0.1 or 1 μM nicotine for 0, 48, 72 and 96 h at 37˚C (31). Finally, 10 μl CCK-8 solution was added to 100 μl RPMI-1640 medium in each well for 2 h. Absorbance values were measured at a wavelength of 450 nm using an automated microplate reader (Molecular Devices, LLC).

Flow cytometry. The death of 16HBE cells ($1x10^6$/100 μl) was analyzed using an Annexin V-FITC and PI staining kit (cat. no. A211-01/02; Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences) and the rate of cell death (% of PI^+ cells) was analyzed with FlowJo software (FlowJo, LLC, version 10.6.0).

Reverse transcription-quantitative PCR (qPCR). Total RNA was isolated from each group of 16HBE cells using TRIzol® (Thermo Fisher Scientific, Inc.; cat. no. 15596026). The concentration of RNA was determined using an ND-2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA by M-MLV Reverse Transcriptase kit (Elk Wuhan Biotechnology Co., Ltd. cat. no. EQ002) with the following temperature protocol: 42˚C for 50 min for the reverse transcription reaction; 99˚C for 5 min to inactivate the reverse transcriptase; and 4˚C to store the reverse transcription product. qPCR was performed with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems; Roche Diagnostics) using a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Data were normalized to the housekeeping gene GAPDH. The mRNA levels were measured using the $2^{-\Delta\Delta Cq}$ method of quantification (32). All qPCR experiments were replicated at least three independent times. The thermocycling conditions used for qPCR were: Initial denaturation at 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The following primer sequences were used: GAPDH, forward 5'-TGACTTCAACAGCGACACCCA-3', reverse 5'-CACCCTGTTGCTGTAGCCAAA-3'; pro-caspase-1, forward 5'-ACAAGACCTCTGACAGCACG-3', reverse 5'-TTCACTTCCTGCCCACAGAC-3'; IL-18, forward 5'-GCTGAAGATGATGAAAACCTGGA-3', reverse 5'-GAGGCCGATTTCCTTGGTCA-3'; and IL-1β, forward 5'-CCAGCTACGAATCTCCGACC-3', reverse 5'-TATCCTGTCCCTGGAGGTGG-3'.

Western blotting. Proteins were extracted from treated 16HBE cells by RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B) and were quantified using the BCA method. Proteins (30 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and were electrophoretically transferred to polyvinylidene fluoride membranes, which were blocked with 5% skimmed milk for 1 h at room temperature. The following primary antibodies were used overnight at 4˚C: Rabbit anti-pro-caspase-1 (1:1,000 dilution, cat. no. ab179515; Abcam), rabbit anti-caspase-1 (1:500 dilution, cat. no. 22915-1-AP; Proteintech Group Inc.), rabbit anti-IL-1β (1:500 dilution, cat. no. A1112; ABclonal Biotech Co., Ltd.), rabbit anti-IL-18 (1:500 dilution, cat. no. A1115; ABclonal Biotech Co., Ltd.), rabbit anti-NLRP3 (1:1,000 dilution, cat. no. ab263899; Abcam), rabbit anti-ASC (1:1,000 dilution, cat. no. ab283684; Abcam), rabbit anti-cleaved N-terminal GSDMD (1:1,000 dilution, cat. no. ab215203; Abcam) and mouse anti-GAPDH (1:500 dilution, cat. no. 60004-1-lg; Proteintech Group, Inc.). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:5,000 dilution, cat. no. BA1054/BA1051; Boster Biological Technology) was used as a secondary antibody for 1 h at room temperature. Immunoreactive protein bands

were detected by ECL hypersensitive chemiluminescence kit (cat. no. P0018M; Beyotime Institute of Biotechnology) with an Odyssey Scanning System (version 3.0, LI-COR Biosciences).

Immunofluorescence analysis. Immunofluorescence staining of 16HBE cells was performed as previously described (33). 16HBE cells ($5x10^5$) plated onto cover glasses were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature. Rabbit anti-caspase-1 (1:50 dilution; cat. no. 22915-1-AP, Proteintech Group Inc.) was added to the fixed 16HBE cells and incubated at 4˚C overnight, followed by incubation with Goat anti-Rabbit IgG F(ab')2 secondary antibodies (dilution, 1:1,000; cat. no. 31234; Invitrogen) for 2 h at room temperature. 4',6-diamidino-2-phenylindole (DAPI, dilution, 1:1,000; cat. no. 62248; Thermo Fisher Scientific, Inc.) was used to stain nuclei at room temperature for 10 min. The images were acquired using a FV3000 confocal fluorescence microscope (Olympus Corporation).

TUNEL assay. 16HBE cells ($4x10^4$ cells/cm^2) were seeded onto cover glasses. After 24 h, the cells were treated with 0.1 or 1 μM nicotine for another 48 h followed by fixing with 4% (w/v) paraformaldehyde for 15 min at room temperature. Then the cells were subjected to a TUNEL assay (DeadEnd Fluorometric TUNEL System; Promega Corporation) according to the manufacturer's instructions. Briefly, cells were incubated with 250 μl of TUNEL labeling solution in one well of a 24-well plate for 1 h at 37˚C covered with aluminum foil. Then cells were incubated with PBS containing DAPI nucleic acid stain (dilution, 1:1,000; 1 mg/ml solution, cat. no. 62248; Thermo Fisher Scientific, Inc.) for 10 min at room temperature to stain nuclei. TUNEL positive (TUNEL+) cells were calculated by counting the number of stained cells in five separate field per slides using a Leica confocal microscope TCS SP2 (Leica Microsystems GmbH).

Statistical analysis. All experiments were repeated at least three times *in vitro* and all data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc.). Data are presented as the mean ± SD. Differences were analyzed for significance by one-way ANOVA followed by Duncan's post hoc test. $P<0.05$ was used to indicate a statistically significant difference.

Results

Nicotine exposure triggers 16HBE cell death. The present study used nicotine-treated 16HBE cells as an *in vitro* model of COPD. To explore the potential role of the primary component of cigarette smoke, nicotine, in the progression of COPD, nicotine was used to treat 16HBE cells. In 16HBE cells incubated with 0.1 or 1 μM nicotine, the proliferation rate significantly decreased compared with the control group (Fig. 1). Conversely, the cell death rate was significantly increased by nicotine (Fig. 2). In order to further characterize nicotine-induced 16HBE cell death, the cells were stained with TUNEL. The results revealed that the number of TUNEL-positive cells was markedly increased in nicotine-treated groups compared with the control group (Fig. 3).

Epithelial cells display characteristic features of pyroptosis after nicotine exposure. To elucidate the relationship between nicotine and pyroptosis, 16HBE cells were used for *in vitro* experiments. The results revealed that 0.1 and 1 μM nicotine treatment significantly induced the mRNA expression levels of pro-caspase-1, IL-1β and IL-18 compared with the control group (Fig. 4A). Furthermore, nicotine promoted the protein expression level of caspase-1, IL-1β and IL-18 in 16HBE cells; however, it had no obvious effect on pro-caspase-1 (Fig. 4B). In addition, 16HBE cells exhibited significant increases in the protein expression levels of NLRP3 and ASC after nicotine administration compared with the control group, as determined by western blotting (Fig. 5A). The NLRP3, ASC and cleaved form of GSDMD was also significantly increased by nicotine in 16HBE cells (Fig. 5A), which is indicative of pyroptosis (34). Furthermore, the increased expression of caspase-1 was also observed in nicotine-treated 16HBE cells by immunofluorescence analysis (Fig. 5B). Taken together, these results suggested that pyroptosis was induced in 16HBE cells upon nicotine administration.

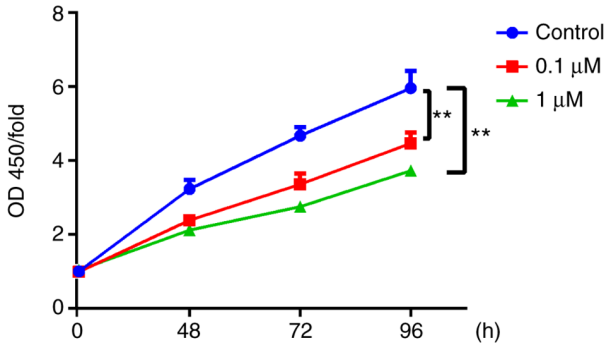

Figure 1. Nicotine suppresses the proliferation of 16HBE cells. 16HBE cells were seeded in 96-well flat-bottom plates at $2x10^3$ cells/well and treated with 0.1 or 1 μM nicotine for 0, 48, 72 and 96 h. Cell proliferation of nicotine-treated 16HBE cells was examined by Cell Counting Kit-8 assay. Data are presented as the mean ± SD (n=3). $^{**}P<0.01$ vs. 0 h. OD, optical density.

Discussion

COPD is a multifactorial disease associated with numerous mechanisms (35,36). The pathogenesis of COPD involves several pathophysiological processes, such as inflammatory response, oxidative stress, apoptosis, protease/anti-proteinase imbalance, production of autoantibodies, changes in cell proliferation and aging (37). The inflammatory response and cell death are two of the most important pathogenic factors associated with COPD (38). As a novel form of programmed cell death, pyroptosis is different from apoptosis, which is classically considered a non-inflammatory form of apoptosis (17,39). Molecularly, apoptosis is executed by activation of the executioner caspases, caspase-3 and caspase-7, downstream of the initiator caspases, caspase-8, caspase-9 and caspase-10 (40). However, whether pyroptosis is associated with COPD pathogenesis remains to be determined.

Cigarette smoke can cause inflammation, which is an important injury factor in lung diseases such as COPD (41). The damage to the lungs caused by cigarette smoke is most directly reflected in the injury of bronchial epithelial cells and alveolar epithelial cells (42). Bronchial epithelial cells are one of the key cell types that mediate inflammation and the 16HBE cell line is a common cell line used to study COPD

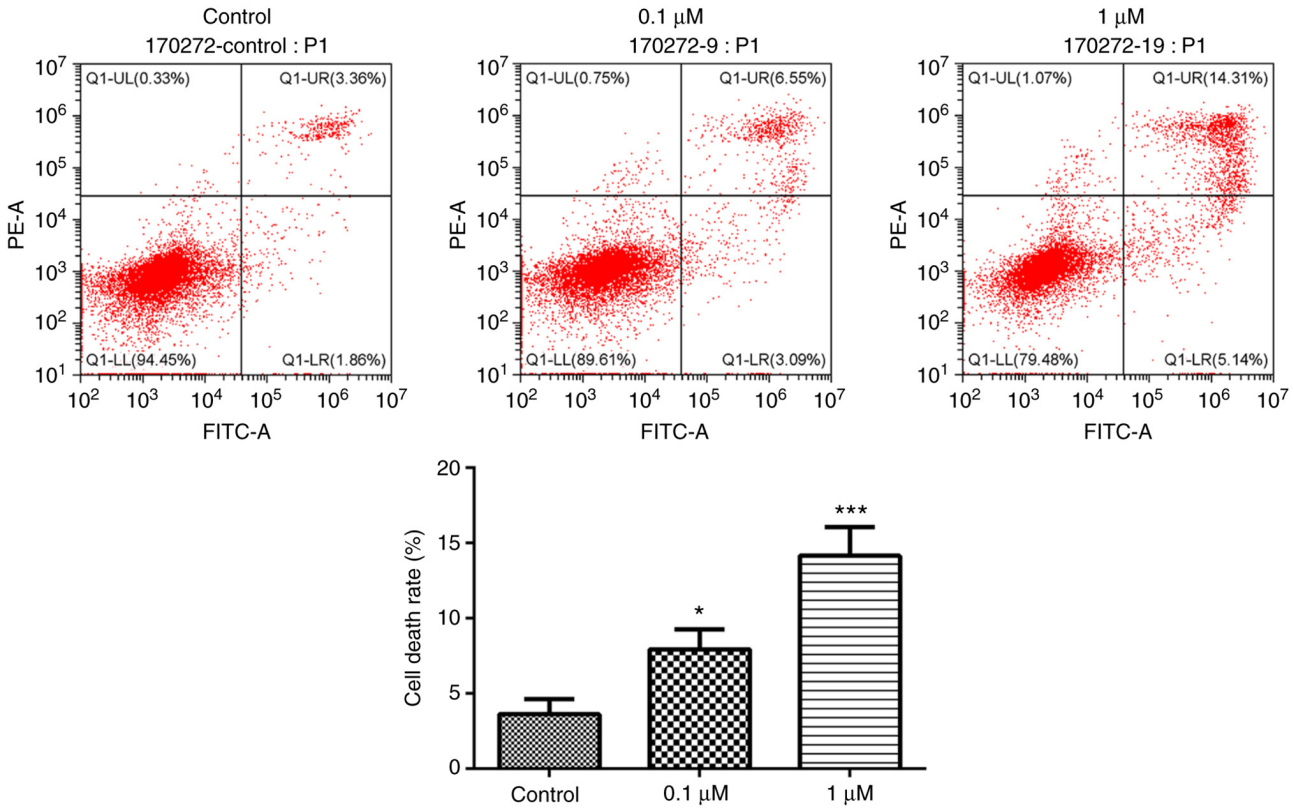

Figure 2. Nicotine induces 16HBE cell death. 16HBE cells were treated with 0.1 or 1 μM nicotine for 48 h. Nicotine-treated 16HBE cell death was evaluated by flow cytometry measuring Annexin V and PI. The number in each quadrant represents the percentage of cells in that compartment. Data are presented as the mean ± SD (n=3). *P<0.05, ***P<0.001 vs. Control.

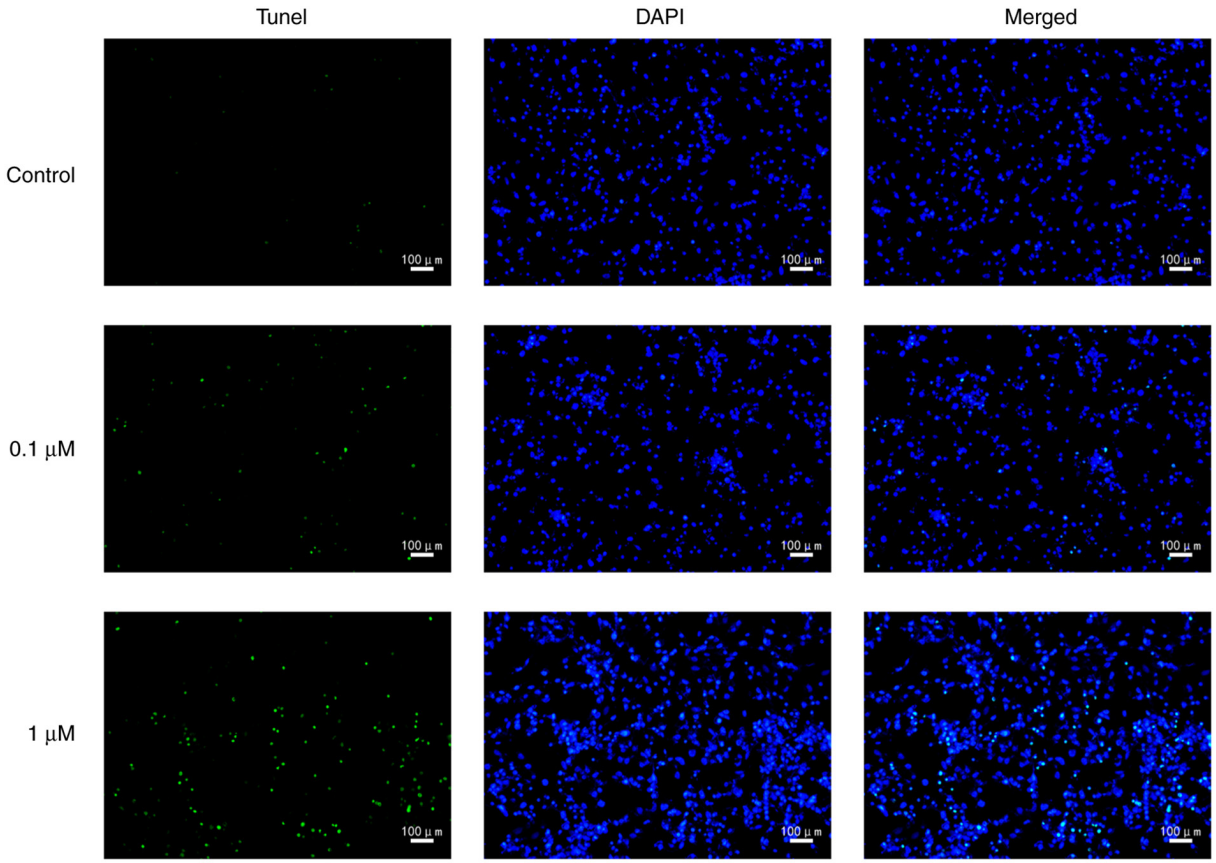

Figure 3. Nicotine induces 16HBE cell death. 16HBE cells were treated with 0.1 or 1 μM nicotine for 48 h. Nicotine-treated 16HBE cell death was examined by TUNEL staining (n=3).

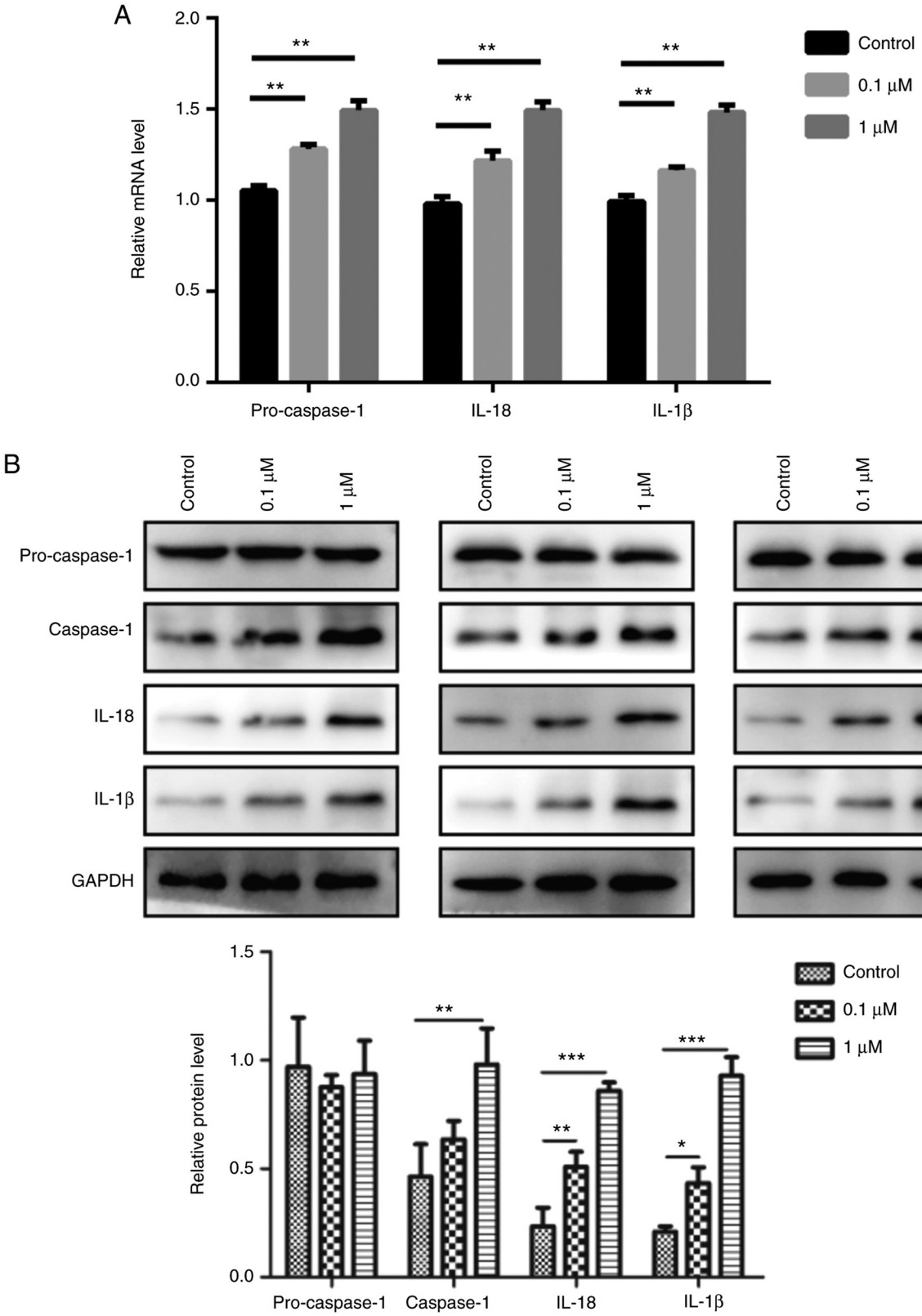

Figure 4. Epithelial cells display characteristic features of pyroptosis after nicotine exposure. 16HBE cells were treated with 0.1 or 1 μM nicotine for 48 h. (A) mRNA expression levels of caspase-1, IL-1β and IL-18 in nicotine-treated 16HBE cells were measured by reverse transcription-quantitative PCR. (B) Protein expression levels of pro-caspase-1, caspase-1, IL-1β and IL-18 in nicotine-treated 16HBE cells were measured by western blotting. GAPDH was used as a loading control. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Control.

pathogenesis *in vitro*. When exposed to harmful substances, such as cigarette smoke, airway epithelial cells are induced to produce various inflammatory cytokines (e.g., IL-18) leading to the recruitment of inflammatory cells (e.g., neutrophils) (43). *In vitro* and *in vivo* studies have demonstrated that smoking causes inflammation and cell death in lung tissue and

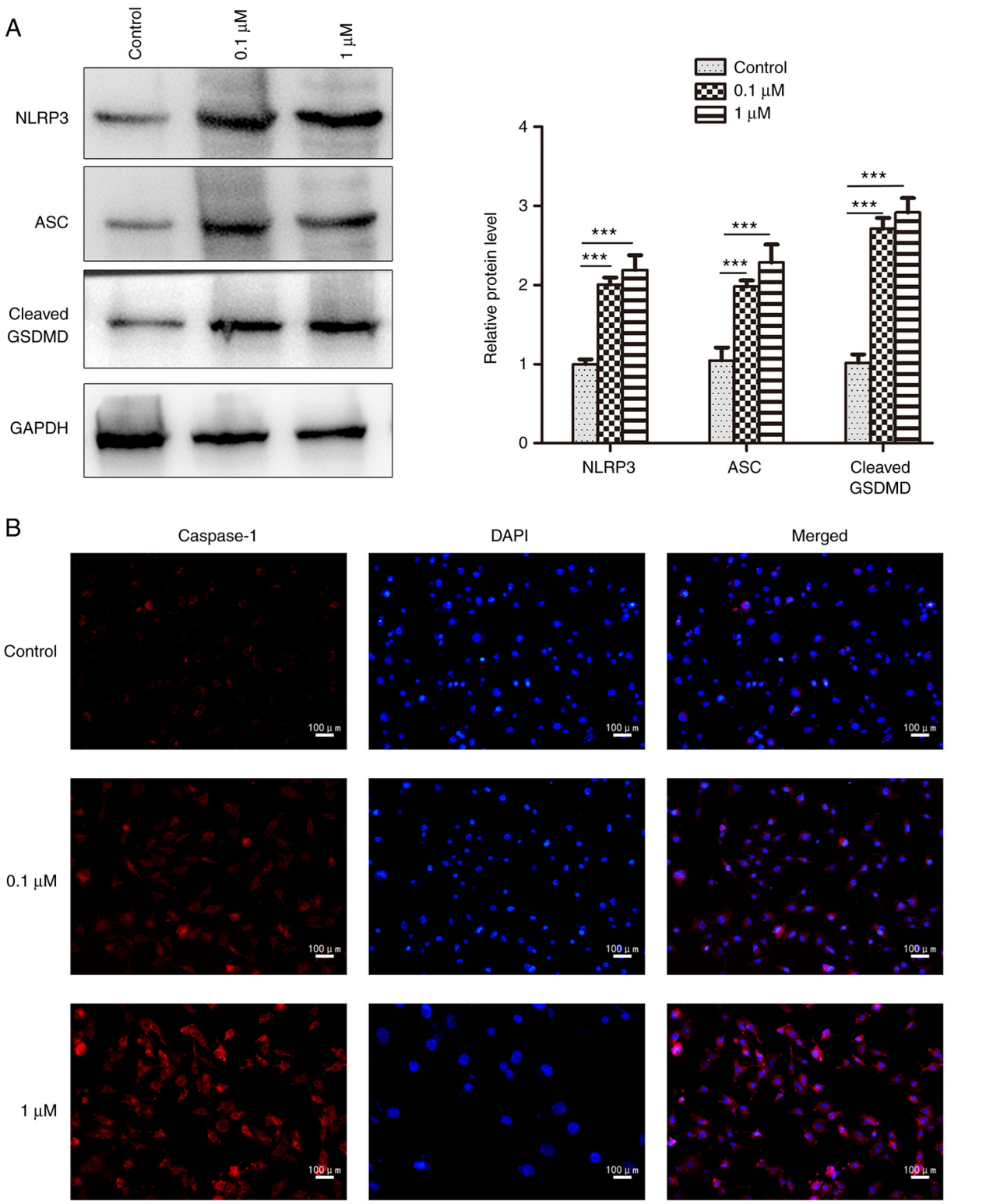

Figure 5. Nicotine increases the expression levels of NLRP3, ASC, GSDMD and caspase-1 in 16HBE cells. 16HBE cells were treated with 0.1 or 1 μM nicotine for 48 h. (A) Protein expression levels of NLRP3, ASC and GSDMD were examined by western blotting. Data are presented as the mean ± SD (n=3). ***P<0.001 vs. Control. (B) Caspase-1 expression was detected by immunofluorescence analysis (n=3). ASC, apoptosis-associated speck-like protein; NLRP3, NOD-like receptor protein 3; GSDMD, gasdermin D.

airway cells (38,41). The present study revealed that treatment with 0.1 and 1 μM nicotine suppressed the proliferation and promoted the death of 16HBE cells, as determined by CCK-8 assay, flow cytometry and immunofluorescence analysis. Furthermore, 0.1 and 1 μM nicotine treatment significantly enhanced the expression levels of caspase-1, IL-1β, IL-18, NLRP3, ASC and GSDMD in 16HBE cells. During pyroptosis, the activated NLRP3 inflammasome can induce the cleavage of pro-caspase-1 into mature caspase-1 (44). In the present study, nicotine treatment significantly increased the

mRNA expression levels of pro-caspase-1 but showed no effect on its protein expression levels. By contrast, nicotine exposure significantly increased the protein expression levels of caspase-1. Thus, it was hypothesized that nicotine promoted the expression of pro-caspase 1, and the increased levels of pro-caspase 1 were then cleaved into caspase-1. These results indicated that nicotine exposure may induce pyroptosis in 16HBE cells, suggesting that pyroptosis could be involved in the progression of COPD. This discovery sheds new light on the mechanism underlying the pathogenesis of COPD.

Bronchial epithelial cells serve an integral role in the airway defense mechanism via the mucociliary system and mechanical barriers, which serve significant roles in the progression of various lung diseases in addition to COPD, such as asthma, lung cancer, pneumonia and pulmonary fibrosis (45). Previous studies have revealed that smoking is significantly prevalent in patients with multiple lung diseases (46-48). However, to the best of our knowledge, whether nicotine exposure induces pyroptosis in these diseases remains to be elucidated. A key mediator of pyroptosis, GSDMB, has already been highly linked to asthma and non-small cell lung carcinoma (49-51). Thus, it was hypothesized that nicotine exposure may induce pyroptosis in bronchial epithelial cells in these diseases; this hypothesis requires further study.

Finally, the present study had some limitations. The *in vitro* experiments were only performed in 16HBE cells; therefore, further experiments should be performed in more cell lines to confirm the findings presented in the current study. Due to limited experimental conditions, nicotine exposure-induced pyroptosis has not been confirmed in *in vivo* experiments. Thus, further experimental research is needed to confirm the results in an animal model of COPD. Furthermore, it is still not clear whether nicotine exposure will induce pyroptosis in patients with COPD; this will be one of our future research directions.

In conclusion, the results of the present study suggested that nicotine exposure suppressed the proliferation and promoted the death of 16HBE cells, which may be associated with the increased expression levels of caspase-1, IL-1β, IL-18, NLRP3, ASC and GSDMD. These findings indicated that nicotine treatment induced pyroptosis in 16HBE cells, which may be associated with the progression of COPD.

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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

YC and YD were responsible for the conception of the present study. RM, YC and YD confirm the authenticity of all the raw data. RM and JZ conducted the experiments, analyzed and interpreted the data. JZ was responsible for statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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