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Short communication

Anti-inflammatory effects of NaB and NaPc in *Acinetobacter baumannii*-stimulated THP-1 cells *via* TLR-2/NF-κB/ROS/NLRP3 pathway

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ABSTRACT

This study evaluated the anti-inflammation effect of the three main short-chain fatty acids (SCFAs) on *Acinetobacter baumannii*-induced THP-1 cells. The three main SCFAs could inhibit *A. baumannii*-stimulated THP-1 cell NF-κB pathway activity and the expressions of NLRP3 inflammasome and GSDMD, and increase autophagy. The three main SCFAs, especially the sodium butyrate (NaB), had the effect of down-regulation of ROS and *TLR-2* expression in THP-1 cells. NaB and sodium propionate (NaPc), but not sodium acetate (NaAc), dramatically suppressed *IL-1β* and *IFN-γ* expression. The results indicated that NaB and NaPc could significantly inhibit the inflammation of THP-1 cells induced by *A. baumannii*, and the inhibitory effect was in the order of NaB > NaPc > NaAC. NaB and NaPc may inhibit inflammation through TLR-2/NF-κB/ROS/NLRP3 signaling pathway.

Keywords: short-chain fatty acids (SCFAs), autophagy, NLRP3, nuclear factor-κB (NF-κB), inflammation

Acinetobacter baumannii is becoming increasingly important as a hospital-acquired pathogen worldwide (1). With the increase of clinical invasive operation opportunities and the widespread applications of antibiotics, hospital infections and drug-resistant strains have increased, resulting in secondary organizing pneumonia, acute lung injury (ALI) and even acute respiratory distress syndrome (ARDS) (2). During ALI/ARDS, acute diffuse alveolar damage occurs, which leads to recruiting of macrophages which are then activated in the lung, and release a mass of cytokine (cytokine storm), such as TNF-α and IL-1β (3). Despite advances in supportive therapy, mortality rates for patients with ALI/ARDS induced by *A. baumannii* remain high.

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In recent years, the role of the "lung-gut axis" in the strategy of "pneumoenteric intestinal treatment" for improving the effect of lung injury treatment has been confirmed by many clinicians (4). Jardou and his colleagues noted that COVID-19 virus infection can cause insufficient production of SCFAs in the intestinal tract of patients, and easily induce ARDS and cytokine storm in patients. Meanwhile, they proposed to use SCFAs such as sodium butyrate (NaB) to prevent and treat cytokine storms and multiple organ failures induced by COVID-19 (5). SCFAs, the main metabolites produced by bacterial fermentation of dietary fibre in the gastrointestinal tract, can be involved in regulating the inflammatory response. SCFAs are saturated fatty acids with a chain length ranging from one to six carbon atoms, including formic acid, acetic acid, propionic acid, and butyric acid. Accumulating evidence suggests that SCFAs have been shown to ameliorate inflammation in inflammatory bowel diseases (IBD) and allergic asthma (6, 7). NaB particularly has been reported to have a prominent anti-inflammatory effect on colitis by modulating inflammatory factors and suppressing the NF-κB pathway activation (8, 9). Additionally, SCFAs have recently been considered to promote Treg cells' differentiation and maturation (10).

SCFAs have been used to treat a variety of inflammatory diseases, including non-alcoholic fatty liver and Crohn's diseases (11). However, the function and mechanism of SCFAs regulating *A. baumannii*-induced acute lung injury remain unclear. In our experiments, we studied the effects of SCFAs including sodium acetate (NaAc), sodium propionate (NaPc) and NaB on inflammation of *A. baumannii*-stimulated THP-1 cells, as well as the regulation of Th1/Th2 and Th17/Treg immune balance. THP-1 cells belong to monocytes, but can be transformed into macrophages after induction by phorbol ester (PMA), which is a common method for preparing macrophages at present. The purpose is to find a new strategy to prevent and cure the ALI/ARDS induced by *A. baumannii*.

EXPERIMENTAL

Bacteria strain and reagents

A. baumannii (ATCC17978), a kind gift from Dr. Zhou Wanqing, grew in the Luria-Bertani medium (LB, Oxoid, UK). Antibodies against LC3 (CST), Beclin-1, Caspase-1, gasdermin D (GSDMD), NLRP3 and NF-κB p65 (BOSTER, China) were employed. Cell nuclear and cytoplasmic protein extraction kit was from Beyotime Biotech Inc (China). The following three different SCFAs were used in this study: NaAc (99 %, Sigma Aldrich, USA), NaPc (99 %, Sigma Aldrich), NaB (99 %, ThermoFisher Scientific, Inc., USA).

Cell culture and treatment

THP-1 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (China) and were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (ThermoFisher Scientific), 100 U mL^{-1} of penicillin, and 100 g mL^{-1} of streptomycin (ThermoFisher Scientific). The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. For experimental purposes, the cells were harvested in a log phase and plated at the density of 1×10^6 cells mL^{-1} medium in 6-well sterile plates (ThermoFisher Scientific). All the cells in experiments were treated with 100 μg mL^{-1} PMA (Sigma Aldrich) for 6 h to induce the macrophage stage. They were divided into 5 groups, including the normal

control (NC) group, *A. baumannii* group, NaAc group, NaPc group and NaB group. The NaAc, NaPc, and NaB groups were pre-treated with 500 μmol L^{-1} NaAc, NaPc, NaB for 24 or 48 h respectively, and then *A. baumannii*, NaAc, NaPc and NaB groups were infected with *A. baumannii* (bacteria/cell = 10:1). After being infected with *A. baumannii*, the cells were continuously incubated for 1, 3 and 6 h. Finally, the cells were collected for mRNA and protein expression analysis. All the experiments were repeated three times.

Western blotting

Proteins were extracted from cells using RIPA lysis buffer with PMSF (Beyotime Biotech, Inc., China), then separated by SDS-PAGE electrophoresis with 15 % gels. After the electrophoresis, the proteins were transferred to PVDF membranes (0.45 μm, Millipore, USA). After being blocked with 5 % skim milk in TBS at 37 °C for 1 h, the PVDF membranes were incubated with primary antibodies for 12 h at 4 °C. Then the PVDF membranes were washed three times with TBST for 15 min and incubated with IgG-HRP (BOSTER) for 1 h at room temperature. After being washed, the immunoreactive bands were analyzed using image analysis software with an ECL system (Millipore). All values were normalized to the loading control, GAPDH.

qRT-PCR

Total RNA was extracted from cells using the Total RNA Extraction Reagent according to the manufacturer's recommendations (Vazyme Biotech Co., Ltd., China). The cDNA was synthesized using a qScript cDNA Synthesis kit (Vazyme Biotech Co., Ltd.) at 37 °C for 15 min and at 85 °C for 5 s. qRT-PCR analysis was executed using a SYBR Green detection

Table I. The sequences of specific primers for qRT-PCR

Genes	Forward(5′→3′)	Reverse(5′→3′)
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
NLRP3	AACAGCCACCTCACTTCCAG	CCAACCACAATCTCCGAATG
Caspase-1	GCACAAGACCTCTGACAGCA	TTGGGCAGTTCTTGGTATTC
IL-1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL-6	CCTTCGGTCCAGTTGCCTTCT	GAGGTGAGTGGCTGTCTGTGT
IL-4	CCGTAACAGACATCTTTGCTGCC	GAGTGTCCTTCTCATGGTGGCT
IL-10	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
IL-17A	CGCAATGAGGACCCTGAGAGA	AGCCCACGGACACCAGTATC
TGF-β	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA
IFN-γ	AATGGCGTGGAGCTGAGA	TGGCAGAGAGGAGGTTGAC
TLR2	GCAAGCTGCGGAAGATAATG	CGCAGCTCTCAGATTTACCC
TLR4	ATGAAATGAGTTGCAGCAGA	AGCCATCTGTGTCTCCCTAA
TLR9	AATTCCCATCTCTCCCTGCT	TCCTTCACCCCTTCCTCTTT

system (Vazyme Biotech Co., Ltd) on a 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The Primer sequences are shown in Table I. The conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 10 s. Analysis of relative gene expression data was performed with the $2^{-\Delta\Delta Ct}$ method.

NF-κB pathway

Nuclear and cytoplasmic proteins extracted from *A. baumannii*-treated THP-1 cells were prepared as follows. The cells (1×10^6 cells mL^{-1}) were pretreated with 500 μmol L^{-1} NaAc, NaPc, and NaB respectively for 24 h. In our experiments, we set up a control group of cells pretreated with 30 μM JSH-23 for 24 h which is the NF-κB inhibitor (MCE, China). Then the cells were co-cultured with *A. baumannii* (bacteria/cell = 10:1) for 3 h. Finally, the cells were collected for NF-κB pathway activation determined by Western blotting. The cells proteins in nuclear and cytoplasmic were separated using the Cell Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's instructions.

Reactive oxygen species (ROS)

THP-1 cells were sub-cultured in 6-well plates and pretreated with NaB, NaPc, NaAc and JSH-23 as the method mentioned above. Cells were then collected with tubes and incubated with DCFH-DA dye at 37 °C for 30 min. Total ROS determination content inside the cells was analyzed by use of the BD Accuri™ C6 Flow Cytometer following the instruction of the ROS assay kit (Beyotime Biotech Inc., China).

Statistical analysis

Data showed as mean ± SEM and obtained from at least three independent samples ($n = 3$). The Student's *t*-test and one-way ANOVA analysis of variance were used to determine the statistical significance of the differences between the values for the various experimental and NC groups using SPSS Statistics 12.0 and GraphPad Prism 8 (GraphPad, USA). $p < 0.05$ was considered a statistically significant difference.

RESULTS AND DISCUSSION

SCFAs inhibited NLRP3 inflammasome and GSDMD

SCFAs, as important regulatory molecules in the immune system, are essential for the maintenance of organism immune homeostasis (12). They are the major metabolites of gut flora degradation of soluble fiber in the colon, among which acetate (C_2), propionate (C_3), and butyrate (C_4) are the major acids produced during carbohydrate and amino acid fermentation (13). At the cellular, three kinds of SCFAs, such as NaB, NaPc and NaAc, could regulate cell growth, differentiation and proliferation as well as induce cell cycle arrest and apoptosis in a cell-specific manner (14).

To evaluate the anti-inflammation effect of SCFAs on THP-1 cells, THP-1 cells were pre-treated with NaB, NaPc and NaAc for 24 and 48 h. The results showed that NaB

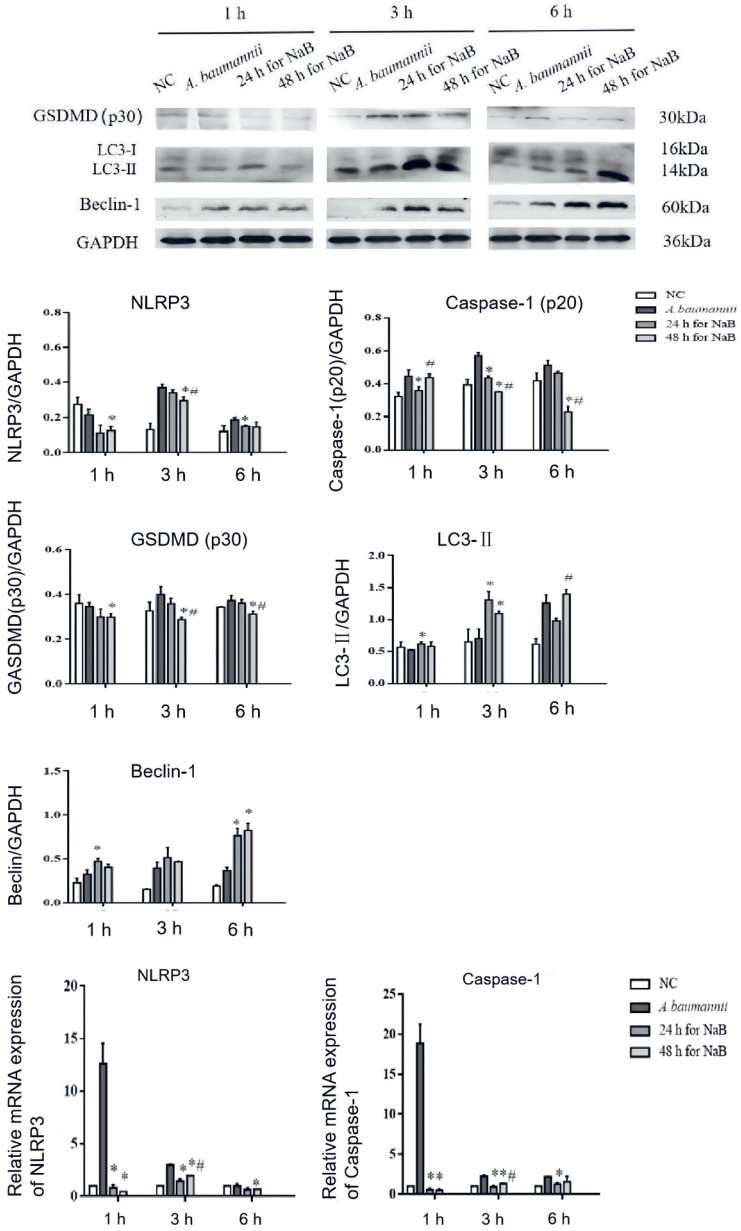

Fig. 1. The expression of NLRP3 inflammasome and autophagy in THP-1 cells. Cells were pretreated with or without 500 μmol L^{-1} NaB for 24 and 48 h. The cells were infected with *A. baumannii* for 1, 3 and 6 h, respectively. a) and b) Western blotting assay, c) qRT-PCR assay. NC – negative control. Results were expressed as mean ± SEM of triplicate replicates ($n = 3$). *$p < 0.05$ *vs*. *A. baumannii* group. #$p < 0.05$ *vs*. 24 h for NaB group.

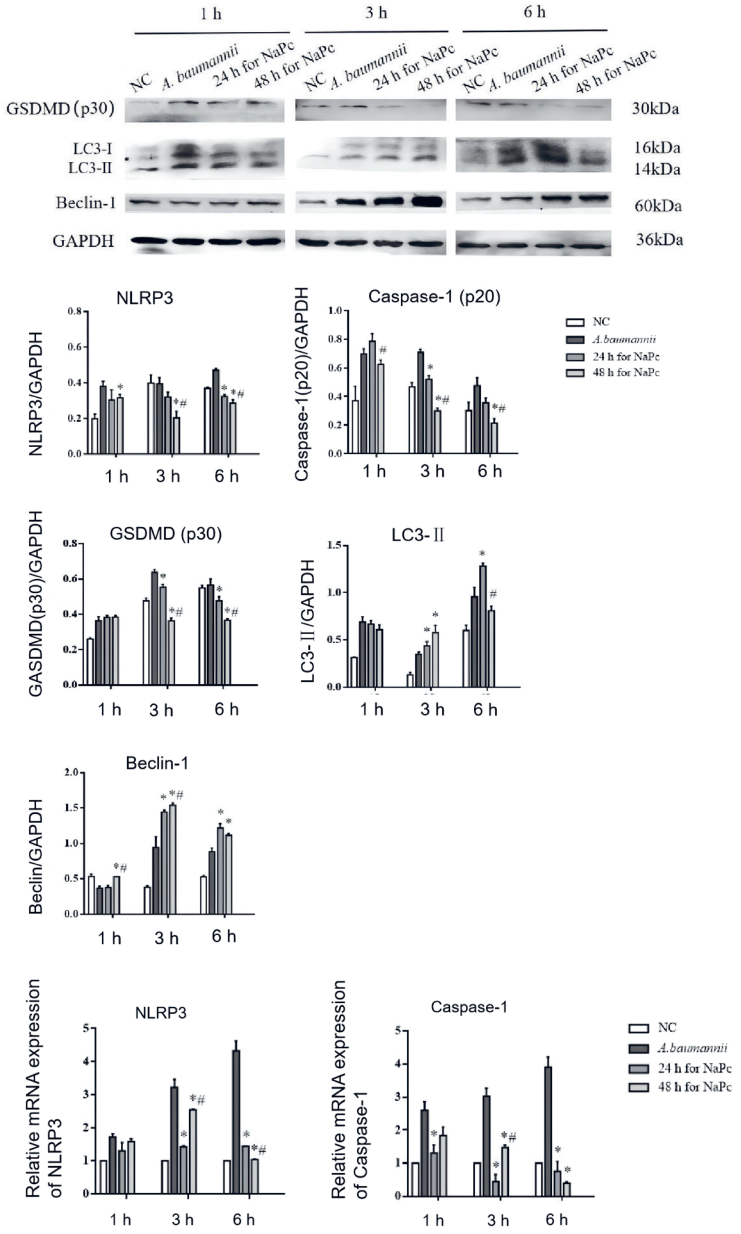

Fig. 2. The expression of NLRP3 inflammasome and autophagy in THP-1 cells. Cells were pretreated with or without 500 μmol L^{-1} NaPc for 24 and 48 h. The cells were infected with *A. baumannii* for 1, 3 and 6 h, respectively. a) and b) Western blotting assay, c) qRT-PCR assay. NC – negative control. Results were expressed as mean ± SEM of triplicate replicates ($n = 3$). *$p < 0.05$ *vs.* *A. baumannii* group. #$p < 0.05$ *vs.* 24 h for NaPc group.

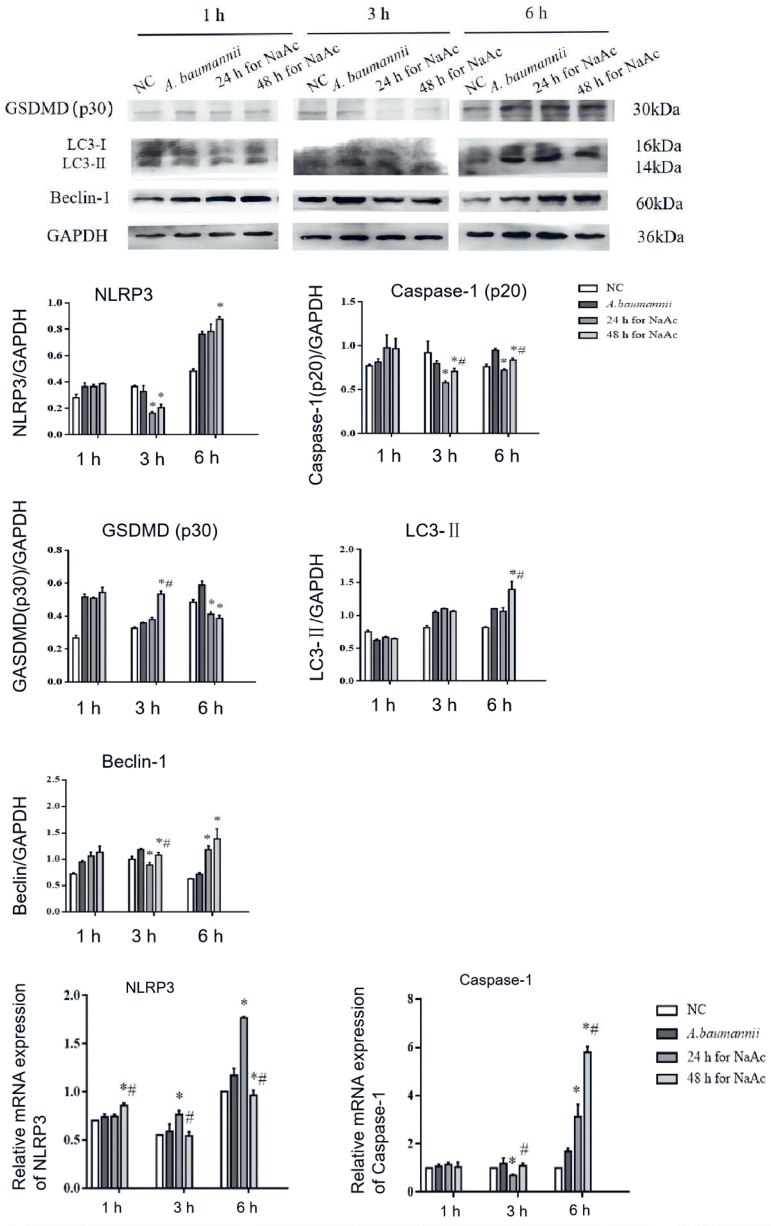

Fig. 3. The expression of NLRP3 inflammasome and autophagy in THP-1-derived macrophages. Cells were pretreated with or without 500 μmol L^{-1} NaAc for 24 and 48 h. The cells were infected with *A. baumannii* for 1, 3 and 6 h, respectively. a) and b) Western blotting assay, c) qRT-PCR assay. NC – negative control. Results are expressed as mean ± SEM of triplicate replicates ($n = 3$). *$p < 0.05$ *vs. A. baumannii* group. #$p < 0.05$ *vs.* 24 h for NaAc group.

obviously inhibited the mRNA expressions of Caspase-1 and NLRP3 over 10-fold compared with *A. baumannii* group infected for 1 h; NaB and NaPc dramatically inhibited the Caspase-1 and NLRP3 expression when infected for 3 h and 6 h ($p < 0.05$). The NaAc had no obvious effect on the Caspase-1 or NLRP3 expression except for being infected for 6 h (Figs. 1–3). Moreover, NaB and NaPc showed significant inhibition in protein expressions of Caspase-1 and NLRP3 in THP-1 cells when infected for 3 h and 6 h ($p < 0.05$), but the NaAc had no obvious inhibitory effect. Consistent with qRT-PCR results, Western blotting analyses showed that NaB and NaPc significantly suppressed NLRP3 and Caspase-1 expressions compared with the *A. baumannii* group.

NLRP3 is activated to combine with ASC and Caspase-1 to form an inflammasome. Caspase-1 is cleaved and activated to cut GSDMD, as the executor of pyroptosis (15). When infected with *A. baumannii* for 3 and 6 h, NaB and NaPc showed significant inhibition in the cleaved-GSDMD (p30) expression compared with *A. baumannii* group ($p < 0.05$). It showed that NaB and NaPc, particularly NaPc, significantly suppressed the GSDMD expression compared with the *A. baumannii* group (Figs. 1–3).

SCFAs induced autophagy

Autophagy is a basic response mode that occurs when eukaryotic cells encounter various stressors, and participates in numerous life activities of the cells and enables the cells to maintain a dynamic equilibrium under various stress conditions. Moreover, cell autophagy could inhibit the activation of the NLRP3 inflammasome, which was related to the ASC activation and pro-IL-1β mature (16, 17). A study by Dai indicated that the accumulation of ROS-damaged mitochondria in THP-1 cells was suppressed by enhancing autophagy (18). A recent study by Ko and his colleagues revealed that NF-κB upregulated autophagy expression in LPS-stimulated macrophages, which in turn induced mitophagic clearance of damaged mitochondria and thereby attenuated NLRP3 inflammasome--dependent IL-1β production (19).

In this study, when treated with NaB and NaPc, the expressions of LC3-II and Beclin-1 dramatically increased in 3 and 6 h compared to the *A. baumannii* treatment ($p < 0.05$). The effect of NaAc on LC3-II and Beclin-1 expression was not obvious (Figs. 1–3). The results indicated that NaB and NaPc might suppress the activation of the NLRP3 inflammasome, ROS and pyroptosis *via* enhancing the cells' autophagy.

SCFAs suppressed ROS

When NLRP3 recognizes ROS, it undergoes self-oligomerization and recruits the adaptor protein ASC, and then recruits and activates the Caspase-1 precursor to the mature Caspase-1. Then the pro-IL-1β was sheared to become mature IL-1β which was secreted extracellularly (20). In this experiment, after being infected with *A. baumannii* for 3 h, the expression of ROS in THP-1 cells increased over 20-fold compared with the NC group. When exposed to NaB for 24 h, the ROS expression had a significant decrease compared to the *A. baumannii* group ($p < 0.05$). It was also found that JSH-23, a key downstream effector of the NF-κB pathway, can suppress ROS production, and the effects of NaB and 30 μM JSH-23 were similar (Fig. 4).

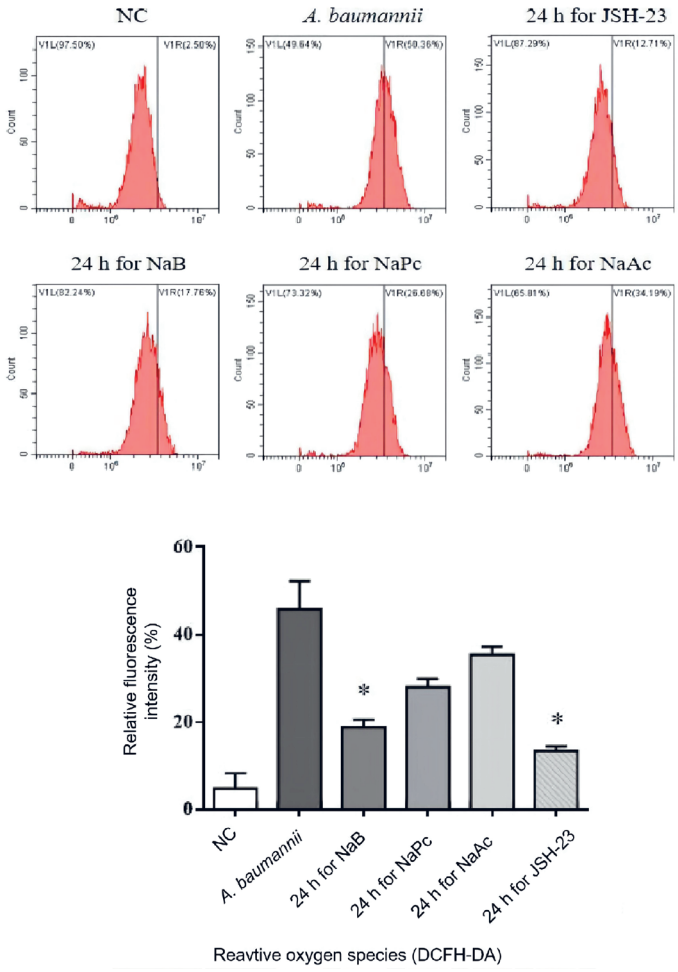

Fig. 4. NaB inhibited the generation of reactive oxygen species (ROS) in THP-1 cells. *$p < 0.05$ *vs. A. baumannii* group.

SCFAs inhibited the NF-κB pathway

NF-κB pathway plays a key role in resisting pathogen invasion, which was up-regulated by Toll-like receptors. Moreover, the incorrect regulation of NF-κB has been linked to inflammatory injury, cancer and autoimmune diseases (21). In this study, we discovered that the three main kinds of SCFAs could down-regulate the activity of the NF-κB pathway (Fig. 5). When pre-treated with NaB, NaPc and NaAc for 24 h, the translocating of NF-κB p65 into the nucleus induced by *A. baumannii* was significantly inhibited ($p < 0.05$). It suggested that the three main SCFAs could inhibit *A. baumannii*-stimulated NF-κB pathway in THP-1 cells.

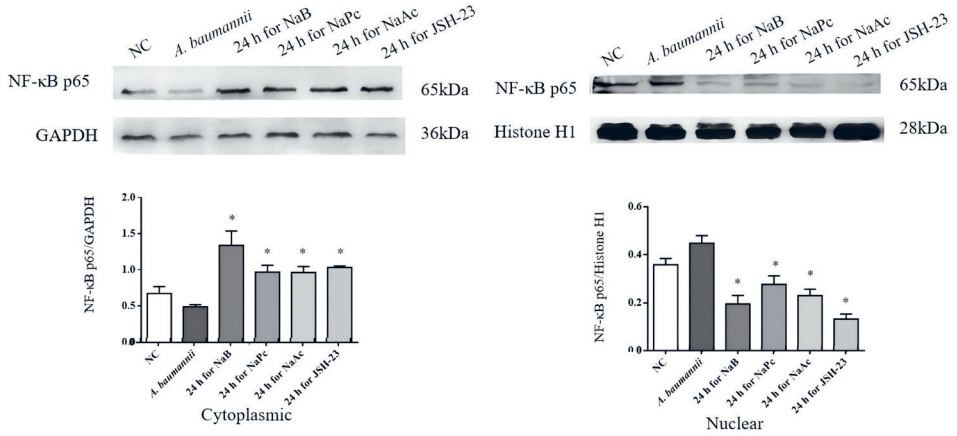

Fig. 5. SCFAs inhibited *A. baumannii*-stimulated nuclear entry of p65 in THP-1 cells. *$p < 0.05$ *vs. A. baumannii* group.

SCFAs regulated Toll-Like receptors and the cytokine expressions

During innate immune responses at lung mucosal sites, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), such as lipopolysaccharides (LPS) and peptidoglycans (PGN), are recognized through pattern recognition receptors (PRR) including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Toll-like receptors, such as TLR-2 and TLR-4, can respectively recognize gram-negative bacterial lipopolysaccharides (LPS) and bacterial lipoteichoic acid (22). In this experiment, the expression of TLR-2 was stimulated by *A. baumannii* infection, while NaB and NaPc could vastly decline its mRNA expression ($p < 0.05$) (Fig. 6). The three main SCFAs had no significant effect on the TLR-4 expression compared to the *A. baumannii* group ($p > 0.05$).

In this experiment, the expressions of IL-4, IL-10 and TGF-β significantly increased when treated with NaB, NaPc and NaAc respectively compared to the *A. baumannii* group,

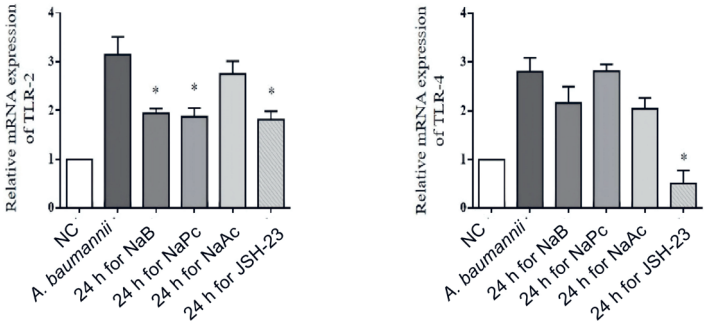

Fig. 6. SCFAs inhibited *A. baumannii*-stimulated TLR-2 activation in THP-1 cells. *$p < 0.05$ *vs. A. baumannii* group.

while the expressions of IFN-γ and IL-1β were dramatically suppressed ($p < 0.05$) (Fig. 7). For the Th1 type cytokines, the expressions of IFN-γ and IL-1β when pre-treated with NaB in 1 and 3 h compared to the *A. baumannii* group ($p < 0.05$); when pre-treated with NaPc for

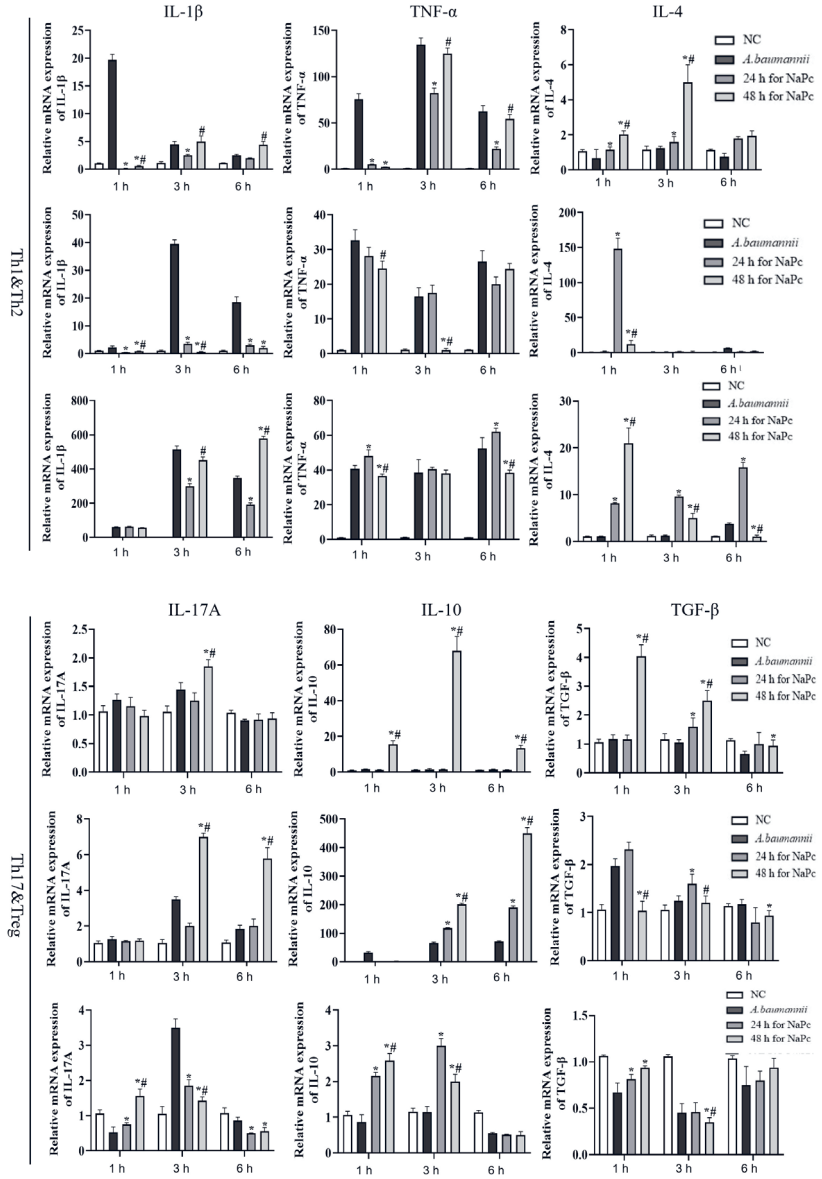

Fig. 7. SCFAs regulate inflammatory cytokines related to immune balance in THP-1 cells. *$p < 0.05$ *vs.* *A. baumannii* group. #$p < 0.05$ *vs.* SCFAs group.

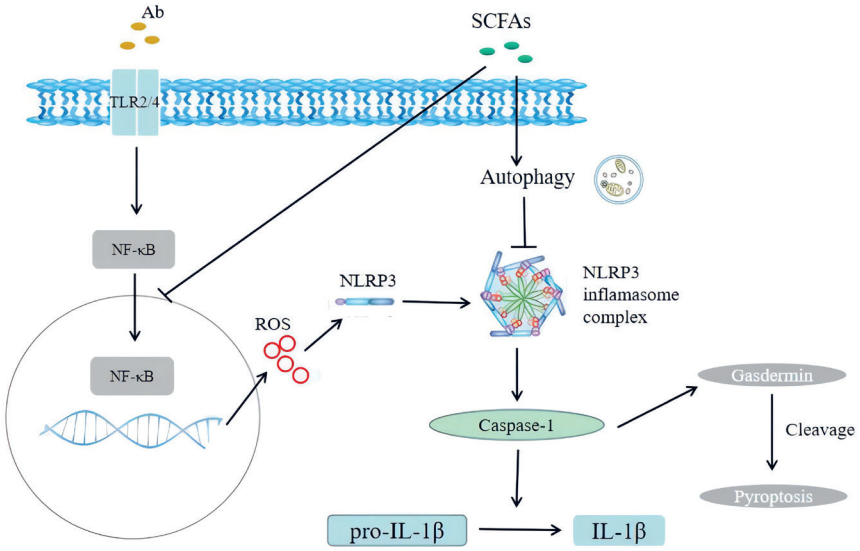

Fig 8. SCFAs, including NaB and NaPc, could enhance THP-1 cell autophagy and restrain the inflammatory cytokines expressions, and suppress activation of NLRP3 inflammasome and pyroptosis *via* TLR-2/NF-κB/ROS/NLRP3 pathway.

48 h, the expressions of IL-1β and IFN-γ significantly decreased compared to the *A. baumannii* group ($p < 0.05$). The results indicated that NaB and NaPc could inhibit the expression of Th1 type cytokines, whereas NaAc had no inhibitory.

Compared with the *A. baumannii* group, when pre-treated with NaB, NaPc and NaAc, the expression of IL-10 dramatically increased ($p < 0.05$); when pre-treated with NaB, the expression of TGF-β also increased dramatically ($p < 0.05$). When pre-treated with NaPc and NaB, the expression of IL-17A was significantly increased ($p < 0.05$). Additionally, NaAc firstly induced IL-17A production in 1 h and then inhibited its production when infected with *A. baumannii* in 3 and 6 h ($p < 0.05$). Overall, the results showed that NaB and NaPc could significantly promote Th1 type cytokines expression and suppress Th2 type cytokines expression, as well as increase the expression of IL-17A which is an important indicator of host innate immunity.

CONCLUSIONS

This study indicates that the SCFAs, including NaB and NaPc, could enhance THP-1 cell autophagy and restrain the inflammatory cytokines expressions, along with suppressing the activation of NLRP3 inflammasome and pyroptosis *via* TLR-2/NF-κB/ROS/NLRP3 pathway. In future studies, the effect of oral NaB and NaPc on the prevention and adjuvant of ALI/ARDS in patients with respiratory tract infection *A. baumannii* could be observed (Fig. 8).

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Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, W. L. and H. H.; methodology, C. S. and Z. Y.-Y. and Z. H.; analysis, D. L.-K., X. Y.; investigation, Y. M. and S. C.; writing, original draft preparation, C. S.; writing, review and editing, W. L. and H. H. All authors have read and agreed to the published version of the manuscript.

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