TRPM8 knockdown relieved inflammatory response and cell apoptosis in pneumonia model induced by Streptococcus pneumoniae in vitro

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ABSTRACT

Streptococcus pneumoniae infection remains a leading cause of pneumonia-related deaths. Transient receptor potential melastatin 8 (TRPM8) exerts crucial roles in lung diseases. We first dissected the role of TRPM8 in pneumococcal pneumonia and the mechanism related to TRPM8 effects. TRPM8 expression and inflammation cytokines level were determined in 15 paired patients and controls. A549 cells were pretreated with si-TRPM8, followed by infection with S. pneumoniae D39 strain (D39). TRPM8 expressions in D39-treated cells were detected and the effect of TRPM8 inhibition on the viability, apoptosis, and inflammation induced by D39 was evaluated. To explore the mechanism underlying TRPM8 effects, cells in D39+si-TRPM8 group were further treated with MAPK activator (Anisomycin, ANIS). TRPM8 was highly expressed in patients and cell models at mRNA or/and protein levels. Cytokines of TNF-α, IL-1β and IL-6 were intensely upregulated in the serum samples of patients and cells infected with D39 (p<0.05). TRPM8 knockdown attenuated the reduced cell viability and increased cell apoptosis (reflected by the upregulation of Bax and downregulation of Bcl-2) in D39 group (p<0.05). The expression level of inflammation cytokines was lower in D39+si-TRPM8 group than D39 group (p<0.05). The protein levels of NF-κB p-p65 and p-p38 MAPK were intensely accumulated in D39 treated cells, while reduced by TRPM8 inhibition (p<0.05). ANIS addition significantly attenuated the altered cell viability, cell apoptosis and inflammation response in D39+si-TRPM8 group (p<0.05). TRPM8 knockdown relieved D39 infection-caused inflammation and cell apoptosis via NF-κB/MAPK signaling.

Introduction

The community-acquired bacterial pneumonia (CABP) remains a major health concern because of its staggeringly high mortality. Streptococcus pneumoniae (S. pneumoniae) infection is the primary cause of CABP, which is presented in 50% of all CABP cases (1). As reported, there are more than 5.6 million adults affected by CABP in the United States annually (2,3). Notably, S. pneumoniae infection is responsible for approximately 30% mortality of cases admitted to ICU (4). Underlying the pathogenesis of pneumococcal pneumonia, S. pneumoniae attachment to airway epithelial cells is the primary event, which results in lung inflammation, reflected by exaggerated pro-inflammatory response and increased immune cell recruitment (5). Despite advances in the diagnosis and treatment options, the mortality and economic costs related to pneumococcal pneumonia remain the global burden.

Emerging researches have suggested that a subset of transient receptor potential (TRP) channels are associated with the development and progression of respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) (6,7). These channels have been proposed to be the therapeutic target for lung-related diseases (8). TRP channel melastatin 8 (TRPM8) is a member of the subfamily of TRP channels and has been reported to be activated by cold and chemical stimuli (9). Sabnis et al. have indicated that TRPM8 variants are implicated in the pathophysiology of asthma by modulating the airway inflammation induced by cold air inhalation (10). The overexpression of TRPM8 has been found in COPD patients, indicating the putative role of TRPM8 in the pathogenesis of COPD (11). With regard to the role of TRPM8 in respiratory diseases induced by external stimuli, we speculated that TRPM8 might play a key role in pneumococcal pneumonia.

Further, recent evidence has shown that the high expression of TRPM8 is associated with the increased MAPK/NF-κB signaling (12). Therefore, we constructed the pneumonia cell model induced by S. pneumoniae D39 strain (D39) and dissected the role of TRPM8 in D39-induced inflammation and apoptosis. TRPM8-mediated mechanism associated with MAPK/NF-κB pathway underlying pneumococcal pneumonia was further determined.

Materials and Methods

Subjects

A total of 15 patients (mean age: 51.3 ± 2.4, range: 20-79 years) with pneumonia induced by S. pneumoniae were included in this study from January 2020 to February 2022. The diagnosis of S. pneumoniae caused pneumonia was confirmed

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based on the chest radiographs and serological response. The patients with chronic diseases were excluded. Another 15 paired healthy participants were included according to physical examination. The blood samples of the eligible patients were collected at admission and the blood collection of healthy ones was conducted during physical examination. The peripheral blood was used for detecting the expression of TRPM8 and the levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6).

Informed consent of all the subjects had been signed prior to the study and this study was approved by the Ethics Committee of our hospital. Our study procedure was performed and complied with the Declaration of Helsinki guidelines.

Epithelial cells and S. pneumoniae D39 strain

Human lung epithelial A549 cell line and S. pneumoniae D39 strain (D39) were obtained from the American Type Culture Collection (ATCC). Cell culture was conducted in a sealed cell incubator with F-12K medium (supplemented with 10% fetal bovine serum) at a routine condition (37°C, 5% CO2). The D39 strains were routinely cultured in Brain Heart Infusion Agar at 37°C.

Cell transfection

TRPM8 knockdown was achieved by siRNA transfection strategy. The siRNA targeting TRPM8 (si-TRPM8, 5'-UCUCUGAGCGCAUCAUUC (dTdT)-3') and scrambled siRNA (si-NC, 5'-AAAGTGTGTGTTTGTCACT-3') (13) were obtained from GenePharma, Shanghai, China. A549 cells in a 6-well plate were maintained at 37°C. Cells at 40-50% confluence were subjected to siRNA transfection by Lipofectamine 2000 (Invitrogen) as per the recommended protocol. TRPM8 expression intervened by siRNAs was tested by qRT-PCR assay.

Cell in vitro model construction

To simulate the pathological condition of induced pneumonia in humans, A549 cells were infected with D39 in vitro per the previous method (14). Briefly, A549 cells were re-seeded in a plastic container and cultured for 10 days until 90% confluence. Then monolayer cells were incubated with D39 at a multiplicity of infection (MOI) of 10 for 4 h.

Real-time PCR (qRT-PCR)

The expressions of TRPM8, NF-κB p65 and p38 MAPK were determined in clinical blood samples and/or cells by qRT-PCR assay. We exploited the TRIzol reagent to achieve total RNA extraction. Then, the first strand of cDNA was reversely transcribed from RNA with the application of PrimeScript RTase (TaKaRa, Dalian, China). cDNA was reversely transcribed from RNA with the application of PrimeScript RTase (TaKaRa, Dalian, China). cDNA was reversely transcribed from RNA with the application of PrimeScript RTase (TaKaRa, Dalian, China). cDNA was reversely transcribed from RNA with the application of PrimeScript RTase (TaKaRa, Dalian, China). cDNA was reversely transcribed from RNA with the application of PrimeScript RTase (TaKaRa, Dalian, China).

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ELISA

Centrifugation (1000 rpm, 20 min) was conducted to harvest the supernatants of blood samples and cell cultures. The pro-inflammatory factors were detected by ELISA kit (Mbio, Shanghai, China) against TNF-α, IL-1β and IL-6 as the manufacturer’s recommendation.

Immunofluorescence

After being infected with the D39 strain, A549 cells were plated on 24-well plate with coverslips overnight at 37°C. Then, cells were treated with 4% paraformaldehyde and 0.5% Triton X-100. The antibodies used for immune staining included primary antibody against TRPM8 (Rabbit polyclonal antibody, 1:1000, Invitrogen), and Alexa Fluor™ 488 coupled goat anti-rabbit IgG antibody (1:1000, Invitrogen). The nuclei counterstaining was conducted with DAPI and the immunofluorescence signaling was observed under a fluorescence microscope.

CCK8 assay

After treatment, the proliferative ability of A549 cells was monitored by a CCK8 WST-8 assay kit (CA1210, Solarbio, Beijing, China). Cell culture (100 μL) was seeded in a 96-well plate and routinely pre-cultured for 24 h. Then, cell cultures (10 μL/well) were further maintained for the indicated time, followed by supplementation with 10 μL CCK8 solution for 2 h-incubation. Cell viability of transfected and non-transfected cells was determined by observing the absorbance of cell cultures at 450 nm.

Flow cytometry

Cells (1×10^6) were seeded in a 6-well plate and subjected to the indicated disposals. Then, cells were harvested and re-suspended. Cell suspension was added with 5 μL Annexin V and PI (propidium iodide) and maintained for 15 min away from light, followed by flow cytometry measurement.

Western blot

Cells were lysed were prepared with the application of RIPA solution, followed by protein protein isolation through centrifugation at 10000 rpm for 15 min. Then, the protein samples were resolved by 10% SDS-PAGE and transferred onto PVDF membranes. Primary antibodies used for western blot included TRPM8 rabbit polyclonal antibody (1:800), Bax monoclonal rabbit antibody (1:800), and Bcl-2 rabbit polyclonal antibody (1:1000), which were obtained from ThermoFisher Scientific (Waltham, MA). Those primary antibodies against rabbit NF-κB p65 (1:800), rabbit phospho-NF-κB p65 (1:800), rabbit p38 MAPK (1:800), rabbit phosphor-p38-MAPK (1:800) and rabbit GAPDH (1:800) were obtained from Cell signaling technology (Danvers, MA).
High expression of TRPM8 in patients with pneumonia induced by *S. pneumoniae*

The expression levels of TRPM8 were evaluated in blood samples of *S. pneumoniae*-induced pneumonia patients. By qRT-PCR assay, Figure 1A showed TRPM8 expression was remarkably increased in pneumonia patients, in comparison with normal ones (p < 0.01). Meanwhile, the serum levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) were obviously elevated in pneumonia patients (p < 0.001, Figure 1B). Thus, we speculated that TRPM8 overexpression was correlated with elevated immune response in pneumonia patients caused by *S. pneumoniae*.

Expressions of TRPM8 in cell models

After the D39 infection, the mRNA expression of TRPM8 was upregulated in A549 cells (p < 0.001, Figure 2A). The immunofluorescence assay revealed that there was an increase in red fluorescence signals after D3 infection (Figure 2B). Similarly, TRPM8 expression at the protein level was robustly elevated in the D3 treatment group compared with controls by western blot (p < 0.001, Figure 2C).

Effect of TRPM8 knockdown on A549 cells infected with D39

A549 cells were pre-treated with si-TRPM8 and si-NC as controls, followed by D39 treatment. Results suggested that TRPM8 expression was distinctly suppressed by si-TRPM8 transfection compared with si-NC treatment (p < 0.001, Figure 3A), indicating the effective transfection. Cell viability of A549 was significantly suppressed by D39 infection, which was attenuated by TRPM8 knockdown (p < 0.01, Figure 3B). Additionally, si-TRPM8 transfection obviously reduced cell apoptosis induced by D39 infection (p < 0.0001, Figure 3C). Meanwhile, the western blot suggested that D39 induced an obvious increase of apoptosis-related protein Bax, which was relieved by si-TRPM8 pretreatment (p < 0.05). Another apoptosis-related protein Bel-2 elicited an opposed expression trend in A549 cells intervened by D39 infection and si-TRPM8 transfection (Figure 3D). Interestingly, the cytokines of TNF-α, IL-1β and IL-6 were markedly increased in D3 treated cells, while were declined by si-TRPM8 supplementation (all p < 0.001, Figure 3E). Thus, TRPM8 knockdown relieved D39 infection-induced cell damage *in vitro*.

Effect of TRPM8 knockdown on NF-κB/MAPK signaling underlying D39 infection

To further dissect the mechanism driving the effect of TRPM8 on the pneumonia cell model, cells were pre-transfected with si-TRPM8 in D39 infected A549 cells, followed by incubated with p38 MAPK activator anisomycin (ANIS) \(^{13}\). RT-PCR assay showed that D39 induced an increase of NF-κB p65 mRNA expression, which was declined by si-TRPM8 transfection (all p < 0.01). The declined expression of NF-κB p65 in the D39+si-TRPM8 group was further reversed by ANIS addition (Figure 4A).

**Statistics**

In the present study, the same experiments were done in triplicate. Data expressed as mean ± SD (standard deviation) were disposed of by SPSS 11.5. Multi-group comparison was achieved with the application of one-way ANOVA and Tukey method. Statistical significance was considered when p < 0.05.

**Results**

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Similarly, the mRNA expression of p38 MAPK tends to increase in the D3 infection group, while declining by TRPM8 knockdown (p<0.001). The mRNA expression of p38 MAPK in the D39+si-TRPM8 group was lower than in the D39+si-TRPM8+ANIS group (p<0.01, Figure 4B). Consistent with these results, western blot analysis indicated that the protein level of NF-κB p-p65 and p-p38 MAPK showed similar expression trends in different groups (Figure 4C). These results suggested that TRPM8 knockdown declined the activation of the NF-κB/MAPK pathway in D39 infected cells.

**Effect of TRPM8 knockdown on cell damage induced by D39 involved with NF-κB/MAPK pathway**

To determine whether the NF-κB/MAPK pathway was involved in TRPM8-regulated cell damage underlying D39 infection, we explored the effect of ANIS supplementation on cell viability, apoptosis and immune response underlying D39 treatment. Results indicated that si-TRPM8 transfection elevated cell viability after D39 infection, which was attenuated by ANIS addition (p < 0.01, Figure 5A). Contrarily, TRPM8 knockdown reduced apoptosis of D39 treated cells compared with si-NC transfected group (p<0.001, Figure 5B). Parallelly, ANIS addition caused the increased protein level of Bax and reduced expression of Bcl-2 in A549 cells of the D39+si-TRPM8 group (p<0.001, Figure 5C). Simultaneously, the levels of TNF-α, IL-1β and IL-6 were elevated in the D39+si-TRPM8+ANIS group compared with the D39+si-TRPM8 group (p<0.01, Figure 5D). Thus, TRPM8 knockdown inhibited D39-induced cell damage via NF-κB/MAPK pathway.

**Discussion**

Pneumococcal pneumonia remains a major concern for its high mortality, and has posed a threat to public health and economic cost. Under the pathological conditions of pneumococcal pneumonia, *S. pneumoniae* attaches to epithelial cells of airways followed by the establishment of the bacteria in the lungs (5). Despite improvement in diagnosis and therapy strategies, considerable efforts are still needed to discover a novel target for pneumococcal pneumonia treatment.

TRP family composed of seven subfamilies has been found to be implicated in various physiological and pathological conditions, including lung diseases (16,17). TRPM8 belonging to the TRP (melastatin) family, is widely expressed in non-neurological tissues, including prostate, testicular, and lung tissues. TRPM8 is sever as a cation channel sensing exogenous ligands such as cold temperature, menthol and icilin (18-20). It is reported that TRPM8 is activated by cold stimulus, which contributes to the high expression of inflammatory cytokines in lung epithelial cells (21). The overexpression of TRPM8 induced by cold temperature has been determined in the ovalbumin-induced asthma murine model (22). A higher expression of TRPM8 is also found in COPD patients in comparison to healthy controls (11). Although the intense expression of TRPM8 has been confirmed in lung epithelial cells elicited by cold temperature, the TRPM8 expression in pneumonia induced by *S. pneumoniae* and the relationship between TRPM8 expression and inflammatory response as well as cell apoptosis has not been elucidated clearly.

To our knowledge, it is the first time to detect the expression of TRPM8, and TRPM8-mediated mechanism in pneumococcal pneumonia. We observed that TRPM8 was upregulated in pneumonia patients and cell models induced by *S. pneumoniae*. Although the precise role of TRPM8 in pneumococcal pneumonia has not been fully understood, based on the previous researches mentioned above, we inferred that the high expression of TRPM8 was correlated with the airway inflammation and cell apoptosis underlying pneumococcal pneumonia. Our data provided evidence that TRPM8 knockdown obviously elevated the viability and inhibited apoptosis of lung epithelial cells underlying D39 infection. Meanwhile, TRPM8 inhibition attenuated the D39-induced accumulation of inflammatory cytokines. TNF-α, IL-1β and IL-6 are essential cytokines that mediate inflammatory response. TNF-α is intensively expressed and induced by various stimuli, which is involved in several biological processes, including cell death and proliferation (23). IL-6...
is a key mediator in the acute phase of inflammation response underlying respiratory inflammatory diseases (24). IL-1β exerts a critical role in the amplification of inflammation response, and its activation is closely associated with NF-κB signaling (25). A previous evidence determined that these cytokines were significantly increased in acute pneumonia induced by lipopolysaccharide, a component of Gram-negative bacteria (26). In agreement with the previous findings, we suggested that the levels of inflammatory cytokines were remarkably elevated in pneumococcal pneumonia patients in vivo and cell models in vitro. The accumulation of inflammatory cytokines was relieved by TRPM8 knockdown. Collectively, TRPM8 was involved in the D39 infection-caused airway inflammation and cell apoptosis of lung epithelial cells.

We further dissected the correlation of TRPM8 effects with NF-κB / MAPK pathway underlying D39 infection. NF-κB is a major transcription regulatory factor, which exerts a regulatory role in inflammation by mediating the expression of cytokines including TNF-α, IL-1β and IL-6 (27). MAPK is an intracellular signaling, which can be activated by external stimuli and further modulates the expression of inflammatory mediators in the airway (28). Many studies have indicated that the NF-κB/MAPK pathway is activated in lung inflammation underlying acute pneumonia (26,29,30). The study of Ko et al. revealed that p38 MAPK and NF-κB p65 phosphorylation were increased in an acute pneumonia cell model (26). The results were consistent with our finding that NF-κB p-p65 and p-p38 MAPK levels were increased in D39-exposed A549 cells. TRPM8 knockdown attenuated the altered expression of p-p65 and p-p38 determined by western blot. These findings indicated that TRPM8 elicited an effect on lung cells through the NF-κB/MAPK pathway.

Overall, TRPM8 was overexpressed in pneumococcal pneumonia patients and cell models induced by S. pneumonia D39. Knockdown of TRPM8 relieved D39-induced inflammation and apoptosis in lung cells through reducing NF-κB/MAPK signaling. Our study may provide insights to the therapeutic potential of TRPM8 in pneumococcal pneumonia. TRPM8 may be utilized as the target for combating pneumonia caused by S. pneumonia.

Author contributions
HG and KS conceived and designed the study. KS and LS performed the experiment and drafted the manuscript. KS performed the literature search and data analysis. LS contributed to the data analysis. HG and KS discussed the results. HG and KS revised the manuscript. All authors reviewed the manuscript.

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Data availability
The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests
The authors have declared no conflicts of interest in this work.

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