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Effect and mechanism of calpains on pediatric lobar pneumonia

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ABSTRACT

Lobar pneumonia, one of the community-acquired pneumonia (CAP), is a common pediatric low respiratory tract infection. Calpains are Ca^{2+}-activated cysteine proteases whose activation mechanism is elusive. The present study was undertaken to detect the role and mechanism of calpains in pediatric lobar pneumonia. The human acute lung infection model (ALIM) was constructed and infected by *Streptococcus*. Enzyme-linked immunosorbent assay (ELISA) was used to measure interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α. We observed the lactate dehydrogenase (LDH) release, calpains activity and calpain inhibitor effects in ALIM. The expression of proliferating cell nuclear antigen (PCNA) protein was quantified by western blotting. Then the effects of calpain 1 and 2 knockdown on expressions of inflammation factors and PCNA protein, LDH release and apoptosis were evaluated in lung MRC-5 cells. In constructed ALIM, expressions of IL-6 ($P < 0.01$), IL-8 ($P < 0.01$), TNF-α ($P < 0.05$) and PCNA protein ($P < 0.05$) were significantly reduced by the calpain inhibitor. Expressions of IL-6, IL-8, TNF-α, PCNA protein and relative LDH release were statistically reduced by the small interfering (si) RNA-calpain 1 and 2 in MRC-5 cells ($P < 0.05$). Calpains silence increased apoptotic cells from 5% (negative control) to more than 20% in MRC-5 cells. The present study suggests that calpains possess a significant effect on inflammations, cell proliferation and apoptosis. Suppression of calpains may provide a potential therapeutic target of lobar pneumonia.

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calpains; calpain inhibitor; inflammations; lobar pneumonia

Background

Lobar pneumonia, one of the community-acquired pneumonia (CAP), is a common pediatric low respiratory tract infection needing hospitalization.[1] According to the statistics of the World Health Organization (WHO), CAP is the leading cause of mortality among children less than 5 y old, which maintains 19% of total pediatric deaths.[2,3] The increasing incidence of lobar pneumonia has been noted over the years with extensive alveolar infiltrates. The considerably serious clinical manifestations cover hyperpyrexia, cough and expiratory dyspnea, which usually result in extra pulmonary multi-system complications. Currently therapeutic strategies on pediatric lobar pneumonia are not standardized.[4] New antibiotics are developed increasingly, however, the morbidity and mortality of lobar pneumonia have not met a marked fall.

Streptococcus continues to be the most frequent pathogen of lobar pneumonia, a leading cause of death globally.[5,6] It is well known that *Streptococcus*-induced cytotoxicity underlies pulmonary tissue injury during lobar pneumonia and determines the outcome of infection.[7] Most of current knowledge about the pathogenesis of *Streptococcus* infection in pneumonia lay the ground from animal experiments and cell culture,[8,9] whereas little can simulate inflammations status of human diseases. A neotype of acute *Streptococcus pneumoniae* infection in human lung tissue was constructed to establish interactions between pathogens and

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pulmonary host cells, which pointed to mechanisms of the pulmonary inflammatory reaction.[10] Thereby, using vital lung specimens from pediatric patients undergoing lung resection, we developed a human acute lung infection model (ALIM) of *Streptococcus* infection to simulate inflammatory responses in pediatric lobar pneumonia at the tissue level. Lactate dehydrogenase (LDH) release has been a primary marker of cell non-specific death,[11] which was used to assess cell survival rate in pediatric ALIM. Inflammatory factors interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α were employed to evaluate inflammations in ALIM, since they were suggestive of excellent indicators of CAP inflammations.[12]

Calpains are Ca^{2+}-activated neutral cysteine proteases which are existed in cytosol of all vertebrate cells.[13,14] Since calpains were first identified in 1964,[15] emerging evidence has implicated that calpains are involved in many kinds of processes as an aggravating factor including the cell proliferation, migration, apoptosis and inflammations.[16-20] Some reports made a hypothesis that activated calpains were due to calpains migration from cytoplasm to cytomembrane.[21,22] The activated mechanism of calpains is elusive, but calpain inhibitor possesses protective effect on a variety of cells and viable tissues. Calpain inhibitor has been applied to alleviate organ injury and multi-system dysfunction in bovine aorta endothelium cells.[23] Many calpain inhibitors were reported to block LDH release[24] by reason of hydrolysis to cell framework proteins.[25,26] Although it is widely accepted that calpains and inhibitors play critical roles in cell functions, the role of calpains in lobar pneumonia is elusive.

The aim of the present study was to detect the role and mechanism of calpains in pediatric lobar pneumonia. The pediatric ALIM was constructed and infected by *Streptococcus* to induce inflammatory responses. Then we observed the calpains activity and calpain inhibitor effects on lobar pneumonia in ALIM. The effect and mechanism of calpain 1/2 knockdown on lobar pneumonia were evaluated in lung MRC-5 cells. The study is expected to provide valid theoretical and experimental basis for treatment of lobar pneumonia.

Results

Construction of pediatric ALIM

Normal lung specimens infected by *Streptococcus in vitro* were employed to construct the human ALIM. The relative LDH level of supernates was used to assess survival rate of lung cells infected by *Streptococcus*. Figure 1A demonstrated that the relative LDH levels showed a time-dependent increase, which was suggestive of time-dependent necrosis of lung cells in the ALIM. Compared to non-infected lung tissue, the relative LDH levels in ALIM were predominantly higher until 48 h ($P < 0.05$). After infection of *Streptococcus* for 48 h, the survival cells in ALIM were more than 60%. In addition, expressions of IL-6 ($P < 0.01$), IL-8 ($P < 0.01$) and TNF-α ($P < 0.05$) in ALIM were significantly higher than non-infected lung tissue (Fig. 1B). Collectively, these results showed that the human ALIM infected by *Streptococcus* was successfully constructed for further study.

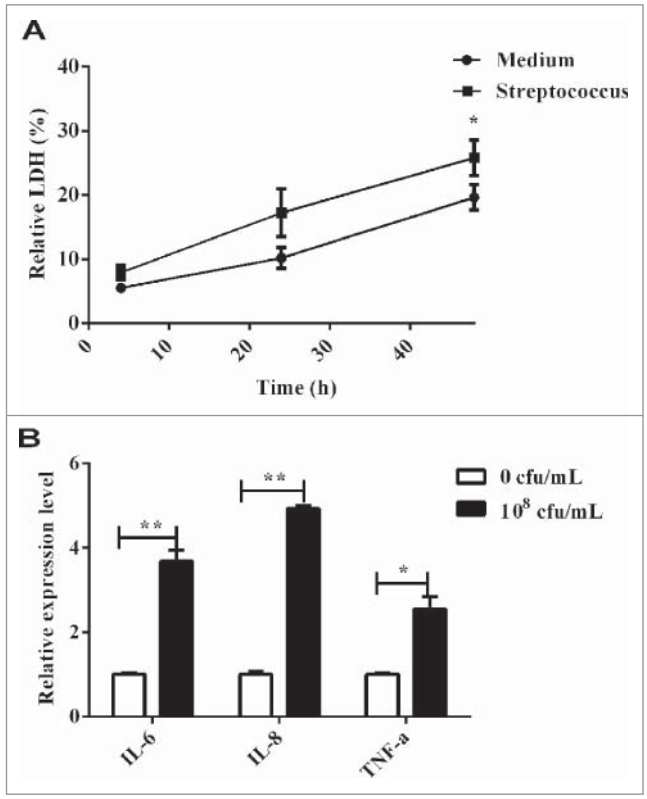

Figure 1. The construction of human ALIM infected by *Streptococcus*. A. The relative LDH vitality of supernates in ALIM at 4 h, 24 h and 48 h. B. The relative expression of IL-6, IL-8 and TNF-α of supernates in ALIM. LDH, lactate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; ALIM, acute lung infection model; cfu, colony-forming unit.*, $P < 0.05$, **, $P < 0.01$.

Calpain activity analysis in ALIM

After establishment of human ALIM, calpain activity in supernates was evaluated. Figure 2A revealed

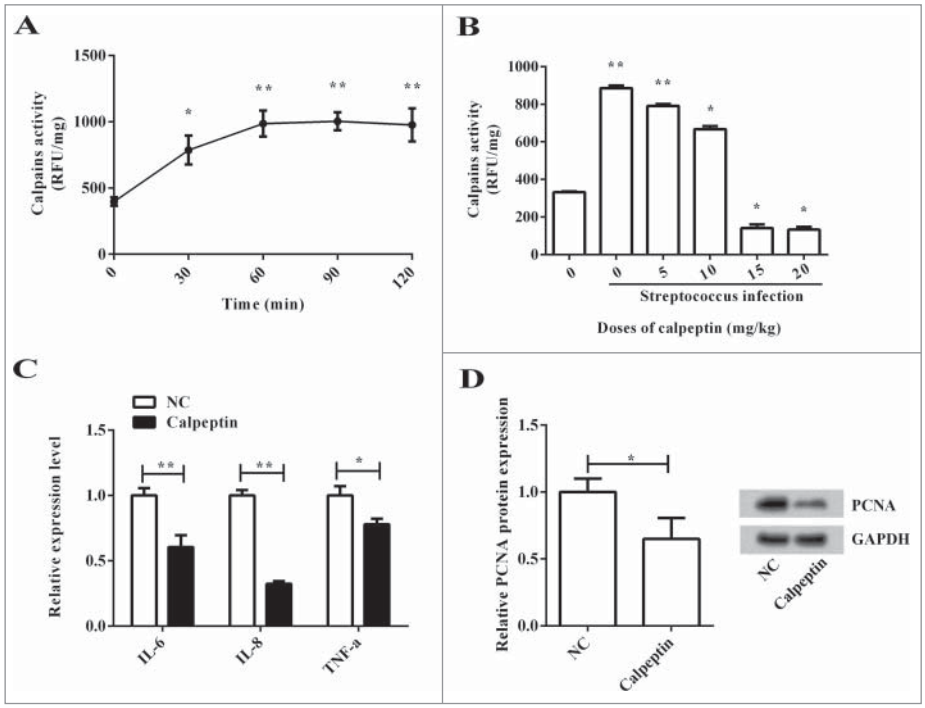

Figure 2. Alterations of calpains activity in the ALIM supernates with the calpain inhibitor (calpeptin). A. Calpain activity in the ALIM supernate maintained on a high level. B. The effect of different doses of calpeptin on calpains activity. C. Expressions of IL-6, IL-8 and TNF-α in ALIM were significantly reduced by calpeptin. D. The expression of PCNA protein was lower when the calpeptin was added. RFU, relative fluorescence units; NC, negative control; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; ALIM, acute lung infection model. *, $P < 0.05$, **, $P < 0.01$.

that the calpain activity in the ALIM supernates was double after 60 min compared with the initial activity and maintained the high level for 60 min. The high calpain activity in the constructed ALIM suggested that there were certain inflammatory responses after *Streptococcus* infection. Then, different doses of calpain inhibitor (calpeptin) were added in the lung tissue homogenates to interfere in the calpains activity. Figure 2B showed that the inhibiting effect on calpains activity was strengthened as high doses of calpian inhibitor were added. We found that 15 mg/kg calpain inhibitor could reduce the calpain activity to the initial level. Thereby 15 mg/kg was designated as the dose of calpain inhibitor in the further study. Expressions of IL-6 ($P < 0.01$), IL-8 ($P < 0.01$) and TNF-α ($P < 0.05$) in ALIM were significantly reduced by the calpain inhibitor compared to non-infected tissue (Fig. 2C). The expression of PCNA protein was lower when the calpain inhibitor was added ($P < 0.05$, Fig. 2D). Thus, the findings revealed that calpain inhibitor had a protective effect in inhibiting cell proliferation of human ALIM.

The effect of calpains on the lung MRC-5 cells

The siRNA-Calpains 1 and 2 were transfected to lung MRC-5 cells and the transfection efficiency was evaluated in Fig. 3A. The relative calpains expression level was statistically decreased both by calpains 1 siRNA and 2 siRNA as compared to negative control cells ($P < 0.05$). Accordingly, it was concluded that silence calpains were effectively and these transferred MRC-5 cells could be used in the further study. Then we monitored expressions of IL-6, IL-8, TNF-α and PCNA protein in transferred lung cells with calpains silence. Expressions of IL-6, IL-8 and TNF-α were significantly reduced by the siRNA-calpains ($P < 0.05$, Fig. 3B) and the expression of PCNA protein was lower compared with MRC-5 cells transferred empty vector siRNA ($P < 0.05$, Fig. 3C). It indicated that calpains promoted the cell proliferation and inflammation of lung MRC-5 cells *in vitro*.

Mechanism of calpains in lung MRC-5 cells

LDH release was used to detect cell membrane permeability in MRC-5 cells. We found that the relative

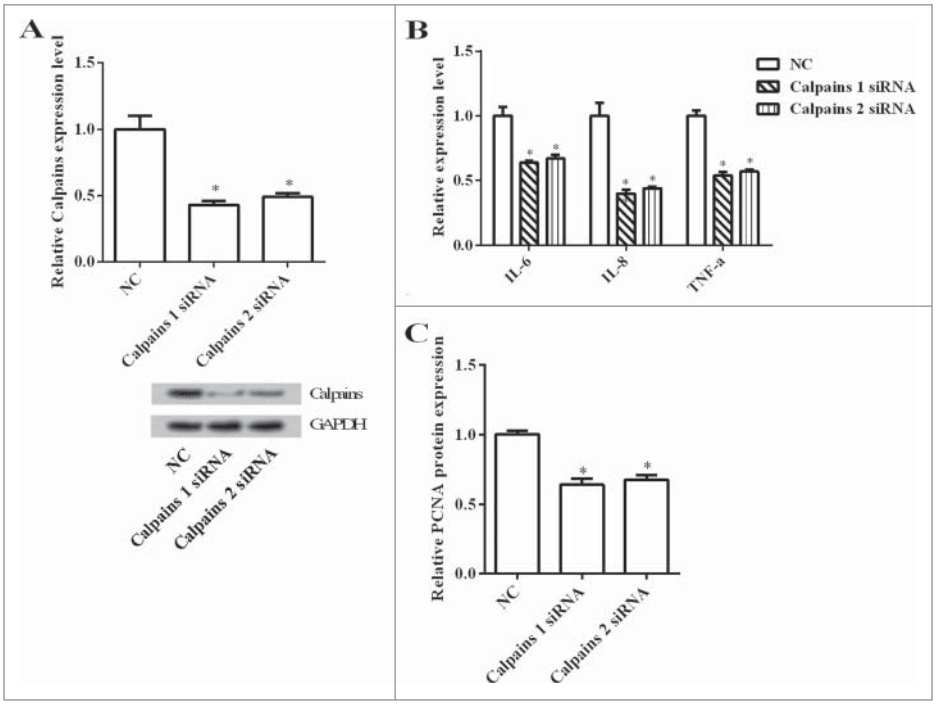

Figure 3. The effect of calpains on the lung cells MRC-5. A. The transfection efficiency of siRNA-calpains 1 and 2 to lung fibroblast MRC-5 cells. B. Expressions of IL-6, IL-8 and TNF-α in transferred lung cells with calpains silence. C. The expressions of PCNA protein in transferred lung cells with calpains silence. NC, negative control; si, small interfering; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF, tumor necrosis factor; PCNA, proliferating cell nuclear antigen. *, $P < 0.05$.

LDH release was reduced by siRNA-calpains compared with the negative control ($P < 0.05$, Fig. 4A). Fig. 4B identified that calpains silence increased apoptotic cells from 5% (negative control) to 26% (calpain 1 siRNA) and 20% (calpains 2 siRNA). Thereby, it claimed that silencing of calpains could improve the membrane permeability and inhibit cell apoptosis in lung MRC-5 cells.

Discussion

To our knowledge, this study provides the first insight into the role and mechanism of calpains in pediatric lobar pneumonia infected by *Streptococcus*. In the constructed human ALIM, calpains aggravated inflammations and cell proliferation, which might induce continuous lung inflammations and formation of lumps. The significant effect of calpains predicted calpains to be the potential target of pediatric lobar pneumonia treatment. Gene silencing and drug intervention on calpains activity have important implications for treatment of lobar pneumonia.

Streptococcus lysates represent a common clinical condition of lobar pneumonia, especially after antibiotic treatment. *Streptococcus* R6 possesses a strong capacity to be transformed to penicillin resistant.[27] In consequence we chose *Streptococcus* to infect the constructing pediatric lobar pneumonia model ALIM. Furthermore, LDH is a major indicator of cell nonspecific death,[11] which is used to assess cell survival rate in the present study. The finding of relative LDH level in pediatric ALIM claimed that ALIM cells died with time dependence in 48 h. Survival cells in ALIM were estimated to be more than 60% at 48 h, which indicated that the ALIM could survive at least 48 h cultured *in vitro*. Additionally, ELISA results showed *Streptococcus* infection significantly stimulated lung tissues to secrete inflammatory mediators IL-6, IL-8 and TNF-α in ALIM. It hints that as a unit of reflecting structures and functions of the alveolar space, the constructed ALIM can be used at further tissue level. Droemann *et al.* [28] also used the similar lung tissue model *in vitro* for the pathogenesis of chlamydia pneumonia and lung cancer. To sum up, the ALIM can serve as the bridge between pediatric lung cells and animal model to detect regulation and intervention of inflammatory processes at the tissue level.

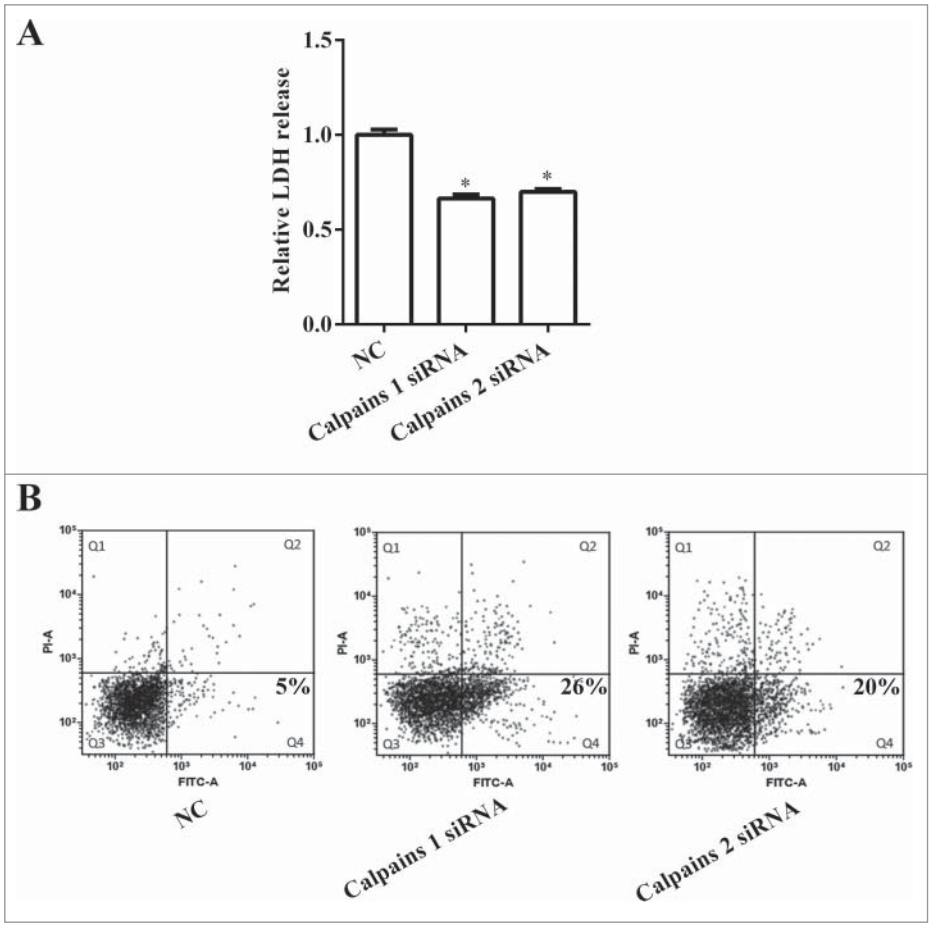

Figure 4. The effect of calpains on membrane permeability and cell apoptosis in the lobar pneumonia. A. LDH release was used to detect cell membrane permeability in MRC-5 cells. B. The flow cytometry identified cell apoptosis in MRC-5 cells with calpains silence. NC, negative control; si, small interfering; LDH, lactate dehydrogenase; PI, Propidium iodide.*, $P < 0.05$.

We proposed in the pediatric ALIM, augmented calpain activity declared calpains were activated in inflammations by *Streptococcus* infection. Calpain inhibitor had a protective effect on ALIM by inhibiting inflammations, which was consistent with the following reports. Calpain inhibitor also possessed cytoprotective properties under the stimulus of hypoxic, toxicosis and septicopyemia.[29-31] Pharmaceutical calpain inhibition observably attenuated mechanical ventilation caused by acute lung injury (ALI).[32] Based on above, calpain inhibitor is potential to be a new and promising target of lobar pneumonia therapy.

To further understand the function and mechanism of calpains in lobar pneumonia, we evaluated the LDH release and apoptosis by knockdown of calpains in lung MRC-5 cells. Our results showed that knockdown of calpains could improve the membrane permeability and increase cell apoptosis in MRC-5 cells. There is extensive evidence that calpains mediate membrane permeability.[25,33] So far, the identified calpain zymolytes contain signal molecule, membrane protein, desmoenzymes and structural protein.[20,34-36] Calpains make cell frame structure instable via hydrolyzing membrane proteins to increase membrane permeability. Besides, it was reported that calpains contributed to mitochondrial permeability transition pore opening after ischemia reperfusion in the rat heart.[37] Collectively, calpains increase membrane permeability such as mitochondria, cytomembrane, leading to activated inflammation factors and eventually causing inflammatory reaction.

Conclusion

In conclusion, the present study seeks to determine the role and mechanism of calpains in pediatric lobar

pneumonia. The human ALIM infected by *Streptococcus* induces the augmented calpains activity, which indicated the inflammation responses in lobar pneumonia. Calpains could increase the membrane permeability, promote cell proliferation and inhibit cell apoptosis in lung MRC-5 cells. These results claim that calpains possess a significant effect on inflammations, cell proliferation and apoptosis. Suppression of calpains may provide a potential therapeutic target of lobar pneumonia.

Materials and methods

Bacterial strains culture

Streptococcus strain R6 (ATCC 6303, Rockville, USA) was applied in the present study. Bacteria were maintained on chocolate agar (AppliChem, Gatersleben, Germany) at 37°C in a humidified atmosphere containing 5% CO_2. *Streptococcus* was incubated in 30 ml of Todd Hewitt Broth (Oxoid, Shanghai, China) overnight and then harvested from the chocolate agar to obtain *Streptococcus* crude extracts. After centrifugation and supernatant removal, the remaining *Streptococcus* was suspended in 10 ml of phosphate buffer saline (PBS) and sonicated. Then the lysate of *Streptococcus* was gathered and stored at −70°C.

Construction of ALIM

Human lung explants were obtained from 5 patients subjected to the local thoracic surgery due to isolated nodules or masses. Participants who were diagnosed with lobar pneumonia by pulmonary function test (PFT)[38] and chest x-ray were enrolled in current study from January, 2015 to January, 2016. The demographics of them were shown in Table 1. These patients were aged 1–5 years, 3 male and 2 female with no clinical or laboratory signs of acute respiratory infection. The PFT parameters in Table 1 and blood gas analysis determining the artery blood oxygen partial pressure (PaO_2) ≥ 60 mmHg and partial pressure of carbon dioxide ($PaCO_2$) < 90 mmHg were indicative of justified lung resection. Patients were administrated intravenously linezolid (Pfizer Inc., New York, USA) for 2–4 w in preceding pneumonia treatment. Pediatric patients underwent lung suppuration resulting from poor cure of lobar pneumonia to lung resection. This study was approved by the Ethics Committee of Guangzhou Medical University and all guardians signed informed consent form.

Table 1. Characteristics and demographics in 5 patients undergoing lung resection.

Parameters	Patients (n = 5)
Gender	
Male	3 (60%)
Female	2 (40%)
Mean age in years	3.6 ± 1.67
FEV_1/VC (%)	69.7 ± 3.80
FEV_1%	75.1 ± 2.29
MVV%	72.3 ± 3.54

FEV_1, forced expiratory volume; VC, vital capacity; MVV, maximum ventilator volume.

Normal lung tissues, at least 5 cm away from the node or mass, were employed to construct the ALIM. They were cultivated with 800 μL Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogn, CA, USA) in 24-well plates. Lung specimens and 10^8 colony-forming unit (cfu)/mL *Streptococcus* were maintained at 37°C in an atmosphere containing 5% CO_2 for 48 h. Lung specimens were fixed at 4°C in the Hepes–glutamic acid buffer mediated organic solvent protection effect (HOPE) solution for 24–48 h.[39]

LDH release

At time points 4 h, 24 h and 48 h, supernates were collected from ALIM infected by *Streptococcus* for LDH release analysis. The LDH vitality was calculated based on standard pyruvic acid (Sigma, USA) linear relation by Aeroset (Abbott, USA) at a wavelength of 340 nm. The LDH level in RPMI 1640 medium was the control.

Enzyme-linked immunosorbent assay (ELISA)

At time point 48 h, supernates of lung tissues were collected and stored in −30°C. Quantitative measurement of IL-6 (RAB0306), IL-8 (RAB0319), and TNF-α (RAB1089) in supernates of ALIM was performed based on the human ELISA kit instructions (Sigma, USA).

Calpain activity

Calpain activity was assayed using an InnoZyme Calpain 1 and 2 Activity Kit (Calbiochem, Germany). Lung tissue homogenates of supernates were collected after infection 48 h and centrifuged at 8,000 rpm for 20 min. The measurement assesses the ability of calpains to digest the substrate DABCYL-TPLKSPPPSPR-(EDANS). Calpain activity was measured fluorometrically at an excitation of 320 nm and an emission of

500 nm, which was shown as relative fluorescence units (RFU). The calpain inhibitor calpeptin (Enzo Life Sciences, Plymouth, USA) was added in the lung tissue homogenates of supernates. Different doses of inhibitor 0, 5, 10, 15, 20 mg/kg were interfered in the calpain activity and the same doses of dimethyl sulphoxide (DMSO, Sigma, USA) were added as the control.

Calpains silence transfection

The small interfering RNA (siRNA)-Calpains 1 and 2 (GenePharma Co, Shanghai, China) was constructed and transfected to human lung fibroblast MRC-5 cells (ATCC, USA). The empty vector siRNA was considered to be the negative control. Cell transfections were carried out using Lipofectamine 3000 reagent (Invitrogen, CA, USA) based on manufacturer's introductions. When cells cultured confluence 80–90%, they were stimulated with 10^8 cfu/mL *Streptococcus* for 1 h. Then cells were collected and stored at −30°C for ELISA and following assays.

Reverse transcription-polymerase chain reaction (RT-PCR)

Lung specimens fixed with HOPE solution at 4°C were removed 50 mg. Total RNA of 50 mg lung tissues and MRC-5 cells transfected by siRNA-Calpains was isolated respectively using Trizol reagent (Invitrogen, USA) and treated with DNaseI (Promega). RT-PCR was performed by following the manufacturer's recommendations of NucleoSpin RNA II kit (Macherey Nagel, Dueren, Germany). For the reverse transcription, Multiscribe RT kit (Applied Biosystems, USA) was employed. The primer sequences were: proliferating cell nuclear antigen (PCNA), forward primer: 5′AAACTAGCTACACTTTC CTC'3, reverse primer: 5′TCACGCCCATGGCCAGGT TG'3; Calpain, forward primer: 5′TCGTGCTCGCCCT TAT GC'3, reverse primer: 5′CTTGTCCAGGTCAAAC TTCC'3; glyceraldehyde-3- phosphate dehydrogenase (GAPDH, Abcam, Cambridge, United Kingdom), forward primer: 5′ATC TGGCACCACACCTTCTACA'3, reverse primer: 5′GTTTGGTGGATGCCACAGGACT'3. PCR amplification was carried out by LightCycler Detection System.[40]

Western blot analysis

Lung homogenates and cell particle were lysed by means of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membrane (Millipore, USA). The proteins used for western blotting were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Applygen Technologies Inc., Beijing, China). The total amount of proteins was quantified by Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Appleton, USA). A Bio-Rad Bis-Tris Gel system was employed to establish the western blot system, in which GAPDH was regarded as an internal control. Primary antibodies PCNA (ab18197), calpain 1 (EPR3319) and calpain 2 (ab39165) were obtained from Abcam (Cambridge, United Kingdom). After incubation with the membrane at 4°C overnight, secondary antibodies were marked by horseradish peroxidase for 1 h at room temperature. Then Images were developed and photographed using Image Lab Software (Bio-Rad, Shanghai, China).

Detection of apoptosis

Apoptotic cells were identified and quantified by flow cytometry with Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (BD PharMingen, Beijing, China). The transfected MRC-5 cells were seeded in 6-well culture plate and washed twice with cold PBS. Then they were co-incubated with serum-free culture medium containing 10 μM dichlorofluorescein diacetate (20 min, 37°C, in dark). Subsequently, samples were collected by a trypsin digestion approach and centrifuged. Then they were resuspended in 100 μl annexin-binding buffer and measured with flow cytometer according to the manufacturer's protocol.

Statistical analysis

Each experiment was carried out in triplicate. All results were presented as mean ± standard deviation (SD). The data were analyzed with GraphPad Prism 6.0 software (GraphPad, San Diego, USA). Values were performed by one-way analysis of variance (ANOVA) with SPSS 19.0 software (SPSS, IL, USA). Statistical significance was defined as $P < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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