RESEARCH ARTICLE

The traditional Chinese patented medicine Qingke Pingchuan granules alleviate acute lung injury by regenerating club cells

Yuanyuan Wu1,2 | Wensi Zhu1,2 | Ainiwaer Rouzi1,2 | Lin Tong1,2 | Linxiao Han1,2 | Juan Song1,2 | Jianwen Ding3,4,5 | Yu Yan1,2 | Miao Li1,2 | Ting Pan1,2 | Jie Liu1,2 | Qin Wang1,2 | Yuanlin Song6 | Jie Shen3,4,5 | Jian Zhou1,2,3,6,7

1Department of Pulmonary and Critical Care Medicine, Shanghai Respiratory Research Institute, Zhongshan Hospital, Fudan University, Shanghai, China
2Shanghai Engineering Research Center of Internet of Things for Respiratory Medicine, Shanghai, China
3Research Center for Chemical Injury, Emergency and Critical Medicine of Fudan University, Fudan University, Shanghai, China
4Key Laboratory of Chemical Injury, Emergency and Critical Medicine of Shanghai Municipal Health Commission, Fudan University, Shanghai, China
5Center of Emergency and Critical Medicine in Jinshan Hospital of Fudan University, Fudan University, Shanghai, China
6Shanghai Institute of Infectious Disease and Biosecurity, Fudan University, Shanghai, China
7Shanghai Key Laboratory of Lung Inflammation and Injury, Shanghai, China

Abstract

Qingke Pingchuan granules (QKPCG), a patented traditional Chinese medicine, clinically, are recommended for acute tracheobronchitis, cough, community-acquired pneumonia, and other respiratory diseases. However, its potential protective effect and mechanism of action in acute lung injury (ALI) have not been explored. We aimed to explore the mechanisms underlying the protective role of QKPCG in ALI. The therapeutic efficacy of QKPCG was investigated in a lipopolysaccharide (LPS)-induced ALI mouse model. Mice were divided into three groups, namely, the Control, LPS, and LPS + QKPCG groups. Mice in the LPS + QKPCG group were administered QKPCG intragastrically as a treatment once a day for a total of three days. QKPCG effectively increased survival and reduced lung injury in treated mice. It significantly reduced the LPS-induced expression of interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1α, and IL-1β. RNA-sequencing followed by real-time quantitative polymerase chain reaction validation suggested a critical role of the secretoglobin family 1A member 1 (Scgb1a1) gene in...
INTRODUCTION

Acute lung injury (ALI) is characterized by acute lung inflammation, tissue damage, and poor oxygenation caused by various pathogenic factors. Delayed ALI intervention may lead to life-threatening acute respiratory distress syndrome (ARDS), a severe form of ALI. The mortality rate of ARDS has been reported to be as high as 40%, even with the interventions available in modern medical facilities, such as optimized mechanical ventilation and fluid therapy.\(^1\)\(^2\) Currently employed clinical strategies mainly focus on reducing lung injury and pulmonary edema, ensuring oxygen supply, and reducing subsequent complications.\(^3\)\(^4\)\(^5\) However, inhibiting the progression of early lung inflammation to prevent ARDS has been suggested as the most effective method.\(^6\) Therefore, more efficient early intervention methods are necessary for slowing down and halting ALI progression.

Qingke Pingchuan granules (QKPCG), a patented traditional Chinese medicine (TCM), are an improved version of the Maxing Shigan decoction used to treat respiratory diseases.\(^7\) QKPCG effectively promotes homeostasis and recovery of lung function and relieves cough and panting. In clinical practice, QKPCG are mainly recommended for acute tracheobronchitis, cough, acute exacerbation of chronic obstructive pulmonary disease, community-acquired pneumonia, and other respiratory diseases. However, the potential role and underlying mechanism of QKPCG in alleviating ALI have not been explored.

Secretoglobin family 1A member (Scgb1a1) encodes a pulmonary surfactant protein, Clara cell 10 kDa protein (CC10), that is, mainly secreted by club cells within the lungs and exerts anti-inflammatory as well as antifibrotic effects following lung injury.\(^8\) In particular, CC10 binds key inflammatory mediators in the airway, including prostaglandins, phospholipase A2, and phospholipase C.\(^9\) Furthermore, CC10 can inhibit phosphorylation of NFκB.\(^10\)\(^11\)\(^12\) Reportedly, the absence of CC10 in infectious lung diseases leads to worse outcomes, whereas its overexpression within airways can limit ventilator-induced lung injury and inflammation.\(^9\) Studies have also shown that exogenous supplementation of CC10 effectively inhibits alveolar macrophage-mediated inflammatory responses and suppresses pyroptosis within the brain of a neonatal rat model with sepsis.\(^13\)\(^14\) Importantly, increased levels of CC10 may be related to the regeneration of club cells.\(^8\)

CC10 and SFTPC are markers of club cells and alveolar type II (AT2) cells, respectively.\(^15\) CC10\(^+\) club cells, SFTPC\(^+\) AT2 cells, and CC10\(^+\)SFTPC\(^+\) bronchioalveolar stem cells (BASCs) are resident lung stem cells, which proliferate and differentiate following lung injury to promote tissue repair.\(^16\)\(^17\)

The expression of chitinase-3 like-protein-1 (CHI3L1), one of the nonenzymatic chitinase-like proteins, can be regulated by CC10.\(^18\) CHI3L1 plays a key role in tissue injury, inflammatory response, and tissue remodeling.\(^19\)

We hypothesized that QKPCG may alleviate ALI by decreasing the disruption of club cells and increasing expression of CC10, with increased CC10 suppressing the NFκB pathway. To verify this hypothesis, we investigated the therapeutic efficacy and underlying mechanisms of QKPCG in an LPS-induced ALI murine model.
MATERIALS AND METHODS

QKPCG preparation

QKPCG comprise 10 crude traditional Chinese medical materials (Gypsum fibrosum, Fagopyrum dibotrys (D. Don) H. Haru, Houttuynia cordata Thunb., Ephedra sinica Stapf, Prunus armeniaca var. anu Maxim., Fritillaria cirrhosa D. Don, Ardisia japonica (Thunb.) Blume, Perilla frutescens (L.) Britton, Eriobotrya japonica (Thunb.) Lindl., Glycyrrhiza uralensis (Fish.). QKPCG were obtained from Changchun Lei Yun Shang Pharmaceutical Co., Ltd (QKPCG batch NO. Z20040047). Quality was ensured by strictly following the standards of the China Food and Drug Administration.

Animals

Eight-week-old male C57BL/6 mice were obtained from the Shanghai Jihui Laboratory Animal Care Co., Ltd and bred in the animal facility of the Zhongshan Hospital at Fudan University. The mice were divided into Control, lipopolysaccharides (LPS), and LPS + QKPCG groups. Mice in the LPS and LPS + QKPCG groups were administered an equal amount of phosphate buffered saline (PBS). Two hours following LPS administration, mice in the LPS + QKPCG group were administered QKPCG (10 g/kg) orally once a day for 3 days. All mice were sacrificed on Day 3. We also used Sftpc-DreER; Scgb1a1-CreER; R26-TLR mice to trace the fate of club cells, AT2 cells, and BASCs during lung repair and regeneration. These mice were obtained from the Bin Zhou laboratory at the Chinese Academy of Sciences.

All animal experiments in this study were approved by the Animal Care and Use Committee of Zhongshan Hospital at Fudan University.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

Lung tissues were fixed in 4% paraformaldehyde for up to 24 h and then embedded in paraffin. Subsequently, the paraffin-embedded lung tissues were stained with H&E as per standard histological staining protocols. For IHC staining, the paraffin-embedded lung tissues were sliced into 5-μm-thick sections. The sections were then incubated with specific antibodies against SCGB1A1 (DF6581, Affinity) and SFTPC (DF6647, Affinity).

Immunofluorescence

The collected lung tissues were fixed in 4% paraformaldehyde for 2 h at 4°C. Tissues were then washed three times using PBS, transferred to a tube containing 30% sucrose (dissolved in PBS), and incubated at 4°C for dehydration. Once lung tissue samples sank to the bottom of the tube, they were embedded and cut into 10 mm frozen sections. The frozen sections were then stained with 4’,6-diamidino-2-phenylindole (Beyotime Biotechnology). Finally, the sections were imaged under a fluorescence microscope (Eclipse C1; Nikon).

Morphological quantification of lung sections

The pathological assessment of lung injury included edema, alveolar, and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, necrosis, and alveolar hyaline membrane formation. Each feature was scored on a 0-to-3-point scale based on the severity of injuries. The score was the average of 10 observations per tissue section.

Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines, including interleukin-6 (IL-6), IL-1α, IL-1β, tumor necrosis factor-α (TNF-α), and transforming growth factor β (TGF-β) in the plasma and bronchoalveolar lavage fluid (BALF) were determined using respective ELISA kits—Mouse IL-6 DuoSet ELISA (DY406; R&D), Mouse IL-1 alpha/IL-1F1 DuoSet ELISA (DY400; R&D), Mouse IL-1 beta/IL-1F2 DuoSet ELISA (DY401; R&D), Mouse TNF-alpha DuoSet ELISA (DY410; R&D), and Mouse TGF-beta 1 DuoSet ELISA (DY1679; R&D), as per the manufacturer’s protocols.

Total RNA extraction, RNA-sequencing, and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the lung tissues of mice from the three groups (N = 3 in each group) using the
Western blot analysis

Lung tissue protein was extracted using radioimmunoprecipitation assay buffer with phosphatase and protease inhibitors (Beyotime Biotechnology). Protein concentration was determined using the bicinchoninic acid, protein assay kit (Beyotime Biotechnology) according to the manufacturer’s protocol. Approximately 30 μg of protein was loaded onto Tris-glycine polyacrylamide gels and then transferred to polyvinylidene fluoride membranes. The membranes were then blocked for 1 h using QuickBlock™ Blocking Buffer for Western Blot (P0252; Beyotime Biotechnology). Membranes were incubated with primary antibodies (SCGB1A1 [DF6581, Affinity], SFTPC [DF6647, Affinity], CHI3L1 [DF7223, Affinity], NFκB [8242S, CST], p-NFκB [3033S, CST], and β-Actin [4970, CST]) overnight at 4°C. Subsequently, the membranes were washed three times using Tris-buffered saline with Tween-20 and incubated with corresponding secondary antibodies (Anti-rabbit IgG, and horseradish peroxidase-linked Antibody [7074S, CST]) for 1 h.

Statistical analyses

All statistical analyses were performed using the GraphPad Prism 8.0 software (GraphPad). One-way analysis of variance was performed for three groups comparisons, whereas Student’s t-test was applied. For comparisons of two groups, p < 0.05 were considered as indicative of statistical significance.

RESULTS

QKPCG significantly alleviate LPS-induced ALI

To assess the therapeutic effects of QKPCG against lung injury, we established an LPS-induced murine model of ALI (Figure 1a). Survival analysis indicated that intragastrical administration of QKPCG significantly reduced LPS-induced mortality in the LPS + QKPCG group (Figure 1b). These results were further confirmed via H&E staining, which revealed less structural damage and lower inflammatory cell infiltration within the lung tissues of the LPS + QKPCG group mice compared to those from the LPS group (Figure 1c). Furthermore, the lung injury score indicated that damage in the LPS + QKPCG group was significantly lower than that in the LPS group (Figure 1d). Taken together, we concluded that QKPCG alleviated LPS-induced ALI and promoted the repair of damaged lung tissue.

QKPCG significantly reduce LPS-induced lung inflammation

To investigate how QKPCG alleviated lung damage in mice, the levels of a series of inflammatory cytokines were determined via RT-qPCR and ELISA. RT-qPCR indicated that the expression of IL-6, IL-1α, IL-1β, TNF-α, and TGF-β1, the key inflammatory factors in the cytokine storm, was significantly increased in the LPS group and significantly reduced in the LPS + QKPCG group (Figure 2a). Further, ELISA assays revealed that
FIGURE 1  QKPCG alleviate LPS-induced lung injury. (a) Experimental groups of C57BL/6 mice. (b) Survival curve. (c) H&E staining of mouse lung tissues. (d) Lung injury score. ****p < 0.0001. H&E, hematoxylin and eosin; LPS, lipopolysaccharide; QKPCG, Qingke Pingchuan granules.

FIGURE 2  Changes of inflammatory cytokine expression in the Control, LPS, and LPS + QKPCG groups. (a) Relative expression of IL-6, IL-1α, IL-1β, TNF-α, and TGF-β mRNA. (b) The concentration of IL-1α, IL-1β, TNF-α, and TGF-β in BALF, as determined via ELISA. (c) IL-6 concentration in BALF and plasma, as determined via ELISA. ns > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; QKPCG, Qingke Pingchuan granules; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor-α.
IL-1α and TNF-α levels were significantly increased in the BALF of mice from the LPS group and decreased in the LPS + QKPCG group (Figure 2b). Furthermore, QKPCG tended to lower the concentration of IL-1β and TGF-β1 in BALF, even though these changes were not statistically significant. IL-6 in the BALF and plasma exhibited the same trend as observed in the RT-qPCR results (Figure 2c).

**Scgb1a1 and Sftpc genes are critical in the QKPCG-mediated repair of ALI**

To further investigate the molecular mechanisms underlying the QKPCG-mediated alleviation of ALI, we performed RNA-sequencing analysis. The numbers of DEGs with log2 fold change ≥1.5 are shown in Figure 3a. As depicted in the Venn diagram, we identified 179 upregulated and 187 downregulated genes across the three groups (Figure 3a,b). Of these, Scgb1a1 and Sftpc were the top two downregulated, and Chi3l1 and Anxa2 were the top two upregulated genes. We subjected DEGs to Gene Ontology (GO) enrichment analysis as per biological process, cellular component, and molecular function categories. The top 10 enriched GO terms are listed in Figure 3c. Furthermore, KEGG pathway analysis yielded the top 10 pathways enriched by DEGs (Figure 3d).

Interestingly, DEG analysis indicated that the expression of Scgb1a1 and Sftpc was significantly increased, while that of Chi3l1 was significantly decreased in the LPS + QKPCG group relative to the LPS group. We then validated the role of these three genes in the QKPCG-mediated repair of LPS-induced ALI. To verify the results of RNA-sequencing analysis, we performed RT-qPCR. Compared to the Control group, the expression of Scgb1a1 and Sftpc was significantly decreased in the LPS group and recovered in the LPS + QKPCG group (Figure 4a). Consistent with these findings, western blot analysis indicated that CC10 and SFTPC expression was significantly decreased in the LPS group and significantly increased in the LPS + QKPCG group (Figure 4b). Western blot analysis quantitative analysis also revealed the same change trend (Figure 4d). Furthermore, IHC results indicated that CC10 and SFTPC protein were extremely decreased in the LPS group but presented...
recovery trend in LPS + QKPCG group (Figure 4c). Further, IHC quantitative analysis also revealed that CC10 and SFTPC protein were significantly reduced after LPS attack, but greatly restored with QKPCG treatment (Figure 4e).

We used Sftpc-DreER; Scgb1a1-CreER; R26-TLR mice to trace the fate of the three cell populations, namely CC10−SFTPC+ AT2, CC10−SFTPC−club, and CC10+ SFTPC+(BASC) populations. tdTomato (red) and ZsGreen (green) fluorescence represented CC10 and SFTPC, respectively.17 As shown in Figure 5a, there were no evident fluorescence signal in LPS group, which meant no alive CC10−SFTPC+ AT2, CC10−SFTPC−club, or CC10−SFTPC+ (BASC) populations. However, applied with QKPCG treatment, the disruption of ZsGreen+tdTomato−club cells in the bronchi and ZsGreen−tdTomato+ AT2 cells in the alveoli after LPS attack significantly decreased. The restoration of ZsGreen+tdTomato+ double-positive cells in bronchoalveolar duct junctions was also noted (Figure 5a). Cell number count of AT2 cells, club cells, and BASCs also proved the less disruption of lung cells after LPS attack with QKPCG treatment (Figure 5b).

These results indicated the decreased disruption of lung progenitor cells/stem cell populations and the restoration of lung function after QKPCG treatment.15,20 Collectively, our results suggested that CC10 and SFTPC may play important roles in the QKPCG-mediated repair of LPS-induced ALI. Furthermore, consistent with RNA-sequencing results, RT-qPCR and western blot analyses showed that the gene and protein levels of CHI3L1 were significantly increased in the LPS group and decreased in the LPS + QKPCG group (Figure 4a–d).

**CC10 alleviates LPS-induced ALI**

Next, to verify the function of CC10 in the QKPCG-mediated alleviation of ALI, C57BL/6 mice were divided into Control, LPS, and LPS + CC10 groups. H&E staining revealed the protective effect of CC10 (Figure 6a). Compared with the mice in the LPS group, those in the LPS + CC10 group exhibited less structural damage in the lungs and inflammatory cell infiltration. The injury score also indicated that the lung injury in the
**Figure 5** Immunofluorescence staining of lung tissue. (a) x63 magnification. (b) Cell number count of AT2 cells, club cells, and BASCs. 

\[ (**p < 0.0001) \]

AT2, alveolar type II; BADJ, bronchioalveolar-duct junctions; BASCs, bronchioalveolar stem cells; TB, terminal bronchi.

**Figure 6** CC10 alleviates lung injury induced by lipopolysaccharide. (a) Hematoxylin & eosin staining. (b) Lung injury score.

\[ (**p < 0.0001) \]

CC10, Clara cell 10; LPS, lipopolysaccharide.
LPS + CC10 group was significantly reduced compared to that in the LPS group (Figure 6b).

**CC10 alleviates LPS-induced lung inflammation**

To investigate the anti-inflammatory function of CC10, we detected the concentrations of IL-6, IL-1α, IL-1β, and TNF-α in BALF. ELISA results indicated that the levels of these inflammatory cytokines were significantly decreased in the LPS + CC10 group compared to in the LPS group (Figure 7). Thus, CC10 could attenuate LPS-induced inflammatory cytokine release for the alleviation of lung injury.

**CC10 inhibits the LPS-induced phosphorylation of NFκB**

NFκB is a family of critical transcription factors that coordinate inflammatory responses as well as cellular differentiation, proliferation, and survival. NFκB activation is one of the major cellular responses to LPS stimulation. Western blot analysis results revealed a significantly reduced level of p-NFκB in the LPS + QKPCG group compared to the LPS group (Figure 8a), indicating that the protective effect of QKPCG may be partially mediated via the suppression of NFκB signaling. A recent study showed that CC10 could suppress the phosphorylation of NFκB to regulate the release of inflammatory factors. Western blot analysis of NFκB and p-NFκB in...
lung tissues revealed that CC10 significantly inhibited the phosphorylation of NFκB (Figure 8b).

**DISCUSSION**

Patented TCM formulations have gained increasing importance in the clinical treatment of various diseases, particularly during early-phase interventions. For example, Lianhua Qingwen capsules, Toujie Quwen granules, and Jinhua Qinggan granules have been shown to reduce lung tissue damage, eliminate virus infection, and slow the progression of diseases at an early stage. To optimize the use of TCM formulations, elucidating their exact mechanisms of action is urgently needed. In the current study, we employed an LPS-induced ALI mouse model to evaluate the therapeutic efficacy of QKPCG. Through a set of analyses involving H&E staining, lung injury assessment, and ELISA, we demonstrated that QKPCG could effectively alleviate LPS-induced lung tissue injury and reduce inflammation infiltration. The decrease of inflammation could greatly improve pulmonary circulation which meant less disruption of endothelial permeability and integrity. Importantly, QKPCG protected mice against LPS-induced death, as indicated by the higher survival in the LPS + QKPCG group than in the LPS group. In other words, our findings provide evidence for the protective effect of QKPCG against lung injury.

Next, we performed RNA-sequencing to screen for genes and pathways implicated in the therapeutic effect of QKPCG. RNA-sequencing results suggested that Scgb1a1 and Sftpc may play key roles in modulating the beneficial effects of QKPCG. We performed a series of experiments to validate RNA-sequencing. In brief, RT-qPCR, western blot analysis, and IHC confirmed the QKPCG-induced upregulation of CC10 and SFTPC following LPS-induced injury. The increased levels of CC10 produced by club cells may be related to the decreased disruption of club cells. To validate this hypothesis and directly observe the restoration of lung club cells, we used Sftpc-DreER; Scgb1a1-CreER; R26-TLR mice, in which the three progenitor/stem cell populations, namely club cells, AT2 cells, and BASCs, are labeled and can be tracked. Compared with the LPS group, we observed a significant recovery of all three populations in the LPS + QKPCG group, which was associated with the decreased disruption of these cells, indicating the restoration of lung function following QKPCG treatment. Taken together, we believe QKPCG can alleviate lung injury and promote lung repair, which may be mediated via decreasing the disruption of club cells and increasing production of CC10.

Next, to validate the possible role of CC10 in QKPCG-induced protection against lung injury, we treated ALI...
model mice with exogenous CC10. H&E staining and ELISA demonstrated that exogenous CC10 supplementation significantly reduced lung damage and inflammatory cytokine secretion in the LPS + CC10 group compared to those in the LPS group. These results confirmed that CC10 can inhibit the LPS-induced inflammatory response.

An accumulating body of evidence has shown that the NFκB pathway plays a central role in LPS-induced inflammation. In our study, we demonstrated that QKPCG can significantly suppress the phosphorylation of NFκB following LPS stimulation, as a potential mechanism underlying its protective effects. Reportedly, the CC10 protein can exert an anti-inflammatory effect by regulating NFκB signaling. These findings led us to hypothesize that QKPCG suppresses the NFκB pathway by upregulating CC10. In concordance with the results of QKPCG treatment, exogenous supplementation of CC10 significantly inhibited NFκB phosphorylation. Taken together, our findings suggest that CC10 inhibits the inflammatory response by blocking NFκB phosphorylation.

Finally, RT-qPCR and western blot analysis revealed that the expression of CHI3L1 was significantly decreased in QKPCG-treated mice, corroborating the RNA-sequencing results. Previous studies have shown that the CHI3L1 protein, secreted by a variety of cells and regulated by inflammatory cytokines such as IL-6, IL-1, and TNF-α, is associated with inflammation. High expression levels of CHI3L1 are reported to indicate a poor outcome. Furthermore, CHI3L1 expression can be regulated by CC10. Taken together with the above results of increased CC10 and decreased CHI3L1 expression, it can be inferred that the anti-inflammatory effect of CC10 may be involved in QKPCG-mediated lung injury repair.

In the present study, we demonstrated that QKPCG effectively reduce lung inflammation, promote the regeneration of club cells, and increase CC10 levels. Moreover, CC10 blocked the LPS-induced phosphorylation of NFκB to alleviate inflammation. Taken together, we provide evidence for the therapeutic efficacy of QKPCG against ALI, mediated at least in part via the upregulation of CC10 protein, as a critical mechanism of action.

**AUTHOR CONTRIBUTIONS**

*Study concept and design:* Jian Zhou, Jie Shen, and Yuanlin Song. *Experiments and data collection:* Yuanyuan Wu, Wensi Zhu, Ainiwaer Rouzi, Lin Tong, Linxiao Han, Juan Song, Jianwen Ding, Yu Yan, Miao Li, Ting Pan, Jie Liu, and Qin Wang. *Data interpretation and statistical analysis:* Yuanyuan Wu, Wensi Zhu, Jian Rouzi, Lin Tong, and Linxiao Han. *Drafting of the manuscript:* Yuanyuan Wu and Jian Zhou. *Study supervision:* Jian Zhou, Jie Shen, and Juan Song.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ETHICS STATEMENT**

All animal experiments in this study were approved by the Animal Care and Use Committee of Zhongshan Hospital at Fudan University (2019-107).

**ORCID**

Yuanyuan Wu  https://orcid.org/0000-0001-6262-1513
Jian Zhou  http://orcid.org/0000-0003-4736-9457

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