

Pudilan Xiaoyan oral liquid regulates tissue inflammation and apoptosis in mice with influenza virus pneumonia

Shan Cao¹, Shuang-Rong Gao¹, Chen Ni¹, Zi-Han Geng¹, Ying-Li Xu¹, Bo Pang¹, Meng-Ping Chen¹, Yu Zhang¹, Shan-Shan Guo¹, Yu-Jing Shi¹, Li-Qi Ni², Kun Wang², Rong-Hua Zhao¹, Xiao-Lan Cui^{1*}, Yan-Yan Bao^{1*} 

¹Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China. ²Jumpcan Pharmaceutical Group Co., Ltd., Taizhou 225400, China.

*Correspondence to: Yan-Yan Bao, Xiao-Lan Cui. Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, No. 16, Nanxiao Street, Dongzhimen, Dongcheng District, Beijing Municipality, PRC, Beijing 100700, China. E-mail: baoyanyan885@163.com; cuixiaolan2812@126.com.

Author contributions

Bao YY and Cui XL designed the study and helped coordinate support and funding. Cao S conducted research and wrote manuscripts. Pang B, Gao SR, Xu YL, Geng ZH, Chen MP, Zhang Y and Ni C participated in the experiments. Guo SS, Shi YJ, Ni LQ, Wang K and Zhao RH participated in the study design. Cao S performed the statistical analysis. Cao S helped draft the manuscript. Bao YY revised the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

Acknowledgments

This research was funded by Scientific and Technological Innovation Project of China Academy of Chinese Medical Sciences, grant number CI2021A04608 and National Natural Science Foundation of China, grant number 82141206.

Peer review information

Traditional Medicine Research thanks all anonymous reviewers for their contribution to the peer review of this paper.

Abbreviations

IAV, influenza A virus; PDL, Pudilan Xiaoyan oral liquid; PRR, pattern recognition receptors; TCM, traditional Chinese medicine; TLR, Toll-like receptor; TIR, toll-interleukin-1 receptor; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; ALI, acute lung injury; ICR, institute of cancer research; PDL-M, PDL moderate-dose; PDL-L, PDL low-dose; SFJD, ShuFengJieDu capsule; OP, Oseltamivir phosphate capsules; Bcl-2, B-cell lymphoma-2.

Citation

Cao S, Gao SR, Ni C, et al. Pudilan Xiaoyan oral liquid regulates tissue inflammation and apoptosis in mice with influenza virus pneumonia. *Tradit Med Res.* 2024;9(6):36. doi: 10.53388/TMR20231010001.

Executive editor: Xi-Yue Liu.

Received: 10 October 2023; Accepted: 30 January 2024;

Available online: 31 January 2024.

© 2024 By Author(s). Published by TMR Publishing Group Limited. This is an open access article under the CC-BY license. (<https://creativecommons.org/licenses/by/4.0/>)

Abstract

Background: The influenza A virus is the primary cause of respiratory infections and poses a global health risk. Pudilan Xiaoyan oral liquid (PDL) exhibits anti-inflammatory and immunomodulatory properties. PDL is commonly employed in clinical practice to manage upper respiratory tract infections. However, there is still much to uncover regarding its potential therapeutic mechanism. **Methods:** Institute of cancer research mice were infected with influenza A virus via nasal drip. The general state of the mice, lung index, and lung index inhibition rate were used to evaluate the efficacy of PDL. Enzyme-linked immunosorbent assay, western blotting, and immunohistochemistry were used to observe the presence of proteins and cytokines in the lung tissue. Apoptosis was evaluated using the TUNEL assay. **Results:** PDL improved the mental state of influenza A virus-infected mice, reduced the lung index, and inhibited viral replication. The expression of interleukin-1 β and tumor necrosis factor- α were decreased, whereas the expression of interleukin-10 in the lung tissue was increased due to PDL treatment. In addition, PDL treatment modulated Toll-like receptor 4 and MyD88 expressions in the lung tissues. PDL significantly reduced apoptosis and decreased cleaved caspase-3 and PARP levels, whereas increased B-cell lymphoma-2 expression in the lung tissue. Notably, the moderate-dose group of PDL exhibited a more pronounced effect. These findings indicate that PDL exerts a protective effect against pneumonia injury in influenza A virus-infected mice. **Conclusion:** PDL inhibited the inflammatory response and regulated apoptosis by regulating Toll-like receptor 4 and MyD88 protein expressions, thereby protecting the lung tissue from viral infection-induced lung tissue injury.

Keywords: Pudilan Xiaoyan oral liquid; influenza A virus; immune response; apoptosis; Toll-like receptors

Highlights

Pudilan Xiaoyan oral liquid is used clinically for the treatment of infectious diseases of the upper respiratory tract. Pudilan Xiaoyan oral liquid is involved in the treatment of viral pneumonia caused by influenza virus infection by attenuating lung tissue inflammation and apoptosis as well as regulating the expression of Toll-like receptor 4 and MyD88.

Medical history of objective

Pudilan Xiaoyan oral liquid is a traditional Chinese medicine preparation that consists of four types of herbs. *Taraxaci Herba* and *Isatidis Radix* are derived from Su-Jing's *Newly Revised Materia Medica* (659 C.E.). *Corydalis Bungeanae Herba* and *Scutellaria baicalensis* are derived from Li-Shizhen's *The Compendium of Materia Medica* (1590 C.E.). In ancient times, it has the function of clearing heat and removing toxin (a common treatment in Chinese medicine. Generally manifested as high fever, redness and heat, constipation, yellow urine and irritability). Modern pharmacological studies have reported that it has a variety of bioactivities, such as antibacterial, antiviral, and immune function-enhancing effects.

Background

Viral pneumonia poses a significant risk to human health as it involves inflammation of the lung parenchyma resulting from a viral infection originating from the upper respiratory tract [1]. Influenza viruses are common respiratory viruses, of which influenza A virus (IAV) is a major pathogen that causes infections in humans and animals [2]. IAV leads to seasonal infections, with 3–5 million cases of severe disease every year as well as sporadic pandemics, which pose a great threat to human health and public health security [3]. Influenza viruses have a wide host range and can spread rapidly. Global influenza prevention and control face numerous challenges. Currently, the main drugs used to treat influenza are anti-influenza virus drugs, and the most effective way to prevent influenza infection and its complications is vaccination against influenza. Currently, antiviral drugs are becoming less effective due to their side effects and the emergence of drug-resistant mutants [4, 5]. Influenza viruses enter the respiratory tract through direct viral infection or weakened immune response [2]. Upon infecting the host, the emergence of pattern recognition receptors (PRRs) within natural immune cells enables the identification of IAV, subsequently triggering a cascade of signalling events that culminates in the production of numerous cytokines. Toll-like receptor 4 (TLR4), a crucial member of the TLR family, plays a significant role in the immune response of organisms. TLR4 actively triggers intracellular signalling pathways, thereby controlling the secretion of pro-inflammatory molecules. TLR4 promotes the inflammatory response by initiating cytokine release [6]. The regulation of these cytokines may affect the severity of inflammatory complications caused by the virus and ultimately reduce the mortality rate [7, 8]. In addition, TLR4 not only induces inflammatory processes but also participates in apoptosis [9–11].

TLR4 is a predominant PRR that primarily regulates inflammation in infectious disease [12]. Moreover, it exhibits a strong association with influenza virus [13–15]. The TLR/MyD88 signalling pathway is closely related to influenza virus pneumonia and plays a vital role in the signalling pathway of TLRs [16]. Numerous studies have demonstrated that the silencing of TLR4 mitigates liver injury induced by lipopolysaccharide (LPS) and diminishes inflammation and apoptosis in the tissue [17]. XIAO-QING LI et al. found that activation of the TLR4/MyD88/NF- κ B signalling pathway reduces inflammatory responses, oxidative stress, and apoptosis, and down-regulates the release of oxidants in skin tissues [18]. Furthermore, the significant involvement of TLR4 in the development of acute lung injury (ALI) has been well documented [19–22]. The inhibitory effects of

Houttuynia cordata polysaccharides on ALI induced by the influenza virus in mice have been demonstrated. These effects are accomplished through the modulation of toll-interleukin-1 receptor (TIR) signalling pathways [23]. LPS functions as an agonist for TLR4, activating the NF- κ B via TIR domain-containing adaptors in both the interferon- β and MyD88 pathways [24, 25]. LPS-induced mice are most commonly used in existing studies on pneumonia. Jinhua Qinggan granules have been shown to have protective effects against LPS-induced ALI in mice. This effect is achieved by reducing inflammation in the lungs through promoting neutrophil apoptosis and regulating the TLR4/MyD88/NF- κ B pathway [26]. TLR4 is primarily known for its ability to recognise bacterial LPS as part of an immune response against bacterial infections [27]. However, its involvement in viral infections has not been well established. Although TLR4 is primarily associated with bacterial recognition, there are indications that it may also recognise viral ligands. TLR4 expression increases or decreases in tissues after viral infection [6, 15, 28, 29, 30, 31]. The role of TLR4 in viral infections, specifically in lung inflammation caused by influenza virus, requires further investigation and understanding [32, 33]. Further research is required to comprehensively explore this relationship and the role of MyD88 in this process.

Formulations of traditional Chinese medicine (TCM) have been extensively studied for their efficacy against influenza viruses. These remedies exhibit a dual mechanism of action: direct suppression of viral replication and mitigation of exaggerated inflammatory reactions, thereby facilitating tissue repair following influenza viral infection. Pudilan Xiaoyan oral liquid (PDL) is a TCM preparation that consists of four types of herbs, namely *Taraxaci Herba*, *Corydalis Bungeanae Herba*, *Isatidis Radix* and *Scutellariae Radix* [34]. PDL clears heat, detoxifies, exerts anti-inflammatory effects, and reduces swelling. Clinical and experimental studies have demonstrated its effectiveness in treating pneumonia. PDL has been shown to have significant viral suppression, symptom relief, and improvement in lung inflammation in COVID-19-infected mice [34]. PDL plays a role in antiviral activity and has broad-spectrum antiviral effects against influenza and parainfluenza viruses. In this study, we focused on the involvement of PDL in viral pneumonia induced by influenza virus infection. This potential mechanism may be associated with the mitigation of inflammation and apoptosis in lung tissues, as well as the regulation of TLR4 and MyD88 expression.

Materials and methods

Viruses and experimental animals

The mouse-adapted influenza virus FM/1/47 (H1N1) was purchased from ATCC (Manassas, VA, USA). The virus was stored in the ABSL-2 laboratory, Institute of traditional Chinese medicine, Chinese Academy of Sciences, in an environment at -80°C . SPF healthy male institute of cancer research (ICR) mice (13–15 g, 4 weeks) and SPF healthy female ICR (13–15 g, 4 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) (male: No. 110011221103182122; female: No. 110011221103182021; SCXK (Beijing) 2021-0006). All mice were housed under standard laboratory conditions and fed specific pathogen-free quality food and purified water. The Animal Ethics Committee of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences approved the animal experiments and the corresponding protocol (2020D007). All experimental steps were performed in strict accordance with the guidelines for the care of laboratory animals (Ninth Edition, National Institute Bethesda Health, Maryland, USA). In addition, this study was conducted in accordance with the ARRIVE standard guidelines while implementing procedures to minimise test animals and potential hazards.

Medicine and reagents

PDL was purchased from Jumpcan Pharmaceutical Co. Ltd. (2012604; Hubei, China). Each bottle of oral liquid contained 10 mL, equivalent to 10.01 g of traditional Chinese medicine. ShuFengJieDu capsule (SFJD) was purchased from Anhui Jiren Pharmaceutical Co., Ltd.

(3201020; Anhui, China). Oseltamivir phosphate capsules (OP) were provided by Yichang HEC Changjiang Pharmaceutical Co., Ltd. (0221903034; Dongguan, China). A protein extraction kit was provided by Beijing Solarbio Science & Technology Co., Ltd. (20220224; Beijing, China). ELISA for TLR4 and MyD88 in mice was performed by LAIZEE BIOTECH Co., Ltd. (RDR-TLR4-Mu; RD-MyD88-Mu; Beijing, China). Mouse Caspase-3 and Mouse B-cell lymphoma-2 ELISA kits were provided by Andy Gene (202302, 202304; Beijing, China). Influenza Virus A Real Time RT-PCR Kit was provided by Shanghai ZJ Bio-Tech Co., Ltd. (20230301; Shanghai, China). Anti-TLR4, anti-MyD88, and anti-PARP antibodies were provided by Proteintech (66350-1-AP, 67969-1-AP, 13371-1-AP; Wuhan, China), while anti-tubulin and anti-influenza A Virus M2 antibodies were purchased from Abcam (GR123235-14 and ab313889; Cambridge, MA, USA). Anti-caspase-3 and anti-B-cell

lymphoma-2 (Bcl-2) antibodies were purchased from Abmart (T40044; T40056, Shanghai, China). Super ECL Plus kit was provided by BIORIGIN (BN16009; Beijing, China).

Infection and treatment

Seventy ICR mice were randomised into the following groups: control, model, OP, SFJD, PDL high-dose, PDL moderate-dose (PDL-M), and PDL low-dose (PDL-L) groups, with half each of male and female mice in each group. Apart from the control group, mice were infected with 35 μ L of 100TCID₅₀ FM1 virus solution through the nose after light anaesthesia. In this study, treatment was started 1 h after infection in mice. The drug was administered to each group by gavage for a duration of 4 days. The dosage of the drug was calculated as 0.2 mL per 10 grams of body weight. Figure 1A shows the details of the administration process. The daily dose of PDL was maintained

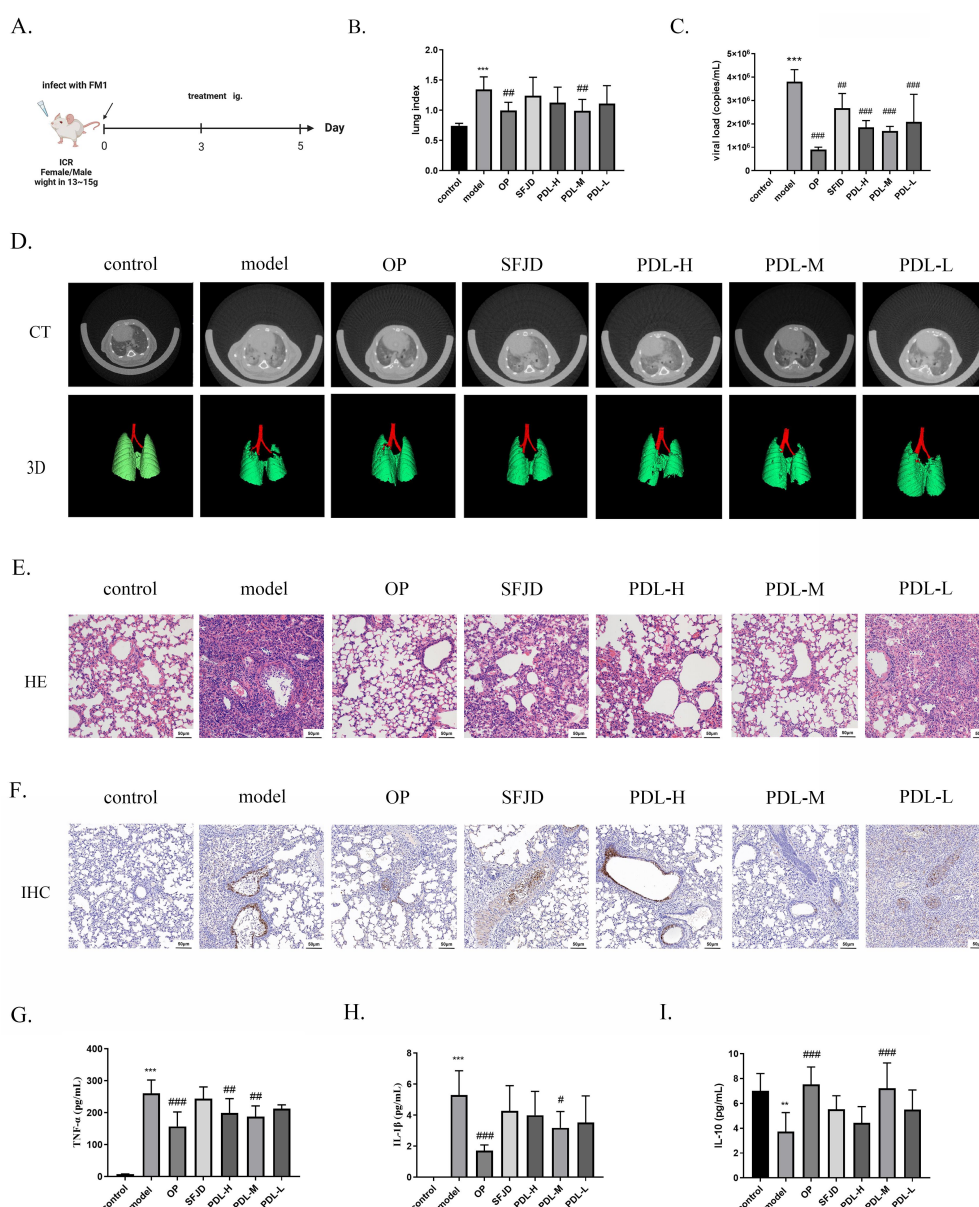


Figure 1 PDL protects against influenza virus infection in mice in vivo. (A) FM1-infected ICR mouse model. (B) Changes in the lung index of mice in each group (n = 10). (C) Virus load of mice in each group (n = 10). (D) Micro CT scanning results of the lung tissues of different groups of mice. (E) Histopathological results of the lung tissues of different groups of mice (×200). (F) IHC results of M2 protein in different groups of mouse lung tissues (×200; positive result: brown). (G) The concentrations of TNF- α in lung tissue (n = 6). (H) The concentrations of IL-1 β in the lung tissue (n = 6). (I) The concentrations of IL-10 in the lung tissue (n = 6). Data are shown as mean $\pm$ SD. Comparison with the control group, * P < 0.05, ** P < 0.01, *** P < 0.001; Comparison with the model group, # P < 0.05, ## P < 0.01, ### P < 0.001. CT, computed tomography; HE, Hematoxylin-eosinstaining; IHC, immunohistochemistry; OP, Oseltamivir phosphate capsules; SFJD, ShuFengJieDu capsule; PDL-H, PDL high-dose group; PDL-M, PDL moderate-dose group; PDL-L, PDL low-dose group; IL, interleukin; TNF, tumor necrosis factor; ICR, institute of cancer research.

according to the dosage for clinical use: the clinical dosage in humans is 30 mL/60 kg per day, and the experimental doses in mice were 11, 5.5, and 2.75 mL/kg/d, equivalent to two, one, and 0.5 clinical doses respectively. All the mice were observed at the end of each day of gavage. SFJD and OP were administered at doses of 1.144 g/kg/d and 27.5 mg/kg/d, respectively. The survival and body weight loss rates were calculated for each group of mice [35].

Lung index and inhibition rate of the lung index

All the mice were weighed and dissected on the 4th day after infection to remove the lungs. The lung index and inhibition rate of the lung index were calculated using the following equations [36]:

$$\text{Lung Index} = \frac{\text{Lung Wet Weight (g)}}{\text{Body Weight (g)}} \times 100 \quad (1)$$

Inhibition rate =

$$\frac{(\text{average lung index of model control group} - \text{average lung index of administration group})}{(\text{average lung index of model control group} - \text{average lung index of normal control group})} \times 100\% \quad (2)$$

Micro computed tomography scan and histopathological screening

Each group of mice was scanned with a micro computed tomography scanner (PE Quantum GX2). On day 4 of infection, the mice were anaesthetised by isoflurane inhalation and CT scans were performed. The parameter settings were 90 kV, 88 mA, and 36 mm, with a scanning time of 2 min per mouse. The fixation process involved immersing the left lung in a 4% solution of polymethyl aldehyde for a minimum of 7 days. Following dehydration using a series of alcohol gradients and paraffin embedding, tissue sections were obtained. The sections were sequentially treated with xylene I and II for 20 min each, followed by 100% ethanol I and II for 5 min each. They were then treated with 75% ethanol for 5 min and rinsed with tap water. The sections were then treated with a haematoxylin differentiation solution and haematoxylin Scott's tap bluing. The films were sealed with a neutral gum and observed under a microscope for inspection, image acquisition, and analysis.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, and the appropriate tissue size was placed in an embedding box for dehydration and embedding. Sections were prepared for histochemical staining (Anti-Influenza A Virus M2 antibody, ab313889; Abcam, Cambridge, MA, USA).

Biochemical analysis

On day 4 post-infection, the mice were dissected and lung tissues were collected to examine the levels of relevant factors and proteins using ELISA. Proteins were extracted from lung tissue using a protein extraction kit. The cytokines interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-10 were analysed using LUMINEX. Additionally, TLR4, MyD88, Caspase-3, and Bcl-2 protein expression levels were assessed by ELISA.

Tunel staining

To detect apoptosis in the lung tissue, we used the TUNEL kit to stain the paraffin sections of lung tissue. Paraffin-embedded sections were stained according to the manufacturer's instructions. Haematoxylin-stained cell nuclei are blue, and DAB staining shows positive apoptotic cell nuclei in brownish-yellow. The number of apoptotic cells was counted at high magnification. At least 500 cells were observed in each slice, and the number of positive cells within every 100 cells was counted as the apoptosis index.

WB and real-time polymerase chain reaction

The protein samples were separated in 10% polyacrylamide gels using electrophoresis after loading equal amounts of protein (60 μ g/lane). After separation, proteins were transferred to Millipore PVDF membranes. To detect specific proteins, cells were incubated

overnight at 4 °C with diluted antibodies against tubulin, TLR4, MyD88, caspase-3, Bcl-2, M2, and PARP. The membranes were washed with TBST three times before incubation with horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG. This secondary antibody was diluted 1:5,000 in TBST and the incubation lasted for 1 h at 37 °C. After another three washes with TBST, protein bands were detected using a Super ECL Plus kit. To analyse the target bands, the scanned images were processed using the ImageJ software. In addition to studying protein expression, the researchers also extracted total RNA samples from the lung tissue using TRIzol reagent. The extraction procedure followed the instructions provided with the Influenza Virus A Real Time RT-PCR Kit. This assay allowed the researchers to analyse samples for specific RNA targets.

Statistical analysis

GraphPad Prism 7.0 software (GraphPad, San Diego, CA, USA) was employed to perform all data processing and analysis. The means $\pm$ SD were used for presenting the data. To evaluate the differences between multiple groups, a one-way analysis of variance was utilized. The significance of the distinctions between groups was assessed using Student's *t*-test. A significance level of *P* < 0.05 was considered to indicate statistically significant differences in the data.

Results

PDL protects against influenza virus infection in mice in vivo

To investigate the efficacy of PDL, we established a mouse model of viral pneumonia caused by nasal drip infection and assessed the disease status of the mice after PDL treatment. On the 4th day after infection the mice were euthanized, and the thoracic cavity of the mice was fully exposed, and the lung tissue was dissected and weighed. The survival rate was 100% in all the groups, whereas the weight loss rates were 28.65, 12.92, 26.08, 24.11, 21.84, and 28.15% in the model, OP, SFJD, PDL high-dose, PDL-M, and PDL-L groups, respectively. Influenza virus infection causes viral pneumonia in mice, and inflammatory exudation increases lung mass; the higher the value of the lung index, the more severe the inflammatory lung lesion [37]. The lung index of mice in the model group was substantially higher than that of mice in the control group, indicating a notable disparity (*P* < 0.001). This suggests that the lung tissue was infected with FM1, and tissue oedema was increased. Furthermore, the lung index was reduced in the other treatment groups compared to that in the model group. Treatment with OP or PDL-M significantly reduced the lung index (*P* < 0.01) (Figure 1B). To assess the extent of viral replication in the lung tissue of mice infected with the influenza virus, we used real-time fluorescent quantitative PCR to assess the viral load in the lung tissue. H1N1/FM1 nucleic acid expression was not detected in the lungs of control mice. Viral RNA was positively expressed in the lung tissues of the model group (*P* < 0.001). Compared to the model group, the viral load was significantly reduced in each dosing group (*P* < 0.001; *P* < 0.01) (Figure 1C).

To further evaluate the role of PDL in influenza virus-infected mice, we evaluated the histopathological status of the lung by micro CT and H&E staining. Micro CT has proven to be a safe and very valid tool for longitudinal visualisation of the lung tissue. The Micro CT results showed uniform density in the control group, and the corresponding three-dimensional stereograms showed intact lung tissue structures with no tissue defects. Diffuse infiltrative shadows and dense enhancing shadows were present in the lung tissues of the model mice, and their 3D stereogram construction showed local defects in the lung tissues and a significant decrease in lung tissue volume compared with the normal group, suggesting the presence of local lesions. After PDL treatment, lung density decreased, dense enhancing shadows reduced, and 3D stereogram construction revealed a reduction in localised defects in the lung tissue and lesion area. Comparing the treatment effects of the three PDL doses, the effect of PDL-M was evident (Figure 1D). As shown in Figure 1E, in the control group, bronchial epithelial cells were intact, alveolar structures were intact, and no inflammatory cells or erythrocyte infiltration was observed in the bronchial lumen

or alveolar cavity. The lung tissue lesions in the model group were severe, with significant thickening of some interalveolar walls, an unclear structure, and obvious infiltration of inflammatory exudates, accompanied by erythrocyte infiltration. The lung tissue sections of the OP group mice were similar to those of the control group, and the lung tissue lesions were mild. In the PDL administration group, the PDL-M group had milder lung histopathological changes, relatively intact bronchial epithelial cells, relatively intact alveolar structures, and reduced erythrocytes and inflammatory exudates compared to the model group. Influenza A virus M2 proteins are type I transmembrane proteins distributed throughout the viral membrane [38]. The immunohistochemistry results revealed no positive expression in the control group, a significant increase in positive expression in the model group, and a decrease in positive expression of M2 protein in the lung tissue after PDL administration, with the best results observed in the PDL-M group (Figure 1F).

There was a notable elevation ($P < 0.001$) observed in the levels of TNF- α and IL-1 β , key inflammatory factors, in the pulmonary tissue of mice belonging to the experimental group when compared to those in the control group. Compared with the model group, the expression levels of the inflammatory factors, TNF- α and IL-1 β , in the lung tissues of mice in each administration group were reduced to different degrees. In addition, the expression levels of inflammatory factors in the OP and PDL-M groups were significantly decreased ($P < 0.05$, $P < 0.01$, $P < 0.001$, Figure 1G, 1H). CD8 $^{+}$ T cells produce the anti-inflammatory IL-10 to reduce and resolve inflammation during an IAV infection [14]. The expression levels of IL-10 in the lung tissues of infected mice were significantly reduced ($P < 0.01$), whereas those in the OP and PDL-M groups were significantly increased ($P < 0.001$; Figure 1I).

Overall, PDL reduced the lung index, decreased viral replication in the lung, inhibited the expression and secretion of inflammatory factors, protected the subunit structure of the lung, and prevented further development of viral pneumonia. In terms of the quantitative effect relationship, PDL-M were effective in treating viral pneumonia.

PDL regulates TLR4 and MyD88 expressions in the lung tissue of

influenza virus mice

M2 protein expression increase in the model group was significant compared to that in the control group and a statistically significant decrease in M2 protein expression after administration of PDL ($P < 0.05$; Figure 2A, 2B). TLRs, which play a crucial role in the body's natural defence against microbial infections, are vital proteins involved in natural immunity [39]. To gain a deeper understanding of how PDL treats viral pneumonia, we studied the expression of TLR4 and MyD88 in the lung tissues. ELISA revealed a significant decrease in the expression levels of TLR4 and MyD88 in the model group compared to that in the control group ($P < 0.001$). However, in the PDL dose group, the expression levels of TLR4 and MyD88 increased slightly, with a particularly significant increase observed in the PDL-M group ($P < 0.001$ and $P < 0.05$; Figure 2C, 2D). Additionally, we assessed the protein expression levels of TLR4 and MyD88 in the lung tissue of mice infected with the influenza virus. Western blotting analysis revealed that the levels of TLR4 and MyD88 proteins were notably reduced in the model group. This decrease in protein expression was statistically significant ($P < 0.05$; Figure 2E–2G). Compared to the model group, the PDL dose group was able to upregulate TLR4 and MyD88 proteins, in which PDL-M significantly upregulated TLR4 and MyD88 protein expression, and the expression in each dosing group was lower than that of the control group ($P < 0.05$; Figure 2E–2G).

PDL protects against apoptosis by inhibiting Caspase-3 expression and promoting Bcl-2 expression

Apoptosis is a well-coordinated process, which in many cases relies on energy and entails the activation of a set of cysteine proteases known as “cysteine specific proteinase” (caspases), and it involves a sophisticated cascade of events that connect the initial stimuli ultimately leading to cell death [40, 41]. The TUNEL kit, which is commonly used for evaluating apoptosis levels in various tissues, was employed to measure apoptosis levels specifically in the lung tissue. As shown in Figure 3A, positive signals of TUNEL staining were detected mainly in the fine bronchial and alveolar epithelial cells of the lungs. The apoptotic index of the model group exhibited a

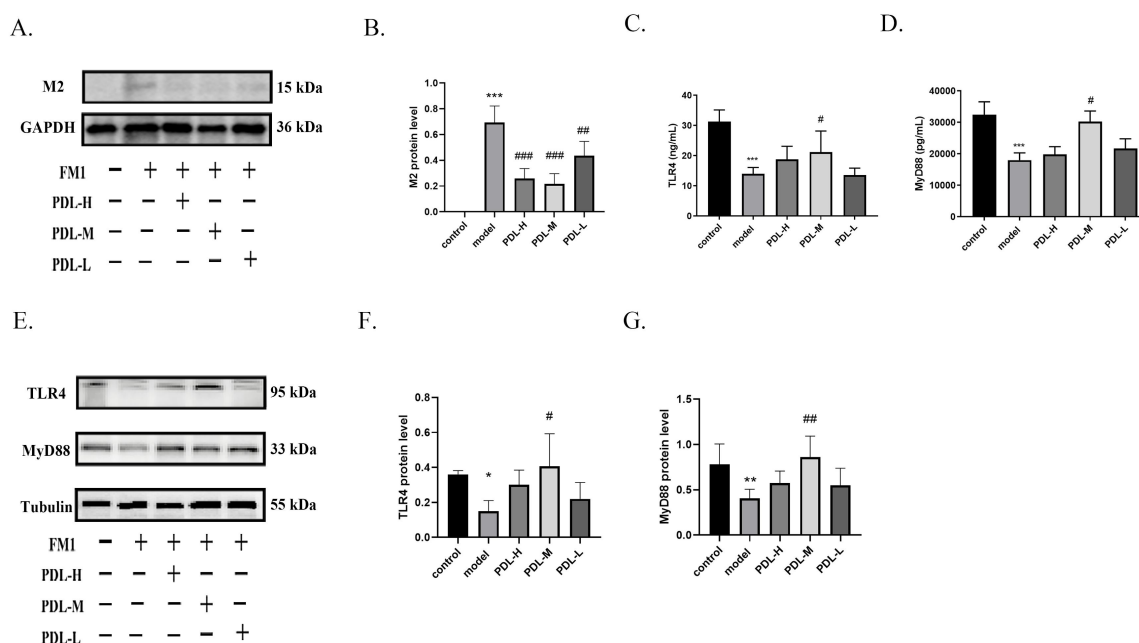


Figure 2 PDL regulates TLR4 and MyD88 expression in the lung tissue of influenza virus mice. (A) Protein expression bands of M2. (B) Protein expression bands of M2 in lung tissues ($n = 4$). (C) ELISA for TLR4 expression in the lung tissue ($n = 6$). (D) ELISA for MyD88 expression in the lung tissue ($n = 6$). (E) Protein expression bands of TLR4 and MyD88. (F) Protein expression of TLR4 in the lung tissues ($n = 4$). (G) Protein expression of MyD88 in the lung tissues ($n = 4$). Data are shown as the mean $\pm$ SD. Comparison with the control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Comparison with the model group, # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$. OP, oseltamivir phosphate capsules; SFJD, ShuFengJieDu capsule; PDL-H, PDL high-dose group; PDL-M, PDL moderate-dose group; PDL-L, PDL low-dose group; TLR, Toll-like receptor.

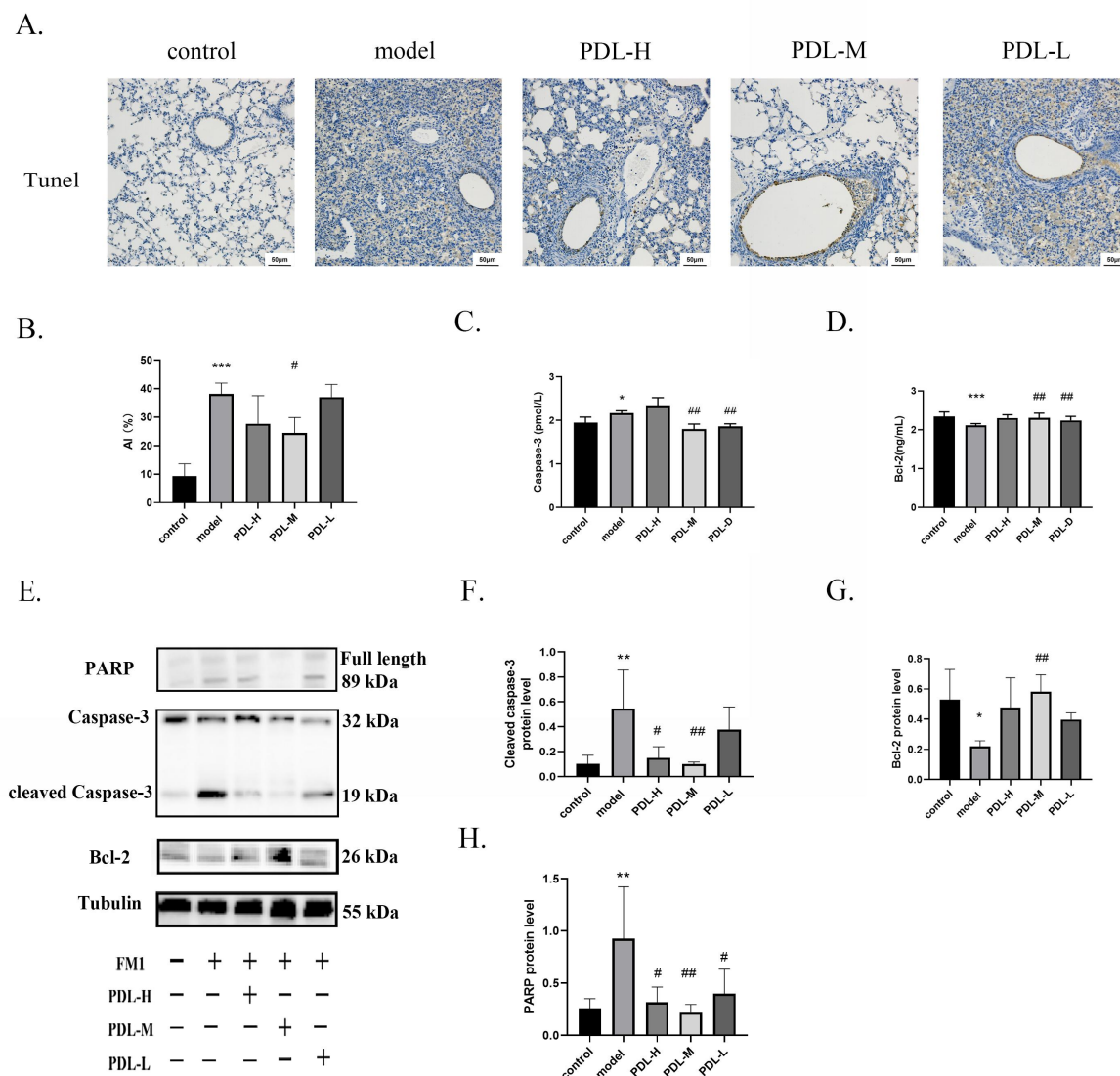


Figure 3 PDL protects against apoptosis by inhibiting caspase-3 expression and promoting Bcl-2 expression. (A) Representative results for TUNEL staining. (B) The results of AI (n = 4). (C) ELISA for caspase-3 expression in the lung tissue (n = 6). (D) ELISA for Bcl-2 expression in the lung tissue (n = 6). (E) Protein expression bands of caspase-3, Bcl-2, and PARP. (F) Protein expression of caspase-3 in the lung tissues (n = 4). (G) Protein expression of Bcl-2 in the lung tissues (n = 4). (H) Protein expression of PARP in the lung tissues (n = 4). Data are shown as the mean $\pm$ SD. Comparison with the control group, * P < 0.05, ** P < 0.01 and *** P < 0.001; Comparison with the model group, # P < 0.05, ## P < 0.01 and ### P < 0.001. OP, oseltamivir phosphate capsules; SFJD, ShuFengJieDu capsule; PDL-H, PDL high-dose group; PDL-M, PDL moderate-dose group; PDL-L, PDL low-dose group; AI, apoptosis index; Bcl-2, B-cell lymphoma-2.

significant increase compared to that observed in the normal control group (P < 0.001); Compared with the model group, the apoptosis index decreased in the PDL dose group, which was significant in the PDL-M group (P < 0.05; Figure 3B). To assess the effect of PDL on apoptosis, we investigated the levels of apoptotic factors (Caspase-3, Bcl-2) in the lung tissue. The results are shown in Figure 3C, 3D, the PDL-M significantly downregulated caspase-3 levels and upregulated Bcl-2 expression in the lung tissue (P < 0.01), which was also validated at the protein level, suggesting that PDL downregulated the expression of the apoptotic protein caspase-3 and upregulated the expression of Bcl-2; the PDL-M group had the best effect (P < 0.05; P < 0.01; Figure 3E–3G). Compared to the control group, PARP protein expression was significantly increased in the model group, whereas PARP expression was significantly decreased and apoptosis was inhibited after PDL treatment (P < 0.05; P < 0.01; Figure 3E, 3H). These results highlight that the protective effect of PDL in mice with viral pneumonia is associated with an attenuated inflammatory

response through protection against apoptosis and modulation of TLR4/MyD88.

Discussion and conclusion

IAV is the leading cause of respiratory infections and poses a global health risk. The antiviral effect of TCM is being increasingly recognised worldwide, and it has played a unique role in clinical treatment. Research has demonstrated that TCM has immunomodulatory properties that can hinder inflammation by regulating the host immune function. Additionally, it has the ability to modulate cell apoptosis induced by the influenza virus, leading to a reduction in lung damage caused by the infection [42]. In clinical practice, PDL has been shown to be useful in the treatment of infectious diseases of the upper respiratory tract [43]. Previous evidence suggests that the herbs in PDL or their major components can inhibit inflammation caused by LPS [44, 45]. The compounds

contained in PDL have been shown to have anti-inflammatory effects both in vivo and in vitro [45–48]. In the present study, an IAV infection model was designed, and mice were treated with PDL. The general state of the mice was observed, and the lung index and inhibition rate were analysed. Viral infection causes inflammatory lesions in the lungs of mice; the higher the lung index value, the more serious the degree of pneumonia. Using the lung index to evaluate the action of drugs is a quantitative concept that can objectively reflect the degree of changes in pneumonia [49]. A pale pink appearance of the lung tissue in the control group was also observed, without oedema or consolidation. In the model group, severe dark brown areas of consolidation were observed, with a significant increase in the lung index and more severe lung tissue lesions. Compared with the model group, the area of dark brown consolidation and lung index in the administration group were reduced. The OP and PDL-M groups showed obvious changes in the lung index and had higher lung index inhibition rates. HE and micro CT findings also demonstrated that PDL can reduce lung tissue lesions and lung injury. These results suggest that PDL can reduce the severity of pneumonia in virus-infected mice. The IAV envelope contains the following three membrane proteins: haemagglutinin, neuraminidase, and M2. M2 ion channels are essential for influenza virus infection of cells [50]. Immunohistochemistry results showed that the positive expression of M2 protein was significantly increased in the model group, and the positive expression was significantly reduced after the administration of PDL, indicating that PDL could attenuate viral replication in cells.

Viral invasion can cause injury and inflammation of respiratory mucosal epithelial cells, which simultaneously initiate the process of infection and resistance to infection in the organism and produces cytokines [51, 52]. Cytokines are critical for cell-to-cell communication and infection resolution in the immune system [51]. The pro-inflammatory cytokine IL-1 β , TNF- α , and IL-10, the anti-inflammatory cytokine, play great roles in influenza virus infection and inflammation. Blocking the expression of IL-1 β could significantly reduce the collection of neutrophils to the airway in the early stage of infection or the peak period of virus replication [53]. IL-1 β collaborates with TNF- α chemotactic neutrophils and other inflammatory cells to enter the lesion site and aggravate tissue damage. IL-10 is a typical anti-inflammatory cytokine. Blocking the action of T cell-derived IL-10 increases the risk of lung inflammation and fatal injury. In the current investigation, the model group exhibited significantly elevated levels of inflammatory factors IL-1 β and TNF- α compared to those in the control group. Moreover, a notable reduction in the expression of IL-10 was observed in the lung tissues of the infected mice. The IL-10 content in the lung tissue increased after PDL treatment. It has been suggested that PDL regulates pro- and anti-inflammatory factors and alleviates excessive immune responses. Notably, among the three PDL doses, the therapeutic effect in the PDL-L group was unsatisfactory. The degree of infection was considered to be more severe, resulting in severe pneumonia; however, the concentration of the drug in the PDL-L group was lower than that of the clinically effective dose, which did not allow it to achieve a good therapeutic effect and alleviate lesions in the lung tissue.

Influenza viruses are recognised by different types of PRRs, leading to the activation of antiviral signals that produce cytokines and chemokines [54]. TLRs recognise specific substances released by invading bacteria and viruses. TLRs act as major barriers against microorganisms and are widely distributed among immune cells and other cell populations. The cell surface receptors TLR2, TLR6, TLR4, TLR3, TLR7, TLR8, and TLR9 can recognise the structural components. In addition, TLR8 and TLR9, which are expressed in endosomes, can identify nucleic acids [30, 31]. Each TLR contains an intracellular TIR domain. TIR domains recruit adaptive proteins, leading to activation of MyD88 or TRIF-dependent signalling cascades. At the same time, the MyD88-dependent pathway produces pro-inflammatory cytokines (IL-1 β , TNF- α) via the transcription factor NF- κ B [31, 54]. Previous studies have shown that TLR4 and MyD88 protein expression increases after influenza virus infection.

Interestingly, the results of the present study showed that the protein expression levels of both TLR4 and MyD88 were lower in the model group than those in the control group, which was consistent with the ELISA results. Abnormal activation of the TLR/NF- κ B pathway, a strong inflammatory response, and immune dysfunction have been observed in patients with severe pneumonia. In contrast, the lung tissue lesions in this study were more severe, corresponding to the severe pneumonia model, and abnormal expression of TLR4 may be correlated with a more severe degree of pneumonia. In addition, severe viral infections lead to apoptosis and TLR4 expression is closely associated with apoptosis [9, 10, 11, 17, 55]. TLR4 has been shown as an important apoptosis regulator, mediating apoptosis in tumour cells through the downstream MyD88/NF- κ B pathway, and interference with TLR4 expression increases apoptosis [56]. Recent studies have demonstrated that attenuation of TLR4 activity hampers the onset of hepatic injury caused by fatty polysaccharides by suppressing both inflammatory responses and apoptosis [17]. Inhibition of the TLR4/MyD88 signalling pathway promotes apoptosis. This may provide a possible cause of abnormal Toll-like protein expression in severe pneumonia. Therefore, apoptosis-related experiments were designed for validation. Based on the TUNEL assay, PDL reduced apoptosis in the lung tissue and played a protective role. The activation of cysteine proteases (caspases) is the most well-known biochemical characteristic of apoptosis in its initial and final phases, detecting of active Caspase-3 in cells and tissues serves as a crucial approach for identifying apoptosis triggered by diverse apoptotic stimuli [57, 58]. Bcl-2 is also a biomarker of apoptosis and plays a role in the inhibition of apoptosis. PARP is a cleavage substrate for cysteine asparaginase (caspase), a core component of apoptosis. In the present study, PDL downregulated the protein expression of caspase-3 and PARP and upregulated Bcl-2 expression. Combined with TLR4 expression and other proteins in the lung tissues, PDL may play a role in inhibiting apoptosis and protecting lung tissues from damage by regulating the TLR4/MyD88 signalling pathway. Notably, we did not perform TLR4/MyD88 overexpression or inhibition experiments to further investigate the relationship between TLR4 and apoptosis. In addition, the host cell type, stage of infection, and whether there is crosstalk between pathways are also important points to investigate, which are relevant aspects to consider in subsequent studies [59, 60].

This study evidenced the potential of PDL to act as an antiviral agent and supported a natural immune response by controlling immune impairment resulting from inflammatory factors and inflammatory infiltration. PDL's ability to inhibit apoptosis and protect the lung tissue may be related to the regulation of TLR4 and MyD88 expressions.

References

1. AlSamman M, Caggiula A, Ganguli S, Misak M, Pourmand A. Non-respiratory presentations of COVID-19, a clinical review. *Am J Emerg Med*. 2020;38(11):2444–2454. Available at: <http://doi.org/10.1016/j.ajem.2020.09.054>
2. Kalil AC, Thomas PG. Influenza virus-related critical illness: pathophysiology and epidemiology. *Crit Care*. 2019;23(1):258. Available at: <http://doi.org/10.1186/s13054-019-2539-x>
3. Sempere Borau M, Stertz S. Entry of influenza A virus into host cells—recent progress and remaining challenges. *Curr Opin Virol*. 2021;48:23–29. Available at: <http://doi.org/10.1016/j.coviro.2021.03.001>
4. Ison MG. Antivirals and resistance: influenza virus. *Curr Opin Virol*. 2011;1(6):563–573. Available at: <http://doi.org/10.1016/j.coviro.2011.09.002>
5. Javanian M, Barary M, Ghebrehewet S, Koppolu V, Vasigala V, Ebrahimpour S. A brief review of influenza virus infection. *J Med Virol*. 2021;93(8):4638–4646. Available at: <http://doi.org/10.1002/jmv.26990>
6. Tang Y, Wu H, Huo C, Zou S, Hu Y, Yang H. Transcriptomic Profiling of Mouse Mast Cells upon Pathogenic Avian H5N1 and

- Pandemic H1N1 Influenza a Virus Infection. *Viruses*. 2022;14(2):292. Available at: <http://doi.org/10.3390/v14020292>
7. Sládková T, Kostolanský F. The role of cytokines in the immune response to influenza A virus infection. *Acta Virol*. 2006;50(3):151–162. Available at: <https://pubmed.ncbi.nlm.nih.gov/17131933/>
 8. Tumpey TM, Basler CF, Aguilar PV, et al. Characterization of the Reconstructed 1918 Spanish Influenza Pandemic Virus. *Science*. 2005;310(5745):77–80. Available at: <http://doi.org/10.1126/science.1119392>
 9. Wu Y, Wang Y, Gong S, et al. Ruscogenin alleviates LPS-induced pulmonary endothelial cell apoptosis by suppressing TLR4 signaling. *Biomed Pharmacother*. 2020;125:109868. Available at: <http://doi.org/10.1016/j.biopha.2020.109868>
 10. Choy KW, Lau YS, Murugan D, Vanhoutte PM, Mustafa MR. Paeonol Attenuates LPS-Induced Endothelial Dysfunction and Apoptosis by Inhibiting BMP4 and TLR4 Signaling Simultaneously but Independently. *J Pharmacol Exp Ther*. 2017;364(3):420–432. Available at: <http://doi.org/10.1124/jpet.117.245217>
 11. Hull C, McLean G, Wong F, Duriez PJ, Karsan A. Lipopolysaccharide Signals an Endothelial Apoptosis Pathway Through TNF Receptor-Associated Factor 6-Mediated Activation of c-Jun NH2-Terminal Kinase. *J Immunol*. 2002;169(5):2611–2618. Available at: <http://doi.org/10.4049/jimmunol.169.5.2611>
 12. Zhang K, Huang Q, Deng S, Yang Y, Li J, Wang S. Mechanisms of TLR4-Mediated Autophagy and Nitroxidative Stress. *Front Cell Infect Microbiol*. 2021;11:766590. Available at: <http://doi.org/10.3389/fcimb.2021.766590>
 13. Martin TR, Wurfel MM. A TRIFfic Perspective on Acute Lung Injury. *Cell*. 2008;133(2):208–210. Available at: <http://doi.org/10.1016/j.cell.2008.04.006>
 14. Zamyatina A, Heine H. Lipopolysaccharide Recognition in the Crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways. *Front Immunol*. 2020;11:585146. Available at: <http://doi.org/10.3389/fimmu.2020.585146>
 15. Shirey KA, Lai W, Scott AJ, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature*. 2013;497(7450):498–502. Available at: <http://doi.org/10.1038/nature12118>
 16. Wei W, Wan H, Peng X, Zhou H, Lu Y, He Y. Antiviral effects of Ma Huang Tang against H1N1 influenza virus infection in vitro and in an ICR pneumonia mouse model. *Biomed Pharmacother*. 2018;102:1161–1175. Available at: <http://doi.org/10.1016/j.biopha.2018.03.161>
 17. Chen S, Tan Y, Xiao X, et al. Deletion of TLR4 attenuates lipopolysaccharide-induced acute liver injury by inhibiting inflammation and apoptosis. *Acta Pharmacol Sin*. 2021;42(10):1610–1619. Available at: <http://doi.org/10.1038/s41401-020-00597-x>
 18. Li X, Cai L, Liu J, et al. Liquiritin suppresses UVB-induced skin injury through prevention of inflammation, oxidative stress and apoptosis through the TLR4/MyD88/NF- κ B and MAPK/caspase signaling pathways. *Int J Mol Med June*. 2018;42(3):1445–1459. Available at: <http://doi.org/10.3892/ijmm.2018.3720>
 19. Zhang R, Ai X, Duan Y, et al. Kaempferol ameliorates H9N2 swine influenza virus-induced acute lung injury by inactivation of TLR4/MyD88-mediated NF- κ B and MAPK signaling pathways. *Biomed Pharmacother*. 2017;89:660–672. Available at: <http://doi.org/10.1016/j.biopha.2017.02.081>
 20. Deng YX, Yang ZW, Gao Y. Toll-like receptor 4 mediates acute lung injury induced by high mobility group box-1. *PLoS One*. 2013;8(5):e64375. Available at: <http://doi.org/10.1371/journal.pone.0064375>
 21. Lyu T, Shen X, Shi Y, Song Y. TLR4 is Essential in Acute Lung Injury Induced by Unresuscitated Hemorrhagic Shock. *J Trauma*. 2009;66(1):124–131. Available at: <http://doi.org/10.1097/TA.0b013e318181e555>
 22. Hu G, Malik AB, Minshall RD. Toll-like receptor 4 mediates neutrophil sequestration and lung injury induced by endotoxin and hyperinflation. *Crit Care Med*. 2010;38(1):194–201. Available at: <http://doi.org/10.1097/CCM.0b013e3181bc7c17>
 23. Zhu H, Lu X, Ling L, et al. Houttuynia cordata polysaccharides ameliorate pneumonia severity and intestinal injury in mice with influenza virus infection. *J Ethnopharmacol*. 2018;218:90–99. Available at: <http://doi.org/10.1016/j.jep.2018.02.016>
 24. Płóciennikowska A, Hromada-Judyczka A, Borzęcka K, Kwiatkowska K. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci*. 2014;72(3):557–581. Available at: <http://doi.org/10.1007/s00018-014-1762-5>
 25. Poltorak A, He X, Smirnova I, et al. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science*. 1998;282(5396):2085–2088. Available at: <http://doi.org/10.1126/science.282.5396.2085>
 26. Zhu Y, Han Q, Wang L, et al. Jinhua Qinggan granules attenuates acute lung injury by promotion of neutrophil apoptosis and inhibition of TLR4/MyD88/NF- κ B pathway. *J Ethnopharmacol*. 2023;301:115763. Available at: <http://doi.org/10.1016/j.jep.2022.115763>
 27. Baharom F, Thomas S, Bieder A, et al. Protection of Human Myeloid Dendritic Cell Subsets against Influenza A Virus Infection Is Differentially Regulated upon TLR Stimulation. *J Immunol*. 2015;194(9):4422–4430. Available at: <http://doi.org/10.4049/jimmunol.1402671>
 28. Ma Q, Huang W, Zhao J, Yang Z, Liu Shen Wan inhibits influenza a virus and excessive virus-induced inflammatory response via suppression of TLR4/NF- κ B signaling pathway in vitro and in vivo. *J Ethnopharmacol*. 2020;252:112584. Available at: <http://doi.org/10.1016/j.jep.2020.112584>
 29. El-Zayat SR, Sibaii H, Mannaa FA. Toll-like receptors activation, signaling, and targeting: an overview. *Bull Natl Res Cent*. 2019;43:187. Available at: <http://doi.org/10.1186/s42269-019-0227-2>
 30. Mabrey FL, Morrell ED, Wurfel MM. TLRs in COVID-19: How they drive immunopathology and the rationale for modulation. *Innate Immun*. 2021;27(7–8):503–513. Available at: <http://doi.org/10.1177/17534259211051364>
 31. Lester SN, Li K. Toll-Like Receptors in Antiviral Innate Immunity. *J Mol Biol*. 2014;426(6):1246–1264. Available at: <http://doi.org/10.1016/j.jmb.2013.11.024>
 32. Kurt-Jones EA, Popova L, Kwinn L, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol*. 2000;1(5):398–401. Available at: <http://doi.org/10.1038/80833>
 33. Rassa JC, Meyers JL, Zhang Y, Kudaravalli R, Ross SR. Murine retroviruses activate B cells via interaction with toll-like receptor 4. *Proc Natl Acad Sci USA*. 2002;99(4):2281–2286. Available at: <http://doi.org/10.1073/pnas.042355399>
 34. Deng W, Xu Y, Kong Q, et al. Therapeutic efficacy of Pudilan Xiaoyan Oral Liquid (PDL) for COVID-19 in vitro and in vivo. *Sig Transduct Target Ther*. 2020;5(1):66. Available at: <http://doi.org/10.1038/s41392-020-0176-0>
 35. Bao Y, Gao Y, Jin Y, Cong W, Pan X, Cui X. MicroRNA expression profiles and networks in mouse lung infected with H1N1 influenza virus. *Mol Genet Genomics*. 2015;290(5):1885–1897. Available at: <http://doi.org/10.1007/s00438-015-1047-1>
 36. Xia L, Shi Y, Su J, et al. Shufeng Jiedu, a promising herbal

- therapy for moderate COVID-19: Antiviral and anti-inflammatory properties, pathways of bioactive compounds, and a clinical real-world pragmatic study. *Phytomedicine*. 2021;85:153390. Available at: <http://doi.org/10.1016/j.phymed.2020.153390>
37. Liu Z, Zhao J, Li W, et al. Computational screen and experimental validation of anti-influenza effects of quercetin and chlorogenic acid from traditional Chinese medicine. *Sci Rep*. 2016;6(1):19095. Available at: <http://doi.org/10.1038/srep19095>
 38. Ren YZ, Li CF, Feng LQ, et al. Proton Channel Activity of Influenza A Virus Matrix Protein 2 Contributes to Autophagy Arrest. *J Virol*. 2015;90(1):591–598. Available at: <http://doi.org/10.1128/JVI.00576-15>
 39. Vidya MK, Kumar VG, Sejian V, Bagath M, Krishnan G, Bhatta R. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *Int Rev Immunol*. 2017;37(1):20–36. Available at: <http://doi.org/10.1080/08830185.2017.1380200>
 40. Elmore S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol*. 2007;35(4):495–516. Available at: <http://doi.org/10.1080/01926230701320337>
 41. Obeng E. Apoptosis (programmed cell death) and its signals-A review. *Braz J Biol*. 2021;81(4):1133–1143. Available at: <https://pubmed.ncbi.nlm.nih.gov/33111928/>
 42. Shim J, Kim J, Tenson T, Min JY, Kainov D. Influenza Virus Infection, Interferon Response, Viral Counter-Response, and Apoptosis. *Viruses*. 2017;9(8):223. Available at: <http://doi.org/10.3390/v9080223>
 43. Feng L, Yang N, Li C, et al. Pudilan xiaoyan oral liquid alleviates LPS-induced respiratory injury through decreasing nitroxidative stress and blocking TLR4 activation along with NF- κ B phosphorylation in mice. *J Ethnopharmacol*. 2018;214:292–300. Available at: <http://doi.org/10.1016/j.jep.2017.07.009>
 44. Ma CH, Zhu LP, Wang J, et al. Anti-inflammatory effects of water extract of *Taraxacum mongolicum* hand-Mazz on lipopolysaccharide-induced inflammation in acute lung injury by suppressing PI3K/Akt/mTOR signaling pathway. *J Ethnopharmacol*. 2015;168:349–355. Available at: <http://doi.org/10.1016/j.jep.2015.03.068>
 45. Mabalirajan U, Ahmad T, Rehman R, et al. Baicalein reduces airway injury in allergen and IL-13 induced airway inflammation. *PLoS One*. 2013;8(4):e62916. Available at: <http://doi.org/10.1371/journal.pone.0062916>
 46. Guo YJ, Luo T, Wu F, et al. Involvement of TLR2 and TLR9 in the anti-inflammatory effects of chlorogenic acid in HSV-1-infected microglia. *Life Sci*. 2015;127:12–18. Available at: <http://doi.org/10.1016/j.lfs.2015.01.036>
 47. Dong S, Zhong Y, Lu W, Li G, Jiang H, Mao B. Baicalin Inhibits Lipopolysaccharide-Induced Inflammation Through Signaling NF- κ B Pathway in HBE16 Airway Epithelial Cells. *Inflammation*. 2015;38(4):1493–1501. Available at: <http://doi.org/10.1007/s10753-015-0124-2>
 48. Shin EK, Kim DH, Lim H, Shin HK, Kim JK. The Anti-Inflammatory Effects of a Methanolic Extract from *Radix Isatidis* in Murine Macrophages and Mice. *Inflammation*. 2009;33(2):110–118. Available at: <http://doi.org/10.1007/s10753-009-9164-9>
 49. Ye Y, Wang H, Liu J, Zhao F, Xu P. Polygalasaponin F treats mice with pneumonia induced by influenza virus. *Inflammopharmacol*. 2019;28(1):299–310. Available at: <http://doi.org/10.1007/s10787-019-00633-1>
 50. Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature*. 2008;451(7178):591–595. Available at: <http://doi.org/10.1038/nature06531>
 51. Gu Y, Zuo X, Zhang S, et al. The Mechanism behind Influenza Virus Cytokine Storm. *Viruses*. 2021;13(7):1362. Available at: <http://doi.org/10.3390/v13071362>
 52. Guo XJ, Thomas PG. New fronts emerge in the influenza cytokine storm. *Semin Immunopathol*. 2017;39(5):541–550. Available at: <http://doi.org/10.1007/s00281-017-0636-y>
 53. Sichelstiel A, Yadava K, Trompette A, et al. Targeting IL-1 β and IL-17A driven inflammation during influenza-induced exacerbations of chronic lung inflammation. *PLoS One*. 2014;9(2):e98440. Available at: <http://doi.org/10.1371/journal.pone.0098440>
 54. Mifsud EJ, Kuba M, Barr IG. Innate Immune Responses to Influenza Virus Infections in the Upper Respiratory Tract. *Viruses*. 2021;13(10):2090. Available at: <http://doi.org/10.3390/v13102090>
 55. Chu H, Shuai H, Hou Y, et al. Targeting highly pathogenic coronavirus-induced apoptosis reduces viral pathogenesis and disease severity. *Sci Adv*. 2021;7(25):eabf8577. Available at: <http://doi.org/10.1126/sciadv.abf8577>
 56. Szajnik M, Szczepanski MJ, Czystowska M, et al. TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. *Oncogene*. 2009;28(49):4353–4363. Available at: <http://doi.org/10.1038/onc.2009.289>
 57. Choudhary GS, Al-harbi S, Almasan A. Caspase-3 Activation Is a Critical Determinant of Genotoxic Stress-Induced Apoptosis. *Apoptosis Cancer*. 2014;1–9. Available at: http://doi.org/10.1007/978-1-4939-1661-0_1
 58. Mazumder S, Plesca D, Almasan A. Caspase-3 Activation is a Critical Determinant of Genotoxic Stress-Induced Apoptosis. *Apoptosis Cancer*. 2008;13–21. Available at: http://doi.org/10.1007/978-1-59745-339-4_2
 59. Tripathi S, Batra J, Cao W, et al. Influenza A virus nucleoprotein induces apoptosis in human airway epithelial cells: implications of a novel interaction between nucleoprotein and host protein Clusterin. *Cell Death Dis*. 2013;4(3):e562–e562. Available at: <http://doi.org/10.1038/cddis.2013.89>
 60. Coleman JR. The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages. *Virol J*. 2007;4:9. Available at: <http://doi.org/10.1186/1743-422X-4-9>