



Original Article

Integrated network pharmacology, transcriptomics and experimental validation to explore mechanisms of Wenyang Jiedu Granule on IAV-induced pneumonia

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ABSTRACT

Objective: Wenyang Jiedu Granule (WYJD) is an effective traditional Chinese medicine (TCM) preparation that has been generally applied for treating respiratory infectious diseases. Clinical observations involving thousands of cases have demonstrated that WYJD could alleviate disease progression and improve symptoms in treating respiratory viral infections, including SARS-CoV-2 and influenza virus. However, the chemical basis and underlying mechanisms of WYJD against influenza A virus (IAV)-induced pneumonia remain to be elucidated. This study aimed to reveal the underlying mechanisms of WYJD in treating IAV-induced pneumonia by a combined strategy of network pharmacology, transcriptomics and experimental validation.

Methods: The pneumonia model was established in BALB/c mice via infection with H1N1 IAV to evaluate the therapeutic effects of WYJD on IAV-induced pneumonia. Firstly, ultra-high performance liquid chromatography-quadrupole Exactive Orbitrap mass spectrometer/tandem mass spectrometer (UPLC-Q Exactive Orbitrap-MS/MS) was employed to analyze the main chemical components in WYJD-containing serum. Subsequently, the effects of WYJD on IAV-induced pneumonia were assessed through pathological observation, plaque forming assay, biochemical analysis, Evans blue staining assay, and immunofluorescence assay. Mechanistically, an integrated approach of network pharmacology and transcriptomics was applied to explore the potential active components, targets and related pathways of WYJD against IAV-induced pneumonia. Fluorescence TUNEL assay, quantitative real-time PCR (qRT-PCR) and Western blotting were utilized for experimental validation and mechanistic studies.

Results: Using UPLC-Q Exactive Orbitrap-MS/MS, a total of 25 prototypes and 15 metabolites were identified in the serum of mice after WYJD administration. WYJD treatment showed protective effects on IAV-induced pneumonia by inhibiting inflammation and lung barrier damage in the IAV-induced pneumonia mice model. Network pharmacology combined with transcriptomics analysis indicated that WYJD exerted therapeutic effects against IAV-induced pneumonia mainly through the synergistic effects of 11 active components, which regulated ten critical signaling pathways via 86 targets. Further experimental validation demonstrated that WYJD could alleviate IAV-induced pneumonia via the IL-17 signaling pathway, Toll-like receptor 7 (TLR7)/Myeloid differentiation primary response gene 88 (MyD88)/mitogen-activated protein kinases (MAPKs)/activator protein 1 (AP-1) signaling pathway and apoptosis.

Conclusion: This study revealed the main active components and mechanisms of WYJD against IAV-induced pneumonia through the IL-17 signaling pathway, TLR7/MyD88/MAPKs/AP-1 signaling pathway and apoptosis, which provides novel insights into the clinical application of WYJD in treating influenza and its complications.

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1. Introduction

Respiratory viral infections pose a significant public health concern globally. The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) since 2019 has had a profound and lasting impact on the global economy and public health. Furthermore, the persistent threat of influenza viruses is exacerbated by recurrent outbreaks and periodic pandemics, which continue to impose significant burdens on global public health and strain healthcare systems worldwide (Gandhi, 2024). According to World Health Organization (WHO) estimates, influenza-related complications claim approximately half a million lives annually worldwide (Javanian, Barary, Ghebrehewet, Koppolu, Vasigala, & Ebrahimpour, 2021). The influenza viruses demonstrate remarkable antigenic variability, with major antigenic shifts typically occurring at approximately decadal intervals. These substantial genetic variations give rise to novel viral strains that evade pre-existing population immunity, consequently triggering new epidemic waves (Webster & Govorkova, 2014). Of particular clinical concern, severe influenza virus infections can progress to life-threatening complications, including acute lung injury, viral pneumonia and acute respiratory distress syndrome, which contribute to considerable morbidity and mortality (Kalil & Thomas, 2019). It is noteworthy that influenza virus infections could trigger excessive cytokine release from lung epithelial cells, which recruit leukocytes and activates adjacent endothelial cells, ultimately resulting in apoptosis of lung epithelial cells and disruption of the lung epithelial-endothelial barrier (Short et al. 2016). In addition, disruption of the lung epithelial-endothelial barrier may promote blood cell infiltration into the alveolar space, thereby resulting in impaired respiratory function (Major et al., 2023). However, mainly antiviral drugs approved for the treatment of influenza, such as oseltamivir, are inhibitors of viral replication but have no direct effect on the underlying inflammation and tissue injury. Therefore, the efficacy of these available drugs remains unsatisfactory (Hanula, Bortolussi-Courval, Mendel, Ward, Lee, & McDonald, 2024). It remains urgent to research and develop effective and safe therapeutic drugs for influenza and its complications.

Traditional Chinese medicine (TCM) has been used to treat respiratory infectious diseases in China for thousands of years, due to its unique clinical advantages, and multi-component and multi-target strategy (Leung et al., 2020). Accumulating evidence has demonstrated that numerous TCM prescriptions exhibit dual therapeutic mechanisms against influenza, including direct antiviral activities through suppressing viral replication, and immunomodulatory effects by mitigating virus-induced hyper-inflammatory responses and associated tissue injury. For example, TCM prescription Xuanbai Chengqi Decoction significantly improved lung tissue damage caused by influenza A virus (IAV) via the regulation of interleukin-6 (IL-6), IL-10, and IL-22, as well as the inhibition of influenza A H1N1 virus replication (Guo et al., 2024). Previous results have also demonstrated that TCM formulas like Lianhuaqingwen Capsules and Haoqin Qingdan Decoction could exert anti-viral and anti-inflammatory activities against respiratory viral infections (Liang et al., 2024; Li et al., 2020a).

Wenyang Jiedu Granule (WYJD, also known as Fuzheng Jiedu Formula/Decoction) is a clinically approved TCM prescription composed of eight herbs in specific proportions. The WYJD prescription is derived from a famous ancient classical TCM formula Si Ni Decoction documented in *Treatise on Cold Damage Diseases* (Shang Han Lun), written by Zhongjing Zhang in the Han Dynasty of China which has been used to treat respiratory infectious diseases for thousands of years. Recently, clinical observations involving thousands of cases have demonstrated that WYJD exhibited significant clinical efficacy in treating patients infected by SARS-CoV-2

through alleviating pneumonia syndrome and reducing the rates of disease progression (Wang et al., 2021; Hua et al., 2025). In clinical practice, WYJD has also been used empirically for influenza treatment with favorable clinical outcomes observed. Experimental studies have also demonstrated that WYJD formula exerted protective effects on LPS-induced acute lung injury via gut-lung axis and IAV-induced acute lung injury by suppressing the NLRP3 inflammasome activation (Li et al., 2025b; Lu et al., 2024). In addition, a total of 29 compounds were simultaneously determined by high-performance liquid chromatography-triple quadrupole tandem mass spectrometry (HPLC-QQQ-MS/MS) in WYJD, including flavonoids, organic acids, alkaloids, and coumarins (Huang et al., 2023). However, the *in vivo* chemical basis, pharmacological effects and underlying mechanisms of WYJD against influenza virus infection are not well understood.

In the present study, a novel approach combining network pharmacology, transcriptomics, and experimental validation was established for the in-depth investigation of WYJD in the treatment of IAV-induced pneumonia. First, a total of 25 prototypes and 15 metabolites from WYJD were identified in WYJD-containing serum using ultra-high performance liquid chromatography-quadrupole Exactive Orbitrap mass spectrometer/tandem mass spectrometer (UPLC-Q Exactive Orbitrap-MS/MS). Subsequently, WYJD was shown to have significant protective effects on IAV-induced pneumonia in the mice model. Then, the network pharmacology and transcriptomics were utilized to explore the mechanisms of WYJD in treating IAV-induced pneumonia, and 11 active components, ten critical signaling pathways, and 86 related targets were selected for further study. Finally, the experimental validation demonstrated that WYJD alleviates IAV-induced pneumonia through the regulation of IL-17 signaling pathway, Toll-like receptor 7 (TLR7)/Myeloid differentiation primary response gene 88 (MyD88)/mitogen-activated protein kinases (MAPKs)/activator protein 1 (AP-1) signaling pathway and apoptosis, which provides new insights into the clinical application of WYJD in treating influenza and its complications.

2. Materials and methods

2.1. Materials

WYJD (lot number: 230601) was manufactured and obtained from Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China). Qualitative and quantitative chemical analysis for quality control of WYJD was performed according to validated methods of the national drug registration standard approved by National Medical Products Administration of China (Supplementary materials).

Intercellular adhesion molecule 1 (ICAM-1) ELISA kit (lot number: 931995617) and vascular cell adhesion molecule 1 (VCAM-1) ELISA kit (lot number: 25219114617) were purchased from Boster Biological Technology (Wuhan, China). Multi-analyte suspension array kit (EQLM-10, lot number: CLEM0102403001) was purchased from Laizee Biotech Co., Ltd. (Shanghai, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit (lot number: A096241205) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Alexa FluorTM 555 (lot number: MPC2412114), Alexa FluorTM 488 (lot number: MPC2409114) and Alexa FluorTM 647 (lot number: MPC2411025) were purchased from Servicebio Technology Co., Ltd. (Wuhan, China). The primary antibodies against zonula occludens-1 (ZO-1, lot number: 00173683, 1:1 000), Occludin (lot number: 00174259, 1:1 000) and Claudin-4 (lot number: 00190124, 1:1 000) were purchased from Proteintech Group, Inc. (Wuhan, China). The primary antibodies against TLR7 (lot number: 1, 1:1 000), p38 mitogen-activated protein kinase (p38) (lot number: 29, 1:1 000), phospho-p38 (p-

p38, lot number: 17, 1:1 000), p44/42 mitogen-activated protein kinase (p44/42 MAPK, Erk1/2, lot number: 35, 1:1 000), p-p44/42 MAPK (p-Erk1/2, lot number: 30, 1:1 000), c-Jun N-terminal kinase (SAPK/JNK, lot number: 18, 1:1 000), p-SAPK/JNK (lot number: 38, 1:1 000), proto-oncogene c-Jun (c-Jun, lot number: 13, 1:1 000), p-c-Jun (lot number: 5, 1:1 000) and β -actin (lot number: 21, 1:1 000) were purchased from Cell Signaling Technology (MA, America). The primary antibody against MyD88 (lot number: 1100385–1, 1:1 000) was purchased from Abcam Limited. (Cambridgeshire, UK). The primary antibodies against B-cell lymphoma 2 (Bcl-2, lot number: H1224, 1:1 000) and Bcl-2 associated X protein (Bax, lot number: C1424, 1:1 000) were purchased from Santa cruz biotechnology (Dallas, USA).

2.2. Animals

For WYJD-containing serum sample preparation, specific pathogen-free SD female rats aged 6–8 weeks (250–300 g) were purchased from Guangdong Vital River Laboratory Animal Technology Co., Ltd. (Certificate number: SCXK Yue 2022–0063), including control group and WYJD treatment group [4.05 g/(kg·d^{−1})], $n = 3$ per group, this dose was given twice the clinical equivalent dose according to the preliminary experiment.

Female mice BALB/c (specific pathogen-free, aged 6–8 weeks) were sourced from Guangdong Vital River Laboratory Animal Technology Co., Ltd. (Certificate number: SCXK Yue 2022–0063). A/PR/8/34 (H1N1) was maintained and propagated by The State Key Laboratory of Respiratory Disease, China. The experiments were approved by the Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou, Animal Ethics License Number: W240001). All animal experiments were strictly followed the guidelines of the International Laboratory Animal Evaluation and Accreditation Management Committee and conducted under biosafety level-2 (BSL-2) conditions.

2.3. Cells

Madin-Darby Canine Kidney (MDCK) cells were kindly provided by the State Key Laboratory of Respiratory Diseases.

2.4. UPLC-Q Exactive Orbitrap-MS/MS analysis of WYJD and WYJD-containing serum

2.4.1. Sample preparation

For WYJD sample preparation, about 0.1 g of WYJD was mixed with 1 mL of water, and steel balls were added for 1 min. The WYJD sample was incubated at -40°C for 2 min and ground at 60 Hz for 2 min. Then the drug sample was ultrasonicated (F-060SD, FUYANG, Shenzhen, China) for 60 min and let stand for 30 min in the ice-water bath. After centrifugation at 12 000 r/min for 10 min at 4°C (TGL-16MS, BIORIDGE, Shanghai, China), the WYJD sample was diluted by 10 times and 200 μL of supernatant was placed into the automatic injection flasks for UPLC-Q Exactive Orbitrap-MS/MS analysis.

After 7 d of adaptive feeding and 12 h of fasting, blood samples were collected from ophthalmic venous plexus at 2 h after PBS or WYJD intragastric administration. Serum samples were obtained by centrifugation at 3 000 r/min for 15 min at 4°C and frozen at -80°C . The 150 μL of serum sample was thawed and mixed with 450 μL of methanol/acetonitrile (2:1, volume percentage). The mixture was then eddied for 1 min, ultrasonicated for 60 min, and let stand for 30 min in the ice bath. After centrifugation at 12 000 r/min for 10 min at 4°C , 500 μL of supernatant was concentrated to dry under nitrogen and then dissolved in 150 μL of water/methanol/acetonitrile (1:2:1, volume percentage). After centrifugation at 12 000 r/min for 10 min at 4°C , 100 μL of

supernatant was placed into the automatic injection flasks for UPLC-Q Exactive Orbitrap-MS/MS analysis. The quality control (QC) sample was prepared by mixing and centrifuging the supernatants of the WYJD sample and the WYJD-containing serum sample in equal volumes.

2.4.2. UPLC-Q Exactive Orbitrap-MS/MS method and data analysis

The chemical characterization of WYJD and WYJD-containing serum was performed on a connected system of ACQUITY UPLC I-Class HF (Waters Corporation, USA)-Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., USA). The chromatographic separation was carried out using an ACQUITY UPLC HSS T3 C₁₈ column (100 mm $\times$ 2.1 mm, 1.8 μm , Waters Corporation, USA) with a flow rate at 0.35 mL/min, 45°C for 16 min. The mobile phase A: 0.1% aqueous formic acid (volume percentage) and mobile phase B: acetonitrile were applied to the gradient elution method, which was set as follows: 5% B (0–2 min), 5%–30% B (2–4 min), 30%–50% B (4–8 min), 50%–80% B (8–10 min), 80%–100% B (10–14 min), 100% B (14–15 min), 100%–5% B (15–15.1 min), 5% B (15.1–16 min). UPLC-Q Exactive Orbitrap-MS/MS acquisitions were performed in data dependent acquisition mode by using positive and negative modes equipped with electrospray ionization source. The instrumental settings of Q Exactive Orbitrap MS/MS were set as follows: Mass scan range was 100–1 500 m/z , ion spray voltage floating was 3 800 V in positive mode and $-3\ 000$ V in negative mode, capillary temperature was 320°C , Aux gas heater temperature was 350°C , Sheath gas flow rate was 35 Arb, Aux gas flow rate was 8 Arb, S-lens radio frequency (RF) level was 8, full MS resolution was 70 000, MS/MS resolution was 17 500, stepped normalized collision energy (NCE) was 10, 20, 40.

The mass spectra data were processed using Progenesis QI v3.0 software (Nonlinear Dynamics, UK) for noise reduction, peak alignment and identification. The compounds were further identified or tentatively characterized by chromatographic elution time, exact molecular mass isotopic distribution and mass fragmentation patterns in comparison with a self-building TCM reference library. The absorbed components and derived metabolites from WYJD in serum sample were characterized by fold change > 10 in the relative intensity of the WYJD treatment group and the control group.

2.5. Animal experiments

To evaluate the effects of WYJD against IAV-induced pneumonia *in vivo*, the mice were in a biological safety cabinet and intranasally inoculated with PBS containing IAV after anesthetized with isoflurane. The mice were randomly grouped into control group (Control, non-infected and given PBS by gavage), IAV group (IAV, infected and given PBS by gavage), oseltamivir group (Oseltamivir, infected and treated with 65 mg/(kg·d) of oseltamivir), according to the pre-experiment setup, low dose of WYJD group (WYJD-L, infected and treated with 2.925 g/(kg·d) of WYJD, half of the equivalent dose of clinical use), medium dose of WYJD group (WYJD-M, infected and treated with 5.85 g/(kg·d) of WYJD, the equivalent dose of clinical use) and high dose of WYJD group (WYJD-H, infected and treated with 11.7 g/(kg·d) of WYJD, twice of the equivalent dose of clinical use). The equivalent dose for mice was calculated based on the clinical dose of WYJD (45 g/d) using body surface area method. For the protection assay, the mice were infected with approximately $2 \times 50\%$ lethal dose (LD_{50}) of IAV ($2 \times 10^{-5}/50\ \mu\text{L}$). Five days of drug administration was performed 2 h after infection and the mice were observed to 15 d. For the antiviral and anti-inflammatory assay, the mice were infected with approximately $1 \times \text{LD}_{50}$ of IAV ($1 \times 10^{-5}/50\ \mu\text{L}$). The mice were daily treated with drugs 2 h after infection and sacrificed for tissue sample collection on the sixth day.

The lung tissues were extracted from mice after anesthesia to observed and weighed. The lung index was calculated as follow:

lung index = (lung weight/body weight) $\times$ 100%. The blood samples of mice were collected into the anticoagulant tube and analyzed by an automatic blood cell analyzer (BC-5000VET, Mindray Medical International Ltd., Shenzhen, China).

2.6. Hematoxylin eosin (HE) staining assay and scoring

The left lung tissues from mice were fixed with 4% paraformaldehyde (PFA) for 24 h, then processed through paraffin embedding and sectioning at 3 μ m thickness. And HE staining was carried out to make pathological sections. The pathological manifestations of lung injury such as infiltration of inflammatory cells in the lung tissue of each mouse were observed under a light microscope and the pathological scores were evaluated by a professional pathologist. The severity of lung injury was quantified using a standardized scoring system according to previously established criteria (Ma, Huang, Zhao, & Yang, 2020).

2.7. Plaque forming assay

Viral loads in lung tissues were quantified using plaque forming assay. The right lung tissues from mice were harvested and homogenized with 0.5 mL of PBS, and the supernatant was collected after centrifugation.

After MDCK cells were seeded into a 12-well plate and grew into a monolayer, the culture medium was discarded and the cell surface was washed twice with PBS. The lung tissue homogenate solutions for testing were diluted to concentrations of 1×10^{-3} , 1×10^{-4} and 1×10^{-5} with the culture medium, respectively. After infection with tested samples for 2 h at 37 °C, the culture medium containing tested samples was discarded and the cell layer was covered with agarose gel containing bovine serum albumin, penicillin and streptomycin, 1 μ g/mL of TPCK-Trypsin and $1 \times$ minimum essential medium. After 48 h of incubation at 37 °C in a 5% CO₂ humidified atmosphere, the cell layer was fixed by 4% paraformaldehyde for 30 min. The overlying agarose gel was removed and the primary antibody of immune mouse serum (self-prepared) was incubated sequentially, followed by coloration with 3-amino-9-ethylcarbazole (AEC) staining kit after the secondary antibody incubation. Viral loads were calculated based on the number of plaques.

2.8. Measurement of protein levels of cytokines

Supernatant of mouse lung homogenate and serum were collected for the measurement of protein levels of cytokines. The protein levels of cytokines (IL-1 β , IL-6, IL-17A, TNF- α , etc.) were measured using a multi-analyte suspension array kit according to the manufacturer's protocol. The acquired fluorescence data were processed and cytokine concentrations were determined using ProcartaPlex Analyst 1.0 software with a five-parameter logistic curve fitting algorithm. Cytokine concentrations were calculated using Procarta Plex Analyst 1.0 software. Cytokines (ICAM-1 and VCAM-1) were measured using ELISA kits according to the instructions.

2.9. Immunofluorescence (IF) staining

After antigen repair, the paraffin sections of lung tissues were blocked with 3% BSA for 30 min to prevent nonspecific binding, incubated with anti-ZO-1 (1:500), anti-Occludin (1:500,) and anti-Claudin-4 (1:500) at 4 °C for 1 h. Then, the paraffin sections were incubated with sheep anti-mouse IgG (H + L), Alexa Fluor™ 555 (1:500), Alexa Fluor™ 488 (1:500), Alexa Fluor™ 647 (1:500) at room temperature for 1 h. Finally, the cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). The IF staining sections were observed using a confocal microscopy.

2.10. Evans blue staining assay

The mice were infected with approximately $1 \times \text{LD}_{50}$ of IAV ($1 \times 10^{-5}/50 \mu\text{L}$) and were daily treated with drugs 2 h after infection. On the sixth day after infection, 0.1 mL of 0.5% Evans blue solution was injected into the tail veins of mice. About 1 h later, mice were anesthetized with tribromoethanol, and the lung tissues of mice was lavaged and collected. The lung tissues were homogenized with 1 mL of PBS. Then 1 mL of formamide solution was added, and the mixture was incubated at 37 °C in an oven for 24 h. The supernatant was collected by centrifugation. To prepare standard solutions, Evans blue was diluted with formamide solution to different concentrations and the mixture were incubated at 37 °C for 24 h as well. The OD values of tested samples and standard solutions at 630 nm were measured and the contents of Evans blue were calculated based on the standard curve.

2.11. Network pharmacology analysis of WYJD-containing serum

The online BATMAN-TCM tool (version 2.0) was used to identify potential targets of WYJD by inputting the compound list in the WYJD-containing serum (Kong et al., 2024; Liao, Zhao, & Guo, 2024). Concurrently, viral pneumonia-associated targets were extracted from the GeneCards database (<https://www.genecards.org/>). Cytoscape 3.9.1 and the STRING database (<https://cn.string-db.org/>) were utilized to analysis and visualize the interaction network relationships between components, targets, and signaling pathways. Subsequently, the DAVID (<https://david.ncifcrf.gov/>) platform was employed to conduct gene ontology (GO) functional enrichment analysis and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of the common targets of WYJD and viral pneumonia.

2.12. Transcriptomics analysis

The total nucleic acid was extracted from mouse lung tissue by Trizol reagent, and tested by agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) to ensure the integrity of the nucleic acid samples. The transcriptomics sequencing was performed on the Illumina Nova Seq X Plus by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China). Bioinformatic analysis was performed using Omicsmart, a dynamic real-time interactive online platform for data analysis (<http://www.omicsmart.com/>). The genes with the parameter of *P* value < 0.05 and absolute fold change > 1.8 were considered as differentially expressed genes (DEGs), and the DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways.

The overlapping pathways enriched in both the network pharmacology and transcriptomics were selected as the potential core mechanisms of WYJD for further experimental validation. The targets of the overlapping pathways and the target-related components were used to build the “component-target-pathway” network of WYJD using Cytoscape 3.9.1.

2.13. Molecular docking and dynamics simulations

Molecular docking predicted the interactions between the candidate active components of WYJD and the essential target proteins using the AutoDock Vina software, which provided the interaction modes and strengths. The water molecules and other nonessential structures were removed before docking. And the stability of the component-protein binding was evaluated through Molecular Dynamics simulations. Molecular docking simulation models were built using the YASARA software and PYMOL software for a 0 – 100 ns simulation based on the above docking conformation, followed by the calculation of the root mean square deviation of atoms and the visualization of the results.

2.14. Quantitative real-time PCR (qRT-PCR)

The total nucleic acid was extracted from mouse lung tissue by Trizol reagent, and reverse-transcribed using PrimeScript™ RT Master Mix. qRT-PCR was performed using ChamQ SYBR qPCR Master Mix. The levels of target genes were normalized to that of β -actin in the lung tissue, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ algorithm as previously described (Pfaffl, 2001). The primer sequences of FOS like 1 (*Fosl1*) mRNA for qRT-PCR were 5'-ATGACCACCCCTCTGACTC-3' (forward), 5'-TCGCC ACTGCTGCTGCTAC-3' (reverse). β -actin: 5'-GATATCGCTGCGCTGG TCG-3' (forward), 5'-CATTCCCACCATCACCCCT-3' (reverse).

2.15. Fluorescence TUNEL assay

The paraffin sections of lung tissues were repaired by protease K, broken with membrane breaking solution, and incubated with the reaction solution which was prepared using a TUNEL kit. And the cell nuclei was labeled with DAPI. The sections stained with TUNEL and DAPI were observed using a confocal microscopy.

2.16. Western blotting

The total protein was extracted from mouse lung tissue using RIPA lysis buffer containing 1% protease inhibitor cocktail and 10 μ mol/L PMSF. The BCA kit was used to detect the protein contents. The protein samples were divided into equal amounts, separated using PAGE gel fast preparation kits, transferred to PVDF membranes (pore size 0.45 μ m), blotted with primary antibodies, and conjugated with the corresponding peroxidase secondary antibody. Signals from antibodies was detected using SuperPico ECL Master Mix (7E1420M5, Vazyme, Wuhan, China).

2.17. TLR7 inhibitor experiment

Enpatoran (M5049), a potent, orally active TLR7 inhibitor, was purchased from MedChemExpress Co. Ltd (HY-123581, 278368, 5 mg, 98.00%, Shanghai, China). Female BALB/c mice were randomly divided into control group (Control), IAV group (IAV), high dose of WYJD group (WYJD-H), M5049 group (M5049) and M5049 combination group (WYJD-H + M5049), five mice in each group. After 2 h infection, high dose of WYJD was given once a day. And 30 min after WYJD administration, 1 mg/(kg·d) of M5049 was given once a day in M5049 group. All mice were sacrificed on the sixth day.

2.18. Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10.2.0. One-way analysis of variance was used to test the homogeneity of variance, and Tukey's T test was used to analyze the significance of the differences between groups in the case of homogeneity of the sample population. Dunnett T3 test was used to analyze the significance of differences between groups in case of uneven variance of the sample population. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of the main chemical components of WYJD and WYJD-containing serum

To clarify the main chemical components of WYJD and WYJD-containing serum, UPLC-Q Exactive Orbitrap-MS/MS and a self-building TCM reference library were used. The base peak chromatograms (BPC) are presented in Fig. 1. A total of 192 compounds

were identified in WYJD (Table S1), and 25 prototypes and 15 metabolites from WYJD were identified in WYJD-containing serum (Table 1). The chemical structures of prototypes and metabolites identified in WYJD-containing serum were shown in Fig. S1, including flavonoids, alkaloids, terpenes, carbohydrates and glycosides, and organic acids and derivatives. The extracted ion chromatograms and mass spectrums of prototypes and metabolites identified in WYJD-containing serum were shown in Fig. S2.

3.2. WYJD showed therapeutic effects on IAV-induced pneumonia

To evaluate the protective effects of WYJD on IAV-induced pneumonia, this study administered five day's WYJD treatment to mice infected with $2 \times LD_{50}$ of IAV, and recorded daily the body weight, survival and clinical signs for 15 d (Fig. 2A). The mice began to exhibit progressive weight loss on the third day and succumbed on the eighth day after IAV infection. However, WYJD treatment mitigated the weight loss, decreased the mortality rate, and extended the survival time of the infected mice (Fig. 2B). Notably, the survival rate of the WYJD-H group increased to 50%.

To evaluate the antiviral and anti-inflammatory effects of WYJD, the pneumonia mice infected with $1 \times LD_{50}$ of IAV were treated with WYJD for 5 d and sacrificed on the 6th day (Fig. 2A). The lung indexes of mice with viral pneumonia might increase, which is mainly associated with the edema of lung tissues caused by viral infection. Compared with the IAV group, the lung indexes of the WYJD treatment groups were significantly reduced (Fig. 2C). As shown in Fig. 2C, HE staining of mouse lung tissues revealed cell death and retraction of ciliated columnar epithelial cells in the IAV group. Additionally, alveolar epithelial cells displayed hyperplasia and hypertrophy, and there was a widening of the lung interstitium accompanied by infiltration of lymphocytes and macrophages in the model group. However, the degrees of lung parenchymal lesions in WYJD treatment groups were decreased, which was manifested in smaller lesion areas and milder lymphocyte infiltration around the lung interstitium. In addition, the viral plaque assay was used to determine the viral replication in lung tissues. The results showed that both medium and high doses of WYJD treatment could significantly reduce the amounts of viral plaques (Fig. 2D).

3.3. Effect of WYJD on inflammatory responses in IAV-induced pneumonia mice

Viral infection could induce strong immune response, leading to the production of antiviral effector proteins to resist viral replication. However, viral infection could also lead to excessive inflammatory response, resulting in cytokines storm and tissue injury. For example, SARS-CoV-2 infection triggers the apoptosis of alveolar epithelial cells and the cytokine storm of IL-1 β , IL-6 and so on (Li et al., 2020b). In this study, blood routine tests were monitored using an automatic blood cell analyzer and the protein levels of multiple cytokines including IL-1 β , IL-6, IL-17A and TNF- α were measured in the lung tissues and serum of mice using multi-analyte suspension array and ELISA kits. As shown in Fig. 3A, the percentages of lymphocytes (%) showed a significant decrease and the percentages of macrophage (%) and neutrophils (%) showed a significant increase in the blood of mice infected by IAV, which is consistent with the symptoms of viral pneumonia, while WYJD treatment could significantly improve the unbalance of blood cells induced by viral infection. As shown in Fig. 3B and 3C, the protein levels of IL-1 β , IL-6 and TNF- α in the lung tissues and serum of the IAV group were significantly increased compared with the control group. Compared with the IAV group, WYJD treatment significantly or trendily inhibited the excessive production of IL-1 β , IL-6 and TNF- α in the lung tissues and serum at the protein levels. In addition,

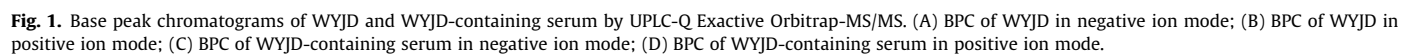


Table 1
Chemical components identified in WYJD-containing serum.

Peak No.	Formula	Identification	Classification	Retention time (min)	Ion mode	Adducts	Measured (m/z)	Theoretical (m/z)	Error ($\times 10^{-6}$)
6	C ₁₂ H ₂₂ O ₁₁	α -Lactose	Carbohydrates and glycosides	0.70	Negative	[M + FA-H] [−]	387.114 9	387.114 4	1.35
48	C ₁₄ H ₁₈ O ₉	Vanillic acid 4- β -D-glucopyranoside	Carbohydrates and glycosides	2.96	Negative	[M-H] [−] , [M + FA-H] [−]	329.088 3	329.087 8	1.59
M1	C ₉ H ₁₂ O ₆ S	Metabolite 2 of hydroxytyrosol	Metabolites (methylation, sulfation)	3.88	Negative	[M-H] [−]	247.028 3	247.028 2	0.33
59	C ₉ H ₁₀ O ₃	4-Hydroxyphenyl-2-propionic acid	Organic acids and derivatives	3.90	Negative	[M-H ₂ O-H] [−] , [M-H] [−]	165.055 1	165.055 7	−3.73
60	C ₁₆ H ₂₂ O ₁₁	Secoxyloganic acid	Terpenes	3.94	Positive	[M + Na] ⁺ , [M + K] ⁺ , [M + NH ₄] ⁺ , [M + H] ⁺	413.105 5	413.105 4	0.17
63	C ₁₆ H ₂₂ O ₁₀	Secologanic acid	Terpenes	4.04	Positive	[M + Na] ⁺	397.110 5	397.110 5	−0.15
M2	C ₉ H ₈ O ₇ S	Metabolite 1 of caffeic acid phenethyl ester	Metabolites (hydrolysis, sulfation)	4.08	Negative	[M-H] [−]	258.992 0	258.991 8	0.61
78	C ₂₄ H ₃₉ NO ₆	Neoline	Alkaloids	4.37	Positive	[M + H] ⁺	438.285 0	438.285 0	0.03
80	C ₁₇ H ₂₄ O ₁₁	Oleoside 11-methyl ester	Terpenes	4.41	Negative	[M-H] [−]	403.125 0	403.124 6	1.11
86	C ₂₄ H ₃₉ NO ₅	Talatisamine	Alkaloids	4.56	Positive	[M + H] ⁺	422.290 0	422.290 1	−0.12
M3	C ₂₇ H ₂₆ O ₁₈	Metabolite 2 of luteolin	Metabolites (glucuronidation, glucuronidation)	4.57	Negative	[M-H] [−]	637.105 9	637.104 6	1.94
M4	C ₂₇ H ₂₆ O ₁₈	Metabolite 1 of fisetin	Metabolites (glucuronidation, glucuronidation)	4.59	Positive	[M + H] ⁺	639.119 9	639.119 2	1.19
M5	C ₉ H ₁₀ O ₇ S	Metabolite 1 of asaraldehyde	Metabolites (demethylation, sulfation)	4.61	Negative	[M-H] [−]	261.007 7	261.007 4	0.94
94	C ₂₁ H ₂₂ O ₁₀	Isocoreopsin	Flavonoids	4.72	Negative	[M-H] [−]	433.114 3	433.114 0	0.54
M6	C ₂₁ H ₂₂ O ₁₃ S	Metabolite 1 of piceatannol	Metabolites (methylation, glucuronidation, sulfation)	4.77	Negative	[M-H] [−]	513.071 3	513.070 8	0.89
M7	C ₂₁ H ₂₂ O ₁₃ S	Metabolite 1 of rhapontigenin	Metabolites (glucuronidation, sulfation)	4.77	Positive	[M + Na] ⁺	537.067 8	537.067 3	0.84
97	C ₂₁ H ₂₀ O ₁₂	7-[(β -D-Glucopyranosyl)oxy]-3',4',5,8-tetrahydroxyflavone	Flavonoids	4.77	Positive	[M + H] ⁺ , [M + Na] ⁺	465.103 1	465.102 8	0.73
M8	C ₂₁ H ₂₀ O ₁₀	Metabolite 1 of liquiritigenin	Metabolites (glucuronidation)	4.80	Positive	[M + NH ₄] ⁺	450.139 4	450.139 5	−0.14
112	C ₂₈ H ₃₄ O ₁₅	Hesperidin	Flavonoids	4.99	Negative	[M-H] [−] , [M + FA-H] [−]	609.183 6	609.182 5	1.74
114	C ₂₂ H ₂₂ O ₁₁	Hispidulin 4'-O- β -D-glucopyranoside	Flavonoids	5.13	Negative	[M-H] [−]	461.109 7	461.108 9	1.72
115	C ₂₂ H ₂₂ O ₁₁	Pratensein 7-O-glucopyranoside	Flavonoids	5.17	Positive	[M + H] ⁺	463.123 3	463.123 5	−0.38
129	C ₂₂ H ₂₂ O ₉	Ononin	Flavonoids	5.45	Positive	[M + H] ⁺ , [M + K] ⁺ , [M + Na] ⁺	431.133 2	431.133 7	−1.01
M9	C ₂₁ H ₂₀ O ₁₀	Metabolite 1 of isoliquiritigenin	Metabolites (glucuronidation)	5.52	Positive	[M + H] ⁺	433.112 7	433.112 9	−0.61
M10	C ₁₆ H ₁₆ O ₇ S	Metabolite 1 of echinatin	Metabolites (reduction, sulfation)	5.87	Negative	[M-H] [−]	351.055 1	351.054 4	1.96
M11	C ₂₇ H ₂₈ O ₁₃	Metabolite 1 of neoglycyrol	Metabolites (hydroxylation, reduction, glucuronidation)	5.90	Negative	[M-H] [−]	559.146 6	559.145 7	1.58
136	C ₄₄ H ₇₀ O ₂₃	Rebaudioside A	Terpenes	6.06	Negative	[M-H] [−] , [M + FA-H] [−]	1 011.430 4	1 011.428 9	1.49
M12	C ₂₂ H ₂₂ O ₁₀	Metabolite 1 of 7-hydroxyflavanone	Metabolites (hydroxylation, methylation, glucuronidation)	6.10	Positive	[M + NH ₄] ⁺	464.155 4	464.155 1	0.59
139	C ₂₀ H ₁₈ NO ₄ ⁺	Berberine	Alkaloids	6.10	Positive	[M + H] ⁺	336.122 9	336.123 6	−2.01
M13	C ₂₄ H ₃₂ O ₁₁	Metabolite 1 of zearalenone	Metabolites (reduction, glucuronidation)	6.19	Positive	[M + H-H ₂ O] ⁺	479.188 8	479.191 1	−4.71
142	C ₁₆ H ₁₀ N ₂ O ₂	Indigotin	Alkaloids	6.23	Negative	[M + FA-H] [−]	307.073 4	307.072 3	3.56
141	C ₁₇ H ₁₂ N ₂ O ₄	Flazin	Alkaloids	6.23	Positive	[M + H] ⁺	309.086 8	309.087 0	−0.69
M14	C ₁₀ H ₁₂ O ₅	Metabolite 1 of xanthoxylin	Metabolites (hydroxylation)	6.75	Positive	[M + H] ⁺	213.075 8	213.075 7	0.21
151	C ₄₂ H ₆₂ O ₁₇	Licorice saponin G2	Terpenes	7.17	Positive	[M + H] ⁺ , [M + Na] ⁺ , [M + K] ⁺	839.405 1	839.406 0	−1.07
153	C ₂₀ H ₂₀ O ₇	Isosinensetin	Flavonoids	7.43	Positive	[M + Na] ⁺ , [M + K] ⁺ , [M + H] ⁺	373.127 8	373.128 2	−1.11
154	C ₁₅ H ₁₂ O ₄	Isoliquiritigenin	Flavonoids	7.46	Positive	[M + H] ⁺	257.080 5	257.080 8	−1.35
156	C ₁₆ H ₁₂ O ₄	Isoformononetin	Flavonoids	7.65	Negative	[M-H] [−]	267.066 4	267.066 3	0.31
M15	C ₂₆ H ₃₈ O ₉	Metabolite 1 of abietic acid	Metabolites (hydroxylation, glucuronidation)	8.19	Negative	[M-H] [−]	493.245 1	493.244 3	1.57

(continued on next page)

Table 1 (continued)

Peak No.	Formula	Identification	Classification	Retention time (min)	Ion mode	Adducts	Measured (m/z)	Theoretical (m/z)	Error (×10 ^{−6})
165	C ₂₁ H ₂₂ O ₈	Quercetagetin 3,5,6,7,3',4'-hexamethyl ether	Flavonoids	8.74	Positive	[M + Na] ⁺ , [M + K] ⁺ , [M + H] ⁺	425.120 2	425.120 7	−1.34
173	C ₂₀ H ₂₀ O ₇	Tangeretin	Flavonoids	9.37	Positive	[M + H] ⁺ , [M + Na] ⁺ , [M + K] ⁺	373.127 9	373.128 2	−0.76
192	C ₃₀ H ₄₆ O ₄	Gypsogenin	Terpenes	11.32	Positive	[M + H] ⁺	471.346 4	471.346 9	−1.04

tion, inflammatory cytokines induced by influenza virus infection could active the alveolar epithelial cells and endothelial cells of microvessels, and lead to high expression of adhesion molecules like ICAM-1 and VCAM-1, which attract more inflammatory cells to accumulate and overactive, forming a cascade effect and leading to serious tissue damage (Sugiyama et al., 2015). The ELISA results showed that the protein expressions of ICAM-1 and VCAM-1 were up-regulated in the lung tissues of IAV-infected mice, while WYJD treatment could reduce the expression of these adhesion molecules (Fig. 3C).

3.4. WYJD alleviated lung barrier damage in IAV-induced pneumonia mice

Excessive inflammatory responses triggered by viral infection can induce pulmonary edema through disruption of alveolar-capillary barrier integrity, potentially leading to acute respiratory distress syndrome and acute respiratory failure (Hook & Bhattacharya, 2024). To determine the effects of WYJD on lung barrier damage induced by IAV infection, we used IF method to observe the tight junction proteins ZO-1, Occludin, and Claudin-4 in mouse lung tissues. As shown in Fig. 4A, the fluorescence intensities of ZO-1, Occludin, and Claudin-4 were lower in the lung tissues of IAV-infected mice, while the fluorescence intensities of these tight junction proteins in the lung tissues of the WYJD treatment groups were increased to varying degrees. And the Western blotting results also indicated that WYJD treatment could increase the protein expression levels of ZO-1, Occludin, and Claudin-4, which is consistent with the results of IF (Fig. 4B).

Additionally, we employed the Evans blue staining method to observe the lung barrier damage directly (Fig. 4C). The contents of Evans blue in the lung tissues of the WYJD treatment groups were significantly lower than those in the IAV group (Fig. 4D).

3.5. Network pharmacology revealed the mechanisms of WYJD against viral pneumonia

A total of 250 targets of components identified in WYJD-containing serum and 2 857 targets of viral pneumonia were obtained. An intersection of two target databases revealed 159 potential therapeutic targets of WYJD against viral pneumonia (Fig. 5A). The protein–protein interaction (PPI) network of the therapeutic targets was constructed using the STRING database and Cytoscape 3.9.1, which identified the ten most prominent nodes (TP53, AKT1, CTNNB1, STAT3, SRC, TNF, EGFR, IL6, TLR4, and BCL2) as primary therapeutic targets (Fig. 5B). In addition, the component–target network revealed that 14 components of WYJD-containing serum might play a significant role in the treatment of viral pneumonia, including berberine, fisetin and luteolin (Fig. 5C). The top ten significantly enriched terms (biological process, BP; cellular component, CC; molecular function, MF) related to the therapeutic targets by GO enrichment analysis were shown

in Fig. 6A, of which the top ten BP terms included negative regulation of apoptotic process. After deleting the pathway not related to viral pneumonia like lipid and atherosclerosis, the IL-17 signaling pathway, Toll-like receptor signaling pathway, apoptosis and MAPK signaling pathway were among the top 20 significantly enriched KEGG pathways (Fig. 6B). In summary, the network pharmacology analysis offers an initial direction for follow-up investigations.

3.6. Transcriptomics identified the genes and pathways related to the therapeutic effect of WYJD against IAV-induced pneumonia

To further elucidating the mechanisms underlying the effects of WYJD against IAV-induced pneumonia, transcriptomics analysis was conducted on the mouse lung tissues of the control group, the IAV group and the WYJD-H group. In all, 4 400 genes showed significant differential expression between the control group and the IAV group. And 581 genes were significantly regulated by WYJD treatment, with 387 genes upregulated and 194 genes downregulated in expression (Fig. 7A and 7B). There are 467 common differentially expressed genes (DEGs) between the control group vs the IAV group, and the IAV group vs the WYJD-H group (Fig. 7C). GO and KEGG pathway enrichment analysis were performed on these 581 genes significantly regulated by WYJD treatment. The top ten GO terms were shown in Fig. 7D, and the BP including the immune system process, defense response, inflammatory response, and myeloid leukocyte migration were significantly enriched. The top 20 signaling pathways were significantly enriched based on the KEGG database, including the IL-17 signaling pathway, MAPK signaling pathway and PI3K-AKT signaling pathway (Fig. 7E).

3.7. Integrated analysis of network pharmacology combined with transcriptomics

A total of ten core signaling pathways related to the therapeutic effect of WYJD against IAV-induced pneumonia, including the IL-17 signaling pathway and MAPK signaling pathway, were enriched in both the network pharmacology and transcriptomics (Fig. 8A). Finally, a total of 86 targets of the ten pathways and 11 target-related components in WYJD-containing serum were selected to build TCM-component-target-pathway network (Fig. 8B). The topological properties indicated that berberine, luteolin, fisetin, isoliquiritigenin, and tangeretin were the top five components in terms of degrees, betweenness centralities and closeness centralities. Therefore, these components were regarded as the candidate active components of WYJD against IAV-induced pneumonia. These core targets lay mainly in the IL-17 and MAPK signaling pathways, which are highly associated with the Toll-like receptor signaling pathway and apoptosis identified by network pharmacology (Fig. 8C) (Lukacs et al., 2010; Sun et al., 2015). Furthermore, IL-17A, MKK6, PTP, HSP72, AP-1, IL-4, CXCL1, CXCL2, CCL2, CCL7, IL-6,

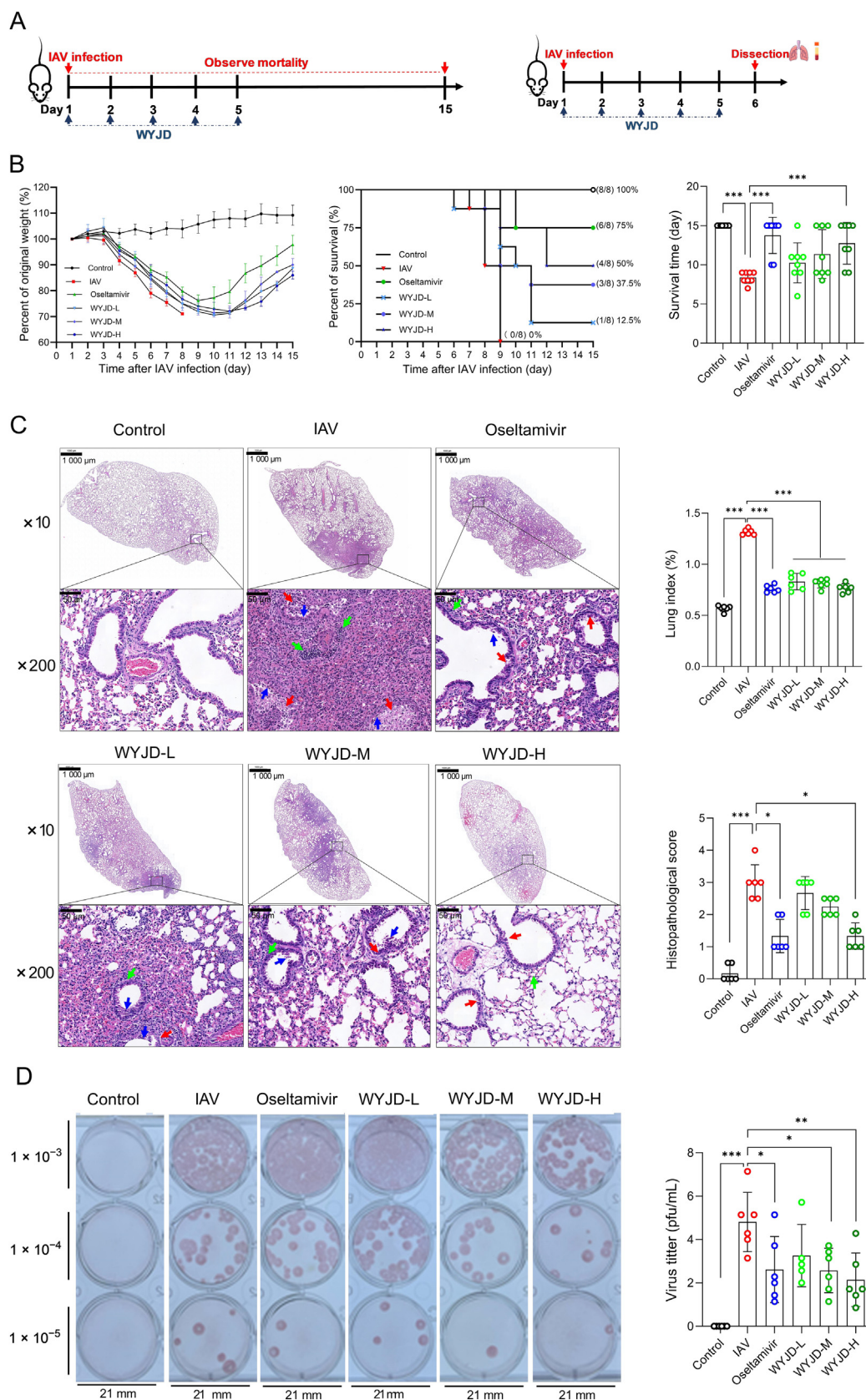


Fig. 2. WYJD showed therapeutic effects on IAV-induced pneumonia. (A) Animal experimental designs. (B) Body weights, survival rates and survival days of mice infected with $2 \times LD_{50}$ of IAV ($n = 8$). (C) Lung indexes, histological observations of lung tissues by HE staining (red: infiltration of inflammatory cells in the bronchus, blue: destruction of the bronchial wall, and green: accumulation of inflammatory cells in the peripheral bronchus), and (D) viral loads of lung tissues by viral plaque assay of mice infected with $1 \times LD_{50}$ of IAV ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs IAV group.

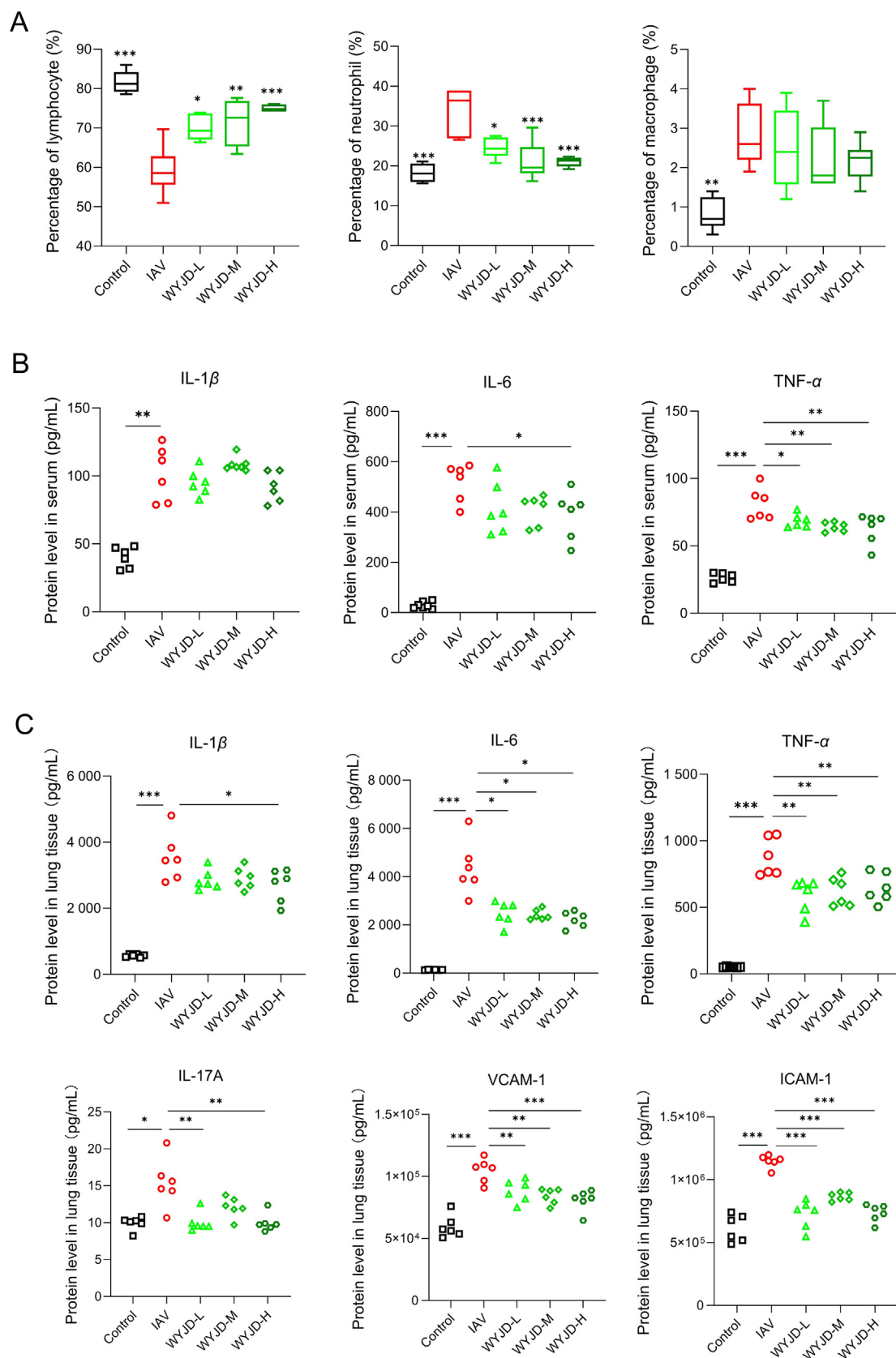


Fig. 3. Effect of WYJD on inflammatory responses in IAV-induced pneumonia mice. (A) Blood routine parameters, (B) protein levels of IL-1 β , IL-6 and TNF- α in serum, and (C) protein levels of IL-1 β , IL-6, TNF- α , IL-17A, VCAM-1 and ICAM-1 in lung tissues of IAV-induced pneumonia mice ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs IAV group.

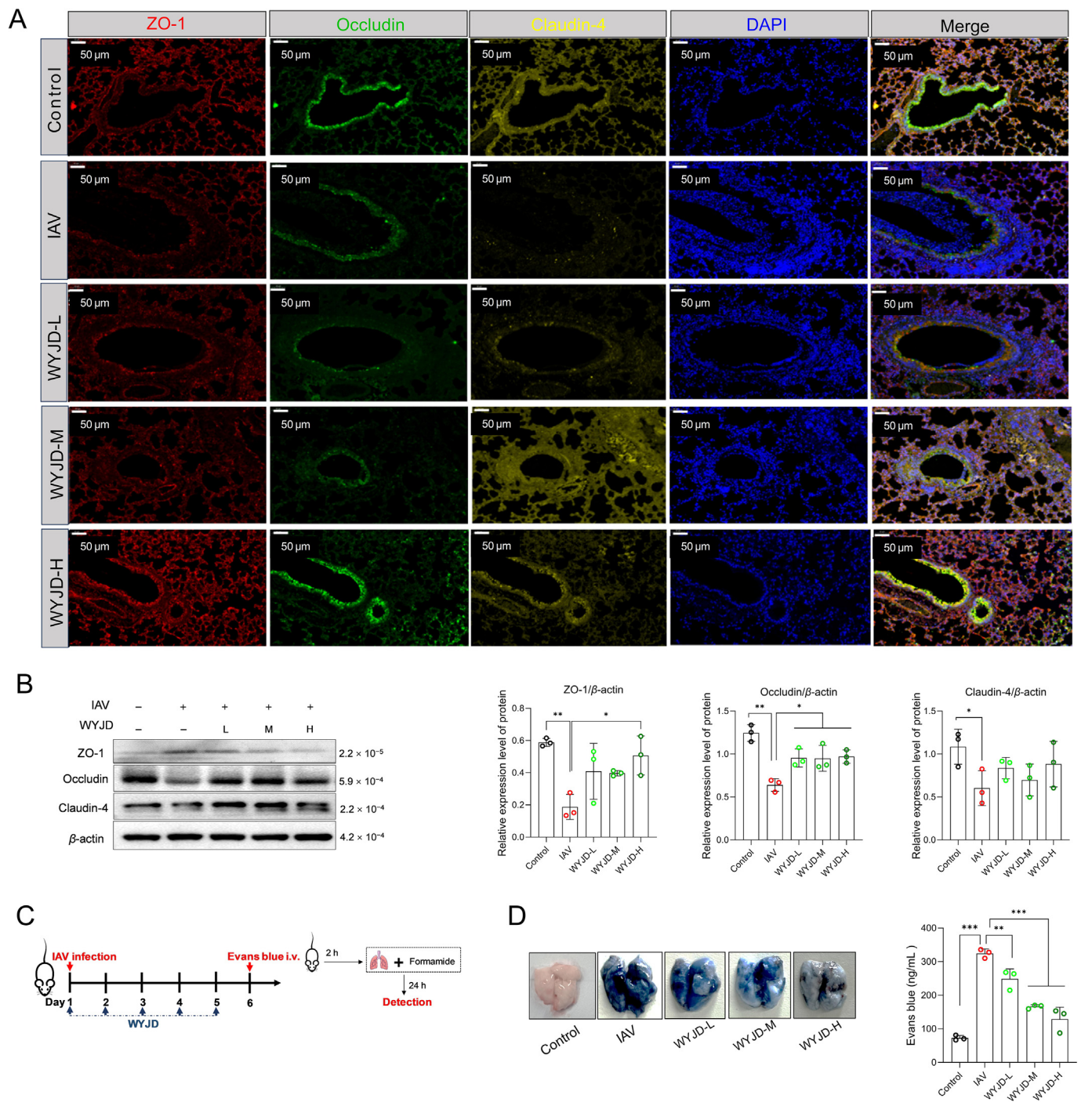


Fig. 4. WYJD alleviated lung barrier damage in IAV-induced pneumonia mice. (A) IF images of the tight junction protein ZO-1, Occludin, and Claudin-4 in the lung tissues, (B) Western blotting results of the tight junction protein ZO-1, Occludin, and Claudin-4 in the lung tissues, (C) animal experimental design for Evans blue staining, and (D) contents of Evans blue in the lung tissues of IAV-induced pneumonia mice ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs IAV group.

G-CSF, MUC5AC, MUC5B, S100A8, and S100A9, which are related to the IL-17 and MAPK signaling pathways, were differentially expressed in transcriptomics and multi-analyte suspension array.

3.8. WYJD regulated the expression of proteins related to the potential signaling pathways

The expression levels of DEGs in transcriptomics that encode proteins related to the IL-17 and MAPK signaling pathways were shown in Fig. 9A. FOS gene family, consisting of *c-Fos*, *FosB*, *FosL1*

and *FosL2*, encode proteins that can dimerize with proteins of JUN family, thereby forming the transcriptional activation factor AP-1. As such, the proteins of FOS family have been inferred to be regulator of multiple cellular processes, such as apoptosis cell death and cell transformation (Galvagni, Orlandini, & Oliviero, 2013; Zhou et al., 2022). In some cases, FosL1 can be activated by viral infections mainly through specific activation of TLR7 and the MAPK signaling pathway (Chen, Li, Liu, Zhang, Liu, & Li, 2023; de Marcken, Dhaliwal, Danielsen, Gautron & Dominguez-Villar, 2019; Jia et al., 2021). Based on the KEGG pathway map, the DEG

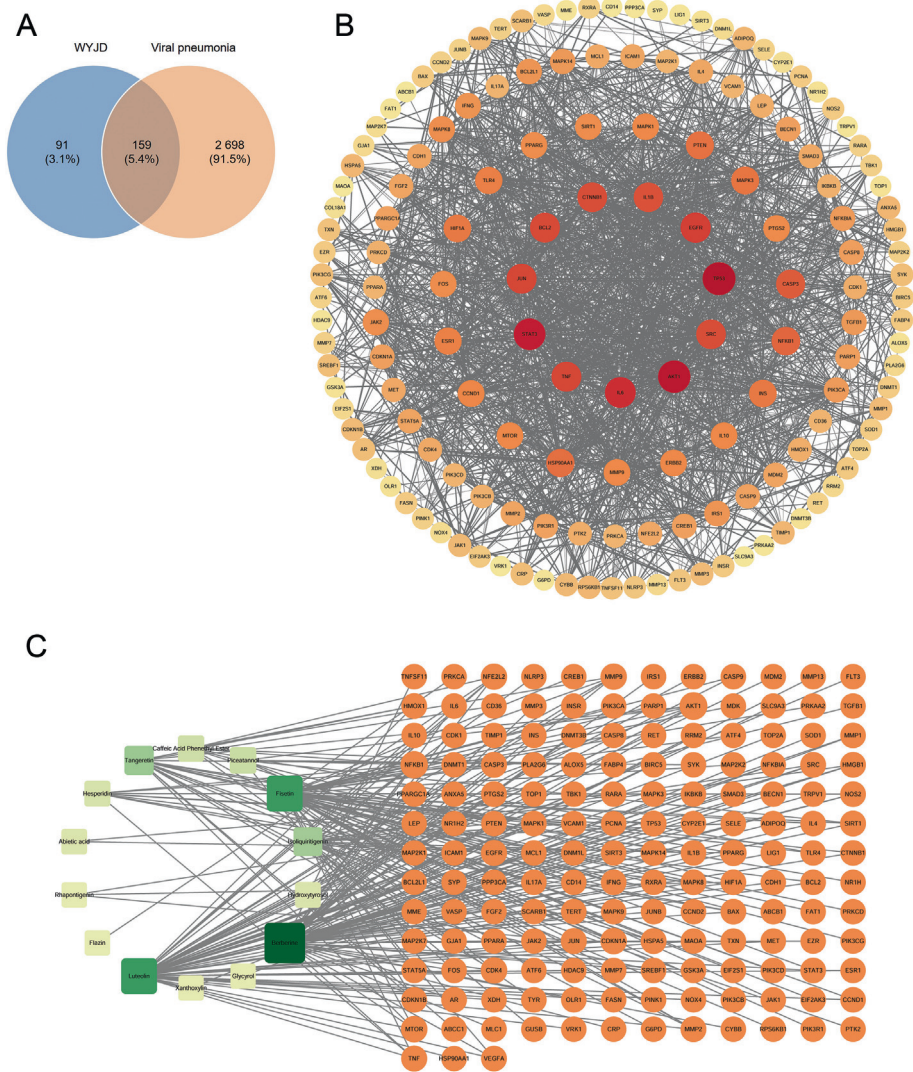


Fig. 5. Identification of the potential mechanisms of WYJD against viral pneumonia. (A) Venn diagram of common targets of WYJD and viral pneumonia. (B) Protein-protein interaction (PPI) network of potential therapeutic targets of WYJD against viral pneumonia. (C) The component-target network of WYJD against viral pneumonia. The green squares represent the possible active components in WYJD-containing serum; the orange circles represent therapeutic targets.

Fosl1-encoded protein AP-1 acted upstream of the proteins encoded by remaining ten DEGs, such as IL-4, IL6 and CXCL2. And the mRNA expression levels of Fosl1 have shown to be down-regulated by WYJD treatment using qRT-PCR (Fig. 9B). Figs. 9C, 9D and 8E shows that IAV infection notably activated the expression of TLR7 and MyD88, increased phosphorylation of ERK1/2, jnk, p38 and c-Jun. However, the activation of TLR7/MyD88/MAPKs/AP-1 were largely abolished by WYJD. Thus, WYJD suppressed the TLR7/MyD88/MAPKs/AP-1 signaling pathway activated by IAV infection.

3.9. WYJD suppressed apoptosis in IAV-induced pneumonia mice

Apoptosis significantly contributes to lung injury caused by viral infections, which closely interacts with the MAPK signaling pathway (Qin et al., 2023; Yang et al., 2024). TUNEL staining was conducted on the lung tissues of mice, and there were obviously more apoptotic signals in the epithelial-endothelial barrier of lung tissues of the IAV group compared with the control group (Fig. 10A). However, the fluorescence signals of apoptosis in the WYJD treatment groups were weaker compared to the IAV group. In addition, western blotting analysis showed that WYJD treatment

could regulate the expression levels of apoptosis related protein Bax and Bcl-2 in mouse lung tissues (Fig. 10B, C).

3.10. WYJD treated IAV-induced pneumonia through TLR7/MyD88/MAPKs/AP-1 signaling pathway

TLR7 plays an important role in IAV infection, which recognizes the ssRNA from influenza and initiates a MAPK signaling cascade through a MyD88-dependent pathway. To further clarify whether WYJD can treat IAV-induced pneumonia through TLR7/MyD88/MAPKs/AP-1 signaling pathway, as Fig. 11A shown, a high dose of WYJD was administrated 2 h after IAV infection, and then M5049 was used to inhibit the TLR7 pathway. Firstly, the lung indexes and histological observations by H&E staining showed that the lung tissue injury in the WYJD-H group, M5049 group and WYJD-H + M5049 group were all alleviated (Fig. 11B). The inhibitory effect of WYJD-H on the levels of IL-1 β , TNF- α and IL-6 in lung tissues were comparable to that of M5049 and WYJD-H + M5049 (Fig. 11C). In addition, WYJD-H, M5049 and WYJD-H + M5049 treatment could increase the protein expression levels of tight junction proteins ZO-1, Occludin and Claudin-4, the indicated the protective effect on lung barrier damage (Fig. 11D and 11E). Mech-

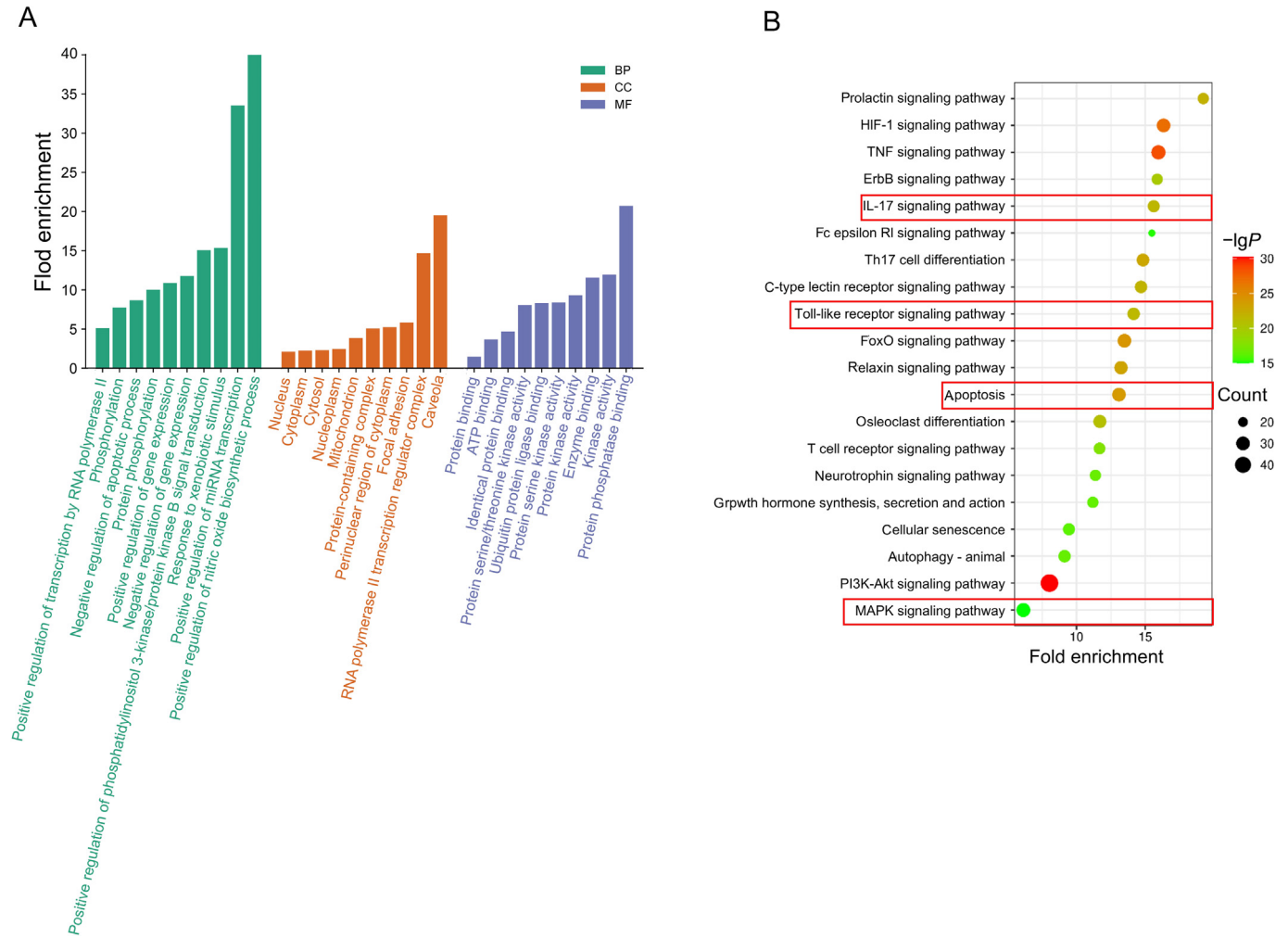


Fig. 6. GO and KEGG pathway enrichment. (A) Top ten GO terms of therapeutic targets of WYJD against viral pneumonia. (B) Top 20 KEGG signaling pathways of therapeutic targets of WYJD against viral pneumonia.

anistically, the Western blotting analysis showed that WYJD-H, M5049 and WYJD-H + M5049 treatment all suppressed the TLR7/MyD88/MAPKs/AP-1 signaling pathway activated by IAV infection.

3.11. Binding activity of candidate active components of WYJD to the essential targets

Based on integrated network pharmacology, transcriptomics, and experimental validation, berberine, luteolin, fisetin, isoliquiritigenin, and tangeretin were regarded as the top five candidate active components of WYJD. Besides, the essential targets associated with these components included TLR7, MyD88, ERK, JNK, p38, c-Jun, Bax, and Bcl-2. Molecular docking and dynamics simulations were performed using AutoDock Vina and YASARA software, yielding 40 docking configurations (Fig. 12A). Lower binding energies indicate higher binding affinity, and notably all five candidate components exhibited binding energies below -7.12 kcal/mol with TLR7, suggesting spontaneous binding interactions. Additionally, M5049, BIRB 796, JNK-IN-8, BAI1 and Bcl-2-IN-9 were selected as corresponding positive controls based on literature reports. And M5049, BIRB 796, JNK-IN-8, BAI1 and Bcl-2-IN-9 exhibited binding energies below -7.6 kcal/mol with TLR7, -8.0 kcal/mol with p38, -8.3 kcal/mol with JNK, -6.5 kcal/mol with Bax, and -8.0 kcal/mol with Bcl-2, respectively. The most favorable docking modes for each target, corresponding to the low-

est binding energies, were visualized using PYMOL software (Fig. 12B).

4. Discussion

In this study, an IAV-induced pneumonia mouse model was established to investigate the effects and mechanisms of WYJD. Treatment with WYJD showed a significant protective effect on the body weight loss and death induced by lethal dose of IAV infection, and ameliorated histological changes in the lung tissues. The pathophysiology of IAV infection involves viral replication and immune activation, which in turn trigger lung inflammation and tissue injury (Herold et al., 2015). Notably, WYJD treatment significantly reduced both lung viral loads and lung inflammation, as evidenced by the downregulation of IL-1 β , IL-6 and TNF- α expressions in serum and lung tissues, and the percentages of lymphocytes, macrophage and neutrophils in whole blood. In addition, the multivalent adhesion of leukocytes, platelets and possible pathogens to lung tissues contributes to lung injury induced by viral infections, and WYJD treatment could also reduce the levels of adhesion molecules ICAM-1 and VCAM-1 to inhibit the migration of inflammatory cells from peripheral blood to the site of inflammation in lung tissues (Xu & Shaw, 2016). These findings demonstrate that WYJD possesses dual therapeutic efficacy with anti-viral and anti-inflammatory activities. Consistent with previ-

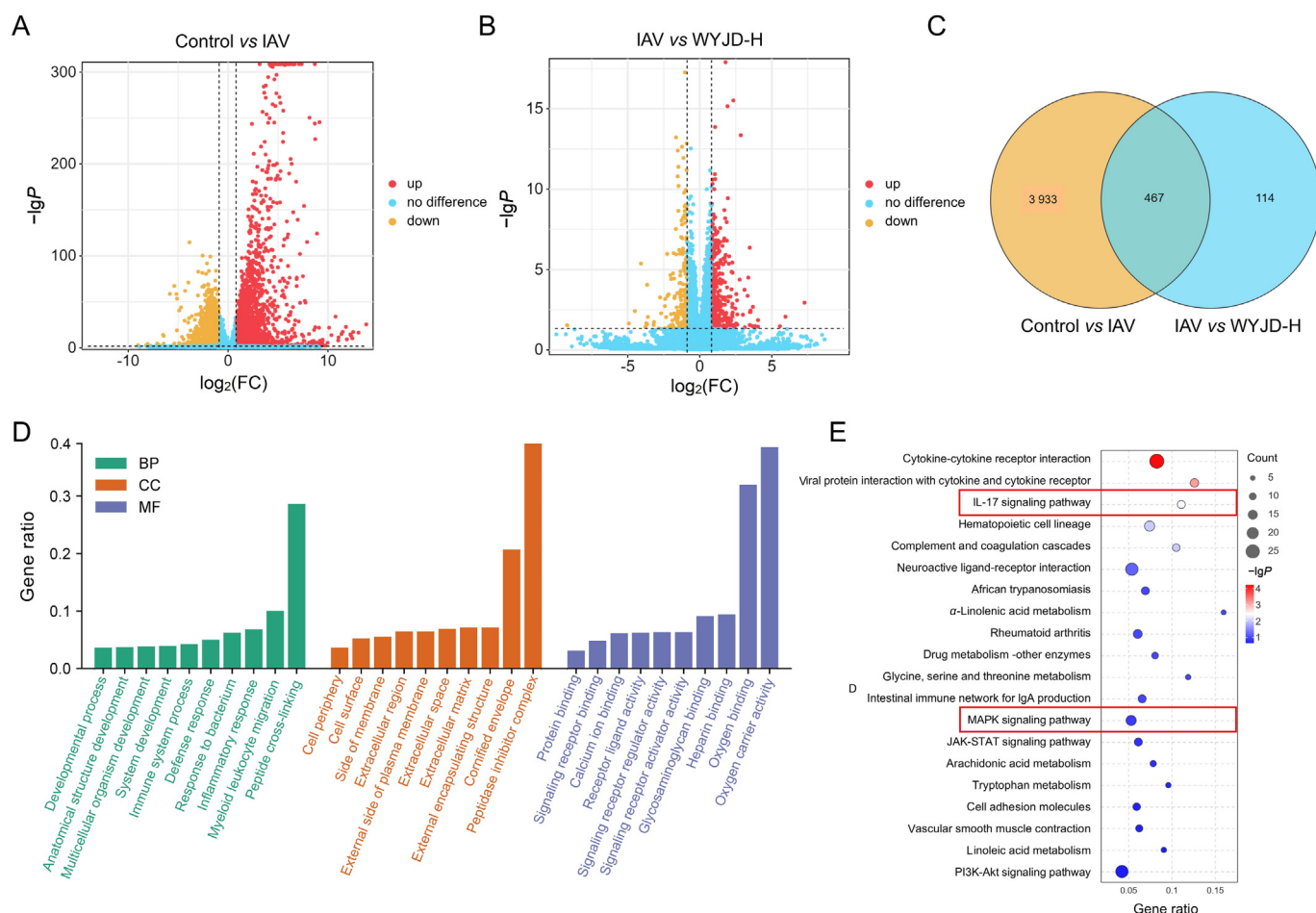


Fig. 7. Transcriptomics identified the genes and pathways related to the therapeutic effect of WYJD against IAV-induced pneumonia. Volcano plots of DEGs between (A) the control group and IAV group, and (B) the IAV group and the WYJD-H group. (C) Venn diagram of the common DEGs of the control group vs the IAV group, and the IAV group vs the WYJD-H group. (D) Top ten GO terms of DEGs between the IAV group and the WYJD-H group. (E) Top 20 KEGG signaling pathways of DEGs between the IAV group and the WYJD-H group ($n = 6$).

ously studies and clinical outcomes of WYJD, our data strongly support that WYJD is an effective therapeutic agent against IAV-induced pneumonia (Li et al., 2025b; Lu et al., 2024; Wang et al., 2021).

Meanwhile, lung barrier damage induced by viral infection is a hallmark pathological feature of viral pneumonia and acute respiratory distress syndrome, contributing to significant morbidity and mortality (Hook & Bhattacharya, 2024). The most evident manifestation of lung barrier damage is increased alveolar permeability, which results in cell and fluid accumulation into the air spaces, impaired gas exchange and pulmonary edema. These pathological changes may severely compromise patients' quality of life (Bos & Ware, 2022). Additionally, lung barrier damage induced by viral infection is associated with secondary bacterial infections, often leading to severe or fatal clinical outcomes (Broggi et al., 2020). Lung index measurement and Evans blue staining assay confirmed that WYJD treatment reduced alveolar permeability and ameliorated lung barrier damage. Specifically, lung barrier is comprised of alveolar epithelial and microvascular endothelial cells that share a basement membrane, and the permeability is regulated by the tight junctional protein complexes between adjacent epithelial and endothelial cells, including ZO-1, Occludin, and Claudin-4 (Chan et al., 2009). This study further demonstrated that WYJD treatment significantly attenuated the downregulation of tight junctional proteins in lung tissues of IAV-infected mice. It is generally recognized that influenza virus infection primarily targets

alveolar epithelial cells and causes direct loss of the tight junctional proteins, inflammatory cytokine production, immune cell recruitment, and cell death, which in turn contributes to lung barrier damage (Uiprasertkul et al., 2007; Walsh et al., 2011). These results suggest that WYJD treatment could protect against IAV-induced lung barrier damage potentially through its anti-viral, anti-inflammatory and anti-apoptotic effects, which further elucidates the mechanisms underlying the clinical efficacy of WYJD in preventing the exacerbation of viral pneumonia.

WYJD is an effective TCM preparation that has been generally applied for treating respiratory infectious disease. However, the underlying mechanisms of its action remain poorly understood. The active components of WYJD were firstly explored by serum chemical composition analysis using UPLC-Q Exactive Orbitrap-MS/MS, and a total of 25 prototypes and 15 metabolites were identified in WYJD-containing serum, including flavonoids, alkaloids, terpenes, carbohydrates and glycosides, and organic acids and derivatives. Multiple chemical components like berberine, luteolin and tangeretin have been widely reported to exhibit immunomodulatory, anti-inflammatory and anti-infective effects, indicating that WYJD might possess complex pharmacological mechanisms through the synergistic effects of multiple components (Chen, Liu, Wang, Lu, & Ye, 2023; Wang, Ling, Yao, Zheng, & Chen, 2020; Xu et al., 2015). In addition, the identified metabolites are primarily Phase II products formed via glucuronidation and sulfation of the prototypes in WYJD, which exhibit enhanced water solubility. However, the pharmaco-

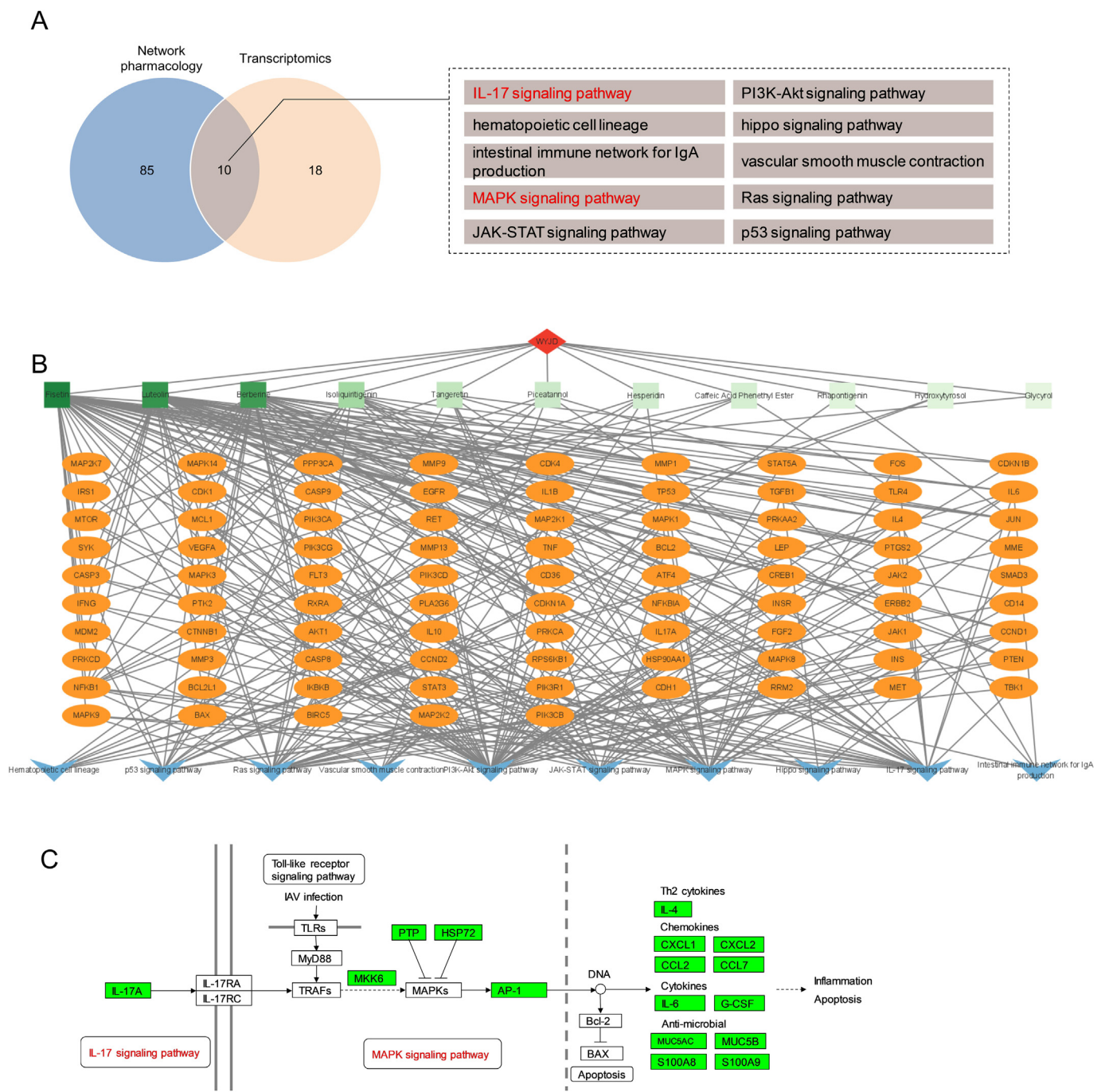


Fig. 8. Integrated analysis of network pharmacology combined with transcriptomics. (A) Venn diagram of the common enriched signaling pathways of transcriptomics and network pharmacology. (B) TCM-component-target-pathway network of the therapeutic effect of WYJD against IAV-induced pneumonia. (C) Candidate pathways related to the therapeutic effect of WYJD against IAV-induced pneumonia. The differentially expressed proteins by transcriptomics and multi-analyte suspension array are labeled in green.

logical activities of these metabolites require further investigation. Therefore, a network pharmacology study based on the components of WYJD-containing serum was performed to find the targets, biological processes, and pathway in play. Negative regulation of apoptotic process was significantly enriched in GO biological process analysis, and the PPI network analysis revealed that apoptosis related target named BCL2 was one of the ten targets with the highest scores. These findings indicate that the biological process of apoptosis might be associated with the effects of WYJD against IAV-induced pneumonia. Programmed cell death is a crucial cellular response frequently observed in IAV-infected tissues, and IAV modulates host cellular

apoptosis through virus-host interactions to support efficient viral replication and propagation, which contributes to severe lung injury (Ampomah & Lim, 2020). Apoptosis related pathways, including the IL-17 signaling pathway, Toll-like receptor signaling pathway, apoptosis and MAPK signaling pathway were also significantly enriched in KEGG pathway analysis. Thus, the network pharmacology preliminarily suggests that WYJD may exert its therapeutic effects by modulating apoptosis and related signaling pathways. Further exploration of the WYJD's therapeutic effects at the gene expression level were conducted by transcriptomics. 467 common DEGs regulated by WYJD were identified, and the IL-17

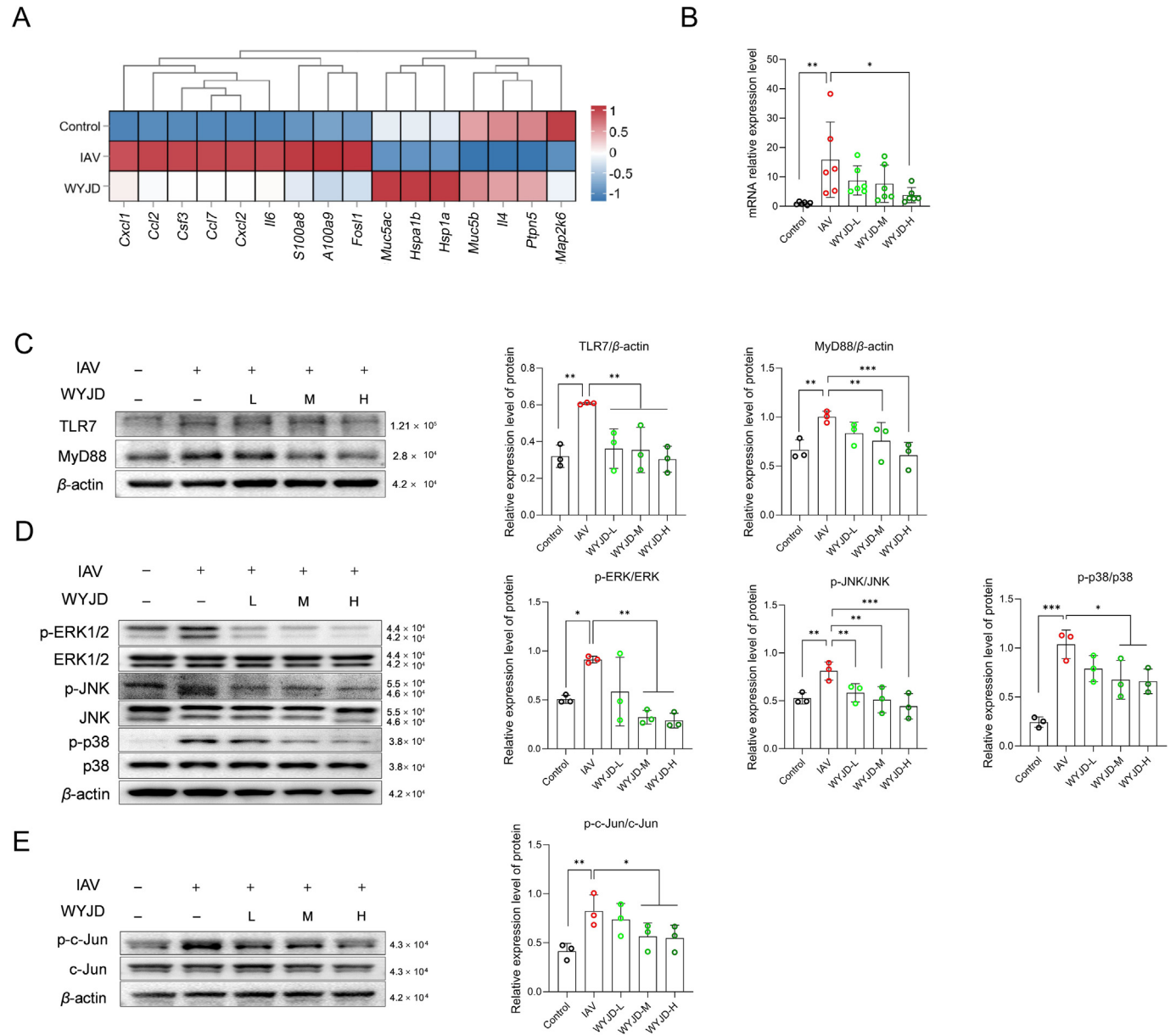


Fig. 9. WYJD regulated the IL-17 and TLR7/MyD88/MAPKs/AP-1 signaling pathways. (A) Heatmap of the expression levels of 11 DEGs in the IL-17 pathway by transcriptomics; (B) mRNA expression levels of *Fos1* in mouse lung tissues by qRT-PCR; (C) Western blotting analysis of TLR7 and MyD88; (D) Western blotting analysis of ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38; (E) Western blotting analysis of c-Jun and p-c-Jun ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs IAV group.

signaling pathway and MAPK signaling pathway were significantly enriched in KEGG pathway analysis. Transcriptomics confirmed some core signaling pathways of WYJD as predicted by the network pharmacology, and suggested that WYJD regulated the expression of certain genes, including *Fos1*, *CXCL1*, *IL-6*, *MUC5AC*, *MUC5B*, *S100A8*, and *S100A9*. The integrated analysis of transcriptomics and network pharmacology final revealed that 11 potential active components regulated 10 critical signaling pathways via 86 candidate targets to accomplish the systemic therapeutic effects of WYJD on IAV-induced pneumonia. Especially, multiple candidate targets lay in the IL-17 and MAPK signaling pathways, including IL-17, FOS, JUN, MAPK1, MAPK9 and MAPK14. WYJD, a classic TCM preparation, exerts therapeutic effects through multi-component, multi-target and multi-channel characteristic of TCM.

Next, the related genes and protein of potential pathways were measured using multi-analyte suspension array, qRT-PCR, and Western blotting. The IL-17 signaling pathway and MAPK signaling

pathway play crucial roles in the immune response against IAV infections, but also mediate lung inflammation and acute lung injury induced by IAV (Li et al., 2011; Li et al., 2025a). Specifically, IL-17A is a common pathogenic molecule regulating disease induced IAV and other respiratory viruses. IL-17A activates the MAPK signaling pathway through its receptors IL-17RA/RC and downstream signal factors like TRAF6. Further, the activation of the MAPK signaling pathway upregulates the expression of apoptosis-related proteins and pro-inflammatory cytokines, which contributes to lung injury. The transcriptomics analysis identified 11 DEGs in the IL-17 signaling pathway. Based on these findings, we inferred that in the lung tissues of IAV-induced pneumonia mice, IL-17 might activate the transcription factor AP-1 encoded by DEG *Fos1* via the MAPK signaling pathway, which in turn regulated the expression of the remaining ten DEGs as downstream targets. Among these downstream ten DEGs, IL-6, G-CSF, CCL2, CCL7, CXCL1, and CXCL2 serve as pro-inflammatory cytokines,

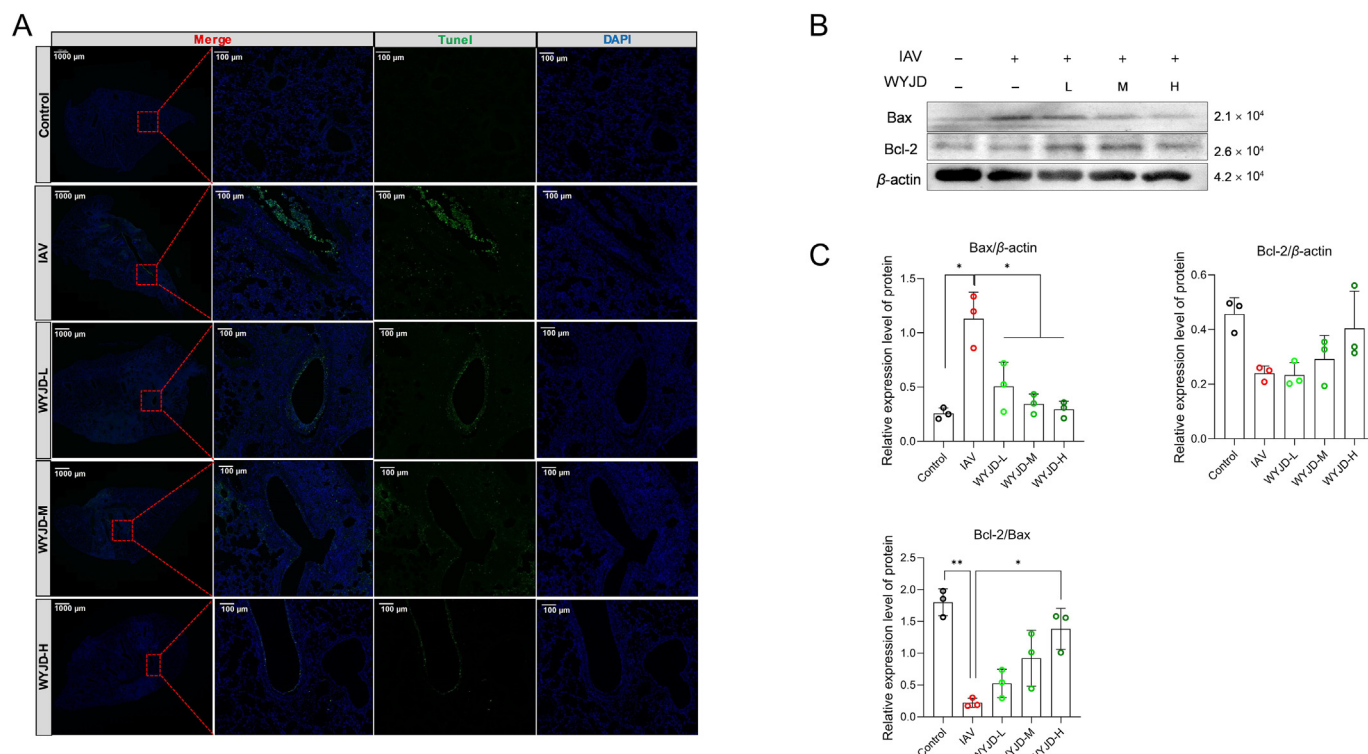


Fig. 10. WYJD suppressed apoptosis in IAV-induced pneumonia mice. (A) Fluorescence images of mouse lung tissues by TUNEL staining. (B, C) Western blotting analysis of Bax and Bcl-2 ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared with the IAV group.

and *MUC5AC* and *MUC5B* serve as mucus hypersecretion associated factors in the pathological process of influenza virus infections (López et al., 2024). Additionally, previous researches have verified that the upregulation of *S100A8* and *S100A9* could further increase the expression of IL-17 and promote lung epithelial cell apoptosis (Bai et al., 2021; Pei, Chen, Qu, & Lu, 2024). Consistent with transcriptomics analysis, we confirmed that WYJD inhibited the expression levels of IL-17A, IL-6 and *Fos11*, suggesting that WYJD could ameliorate IAV-induced pneumonia through the IL-17 signaling pathway.

Meanwhile, *Fos11* as a member of *FOS* gene family can encode proteins that dimerize with JUN protein family, thereby forming the transcriptional activation factor AP-1 (Galvagni, Orlandini, & Oliviero, 2013). AP-1 can bind to a common DNA site and regulate different target genes that execute distinct biological functions, including cell death, immune and differentiation (Shaulian & Karin, 2002; Trop-Steinberg & Azar, 2017). In the cases of IAV infections, the ssRNA from IAV can be recognized by TLR family members, including TLR7/8 (Kanno et al., 2013). TLR7, a classic pattern recognition receptor localized on the cellular endosomes, can activate the MAPK signaling pathway through MyD88-dependent or independent pathways (O'Neill, Golenbock, & Bowie, 2013; Wimmers et al., 2021). Then, the activation of JNK, ERK and p38 in the MAPK pathway regulates the expression and phosphorylation of FOS and Junfamily proteins, leading to the formation the transcription factor AP-1. This complex subsequently induces the expression of various functional proteins, including inflammatory cytokines and apoptosis related proteins, ultimately contributing to the inflammation, apoptosis and tissue damage (Ludwig, Ehrhardt, Neumeier, Kracht, Rapp, & Pleschka 2001; Wan et al., 2022). Additionally, previous reports have shown that the MAPK signaling pathway induced by IAV infection activates the transcription factor Gli1, resulting in a direct downregulation of intercellular junction proteins, such as E-cadherin, ZO-1, and

Occludin (Ruan et al., 2020). Interestingly, some clinical data showed that the *TLR7* mRNA expression and IL-17 levels in peripheral blood mononuclear cells were higher in the acute respiratory distress syndrome group than in the control group, and that TLR7 activation in plasmacytoid dendritic cells could contribute to the Th17 differentiation in viral host defense (Chu, Liu, & Tang, 2024; Yu et al., 2010). Consistent with the results of clinical studies, *in vitro* experiments also indicated that TLR7 activation stimulated the production of IL-17 (Lombardi, Van Overtvelt, Horiot, & Moingeon, 2009; Stojić-Vukanić et al., 2016). However, the links between the TLR7 pathway and the IL-17 pathway are still unclear. Western blotting analysis suggested that WYJD could inhibit the expression of TLR7, and in turn suppress the downstream activation of MyD88, MAPKs and AP-1 induced by IAV infections in lung tissues. Further, we performed the TLR7 inhibitor experiment to confirm the mechanism of WYJD which is mainly focused on TLR7 to suppress its downstream signaling pathways, thereby retarded the pathological process of IAV-induced pneumonia. These findings indicate that WYJD alleviates inflammation, apoptosis, and lung tissue damage by inhibiting TLR7/MyD88/MAPKs/AP-1 signaling pathway, thereby mitigating influenza progression and preventing its severe complications (Fig. 13).

Then, the top five candidate active components of WYJD selected by network pharmacology were docked with key proteins. Berberine, luteolin, fisetin, isoliquiritigenin, and tangeretin all had high docking activities with TLR7, MyD88, ERK, JNK, p-38, c-Jun, Bax, and Bcl-2, suggesting that active components of WYJD might directly bound to these key proteins to regulate TLR7/MyD88/MAPKs/AP-1 signaling pathway and apoptosis.

However, this study does have several limitations. First off, more functional validation experiments involving the silencing or activation of key signaling factors in cellular or animal models need to be performed. More convincing results could be obtained by using genetic manipulation or agonist/inhibitor-induced models.

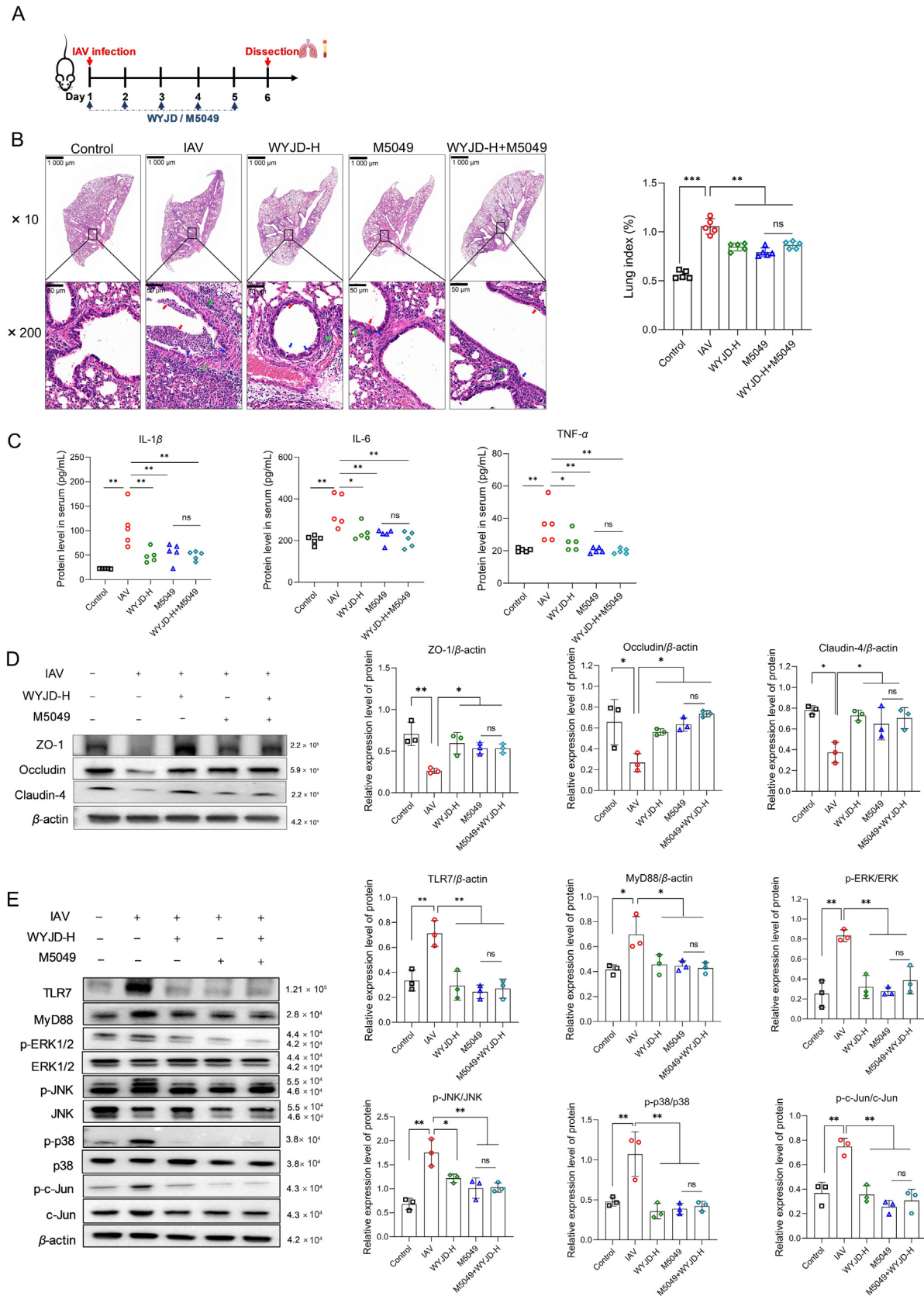


Fig. 11. WYJD treated IAV-induced pneumonia through TLR7/MyD88/MAPKs/AP-1 signaling pathway. (A) Animal experimental design for the TLR7 inhibitor experiment, (B) lung indexes and histological observations of lung tissues by HE staining, (C) protein levels of IL-1β, IL-6 and TNF-α in lung tissues, (D, E) Western blotting analysis of ZO-1, Occludin, Claudin-4, TLR7, MyD88, ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-p38, c-Jun, and p-c-Jun ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared with the IAV group.

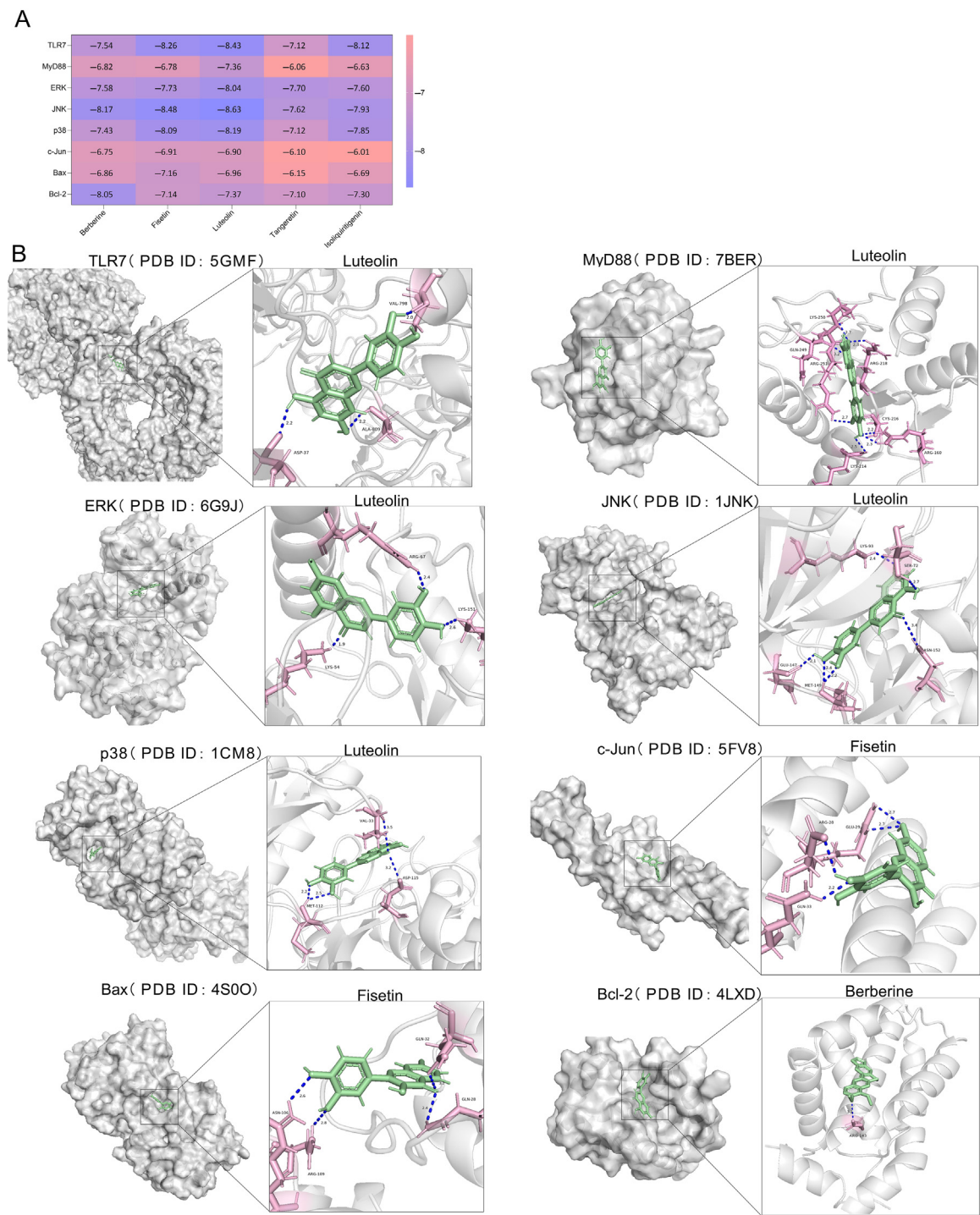


Fig. 12. Results of molecular docking and dynamics simulations. (A) Binding energies (KJ/mol) of the candidate active components of WYJD to the essential targets. (B) 3D docking modes with the lowest binding energies for each target.

Furthermore, the complex chemical composition of WYJD need further investigation using more qualitative and quantitative analysis techniques, and the pharmacological effects as well as molecular targets of its active components and metabolites require direct validation using the single component. In addition, given the differences among species, further clinical trials and experiments using multiple models will be necessary to fully characterize the therapeutic mechanism of WYJD.

5. Conclusion

This study provides compelling evidence that WYJD exerts significant therapeutic effects against IAV-induced pneumonia by attenuating lung inflammation and tissue damage, which is consistent with its traditional clinical applications. After 25 prototypes and 15 metabolites were firstly identified in the WYJD-containing serum, we innovatively used a combined strategy of

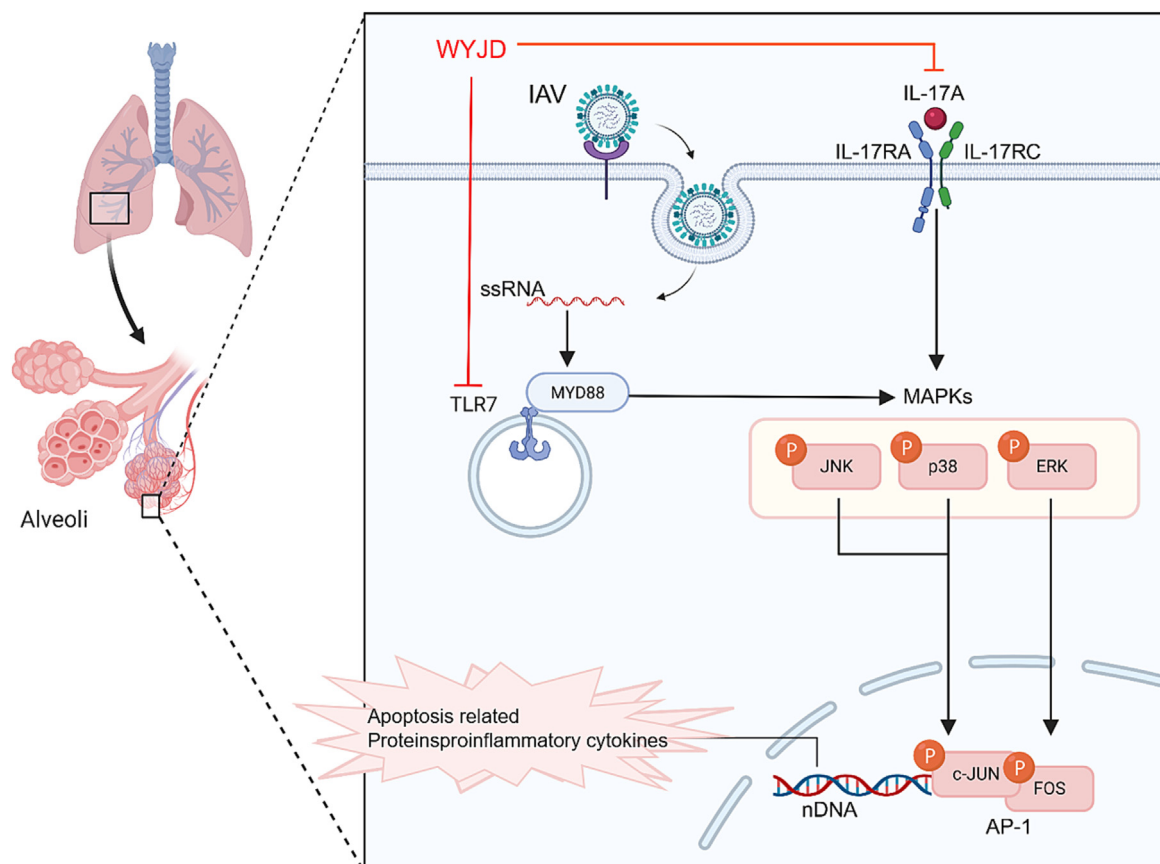


Fig. 13. Potential mechanisms by which WYJD ameliorates IAV-induced pneumonia.

network pharmacology, transcriptomics and experimental validation. Integrated network pharmacology and transcriptomics analysis reveals that 11 active components, ten key signaling pathways, and 86 candidate targets mainly contribute to the effects of WYJD against IAV-induced pneumonia. Further exploration demonstrates that WYJD can alleviate IAV-induced pneumonia through the regulation of the IL-17 signaling pathway, TLR7/MyD88/MAPKs/AP-1 signaling pathway, and apoptosis. Among the 11 active components, berberine, luteolin, fisetin, isoliquiritigenin, and tangeretin may serve as the core constituents mediating the effects of WYJD on TLR7/MyD88/MAPKs/AP-1 signaling pathway and apoptosis. These findings provide novel insights for the clinical application and of WYJD and other TCMs, while also offering new directions for future fundamental research.

CRediT authorship contribution statement

Shengle Qin: Writing—original draft, Investigation, Formal analysis, Data curation. **Taoyu Chen:** Writing—review and editing, Project administration, Methodology. **Yutao Wang, Hongxia Ke:** Methodology, Investigation. **Jingyan Xin, Wenlong He, Yi Guo, Zemiao Niu, Qiaoli Hua:** Methodology. **Zifeng Yang, Yuntao Liu:** Conceptualization, Project administration. **Zhongde Zhang:** Conceptualization, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chmed.2026.02.004>.

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