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An integrated network pharmacology and RNA-Seq approach for exploring the preventive effect of Lonicerae japonicae flos on LPS-induced acute lung injury

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ABSTRACT

Ethnopharmacological relevance: Lonicerae japonicae flos (LJF, the dried flower bud or newly bloomed flower of Lonicera japonica Thunb.), a typical herbal medicine, targets the lung, heart and stomach meridian with the function of clearing heat and detoxication. It ameliorated inflammatory responses and protected against acute lung inflammation in animal models. Acute lung injury (ALI) is a kind of inflammatory disease in which alveolar cells are damaged. However, a network pharmacology study to thoroughly investigate the mechanisms preventing ALI has not been performed.

Aim of the study: In this study, we examined the main active ingredients in LJF and the protective effects of LJF on LPS-induced ALI in rats.

Materials and methods: First, the main active ingredients of LJF were screened in the TCMSP database, and the ALI-associated targets were collected from the GeneCards database. Then, we used compound-target and target-pathway networks to uncover the preventive mechanisms of LJF. Furthermore, we assessed the preventive effects of LJF in an LPS-induced rat model with the RNA-Seq technique to validate the possible molecular mechanisms of the effects of LJF in the treatment of ALI.

Results: The network pharmacology results identified 28 main active compounds in LJF, and eight chemical components highly related to the potential targets, which were potential active compounds in LJF. In all, 94 potential targets were recognized, including IL6, TNF, PTGS2, APP, F2, and GRM5. The pathways revealed that the possible targets of LJF involved in the regulation of the IL-17 signalling pathway. Then, in vivo experiments indicated that LJF decreased the levels of proinflammatory cytokines (TNF, IL-1, and IL-6) in serum and bronchoalveolar lavage fluid, decreased the levels of oxidative stress factors (MDA and MPO) and increased the activities of SOD and GSH-Px in lung tissue. The RNA-Seq results revealed that 7811, 775 and 3654 differentially expressed genes (DEGs) in Ctrl (control group), ALI-LJF (Lonicerae japonicae flos group) and ALI-DXSM (dexamethasone group), respectively. KEGG pathway analysis showed that the DEGs associated with immune response and inflammation signalling pathways and the IL-17 signalling pathway were significantly enriched in LJF. Compared with those in ALI, the expression of CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, MMP9 and TFFAP3, which are involved in the IL-17 signalling pathway, were significantly decreased in the LJF group according to the qRT-PCR analyses.

Conclusions: In view of the network pharmacology and RNA-Seq results, the study identified the main active ingredient and potential targets of LJF involved in protecting against ALI, which suggests directions for further research on LJF.

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1. Introduction

Acute lung injury (ALI) is a clinical syndrome characterized by indirect or direct lung injury with gas exchange dysfunction and alveolar capillary barrier disruption (Guo et al., 2014), which is mainly caused by hypoxemia, respiratory distress and pulmonary oedema and results in acute respiratory distress syndrome (ARDS) (Matthey et al., 2005). LPS, as a common pathogenic factor associated with ALI, is the main constituent of the gram-negative bacterial cell wall (Johnson et al., 2010). The major pathologies associated with ALI are pulmonary oedema, inflammatory infiltration, and hyperplasia of pulmonary microvascular endothelial cells caused by inflammatory factors (Levy et al., 2014). The pulmonary infectious cascade is aggravated by abundant exocytosis of proinflammatory cytokines and the development of protein-enriched areas of oedema (Rubinfeld et al., 2005). Therefore, the efficient control of inflammatory responses is a key tactic for treating ALI.

Lonicerae japonicae flos (LJF, the dried flower bud or newly bloomed flower of *Lonicera japonica* Thumb.) is a traditional Chinese medicine (TCM) that modulates inflammatory responses (Lee et al., 1998; Tae et al., 2003; Xu et al., 2007; Kao et al., 2015). To date, more than 140 compounds in LJF have been isolated and identified, including organic acids, flavones, triterpenoid saponins, and iridoids (Shang et al., 2011). LJF was first grown in the Song Dynasty in China (chronicled in “Su Shen Nei Han prescription”), shows channel tropism of the lung, heart and stomach meridian. According to Sheng Nong’s classic herbal text (Shen Nong Ben Cao Jing), it is used in clinical applications of TCM for clearing heat and detoxification. Modern pharmacological studies have shown its broad biological activities, such as antibacterial, anti-inflammatory, anti-endotoxin, antiviral, and antipyretic activities (Shang et al., 2011). LJF has been included in the “Pharmacopoeia of the People’s Republic of China” and is employed to treat nephritis, pneumonia, acute tonsillitis, chronic enteritis, acute mastitis, and other conditions in the clinic. A previous study revealed that pretreatment with LJF inhibits inflammatory factors, decreases the expression of IL-1β, IL-6 and TNF-α in the lung and protects against lung inflammatory cytokine release by LPS induction (Kao et al., 2015). Shuang-Huang-Lian includes Lonicerae japonicae flos, scutellaria baicalensis Georgi and forsythia suspensa and reduces LPS-induced ALI in mice by inhibiting the overproduction of proinflammatory cytokines (Fang et al., 2015). However, the protective effect and the underlying mechanism of LJF in treating ALI are still unknown. Recently, LJF, as a main ingredient in Chinese medicine, has also been employed extensively to prevent and treat COVID-19 (Luo et al., 2020; Ni et al., 2020; Li et al., 2020).

TCM is characterized by multiple chemical components, multiple targets and multiple effects, and network pharmacology studies the complex relationships between medicines, targets, diseases, and pathways (Hopkins et al., 2008). Network pharmacology can identify multiple components, multiple targets, and multiple pathways, which provides a means to comprehend the therapeutic mechanisms of TCM in the treatment of miscellaneous diseases (Zhou et al., 2016; Zhang et al., 2019). Therefore, we applied network pharmacology to identify the main effective components and the potential targets of LJF and reveal their mechanisms in the treatment of ALI. First, the TCMSP database was used to identify the active ingredients of LJF. Second, the ALI-related targets were collected from the GeneCards database. After the retrieval of known ALI-related targets, pathway enrichment analysis of the targets that overlap between LJF and ALI and network analysis of LJF were performed to explore the treatment of ALI. Finally, by combining in vivo experiments with the RNA-Seq technique, we investigated the protective effects of LJF against LPS-induced ALI.

2. Materials and methods

2.1. Network pharmacology analysis

2.1.1. Active compounds and target screening

The chemical ingredients of LJF were collected from the Traditional Chinese Medicine System Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php) (Ru et al., 2014). In all, 236 chemical components of LJF were acquired from the TCMSP database (Table S8). We employed drug-likeness (DL ≥ 0.18) and oral bioavailability (OB ≥ 30%) as criteria to filter the active ingredients of LJF. The targets were predicted by the SEA database (http://sea.blklab.org/) and the SwissTargetPrediction database (http://www.swisstargetprediction.ch/).

2.1.2. Network construction

The potential targets of the active ingredients of LJF were identified by constructing a component-target network with Cytoscape 3.7.1 software. The ALI-related targets were collected from the GeneCards database, and consistent targets for ALI and active ingredients were screened. The corresponding targets were employed to construct the protein-protein interaction network with Cytoscape 3.7.1 software. The corresponding targets were then used for enrichment analysis with the KOBAS3.0 platform (Xie et al., 2011), and then the target-pathway network was constructed by Cytoscape 3.7.1 software.

2.2. Experimental verification

2.2.1. Samples and sample preparation

Twelve batches of LJF were collected in China. All herbs were authenticated by Professor Sheng Hua Wei and stored at Guizhou University of Traditional Chinese Medicine. HPLC fingerprinting was employed to identify LJF. The LJF aqueous extracts were prepared by using distilled water for reflux extraction. The ratio of material to liquid was 1:20. Over 1.5 h, the LJFs were extracted 3 times, and then the combined extracted filtrate was evaporated in a water bath to obtain the aqueous extract, which was kept at −20 °C until use. Prior to HPLC analysis, the solution was filtered through a 0.45 μm membrane. The chromatographic fingerprinting of LJF showed 14 main peaks, which were identified as 2 (neochlorogenic acid), 5 (chlorogenic acid), 6 (chlorogenic acid), 10 (loganin), 11 (isochlorogenic acid B), 12 (isochlorogenic acid A), and 14 (isochlorogenic acid). The method could be used to identify the characteristics of LJF (Fig. S1).

2.2.2. Animal model

A total of 48 male Sprague-Dawley (SD) rats (weight, 180–220 g; age, 6 weeks) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China) and given standard animal chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee of Guizhou University (approval number GZU-2017099) and were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. Based on the experimental methodology of pharmacology, the rats were randomized into four groups: the control group (treated with an equal volume of PBS, Ctrl; n = 12); model group (5 mg/kg LPS via intraperitoneal injection, ALI; n = 12); Lonicereae japonicae flos group (administration of Lonicereae japonicae flos via stomach perfusion at 1.584 g/kg for six days and intraperitoneal injection of 5 mg/kg LPS, ALI-LJF; n = 12); and dexamethasone group (administration of dexamethasone via stomach perfusion at 5 mg/kg for six days and intraperitoneal injection of 5 mg/kg LPS, ALI-DXMS; n = 12). Six hours after the LPS challenge, the rats were anaesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g). For further analyses, the bronchoalveolar lavage fluid (BALF), lung tissue and blood were collected and stored at −80 °C.
2.2.3. ELISA analysis

After treatment, the levels of IL-1β, IL-6, and TNF-α in the serum and BALF were determined with an enzyme-linked immunosorbent assay (ELISA) kit, and an ELISA kit was used to determine the levels of GSH-Px, MPO, MDA, and SOD in the lung tissue. All data are presented as the mean ± SD. GraphPad Prism 6 software was employed for one-way ANOVA, and a P-value ≤ 0.05 was confirmed to be statistically significant.

2.2.4. Histologic examination

The rat lung tissues were fixed in 4% formaldehyde, embedded in paraffin and cut into 5 μm sections. Then, the sections underwent haematoxylin-eosin (H&E) staining. The changes in pulmonary histopathology were visualized under a microscope, and the pathological scores were obtained. The degree of pulmonary injury was evaluated according to a previous report (Liu et al., 2018).

2.2.5. RNA-seq analysis

Total RNA was extracted with TRIzol reagent (TIANGEN, China), and detected on a 1% agarose gel. The purity, concentration, and integrity of the total RNA samples were assessed prior to further analysis. After cluster generation, the library preparations were sequenced on the Illumina HiSeqTM 4000 platform by Biomarker Technologies (Beijing, China), and the raw reads were generated. Then, the raw reads were filtered by removing adapter and poly-N sequences and inferior quality reads from the raw reads. The clean reads were mapped to the Rattus norvegicus (Rnor_6.0) reference genome sequence by the HISAT2 tools. The levels of quantitative gene expression were estimated by determining the fragments per kilobase of transcript per million fragments mapped. Gene expression analysis of the different groups was performed by DESeq2. Genes with a P-value < 0.05 were defined as differentially expressed genes (DEGs). Then, we used the KOBASE3.0 platform to perform the enrichment analysis of the DEGs (Xie et al., 2011). The volcano plot and heatmap were generated with the OmicShare online tools (http://www.omicshare.com/tools). Venn diagrams were generated by the Draw Venn Diagrams online tools (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.2.6. qRT-PCR

Total RNA was extracted from lung tissues with TRIzol according to the manufacturer’s instructions (TIANGEN, China), and the isolated RNA was reverse-transcribed into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, China). Quantitative real-time (qRT)-PCR was performed with the LightCycler 96 Real-Time PCR System (Roche) and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, China). The mRNA expression of BCL2A1, CXCL1, CXCL2, CXCL6, DNAJC5, FADD, KIT, IFNG, IL1R1, IL6, IL17A, IL17F, IL17C, ITGAS, MMP9, NFKBIA, NLOC4, 1PP1R15A, THBS1, TNFAIP3, TRADD, and GAPDH were detected by qRT-PCR in the lung tissues. The genes primer sequences (Table S9) for qRT-PCR were designed the primer3 platform (http://frodo.wi.mit.edu/primer3/). All qRT-PCR was repeated three times, the expression levels of candidate genes were determined using the 2−ΔΔCT method. Expression levels were normalized against the reference gene GAPDH. Data are represented as mean values ± SD, and GraphPad Prism 6 software was employed for one-way ANOVA, *P < 0.05, **P < 0.01.

3. Results

3.1. Active compounds and target screening

In total, 23 ingredients in LJF fulfilled the two criteria simultaneously (DL ≥ 0.18 and OB ≥ 30%). However, five ingredients (scolymoside, hederaagenol, beriguard, hyperin, and lutelolin-7-o-glucoside) did not meet the criteria of OB ≥ 30%, but they were also presented as candidates. Hence, 28 main ingredients were obtained as potentially active constituents in LJF, including flavonoids and organic acids (Table S1). These 28 active components were used to identify the targets in SwissTargetPrediction and SEA. After removing duplicates, we finally acquired 212 targets (Table S2). The ALI-related genes were collected from the GeneCards database, and 1047 ALI-related genes were identified (Table S3). The shared targets of the 28 active compounds and ALI-related genes were identified by generating Venn diagrams. Ultimately, 94 genes were identified as both the targets of active ingredients and ALI-related genes.

3.2. Compound-target network construction and analysis

The 94 identified genes were used to construct the compound-target network with Cytoscape 3.7.1 software. Among the active compounds in LJF, eight active compounds demonstrated a higher number of connections and were connected with more than 20 targets, including ethyl-linolenate, hederagenol, chrysosertol, kaempferol, luteolin, mandenol, quercetin and ZINC03978781. The network analysis showed that one compound in the herbs can be linked with more than one target. In the compound-target network, ALOX5 (arachidonate 5-lipoxygenase) was simultaneously targeted by 11 active ingredients, AKR1B1 (aldo-keto reductase family 1 member B1) was targeted by 10 active ingredients, and PTGS2 (prostaglandin G/H synthase 2) was targeted by 10 active ingredients (Fig. 1). In the PPI network, eleven targets were linked (Fig. 2.), including IL6 (degree = 55), TNF (degree = 45), PTGS2 (degree = 38), APP (degree = 31), F2 (degree = 24), GRM5 (degree = 22), HSP90AA1 (degree = 21), PPARG (degree = 21), NR3C1 (degree = 20), OPRM1 (degree = 20), and AGTR1 (degree = 20). Thus, IL6, TNF, and PTGS2 might be identified as hub genes.

3.3. Target-pathway network

The 94 potential target proteins were subjected to enrichment analysis by Kobas3.0. As a result, 84 significant pathways related to LJF were identified by Kobas3.0 (Table S4). Then, the target-pathway network was constructed (Fig. S2), containing 96 targets and 84 corresponding pathways. It was obvious that the targets for LJF were mainly interlinked in three pathways, including metabolic pathways (degree = 25), neuroactive ligand-receptor interaction pathways (degree = 23), and pathways involved in cancer (degree = 19). Moreover, we identified the calcium signalling pathway (degree = 12) and NOD-like receptor
signalling pathway (degree = 10). Fortunately, we also observed that these targets participated in pathways linked to immune and inflammatory signalling pathways, including human cytomegalovirus infection-associated pathways, the IL-17 signalling pathway, the PPAR signalling pathway, the PI3K-Akt signalling pathway, human T-cell leukaemia virus 1 infection-associated pathways, pathways involved in inflammatory mediator regulation of TRP channels, the Jak-STAT signalling pathway, natural killer cell-mediated cytotoxicity-associated pathways, T cell receptor signalling pathway, NF-kappa B signalling pathway, TNF signalling pathway and the Toll-like receptor signalling pathway.

3.4. LJF inhibits the expression of IL-6, TNF-α, and IL-1β in LPS-induced ALI rats

To detect the anti-inflammatory effects of LJF, we examined the levels of IL-6, TNF-α, and IL-1β in serum and BALF after treatment with LPS, LJF and DXSM. The results showed that exposure of serum and BALF to LPS increased the production of these inflammatory cytokines (P < 0.05). However, production of these inflammatory cytokines (IL-6, TNF-α, and IL-1β) were significantly inhibited by LJF and DXSM (P < 0.05 (Fig. 3A and B). Then, the level of oxidative stress was measured, and LPS-induced ALI could increase the levels of MDA and MPO and decrease the activities of SOD and GSH-Px in the lung tissue (P < 0.05) (Fig. 3C). Interestingly, the levels of SOD and GSH-Px were significantly enhanced by LJF and DXSM (P < 0.05).

3.5. LJF alleviated pulmonary morphological damage in LPS-induced ALI rats

H&E staining was used to detect the pathological changes in the lungs. After treatment with LPS, the rat lung tissue showed increases in inflammatory cell infiltrates and alveolar histological structure damage compared with the lung tissue in the Ctrl group. In our study, the ALI group showed severe alveolar oedema fluid accumulation, alveolar capillary congestion and bronchial epithelial detachment (Fig. 4B). LJF was superior to DXSM in alleviating LPS-induced ALI (Fig. 4C and D). As expected, LJF significantly alleviated the pulmonary morphological damage caused by LPS.

3.6. RNA-seq analysis

3.6.1. Identification of differentially expressed genes (DEGs)

Furthermore, we explored the genes and pathways by which LJF protected against ALI in rats using RNA-Seq of rat lungs from the Ctrl, ALI, ALI-LJF, and ALI-DXMS groups. The RNA from the three replicate lung samples from the Ctrl, ALI, ALI-LJF, and ALI-DXMS groups was sequenced. In all, 18,657 genes (56.74% of the 32,883 genes in Rnor_6.0) were identified, which were expressed in at least one sample (FPKM cut-off value 0.01). The number of genes expressed in the lungs...
of rats with ALI was 17,950 in the Ctrl group, 17,826 in the ALI group, 17,487 in the ALI-LJF group and 17,681 in the ALI-DXMS group (Table S5). To determine the differentially expressed genes (DEGs), a P-value < 0.05 was used as the cut-off value for gene expression in the Ctrl, ALI, ALI-LJF, and ALI-DXMS groups, which was detected by pairwise comparisons between the ALI group and the Ctrl, ALI-LJF, and ALI-DXMS groups. As a result, 7811 DEGs in rat lung were identified after LPS stimulation, whereas 775 and 3654 DEGs were identified in rat lung tissue treated with LJF and DXSM, respectively. Overall, 1688 upregulated and 1966 downregulated DEGs were identified in the ALI vs. ALI-DXMS groups, and 340 upregulated and 435 downregulated DEGs were identified in the ALI vs. ALI-LJF groups (Fig. 5 A–C). In brief, after removing the duplicate genes, 509 DEG genes were associated with the ALI group that were also affected in the Ctrl, ALI and ALI-LJF groups; 2546 genes were associated with the Ctrl, ALI and ALI-DXMS groups; and only 298 key DEG genes were associated with the Ctrl, ALI, ALI-LJF and ALI-DXMS groups (Fig. 5 D).

To characterize these differentially expressed genes, trend analysis was applied to determine the expression patterns of the 509 DEGs in the Ctrl, ALI and ALI-LJF groups (Table S6). In the ALI vs. Ctrl groups, the expression of 255 genes displayed an initial increase, but there was a decrease in the ALI vs ALI-LJF groups; however, the expression of 217 genes exhibited a reduction in the ALI vs. ALI-LJF groups, but there was
a subsequent increase in the ALI vs. ALI-LJF groups (Fig. 6). Specifically, eight genes (DYRK1A, CA4, IL6, PTAFR, ARG1, MGLL, LTBR4, and TYMP) among the DEGs of the ALI-LJF group were predicted as targets of active ingredients of LJF.

3.6.2. Verification of RNA-Seq results by qRT-PCR

To verify the reliability of the gene expression data obtained by RNA-Seq, eight genes (IL1R1, DNAXCS, THBS1, NLPLC4, BCL2A1, PPPI1R1A5, KIT, and ITGAS) were randomly selected for qRT-PCR detection. The qRT-PCR results showed that the tendency of gene expression was consistent with the RNA-Seq results. For each gene, the qRT-PCR expression results showed a similar tendency to the RNA-Seq results (Fig. S3). The results showed that the RNA-Seq results were credible in this study.

3.6.3. Pathway enrichment analysis

To thoroughly investigate the potential pathways involved in the immune and inflammatory responses, the Kobas3.0 platform was employed for KEGG pathway analysis of these DEGs. The KEGG analysis showed that 40 KEGG pathways were significantly enriched for these DEGs (Table S7, P < 0.01), which mainly participated in pathways linked to the immune system, inflammatory pathways, and infectious diseases. The infectious disease-associated pathways were involved in human cytomegalovirus infection, bacterial invasion of epithelial cells, Epstein-Barr virus infection, Yersinia infection, Kaposi sarcoma-associated herpesvirus infection, and herpes simplex virus 1 infection. These immune- and inflammation-associated pathways mainly included the IL-17 signalling pathway, TNF signalling pathway, NF-κB signalling pathway and Jak-STAT signalling pathway (Fig. 7). Within the four classic immune pathways (rno04657, rno04668, rno04064, and rno04630), we identified 24 candidate genes associated with ALI: CXCL2, CXCL1, CXCL6, IL10, LIF, IL12RB2, IL22, NFKBIA, IFNG, IL12A, IL6, BIRC2, IL17A, IL17C, IL17F, CXCL12, IL1R1, TRADD, MMP9, CCND1, IL19, FADD, BCL2A1, and TNFAIP3.

3.6.4. LJF treatment activated the IL-17 signalling pathway

In this study, IL-17 signalling pathway involved in thirteen DEGs, including CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, MMP9, TNFAIP3, FADD and TRADD. LJF significantly inhibited CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, MMP9, and TNFAIP3 mRNA expression in lung tissue homogenates according to RNA-Seq, which indicates that the IL-17 signalling pathway is critical for treatment of LPS-induced ALI with LJF (Fig. S4). Interestingly, the involved IL-17 family members, including IL-17A, IL-17C and IL-17F, played a significant role in the acute inflammatory responses (Fig. S4). Consistent with the RNA-Seq data, the expression of CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, MMP9 and TNFAIP3 in lung tissue was significantly decreased compared with that in the ALI and LJF groups according to the qRT-PCR analyses (P < 0.05) (Fig. 8). The expression of TRADD and FADD was increased compared with that in the ALI and LJF groups by the qRT-PCR analyses (Fig. 8), and associated with apoptosis in the IL-17 signalling pathway (Fig. S4).

4. Discussion

We employed network pharmacology to determine the potential active ingredients and targets of LJF. Then, we performed compound-target and target-pathway network analyses to explore the mechanisms of LJF. Furthermore, an LPS-induced rat model was constructed to evaluate the effect of LJF in the treatment of ALI. According to the degrees of the nodes in the compound-target network, we identified eight compounds as potential active ingredients that might participate in the regulatory processes of LJF in ALI. Obviously, the active ingredients chrysosoriel (Wei et al., 2019), luteolin (Runguang et al., 2018), kaempferol (Chen et al., 2012; Qian et al., 2019), and quercetin (Wang et al., 2018) have well-established roles in lung inflammation. ALI-related genes were acquired using the GeneCard database. Finally, 94 key genes were identified to build the protein interaction network. Some genes, such as IL6, TNF, PTGS2, GRM5, and PPARG, have been associated with the pathogenesis of ALI. NF-κB regulates the expression of diverse inflammatory cytokines, such as TNF-α and IL-6, which play significant roles in LPS-induced ALI (Chu et al., 2016). The expression of PTGS2 (COX-2) is increased by proinflammatory factors and associated with NF-κB (Hu et al., 2011).

KEGG pathway analysis revealed that these targets were linked with immune and inflammatory signalling pathways, such as the IL-17 signalling pathway, PI3K-Akt signalling pathway, PPAR signalling pathway, Jak-STAT signalling pathway, natural killer cell mediated cytotoxicity-associated pathway, T cell receptor signalling pathway, NF-κB signalling pathway, TNF signalling pathway and Toll-like receptor signalling pathway, indicating the involvement of inflammatory signalling pathways in the development of ALI. The IL-17 family members IL-17A–F play a significant role in acute and chronic inflammatory responses in ALI in vitro and in vivo, indicating that glycyrrhizic acid induces autophagy via the PI3K/AKT/mTOR pathway (Qu et al., 2019). The cystic fibrosis transmembrane conductance regulator ameliorated LPS-induced ALI by inhibiting autophagy via the PI3K/AKT/mTOR pathway in mice (Sui et al., 2020). Recombinant human brain natriuretic peptide protected rats against trauma-induced ALI by inhibiting the JAK/STAT signalling pathway in rats (Song et al., 2015).

Moreover, in animal experiments, LJF showed good efficacy, which was consistent with its effects on ALI. TNF-α, IL-6 and IL-1p are critical mediators of pulmonary inflammation in ALI (Giebel et al., 2007). Our data indicated that pretreatment with LJF decreased the concentrations of these cytokines (TNF-α, IL-6 and IL-1p), indicating that LJF has a significant anti-inflammatory effect on LPS-induced ALI in rats. Oxidative stress plays an important role in the process of LPS-induced ALI (Siebanoff et al., 2005). Moreover, LJF effectively increased the activities of oxidative stress factors (SOD and GSH-Px) in the lungs and reduced the levels of oxidative stress factors (MDA and MPO) in the lungs of LPS-induced ALI mice. These results showed that LJF has powerful antioxidant effects on LPS-induced ALI mice.

Furthermore, RNA-Seq identified genes and pathways activated in response to LJF involved in intervention in ALI. There were 7811/775/
3654 DEGs among the Ctrl, LJF, and DXSM groups compared to the ALI group, respectively, that met the criterion of a P-value < 0.05. The qRT-PCR analysis showed that the trends for eight genes (IL1R1, DNAJC5, THBS1, NPL0C4, BCL2A1, PPP1R15A, KIT, and ITGA5) were consistent with the RNA-Seq results. These findings indicated that the RNA-Seq results were credible. By comparative analysis, we found that there were more differentially expressed genes in the ALI, ALI-LJF and ALI-DXSM groups. In all, 192 DEGs were unique to the ALI-LJF group, and 298 DEGs were shared among the three groups. Through KEGG enrichment analysis, we found that the IL-17 signalling pathway was significantly enriched in the ALI-LJF group. Thirteen genes were found, such as CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, TRADD, MMP9, FADD, and TNFAIP3, that were affected by LJF and were involved in the IL-17 signalling pathway.

In this study, LJF significantly inhibited the LPS-stimulated down-regulation of CXCL2, CXCL1, CXCL6, NFKBIA, IFNG, IL6, IL17A, IL17F, IL17C, MMP9, and TNFAIP3 mRNA expression in lung tissue homogenates according to RNA-Seq and qRT-PCR, which indicates that the IL-17 signalling pathway is critical for treatment by LJF of LPS-induced ALI. Studies have indicated that CXCL1/CXCL2 contributes to recruiting neutrophils to tissues in response to LPS (De Filippo et al., 2013). CXCL1 can recruit neutrophils to the infection region to kill pathogens in ALI (Meng et al., 2018). Immune-modulating CXCL6 was identified in pediatric airway epithelium infected with hRSV (Touzelet et al., 2020).
IL-17A is involved in LPS-induced ALI and ameliorates pathological developmental by activating NF-κB P65 and recruiting neutrophils, which amplifies the cascade effect of inflammation in lung tissues (Song et al., 2017). Acute and chronic inflammation is activated by inflammatory cytokines, such as IL-17A and IL-17F, which accelerate the initiation of inflammatory cytokine production (Jin et al., 2015). An in vivo study demonstrated an increase in MMP9 in BALF in a lung injury model (Eichler et al., 2003), and mice that were deficient in MMP9 had less severe lung injury than wild-type mice (Warner et al., 2001). The deletion of TNFαFAP3 in macrophages reversed daphnetin-elicted inhibition of the immune response (Yu et al., 2014). LJF increased TRADD and FADD mRNA expression in lung tissue homogenates, and associated apoptosis in the IL-17 signalling pathway. Death receptors, such as Fas and TNF-α, bind to the associated TNFR-associated FAS-associated with a death domain (FADD) and protein with death domain (TRADD) respectively, and subsequently activate caspase-8 to induce apoptosis (Zhou et al., 2017). Accordingly, the genes affected by LJF might ameliorate LPS-induced ALI and might be potential targets for the treatment of ALI.

5. Conclusion

In summary, by combining systematic network pharmacology with RNA-Seq technology, we revealed the gene expression changes that were affected by LJF in LPS-induced ALI. First, a set of 28 ingredients was screened from LJF that acted on 212 targets. ALI association analysis identified 94 targets of LJF and enrichment of 84 significant pathways, including the IL-17 signalling pathway, PI3K-Akt signalling pathway, PPAR signalling pathway, and Jak-STAT signalling pathway. Second, we studied the anti-inflammatory and antioxidative effects of LJF in LPS-induced ALI rats. LJF attenuates pulmonary oedema, decreases the production of inflammatory mediators, and increases the activities of antioxidant enzymes. Third, RNA-Seq analysis confirmed the gene changes after treatment with LJF in LPS-induced ALI. Pathway enrichment analysis identified the ALI-related targets of LJF involved in the IL-17 signalling pathway. Accordingly, we found that LJF protection effectively repaired lung injury and reversed unnatural gene expression involving immune and inflammatory signalling pathways.

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Guizhou University (Approval number GZU-201709).

Author contributions

Chang Liu and Ying Zhou conceived and designed the experiments; Chang Liu and Tingting Feng conducted the experimental work and analysis; Min Zhang, Zhi Zhou, and Zhigang Yin developed the animal analysis; Min Zhang, Zhi Zhou, and Zhigang Yin developed the animal analysis; Chang Liu and Tingting Feng conducted the experimental work and analysis; Min Zhang, Zhi Zhou, and Zhigang Yin developed the animal analysis; Min Zhang, Zhi Zhou, and Zhigang Yin developed the animal analysis; Min Zhang, Zhi Zhou, and Zhigang Yin developed the animal analysis; Ying Zhou provided major revisions and comments to the manuscript. All authors reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| LPS          | lipopolysaccharide |
| TNF-α        | tumor necrosis factor α |
| IL           | interleukin |
| ALI          | acute lung injury |
| TCMP         | Traditional Chinese Medicine |
| SD           | Sprague-Dawley |
| LIF          | Lonicerae japonicae flos |
| ARDS         | respiratory distress syndrome |
| ELISA        | enzyme linked immunosorbent assay |
| H&E          | haematoxylin-eosin |
| DEGs         | differentially expressed genes |
| qRT-PCR      | Quantitative real-time-PCR |
| ALOX5        | arachidonic 5-lipoxygenase |
| AKR1B1       | aldo-keto reductase family 1 member B1 |
| PTGS2        | prostaglandin G/H synthase 2 |
| BALF         | bronchoalveolar lavage fluid |

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113364.

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