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Network pharmacology and experiments in vitro reveal that the Paeonia veitchii Lynch and its active ingredient Punica granatum Linn ameliorate IMQ-induced psoriasis in mice via TLR4/NF-κB signaling pathway

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Research Article

Keywords:

Posted Date: April 5th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4186847/v1

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Additional Declarations: No competing interests reported.

Abstract

Pharmacological relevance

Paeonia veitchii Lynch is a traditional Chinese medicine in our country. It has the function of clearing heat and cooling blood, dispersing blood stasis and relieving pain. Its main active ingredient, *Punica granatum Linn*, is a herbaceous polyphenol with many biological properties, such as anti-oxidation, anti-diabetes, anti-cancer and inducing apoptosis. Previous studies have demonstrated a significant therapeutic effect of Punica granatum Linn on psoriasis, although the underlying molecular mechanisms are unknown.

Aim of the study

We screened the active components of Paeonia veitchii Lynch by network pharmacology. Weveriied them in vitro to identify the potential molecular mechanism of Paeonia veitchii Lynch in treating psoriasis.

Materials and methods

Retrieve target genes associated with psoriasis from the GEO database. Using the clinical bioconfidence analysis platform Sangerbox for Gene Ontology (GO) and Encyclopedia of Genomes (KEGG) pathway enrichment. Next, the protein-protein interaction network (PPI) and the Paeonia veitchii Lynch-compound-target network were done with Cytoscape 3.9.1. To find the most essential active ingredient in the treatment of psoriasis, the main active ingredient and its core target were analyzed by molecular docking. Network pharmacological results were verified by in vitro experiments. A mouse model of psoriasis was induced with imiquimod and constructed by observing changes in skin lesions on the back of mice on a daily basis, performing PASI scores and histopathology observation. The levels of IL-6 and TNF-α in serum were measured by Elisa, and the expression of TLR4/NF-KB pathway was evaluated by Western Blot.

Results

348 differentially expressed genes with high correlation to psoriasis were screened, and 23 active components, corresponding to 150 target genes, were obtained by searching “Paeonia veitchii Lynch” from the database. Catechin, Baicalein, β-sitosterol, Punica granatum Linn, Lactobacillus, paeoniflorin, paeonol, sitosterol and stigmasterol are the main active components in Paeonia veitchii Lynch, in psoriasis has more critical significance. In addition, CCNB1, CXCL8, PCNA and S100A9 may be important targets in treating psoriasis. The molecular docking showed that Punica granatum Linn was main active component of Paeonia veitchii Lynch in treating psoriasis. KEGG results indicated that TLR4/NF-κb pathway might be the potential mechanism of TLR4/NF-κb. Western Blot results showed that Punica granatum Linn down-regulated the protein levels of TLR4 and P65.

Conclusions

Our results suggest that Punica granatum Linn may be an essential basis for the treatment of psoriasis by Paeonia veitchii Lynch, and TLR4/NF-κb pathway may be the common pathway of Paeonia veitchii Lynch and Punica granatum Linn.

1. Introduction

Psoriasis (PSO) is a common chronic inflammatory skin disease mediated by many kinds of immune cells, which often presents as erythema of the skin with several layers of silvery-white scales on the surface[1]. Psoriasis can occur in all age groups, but the majority of adolescents, and the prevalence of male slightly higher than female. According to the investigation, the global incidence of psoriasis is 0.9% ~ 8.5%, the incidence of our country is 0.5% ~ 4.6% [2]. In 2019, about 40 million people worldwide had psoriasis, of which 4.6 million were new cases. The prevalence of psoriasis varied from region to region. For example, the prevalence of psoriasis was 0.11% in East Asia, 1.58% in Australia and 1.52% in Western Europe. In all countries and regions, the lowest prevalence of psoriasis was only 0.05% in Taiwan area of China[3]. Psoriasis is an inflammatory skin disease affecting over 40 million adults and children worldwide. It has a long course, is easy to recur and is difficult to cure completely[4]. It can damage the skin, mental health and even the entire body organs of the patients. The skin damage can spread to the whole body and is related to many kinds of complications, such as heart metabolic disease, stroke, chronic kidney disease, inflammatory arthritis, depression and lymphoma[5].

The aetiology of psoriasis is complex and may be related to a various factors such as genetics, infections, immune dysregulation, mental stress, drugs, etc. The pathogenesis of psoriasis is an immune response involving a variety of immune cells, with T-lymphocyte mediation being the mainstay, leading to excessive proliferation of epidermal cells as well as different degrees of keratinocyte aberrant differentiation, inflammation, and angiogenesis[6].The early studies of psoriasis mainly focused on the growth disorder of KCS, with the deepening of the study, the abnormal proliferation of KCS may be due to the innate and adaptive immune disorders. Fitch et al. [7]and Kastelein et al[8]. discovered and proposed the theory of "IL-23/Th17 pathway" in the mechanism of psoriasis[9], and this signalling pathway is still the core theory of psoriasis pathogenesis and an important target for drug development. After skin damage, dendritic cells (DCs) and other antigen-presenting cells activate T-helper cells (Th), especially Th17, by presenting antigens and releasing pro-inflammatory factors such as interleukin (IL)-23. The release of large amounts of IL-17 from Th17 stimulates the over-proliferation of keratinocytes. It up-regulates the expression of inflammatory cytokines, which further activate the activation of DCs and Th17, thus amplifying the inflammatory response of the "IL-23-Th17" axis.

At present, the treatment of psoriasis includes local therapy, physical therapy and systemic therapy, among which systemic therapy includes conventional systemic therapy and biological agent therapy[10]. The traditional drugs for treating psoriasis mainly include methotrexate, cyclosporine, retinoic acid, and so on. Although these drugs can control symptoms, they are difficult to cure and have some side effects. Biological agents have a precise therapeutic effect and only play a role in pathogenic factors, but biological agents are expensive and have a limited effect. With the development of Chinese medicine,

Chinese medicine treatment has become more and more important. Therefore, it is possible to use the Chinese herbal compound preparation with good clinical efficacy as an alternative or supplementary drug in treating psoriasis[11]. The treatment of psoriasis with herbal medicines should attract more attention. In this study, we used IMQ-induced psoriasis model to observe whether Punica granatum Linn has a therapeutic effect on psoriasis. We constructed the drug-target network of Paeonia veitchii Lynch, and conducted KEGG pathway analysis and network topology analysis to search for valuable active components and molecular pathways based on common targets, the results were verified on IMQ-induced psoriasis model.

2. Materials and methods

2.1. Animals

Twelve 8-week-old SPF female BALB/C mice weighing 18-22g purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (License No. : SCXK 2020-0030). All animals lived in the SPF laboratory animal center of Sichuan Lisno Biotechnology Co., Ltd. (License No. : SYXK (Sichuan)2021 – 246). All experiments carried out strictly following the ethical principles of animal experiments. All experiments were approved by the animal experimental ethics committee of Sichuan Lilaisno Biotechnology Co., Ltd. Animal ethics reference number: LLSN-2023120.

2.2 Drugs, chemicals, and antibodies

Punica granatum Linn (Med Chem Express) ; imiquimod cream (Sichuan Mingxin Pharmaceutical Co., Ltd.) ; White Vaseline (Qingdao Jinqi Biotechnology Co., Ltd.) ; hematoxylin dye (Wuhan Seville Biotechnology Co., Ltd.) ; Eosin Dye (Hefei Bomei Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse TNF-α Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., Ltd.), mouse IL-6 Elisa Kit (Shanghai Zhuo Cai Biotechnology Co., bCA protein concentration assay kit (Shanghai Biyuntian Biotechnology Co., Ltd.) ; first antibody and second antibody eluent (Wuhan Seville Biotechnology Co., Ltd.) ; GAPDH antibody (Wuhan AIBOTEK Biotechnology Co., Ltd.) ; P65 antibody (Affbiotech, USA) ; TLR4 antibody (Proteintech, USA)

2.3 Animals and experimental models of psoriasis

Suppliers of Punica granatum Linn: MedChemExpress. Batch number: HY-B0183-271638. Purity: ≥98.0%.The source is Pentaphyllum, and the extraction and preparation methods are confidential to the company's research and development and cannot be provided.

Dissolve 1g ellagic acid (Punica granatum Linn) in 100 mL of DMSO (can be divided into three times 50,30,20), combine the filtrate, and volume 100 mL to obtain 100 mL of 0.01g/mL extract. High dose group: 0.2mL, low dose group diluted 1x, 0.2mL.

Twelve SPF mice were randomly divided into a blank control group, a model group, an Punica granatum Linn high-dose group (100 mg/kg), and an Punica granatum Linn low-dose group (50 mg/kg), with three mice in each group, and were acclimatised and reared for 1 week. One day before the experiment, the hair of the mice shaved in the range of 2 × 3 cm on the back. The mice in the model group and the treatment group were smeared with 62.5 mg imiquimod cream in this area. In contrast ,the control group was smeared with the same amount of Vaseline every day, at the same time continuous administration for 7 days. Punica granatum Linn (100mg/kg) was given by Gavage once a day in the high-dose group, and Punica granatum Linn (50mg/kg) was given by Gavage once a day in the low-dose group with a dose of 0.2 ml. Normal saline was given to the control group and model group once a day, 0.2 ml each time. We constructed the mouse model and administered the drug for seven consecutive days, during which time we observed the skin changes on their backs.

After anesthesia with sodium pentobarbital on day 8, the mice in each group were decapitated and executed, and a dorsal lesion area of about 1cm*1cm in size was selected from each mouse for histopathological sectioning, which was placed in 4% paraformaldehyde solution for preservation; and another dorsal lesion area of about 1cm*1cm in size was taken for Western blotting, which was put into a sterile and enzyme-free freezing tube and placed in a -80℃ refrigerator for spare. The eyeballs of mice in each group were removed to collect blood, and the blood samples of mice were collected. After standing at room temperature for 40 minutes, the blood samples were centrifuged (3000 rpm, 4℃, 10 minutes), and the upper layer of serum was collected, which was used for ELISA.

2.4 Network pharmacology-based analysis

2.4.1 Psoriasis highly relevant differentially co-expressed genes (DEG s) acquisition and biological function enrichment analysis

Acquisition of DEGs for psoriasis: The GSE166388 dataset was screened for DEGs using the R software package limma (version 3. 40. 6) according to the criteria of $|\log2Fc| \geq 1$, $P < 0.05$, and heatmaps as well as volcano diagrams were plotted.

Screening of gene modules with the highest psoriasis correlation: based on the normalized gene expression matrix, the weighted gene co-expression network analysis (WGCNA) package and limma package used to cluster the samples, calculate the soft threshold, and construct the scale-free co-expression network. The association between genes and modules evaluated by gene significance and module significance, and the gene modules with the highest psoriasis correlation were screened.

Functional enrichment of highly correlated DEG s in psoriasis: the DEG s obtained in the previous section intersected with the modules with the highest correlation in the module correlation analysis (highly correlated DEG s). The R software package clusterProfiler used to analyze the GO functional enrichment

of biological processes, molecular functions, and cellular components of the highly correlated DEG s; and the KEGG functional enrichment of the gene pathways.

2.4.2 Screening for key genes in psoriasis pathogenesis

The selected psoriasis highly correlated DEGs were imported into the STRING to plot the PPI network, and the confidence score was set greater than 0. 4. Using Cytoscape's CytoHubba plugin, four Hub gene algorithms, MCC, DEGREE, EPC and CLOSENESS, were used to obtain the key psoriasis genes by taking the intersection of the top 10 genes from the results of the four algorithms.The expression levels of the selected key genes in the pathogenesis of psoriasis were verified using the external psoriasis gene chip GSE2737 as the verification set.

2.4.3 Correlation analysis between the expression level of key genes for psoriasis pathogenesis and the proportion of immune-infiltrating cells in skin lesion tissues

The percentage of 22 immune cells in all samples was calculated and visualized by the CIBERSORT algorithm at the $P < 0.05$ criterion; differences in immune cells between psoriasis lesion tissue samples and healthy control skin tissues were analysed and visualized by box-and-line plots. Pearson correlation analysis was performed between key genes and immune infiltrating cells.

2.4.4 Screening of active ingredients and target genes of drugs

We searched the China National Knowledge Infrastructure, Wanfang Database and Weipu database for the past 15 years, and established the database through the auxiliary platform of traditional Chinese medicine (TCM) inheritance, which will screen the information of 178 prescriptions, after screening the frequency of use of traditional Chinese medicine, it was found that the frequency of use of radix rehmanniae and Paeonia veitchii Lynch was the highest, therefore this topic chooses“ Paeonia veitchii Lynch” this one traditional Chinese medicine to carry on the follow-up research.

With the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform(TCMSP, http://tcmspw.com/tcmsp. php), the active ingredients and their corresponding targets were screened, and the search term was "Paeonia veitchii Lynch", and the screening condition was oral bioavailability (OB) ≥ 30%. The search term was "Paeonia veitchii Lynch", and drug likeness (DL) ≥ 0.18 and oral bioavailability (OB) ≥ 30% were used as criteria for screening. We continued to search the BATMAN-TCM database, supplemented the active ingredients obtained from TCMSP with the research results of related literature, and searched for information on potential targets related to the active ingredients through the TCMSP database. The obtained targets were de-emphasized and the target names were standardized with the UniProt database (https://www.uniprot.org/).

2.4.5 "Drug-component-target" network construction

Using Cytoscape 3.9.1, we imported the active ingredients of Paeonia veitchii Lynch and their corresponding psoriasis-related target information, constructed the Paeonia veitchii Lynch active

ingredient-psoriasis target network, and analyzed the network topology parameters by using the Cytohubba plug-in to rank the node degrees of freedom (degree), and screened out the top 9 major active ingredients according to the degree value. The plant name has been checked with “World Flora Online” (www.worldfloraonline.org)

2.4.6 Molecular docking

To verify the reliability of the results by molecular docking. The psoriasis-related critical active ingredients in Paeonia veitchii Lynch were ligands, and the important targets corresponding to the active ingredients were receptors. The two-dimensional structure files of the ligands were downloaded from PubChem database, and the compounds were optimized using Chem3D software and saved in MOL2 format. The protein crystal structure of the receptor obtained in the PDB database, and de-watering and de-functionalization performed using PyMol software. After that, protein molecules were given H treatment and ligand and receptor files were converted to pdbqt files using Auto Dock; receptor active pockets were defined and saved in position. Subsequently, molecular docking was achieved by Autodock Vina 1.1.2, with the docking parameters set to default values, and the docking heatmap was output. At last the results were envisioned by Pymol 2.5.4 .

2.5 Experimental validation

2.5.1 Skin PASI scores in mice

We observed the changes of the skin lesions on the backs of mice daily and recorded the indicators of erythema (D), scaling (I), and hypertrophy of the lesions (E) as well as the total scores, and assessed the severity of the lesions according to the PASI scores, which were based on the criteria shown in Table 1.

Table 1
Mouse skin PASI score

Erythema (D)	Scaling (I)	Degree of lesion hypertrophy (E)
0 points: no erythema seen	0 points: no scales on the surface	0 points: no thickening of lesion
1 point: erythema is light red	1 point: some lesions covered with light scales	1 point: lesions are slightly thickened compared with normal skin
2 points: bright red erythema	2 points: most of the lesions are covered with flaky scales	2 points: lesions are moderately elevated compared with normal skin, with rounded or sloping edges
3 points: dark red erythema	3 points: almost all lesions covered with thick scales	3 points: thickening of the lesions, with more pronounced elevation than normal skin
4 points: erythema is very dark red	4 points: all lesions are covered with thick, stratified scales	4 points: lesions are highly thickened, with very pronounced elevation compared to normal skin

2.5.2 HE staining

Fixed mouse skin tissues were taken and dehydrated according to ethanol concentration from low to high; transparent for 2 hours; dipped in wax; embedded in paraffin; sectioned; patched; deparaffinized; stained with hematoxylin for 5 minutes; rinsed in tap water; differentiated (ethanol hydrochloride); washed well in warm water for 5 minutes; restained in eosin solution; dehydrated (concentration from low to high); and blocked after transparent in xylene.

2.5.3 ELISA for IL-6 and TNF-α expression levels

The ELISA kits used to detect the expression levels of IL-6 and TNF-α in the serum of various groups of mice. The specific steps were as described in the instruction manual: add the standard and the samples to examine in the kit; incubate at 37℃ for 60min; wash the plate with PBST; add the secondary antibody of the enzyme marker; incubate at 37℃ for 60min; wash the plate and add the color-developing solution, and then add the terminating solution after 15min, and then use the enzyme meter to determine the absorbance (OD) of the samples at 450nm, and then calculate the concentration according to the regression equation of the standard curve.

2.5.4 Western blotting to detect the expression of proteins of skin lesion-related pathways in mice

Mouse skin tissue samples were removed and placed in 2 mL grinding tubes. Two 3 mm grinding beads and RIPA lysis solution (according to the mass ratio of samples: lysis solution = 1:10) were added into each tube, and placed in a high-speed low-temperature tissue grinder (temperature − 20 ℃, grinding for 4 times, each time for 60 s); taken out and put in the refrigerator at 4 ℃ for 30 min to carry out lysis, and taken out and put into the centrifugal machine to centrifugate for 10 min at 4 ℃ and 12000 rpm; after centrifugation, the supernatant was taken, and the protein concentration was determined by BCA protein quantification kit. After 30 min, the sample was put into a centrifuge (4 ℃, 12000 rpm for 10 min); after centrifugation, the supernatant was taken and the protein concentration was determined by BCA protein quantification kit. The protein concentration was determined by BCA protein quantification kit. Samples were prepared with sample buffer and protein, and the protein was denatured by boiling water bath; the protein was separated by SDS PAGE gel electrophoresis, and the protein in the gel was transferred to PVDF membrane; the membrane was closed by shaking at room temperature with 5% skimmed milk for 2 h, and then the primary antibody was added (concentration of the primary antibody: P65 1:500; TLR4 1:2000; GAPDH 1:50,000), and the membrane was incubated at 4 ℃ for the whole night; The membrane was washed 3 times with TBST, the secondary antibody (dilution concentration: 1:5000) was added and incubated for 2 h at room temperature; the membrane was washed 3 times with TBST for 10 min each time; the membrane was exposed and developed by ECL chemiluminescence, and photographs were taken; the bands were exposed with Tanon Fluorescence Image Analysis System Software V2.0 using GAPDH as the internal reference, and the results were scanned with Gel-Pro analyzer4 software. The results were scanned by Gel-Pro analyzer 4 software, and the exposure results were expressed as the IOD of the target protein.

2.5.5 Statistical analysis

Using SPSS 17.0 for statistical analysis. Data were presented as Mean ± SD, One-Way ANOVA test was applied for comparison between multi-sample means, LSD test was used for chi-square, Tamhane's T2 test was used for non-chi-square, and the test results were considered to be significant for differences between groups at $p < 0.05$.

3. Result

3.1 Network pharmacology analysis

3.1.1 Psoriasis highly relevant differentially co-expressed genes (DEG s) acquisition and biological function enrichment analysis

1181 psoriasis DEGs were screened, involving 564 upregulation of genes and 617 downregulation of genes (Fig. 1A.B). WGCNA analysis showed that 24 co-expression modules were obtained, among which the highest psoriasis relevance was found in the darkolivegreen4 module, which contained a total of 1807 genes (Fig. 1C). The obtained DEGs were intersected with the module with the highest relevance. A total of 348 highly relevant DEGs for psoriasis were finally obtained.

GO enrichment analysis showed (Fig. 1D) that in terms of biological processes, psoriasis highly relevant DEGs were mainly enriched in mitochondrial translation elongation, mitochondrial translation termination, response to other organisms, translation termination, and response to external biological stimuli, and in terms of molecular functions they were mainly enriched in mitochondrial endosomes, organellar ribosomes, mitochondrial ribosomes, organellar endosomes, and mitochondrial protein complexes; In cellular components, they were mainly enriched in structural composition of ribosomes, endopeptidase activity, the activity of serine peptidase, the activity of serine endopeptidase and the effect of peptidase on l-amino acid peptide. The results of KEGG (Fig. 1E) showed that the highly relevant DEGs related to psoriasis were enriched in NF- κB pathway, Toll-like receptor pathway, cytokine-cytokine receptor interactions, P53 pathway, and IL-17 pathway, etc.

c.(A) Volcano map of differentially expressed genes.(B) Heat map of differentially expressed genes.(C) Module correlation bar chart.(D) GO enrichment analysis lollipop diagram.(E) KEGG enrichment analysis circle diagram.(F) The top 20 genes of the four algorithms.

3.1.2 Screening for key genes in psoriasis pathogenesis

Four key genes for psoriasis pathogenesis, PCNA, CXCL8, S100A9 and CCNB1, were finally obtained (Fig. 1F). The analysis results of GSE2737 chips selected as a validation set showed that the differences in the expression levels of PCNA, CXCL8, S100A9, and CCNB1 were statistically significant in psoriasis lesion tissues and normal skin tissues ($P < 0.05$). All the key genes were significantly up-regulated in psoriasis patient's tissue samples (Fig. 2A).

3.1.3 Correlation analysis between the expression level of key genes for psoriasis pathogenesis and the proportion of immune-infiltrating cells in skin lesion tissues

CD8 + T cells, helper T cells, and unactivated dendritic cells had the highest average percentage and the most pronounced immune infiltration in skin lesion tissue samples from psoriasis patients. Activated dendritic cells and neutrophils were significantly higher in psoriatic lesion tissues. In contrast, naive B cells, activated NK cells, and monocytes appeared to be reduced to varying degrees in psoriatic lesion tissues. There was a positive correlation between γ δ T cells and activated CD4 memory T cells (r = 1. 00); and a negative correlation between neutrophils and activated NK cells (r = -0. 93). The key psoriasis genes PCNA and CCNB1 were positively correlated with neutrophils and activated dendritic cells (r = 0. 87, 0. 83, P < 0.05; r = 0. 86, 0. 85, P < 0.05, respectively), and S100A9 and CXCL8 were negatively correlated with monocytes (r = -0. 80, -0. 78, P < 0.05, respectively). (Fig. 2BCDE).

3.1.4 Drug active ingredients and target genes

The TCMSP and BATMAN-TCM databases analysed the Chinese herbal medicine "Paeonia veitchii Lynch". With the screening conditions, 29 active ingredients were obtained from the TCMSP database, and 32 active ingredients of Paeonia veitchii Lynch were screened from the BATMAN-TCM database. The results of the two databases were merged, the duplicates were removed, and a total of 23 active ingredients corresponding to 150 target genes were obtained.

3.1.5 "Drug-component-target" network

Using Cytoscape 3.9.1, the network structure diagram of "Paeonia veitchii Lynch - active ingredients - targets" was established, and the Cytohubba was utilized to select the top 9 active ingredients based on the degree value, which were catechin, baicalin, β-sitosterol, Punica granatum Linn, lactobacillus, paeoniflorin, tanshin, glutosterol, stigmasterol, paulownia glycosides, etc. The results were summarized as follows: salvinorin, sterol, and stigmasterol. Among them, CCNB1, CXCL8, PCNA, and S100A9 with larger values may be important targets for psoriasis, and suggest that Paeonia veitchii Lynch can be effective in the treatment of psoriasis through the combined efficacy of multiple targets (Fig. 3A).

3.1.6 Molecular docking

The key effective active ingredients, catechin, baicalein, β-sitosterol, Punica granatum Linn, lactacystin, paeoniflorin, salvinorin, glutosterol, and stigmasterol were combined with the core target proteins, CCNB1, CXCL8, PCNA, and S100A9 two by two and molecularly docked. Among the 36 docking results, all molecules were docked with the binding affinity of < − 4.25 kcal /mol, suggesting that the binding ability of the receptor and ligand is strong, and the smaller the value of binding affinity, the more stable the binding conformation is, and the greater the possibility of interaction. Therefore, Punica granatum Linn, which has the strongest binding ability and the most stable binding effect, was chosen as the most promising traditional Chinese medicine for the treatment of psoriasis searched in this study. (Fig. 3B-D)

3.2 Experimental validation

3.2.1 Effect of Punica granatum Linn on the appearance of skin lesions and PASI scores in psoriasis mice

No erythematous scales and skin lesion hypertrophy were seen on the back of mice in all groups on day 1 of modelling. On day 2, mice in the IMQ modelling group showed slight erythematous scales on the backs, which were not seen in the other groups. On day 4, most of the lesions in the model group were covered with flaky scales and showed signs of lesions that were slightly higher than the skin and slightly dry and wrinkled skin. With the increase of modelling time, the severity of skin lesions in the model group was positively correlated with the modelling time, and the disease progressed more slowly in the Punica granatum Linn-treated groups than in the model group. In contrast with the model group, scales and thickness of skin lesions on the back of mice in the two Punica granatum Linn-treated groups were reduced, with the treatment effect being more pronounced in the low-dose group of Punica granatum Linn. The PASI scale showed the same results (Fig. 4AB)

3.2.2 Effect of Punica granatum Linn on the histopathology of skin lesions in psoriatic mice

Microscopic results showed that model group can see more obvious pathological changes, mice skin tissue local areas of hyperkeratosis, thickening of the stratum corneum, local areas of hyperkeratosis, residual nuclei in the stratum corneum, thickening of the epidermis, can be seen to increase the number of echinocandle cell layer, the dermis of the skin tissue can be seen in the inflammatory cell infiltration, with the nucleus of rounded dark staining of the lymphocytes and the rod-shaped lobular nucleus of the neutrophilic example of the cell is predominantly, basically in line with the psoriasis-like changes.

In contrast with model group, the lesions in both low-dose and high-dose groups of Punica granatum Linn were a slight decrease, the epidermal thickness was a significantly decrease, the hyperkeratosis and hypertrophy of the stratum spinosum were significantly improved, and the infiltration of inflammatory cells was gradually reduced. The improvement of the lesions in the high-dose group was more obvious. The above results suggest that Punica granatum Linn can alleviate imiquimod-induced skin inflammation in psoriasis mice, and the high dose group was more effective than the low dose group. (Fig. 4C)

3.2.3 Effect of Punica granatum Linn on serum levels of IL-6 and TNF-α in psoriasis mice

The results, as shown in the figure, showed that the concentrations of IL-6 and TNF-α in the serum of mice in the model group were significantly increased ($P < 0.01$). High-dose Punica granatum Linn had a significant inhibitory effect on both IL-6 and TNF-α in mice ($P < 0.05$). The above results suggested that high-dose Punica granatum Linn significantly inhibits IL-6 and TNF-α in the serum of imiquimod-induced psoriasis mice (Fig. 4D)

3.2.4 Effect of Punica granatum Linn on TLR4/NF-κB signaling pathway in skin lesion tissues of psoriatic mice

After the protein blotting test, in contrast with the control group, TLR4 in the skin of mice in the IMQ group was greatly elevated ($P<0.01$), and the addition of Punica granatum Linn significantly inhibited expression of TLR4 in both high and low-dose group, and the difference was statistically significant ($P<0.05$), which suggests that Punica granatum Linn has a significant inhibitory effect on TLR4 protein expression in the skin tissue of psoriasis mice induced by imiquimod, and that the inhibitory effect of high dose of Punica granatum Linn is more significant than low dose group. TLR4 protein expression in the skin lesion tissues of mice with imiquimod-induced psoriasis, and the inhibitory effect of Punica granatum Linn in the high-dose was more significant than that in the low-dose.

In contrast with the blank group, the expression of NF-κB (p65) in the IMQ model group was greatly elevated ($P < 0.01$); in contrast with the model group, the relative expression of NF-κB (p65) in the skin of mice in all doses of Punica granatum Linn groups was reduced, and differences were statistically significant ($P < 0.05$) in groups except for low-dose group of Punica granatum Linn ($P > 0.05$). The above results suggest that high-dose Punica granatum Linn has a certain inhibitory effect on NF-κB (p65) protein expression in skin lesion tissues of imiquimod-induced psoriasis mice. (Fig. 4E)

4. Discussion

Psoriasis is characterized by chronic scaly papular plaque, skin damage that can be generalized throughout the body, and is associated with many comorbidities, such as cardiometabolic disorders, stroke, chronic kidney disease, inflammatory arthritis, depression, and lymphoma[4]. Long course and repeated attack of psoriasis can reduce the quality of life of patients, resulting in a heavy burden on society and individuals. As a common herb for clearing heat and cooling the blood, Paeonia veitchii Lynch is widely used in the treatment of psoriasis caused by blood-heat.[12–14] The results of network pharmacology indicate that Punica granatum Linn is the material basis for the main pharmacological effect of Paeonia veitchii Lynch, therefore, the present study verified the ability of Punica granatum Linn in the anti-psoriasis activity through the experiments of the animal model.

In this study, we started with the CIBERSORT algorithm to screen the key genes for psoriasis pathogenesis, to investigate the relationship between the key genes for psoriasis pathogenesis and immune infiltrating cells, and to screen traditional Chinese medicines with therapeutic potential for psoriasis. After further analyzing and screening the 348 DEGs, we obtained four key psoriasis genes: PCNA, CXCL8, S100A9 and CCNB1. PCNA is a metabolic protein related to DNA replication, repair and cell cycle progression, and it plays an essential role in cell proliferation[15], reflecting the abnormalities in the proliferation and differentiation of psoriasis epidermal cells, and it can be used as the most critical gene for assessing the progression of psoriasis. CXCL8, or IL-8, is an essential pro-inflammatory factor involved in the pathogenesis of psoriasis[16], making it a potential therapeutic target for psoriasis[17]. It is involved in regulating keratinocyte proliferation, neutrophil infiltration and angiogenesis in psoriasis.

CXCL8 receptors are found in keratinocytes of psoriatic lesions, and CXCL8 activates keratinocytes through autocrine secretion, and produces and releases inflammatory mediators, which contribute to the migration of neutrophils to the lesion site[18, 19]. Some studies have shown that CXCL8 protein levels are positively correlated with PASI scores, further suggesting that CXCL8 may be closely related to the severity of psoriasis. CXCL8 may be involved in the development of psoriasis, and in the future, it may be used as an effective indicator to predict the severity of psoriasis, which may provide a useful reference value for psoriasis and clinical diagnosis. S100 calcium-binding protein A9 (S100A9) is mainly expressed in neutrophils, monocyte-macrophage cell lines, and diseased keratinocytes[20]. S100A9 and S100A8 form a dimer that binds target proteins, transmits calcium signals, and regulates the concentration of calcium ions in the cytoplasm[21], which is involved in the processes of cell proliferation and differentiation, inflammation, and apoptosis. The S100A8 and S100A9 genes have been identified near the psoriasis susceptibility locus PSORS4[22], and their expression has been found in the early stages of psoriasis[23]. Serum levels of S100A8/A9 in psoriasis patients correlate significantly with disease activity, suggesting that these S100 proteins are potential causative factors in psoriasis.[24]. The pathogenesis of psoriasis has a closely connection with the cell cycle of keratinocytes, and CCNB1 is a cell cycle-related gene. Some studies have shown that CCNB1 and CDC20 are highly expressed in psoriasis lesions, which further suggests that CCNB1 may be closely related to the development of psoriasis vulgaris[25]. In conclusion, these key target genes in psoriasis may play important roles in regulating keratinocyte proliferation and differentiation, participating in inflammatory response, maintaining normal skin barrier function, and regulating cytoplasmic calcium ion concentration.

Immune infiltration analysis of 22 immune cells in the skin lesion tissue samples was performed by the CIBERSORT reverse convolution algorithm. From the results, it can be seen that helper T cells, unactivated dendritic cells, and CD8 + T cells had the highest percentage, and helper T cells and unactivated dendritic cells were also significantly elevated in the case samples. Multiple immune cells were elevated in psoriasis case samples, suggesting their importance in psoriasis. Immature DCs are very few in inflammatory diseases and the ability of migration is great; mature DCs can activate the initial T cells effectively, which is the central link to start, regulate and maintain the immune response[26, 27]. Immunological and inflammatory stimuli are essential for the development of psoriasis, and an imbalance in peripheral blood Th1/Th2 balance and activation of related signaling pathways are the main drivers of psoriasis in both the initiation and amplification phases of psoriasis development[28]. Among them, Th1 mainly secretes IL-2 and IFN-γ, which participate in cellular immunity and inflammation[29], while Th2 secretes IL-4 and IL-10, which participate in humoral immunity and inhibit inflammation[30]. Under normal circumstances, the relative balance of Th1/Th2 in the peripheral blood plays an immunoprotective role, while its imbalance will cause excessive inflammatory reactions[31]. Immune cell correlation analysis showed a significant positive correlation between γδ T cells and activated CD4 memory T cells, suggesting that the synergistic effect of these two immune cells has an important impact on psoriasis pathogenesis. An important feature that distinguishes memory T cells from initial T cells is the ability to produce cytokines efficiently and rapidly, and is a key mechanism by which CD4 + memory T cells exert their anti-infective effects. Studies on human psoriasis have shown

that plasmacytoid dendritic cells contribute to psoriasis through the secretion of IFNα, and that IFNα induces the secretion of IFNγ and the psoriasis-associated growth factor IGF-1 by γδ T cells. In addition, γδ T cells also secrete TNF-α, IL- 17A and other cytokines related to the development of psoriasis, and IL-8, CCL3, CCL4, CCL5 and other inflammatory factors that chemotaxis inflammatory cells to the local area of the skin, and γδ T cells may play a key role in the disease progression of psoriasis. It can be hypothesized that the synergistic effect of these two types of immune cells may have an important influence on the pathogenesis of psoriasis, which can be verified by relevant experimental studies. The key psoriasis genes PCNA and CCNB1 were positively correlated with neutrophils and activated dendritic cells, and S100A9 and CXCL8 were negatively correlated with monocytes. It is suggested that the key psoriasis genes are related to the immune cell infiltration mechanism of psoriasis.

The mechanism of action of Paeonia veitchii Lynch on psoriasis was analyzed by using network pharmacology methods. The analysis and screening yielded 23 active ingredients, including catechin, baicalein, β-sitosterol, Punica granatum Linn, paeoniflorin, and salvianolic acid, and 150 targets of action, including CCNB1, CXCL8, PCNA, and S100A9, in this drug. The molecular docking results confirmed that Punica granatum Linn had the strongest binding ability and the most stable binding effect. KEGG showed that the highly relevant DEGs for psoriasis were enriched in NF- κB pathway and TLR4 pathway. Therefore, we constructed an IMQ-induced psoriasis model to validate the efficacy of the active ingredient. TLR4 plays an essential role in various inflammation-related diseases. Meanwhile, IMQ, as a TLR agonist, over-activates the TLR4 pathway, resulting in a prolonged and excessive inflammatory response. NF-κB, as a major pathway downstream of TLR4, was also over-activated in IMQ-stimulated and psoriasis patients. Western blotting results showed that NF-κB pathway was included in IMQ-induced inflammatory skin injury in mice, and Punica granatum Linn was able to alleviate IMQ-induced skin injury in mice by inhibiting the over-activation of TLR4/NF-κB signaling pathway. Mouse skin damage by restraining the over-activation of TLR4/NF-κB signaling pathway.

NF-κB p65 plays a key role in mediating a positive feedback loop in psoriasis, where its activation moves to the nucleus and stimulates the transcription of proliferative and inflammatory regulatory genes[32]. As a protein transcription factor, NF-KB is involved in regulating inflammation and some complicated biological processes. It is a key regulator of various immune and inflammatory responses, cell proliferation and differentiation. It promotes the expression of cytokines involved in psoriasis, involving IL-6, IL-1β, and TNF-α, among others. The results confirm Punica granatum Linn ameliorates histopathological changes and reduces the production of TNF-α and IL-6 by inhibiting the expression of TLR4 and the activation of NF-κB.

5. Conclusion

Our study showed that Punica granatum Linn improved Pso symptoms in mice. Punica granatum Linn may ameliorate the histopathological changes of Pso by restraining the expression of TLR4 and the activation of NF-κB and decreased the production of TNF-α and IL-6. Punica granatum Linn is an

important basis for the treatment of psoriasis by Paeonia veitchii Lynch, and the TLR4/NF-κB pathway may be a common pathway of action for Paeonia veitchii Lynch and Punica granatum Linn.

Declarations

Funding

Project Supported by the National Natural Science Fundation of China(Grant No.81773312)

CRediT authorship contribution statement

Suyue Pan: Writing – original draft, Writing – review & editing. Qiao Huang: Project administration, Visualization, Writing – original draft. Pu Wang: Project administration. Min Hu: Methodology, Validation. Weijia Li: Validation. Yi Peng: Validation. Lingyu Liu: Data curation. Qianfan Jiang: Data curation. Jiahui Qi: Project administration. Yuqing He: Funding acquisition, Supervision.

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.

Ethical approval and consent to participate

All experiments carried out strictly following the ethical principles of animal experiments. All experiments were approved by the animal experimental ethics committee of Sichuan Lilaisno Biotechnology Co. , Ltd. Animal ethics reference number: LLSN-2023120.

Declaration of purpose: We screened the active components of Paeonia veitchii Lynch by network pharmacology. Weveriied them in vitro to identify the potential molecular mechanism of Paeonia veitchii Lynch in treating psoriasis.

Research Processes: In this study, we analysed psoriasis gene chips based on bioinformatics methods, explored the immune infiltration mechanism of key psoriasis genes using the CIBERSORT deconvolution algorithm, screened out the main active components of Paeonia lactiflora by applying cyberpharmacology, and explored the mechanism of action of the psoriasis-associated TLR4/NF-κB signalling pathway by in vivo animal experiments.

Risks and benefits: Researchers will explain potential physical, psychological, or social risks to participants and inform participants of how to avoid or mitigate these risks. Also, researchers will explain to participants the benefits they may gain from the research, which may be a contribution to the individual or to society as a whole.

Confidentiality and anonymity: I understand that any information collected during this study about any information will be kept confidential and used only for the purposes of this study. My participation is

voluntary and I have the right to withdraw from the study at any time without penalty.

Results of the study: Our results suggest that Punica granatum Linn may be an essential basis for the treatment of psoriasis by Paeonia veitchii Lynch, and TLR4/NF-κ b pathway may be the common pathway of Paeonia veitchii Lynch and Punica granatum Linn.

Consent for publication

Not Applicable.

Availability of date and materials

Data and materials will be made available on request. If anyone would like to obtain data from this study, please contact the first author Pan Suyue, E-mail P791534202@163.com.

Acknowledgements

We would like to thank the School of Public Health of Guangdong Medical University for supporting us in completing our project!

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Figures

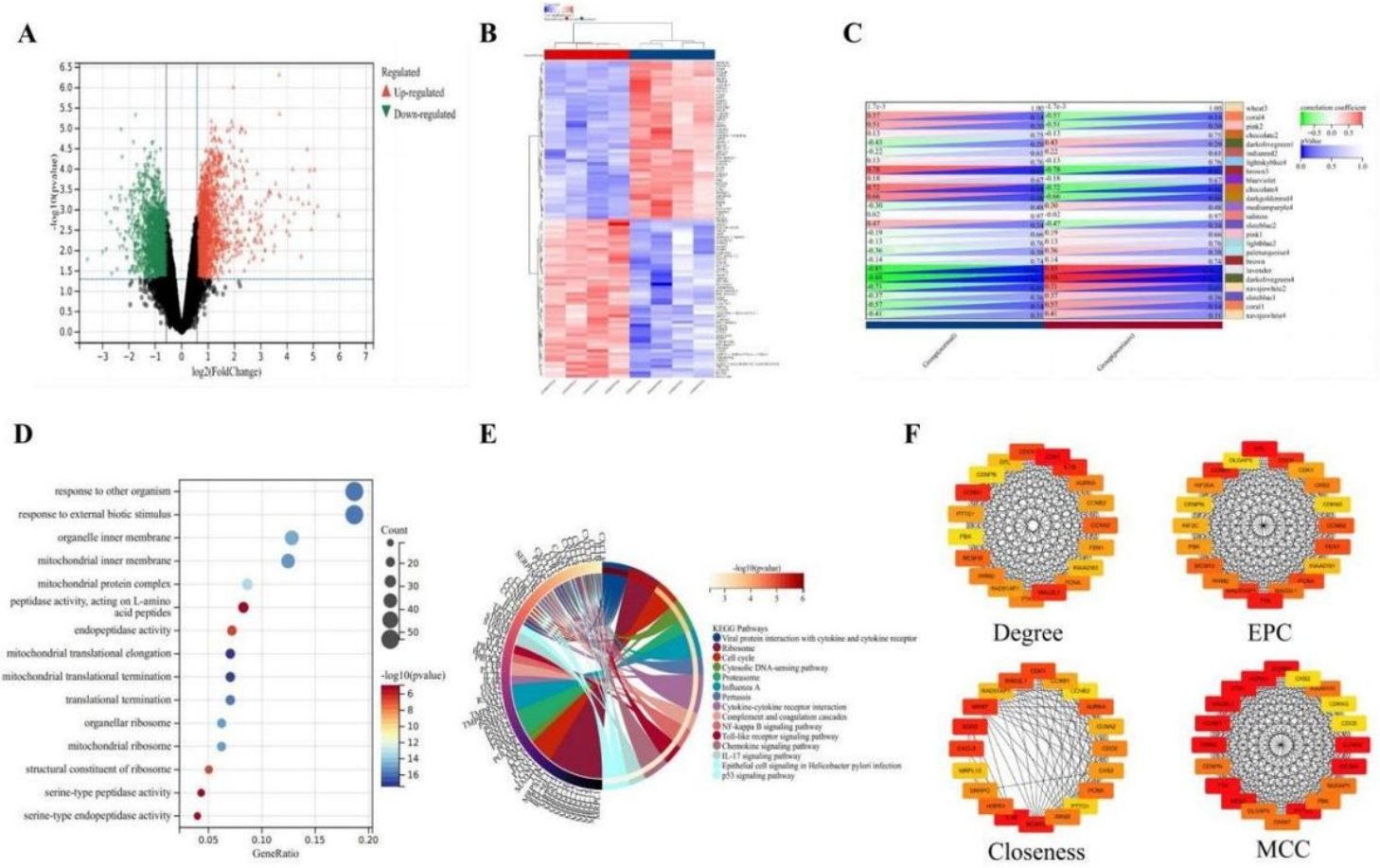

Figure 1

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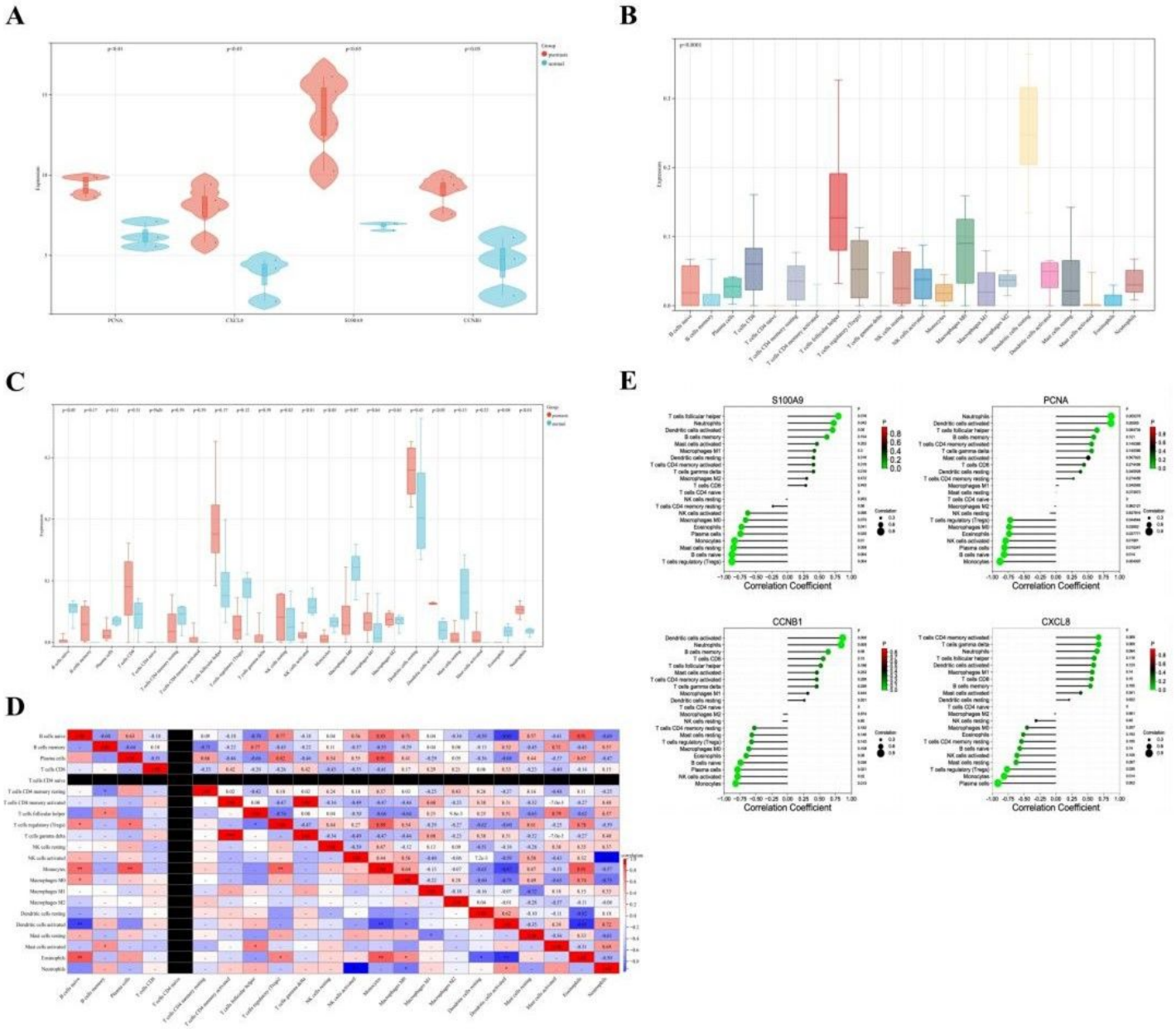

Figure 2

(A) Hub gene expression levels in GSE2737.(B) The box diagram of immune cell distribution.(C) The proportion box diagram of 22 kinds of immune cells.(D) The cell correlation matrix diagram.(E) The correlation map between key genes and immune cells.

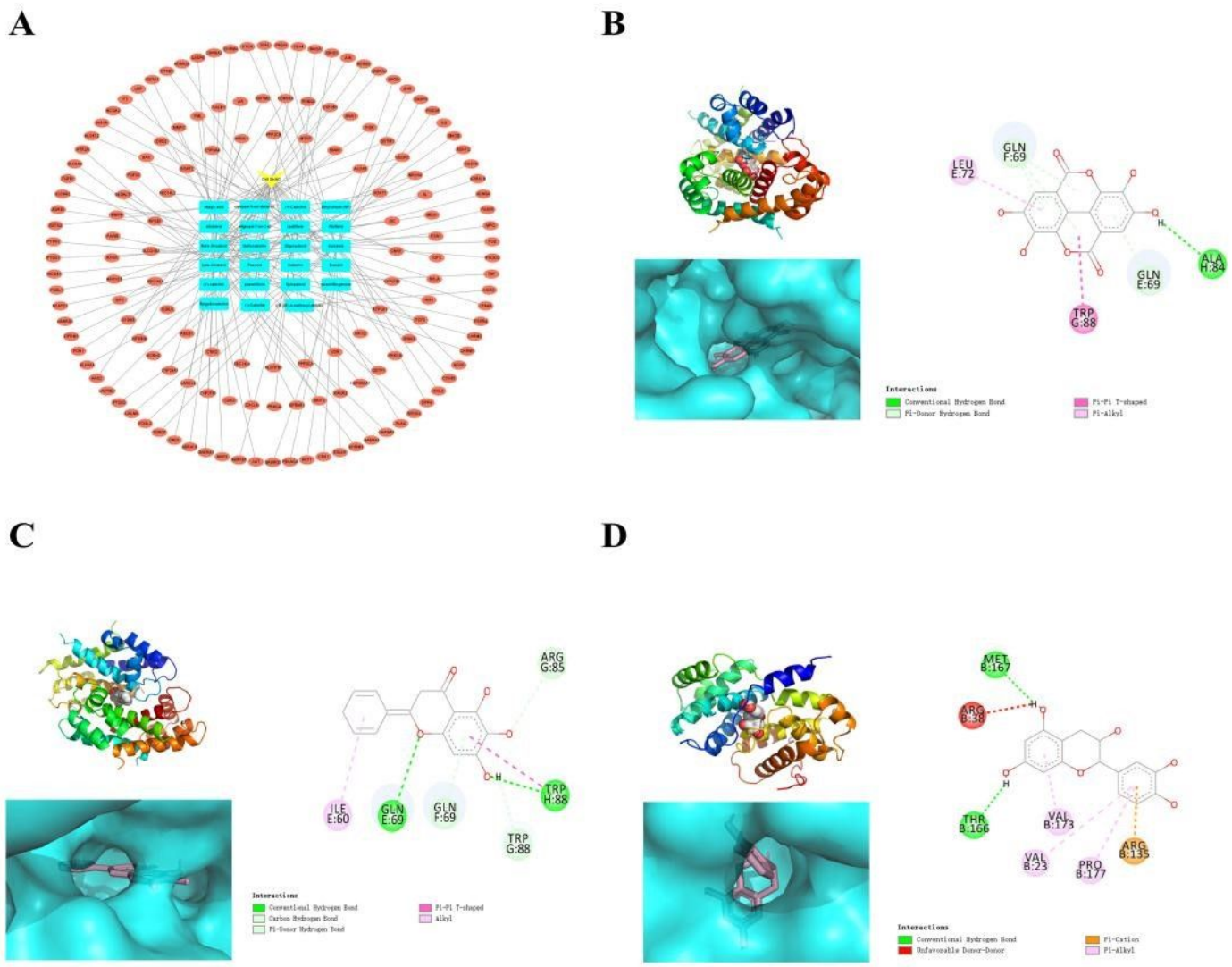

Figure 3

(A) "Paeonia veitchii Lynch - active ingredients - targets" network construction.(B) Molecular docking of S100A9 and Punica granatum Linn.(C) Molecular docking of S100A9 and xanthosides.(D) Molecular docking of CCNB1 and catechins.

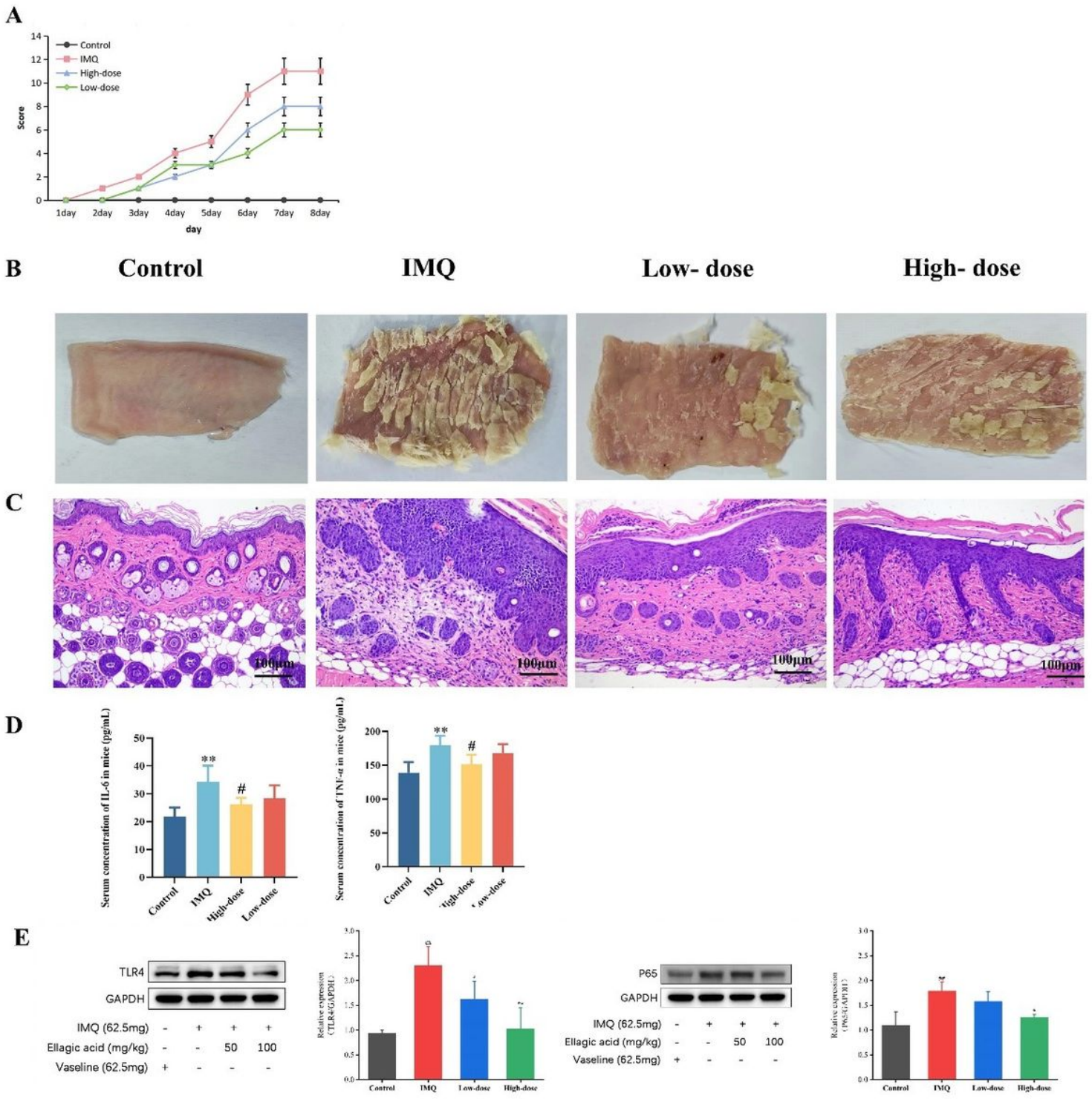

Figure 4

(A) PASI scores of skin lesions in psoriasis model mice by Punica granatum Linn.(B) Dorsal skin surface conditions in psoriasis-like mice by Punica granatum Linn.(C) Pathological changes in HE staining (200x) of mouse back skin tissue.(D) Effect of Punica granatum Linn on serum levels of IL-6 and TNF-α in mice with psoriasis.(E) Effect of Punica granatum Linn on TLR4/NF-κB signalling pathway in skin lesion tissues of psoriasis mice.

Supplementary Files

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