Yinxieling decoction ameliorates psoriasis by regulating the differentiation and functions of Langerhans cells via TGF-β1/PU.1/IL-23 signal axis

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Abstract

Langerhans cells(LCs) play a critical role in skin immune responses and the development of psoriasis. Yinxieling(YXL) is a representative herbal medicine formula for the treatment of psoriasis in South China. It was confirmed to improve psoriasis without obvious side effects in the clinic. Here we attempted to clarify whether and how YXL regulates the differentiation and functions of LCs in Imiquimod(IMQ)-induced psoriasis-like C57BL/6 mouse model in vivo, and induced LCs in vitro. The Psoriasis Area Severity Index (PASI) score was used to evaluate efficacy of YXL for IMQ-induced psoriasis-like mice. Flow cytometry was utilized to analyze the effects of YXL to regulate the differentiation, migration, mature and antigen presentation of LCs. The results show that YXL significantly alleviated skin inflammation, as reduced in PASI score and classic psoriatic characteristics in pathological sections. Although there was not any effect on the proportion of total DCs in the skin draining lymph nodes, the expression of epidermal LCs and its transcription factor PU.1 was significantly inhibited, and its migration to draining lymph nodes and maturation were also inhibited. Further data showed that the number of antigen-carrying LC in the epidermis increased, indicating that YXL could effectively inhibit the antigen presentation of LCs. YXL also significantly inhibited the differentiation of LC in vitro. Further data showed that YXL decreaded the relatve expression of TGFβmRNA and IL-23 mRNA. Thus, YXL allievates psoriasis by regualting differentiation, migration, maturation and antigen presentation via TGFβ/ PU.1/IL-23 signal axis.

Ψινξιελινγ δεχοχτιον αμελιορατες πσοριασις βψ ρεγυλατινγ τηε διφφερεντιατιον ανδ φυνχτιονς οφ Λανγερηανς χελλς ια ΤΓΦ-β1/ΠΥ.1/ΙΛ-23 σιγναλ αξις

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ABSTRACT

Langerhans cells(LCs) play a critical role in skin immune responses and the development of psoriasis. Yinxieling(YXL) is a representative herbal medicine formula for the treatment of psoriasis in South China. It was confirmed to improve psoriasis without obvious side effects in the clinic. Here we attempted to clarify whether and how YXL regulates the differentiation and functions of LCs in Imiquimod(IMQ)-induced psoriasis-like C57BL/6 mouse model in vivo, and induced LCs in vitro. The Psoriasis Area Severity Index (PASI) score was used to evaluate efficacy of YXL for IMQ-induced psoriasis-like mice. Flow cytometry was utilized to analyze the effects of YXL to regulate the differentiation, migration, mature and antigen presentation of LCs. The results show that YXL significantly alleviated skin inflammation, as reduced in PASI score and classic psoriatic characteristics in pathological sections. Although there was not any effect on the proportion of total DCs in the skin draining lymph nodes, the expression of epidermal LCs and its transcription factor PU.1 was significantly inhibited, and its migration to draining lymph nodes and maturation were also inhibited. Further data showed that the number of antigen-carrying LC in the epidermis increased, indicating that YXL could effectively inhibit the antigen presentation of LCs. YXL also significantly inhibited the differentiation of LC in vitro. Further data showed that YXL decreaded the relatve expression of TGFβmRNA and IL-23 mRNA. Thus, YXL allievates psoriasis by regualting differentiation, migration, maturation and antigen presentation via TGFβ/ PU.1/IL-23 signal axis.

KEYWORDS

Yinxieling, Psoriasis, Langerhans cells, Migration, Maturation, Antigen presentation

INTRODUCTION

Psoriasis is a common chronic skin inflammation. The condition is complex and stubborn, prone to recurrent attacks. The main clinical manifestations are erythema, scaly and epidermal thickening, which can take place throughout the body. At present, the incidence of psoriasis in the world is about 2%-3%[1]. In 2014, the organisation passed a resolution recognising psoriasis as a "chronic, non-communicable, painful, disFIGUREuring, and disabling disease for which there is no cure"[2]. Beyond skin inflammation, psoriasis is thought to be as a unique autoimmune and auto inflammatory disease[3]. The immune response is mainly expressed in cytokines: type 1 (Th1) and type 17 (Th17) T cells are activated by IL-12 and IL-23 secreted by dendritic cells(DCs) in the skin[4]. Through various cytokines cause a chronic inflammatory state and alter epidermal hyperproliferation, differentiation, apoptosis, and neoangiogenesis that produce the cutaneous findings seen in this disease[5]. Therefore, DCs play an important role in the pathogenesis of psoriasis.

In general, most DCs are in an immature state and express low levels of co-stimulators and adhesion factors. Immature DCs has strong antigen phagocytosis ability, and will differentiate into mature DCs when taking up antigen or stimulated by certain factors. In this process, DCs migrate from the antigen-exposed peripheral tissues into secondary lymphoid organs, stimulating T cells to launch an immune response[6]. Langerhans cells (LC) are a subpopulation of dendritic cells in the process of differentiation and development, scattered in the basal layer and the spinous layer. It can capture and process antigens invading the skin and deliver them to T cells, stimulating the proliferation and activation of specific T cells. The isolation and identification of Langerhans cells were determined by the expression of HLA-DR, CD1a and Langerin (CD207)[7].

At steady state, the LC of the epidermis constantly probes the environment for invading pathogens by expanding and contracting its dendrites[8, 9]. In an inflammatory state, keratinocytes locally produce proin-

flammatory cytokines that promote LC maturation and movement to lymph nodes within 3-4 days after these antigenic stimulation and changes in the cytokine microenvironment[10, 11]. LC dissociates from neighboring cells by down-regulating E-cadherin and up-regulatingα 6-integrin, which enters the dermis of the skin through the basement membrane.

Once in the dermis, LC up-regulation the chemokine receptor CCR7,which can access the dermal lymphatic vessels, then enter dermal lymphatic vessels in a CCR7-dependent manner, and finally enter the T-cell region of lymph nodes and initiate adaptive immune responses[12-14]. Dermal LC are regarded as responsible for activating both the Th1 and Th17 infiltrate by secreting IL-12 and IL-23, respectively. This mixed cellular response secretes cytokines and leads to a cascade of events involving keratinocytes, fibroblasts, endothelial cells, and neutrophils that create the cutaneous lesions seen in psoriasis[15].

Owing to the characteristics of multi-component and multi-target, traditional Chinese medicine has more prominent advantages in the treatment of psoriasis, and there are many kinds of traditional Chinese medicine prescriptions put into clinical use. Herbal medicine formula *Yinxieling* (YXL) is optimized and condensed from the empirical prescription of Guowei Xuan, an national famous old herbalist doctor. The whole prescription is composed of ten herbs. It has the effects of nourishing blood and moistening dryness, cooling blood and detoxifying, removing blood stasis and dredging collateral, and has been used for psoriasis vulgaris more than twenty years in Guangdong provincial hospital[16]. Previous studies have demonstrated that YXL affects psoriasis microenvironment by regulating macrophage polarization by affecting NF-κB, STAT1 and STAT6 signaling pathways[17]. It can further alleviate psoriasis by inhibiting the phosphorylation of PI3K/Akt/mTOR pathway and affecting the immune response of NK cells in patients with psoriasis[18, 19].

Based on the clinical efficacy of Herbal Medicine formula Yinxieling (YXL), this study aims to investigate its effect on psoriasis-like C57BL/6 mice. Langerhans cells (LCs) play an important role in the occurrence and development of psoriasis. So we also explore the mechanism of YXL on the differentiation and functions of LCs in psoriasis-like mice. So as to provide experimental basis for better treatment of psoriasis with traditional herbal medicine

MATERIALS AND METHODS

2.1 Preparation of herbal extracts for Yinxieling.

According to the normal clinical dosage, A: RADIX REHMANNIAE (*Rehmannia glutinosa Libosch.*), B: RHIZOMA SMILACIS GLABRAE: (*Smilax glabra* Roxb.), C: FRUCTUS MUME (*Prunus mume*(Sieb.) Sieb.etZucc.), D: HERBA SARCANDRAE (*Sarcandra glabra*(Thunb.) Nakai), E: RADIX ARNEBIAE/RADIX LITHOSPERMI (*Lithospermum erythrorhizon Sieb. et Zucc.*), F: RADIX PAEONIAE RUBRA (*Paeoniae Radix Rubra*), G: RHIZOMA CURCUMAE (*Curcuma phaeocaulis Val.*), H: RHIZOMA CHUANXIONG (*Ligusticum chuanxiong Hort.*), I: RADIX ANGELICAE SINENSIS (*Angelica sinensis(Oliv.)Diels*) , J: RADIX GLYCYRRHIZAE (*Glycyrrhiza uralensis Fisch.*) with a weight ratio of 20:15:15:15:15:9:9:9:6:6. All these herbs were afforded by Guangdong provincial hospital of Chinese Medicine (Guangzhou, China) and kept in 4 refrigeration storage. The above herbs were soaked in 10 times the amount of water for 30min, and extracted for 1h, then filtered. The extraction process was repeated twice and filtrate was incorporated. The extract was concentrated to 200 mL by vacuum rotary evaporation at 65, anhydrous ethanol was added to adjust the alcohol concentration to 65%, and stood overnight at 4. The supernatant was concentrated to extract form, freeze-dried into powder and sealed at 4.

UPLC qualitative analysis.

UPLC analysis were performed to control the quality of the extration of YXL, using a Waters Acquity Ultra performance LC system (Waters, MA, USA), including quaternary solvent manager, sampler manager, column compartment and PDA detector, equipped with Waters Empower software. The separation was carried out on an Acquity UPLC BEH C18 column (100 mm $\times$ 2.1 mm i.d, 1.7 μm, Waters Corp., Milford, MA, USA). The column temperature was set at 40 ^{o}C. The mobile phase consisted of 0.1 % formic acid (A) and acetonitrile (B). The gradient condition was as follows: 0-5 min, 4% B; 5-6 min, 4-8 % B; 6-9 min, 8-9

% B; 9-14 min, 9-10 % B; 14-21 min, 10-19 % B; 21-24 min, 19%B; 24-26 min, 19-21 % B; 26-27 min, 21-70 % B; 27-31min, 70%B, and finally reconditioning the column with 4 % B isocratic for 10 min after washing column with 100 % B for 4min. The flow rate was set at 0.35 mL min-1 and the injection volume was set at 5 μL. The detection wavelength was set at 290nm. FIGURE S1.

Mice and treatment

Male C57BL/6 mice with SPF grade of 7-9 weeks of age were purchased from Guangdong Experimental Animal Center (Guangzhou, China). During the experiment, the animal room and feed were provided by the Guangdong Academy of Traditional Chinese Medicine Sciences. The animal room was dry and ventilated, the ambient temperature was controlled between 22-26, the humidity was 50%-70%, drinking water and eating were free, and the light and darkness cycle was carried out for 12h, which matched the requirements of the animals to be tested.

Mice were randomly divided into 6 groups (n = 3 per group, repeat 3 times): control group, Imiquimod (IMQ) group, IMQ + Cyclosporin (CsA) group, YXL high dose group (IMQ+YXL-H), YXL medium dose group (IMQ+YXL-M) medium and low dose group (IMQ+YXL-L). All the mice had been oral treated for 7 days. Among them, oral treated group were given 25 mg/kg CSA, 3.0 g/kg(equal to about 17g raw herbs), 2.0 g/kg and 1.0 g/kg YXL, which were dissolved in distilled water, while control group and IMQ group were only given distilled water.

From day 3rd, All the IMQ treated mice were topically treated daily with 50 mg 5 % Imiquimod cream (Sichuan Mingxin Pharmaceutical Co., Ltd. Sichuan, China) on shaved back skin for five consecutive days. And the control group was given the base material.

Assessment of psoriasis-like dermatitis

IMQ was applied on the 3rd day after intragastric administration, and the psoriasis lesions and disease severity were scored on the 4th day. PASI score was modified for evaluating the mouse psoriasis-like skin lesions from human psoriasis area and severity index clinical scoring system. The modified PASI had three parameters, including skin erythema, scaling and thickness, which were used to assess the severity of the IMQ-induced psoriasis-like dermatitis. These indexes were evaluated independently using a defined rating system (0: none, 1: mild, 2: moderate, 3: marked, 4: severe)[20]. PASI score was the sum of these three numerical values.

Body weights were recorded daily at the first day when IMQ was treated on the mice. Weight gain or reduction was calculated, comparing to the first day body weight.

2.5 Tissue preparation and histological analysis

On the experimental 8th day, the mice were anesthetized with 1.5% phenobarbital sodium. Skin lesions were cut for histopathological examination. All the skin samples had been fixed in 4% neutral-buffered formaldehyde solution for 48h and processed for paraffin embedding. Sections were stained with Hematoxylin-Eosin (HE).

2.6 Flow cytometer analysis.

2.6.1 Lymph nodes cell preparation and stimulation.

Skin draining lymph nodes were collected from the axillas of each mouse and put into the precooled wash buffer (2 % FBS and 0.2 % 0.5 M EDTA in 1*PBS). All lymph nodes were ground with the frosted surface of glass slides and passed through 70 μ m strainers to generate the single-cell suspension.

2.6.2 Epidermics cell preparation and stimulation.

The skin on the back of mice (2 cm*2 cm) was cut and placed in a 60-mm petri dish with PBS buffer and placed on ice. The excess subcutaneous tissue under the skin was gently scraped off with a scalpel blade, washed once with 75% ethanol quickly, and washed twice with PBS. The epidermis was placed in a 60mm

petri dish with 0.25% dispase (0.025g dispase+10 mL 1*PBS), incubated at 37 for 1h, and then the dermal part of the skin was gently scraped off. After the epidermis was cut into pieces, 700 μ L DNase (1 mg/mL) and 6.3 mL 1 *PBS were added and incubated at 37 for 50-60min. Digestion was terminated with a small amount of staining buffer(2 % FBS in 1*PBS), and epidermal cells were collected and counted after filtration with 100 μ m and 40μ m filters.

2.6.3 Flow cytometric detection of DC and LC in lymph nodes and epidermis. One million cells per staining were incubated with BD Pharmingen Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block) (clone: 2.4 G2, BD) for five minutes to block non-antigen-specific binding of immunoglobulins. Then cells were stained for cell surface and intracellular markers with the following conjugated monoclonal antibodies: MHC Class II (I-A/I-E) Monoclonal Antibody, eFluor 450 (clone: M5/114.15.2, eBioscience), CD207 (Langerin) Monoclonal Antibody, PE (clone: eBioL31, eBioscience), Alexa Fluor® 647 anti-SPI1 (PU.1) Antibody (clone: 7C2C34, Biolegend), BD Pharmingen FITC Rat Anti-Mouse CD103 (clone: M290, BD Pharmingen), BD Pharmingen Alexa Fluor(r) 647 Rat Anti-Mouse CD45R (clone: RA3-6B2, BD Pharmingen), BD Pharmingen PerCP-Cy5.5 Rat Anti-CD11b (clone: M1/70). Cells were analyzed with FACS Aria III (BD Biosciences, San Jose, CA, USA) and FlowJo software version 10.0 for Microsoft (TreeStar, Sam Carlos, CA).

2.7 Effect of YXL on LC migration

Fluorescein isothiocyanate (FITC) was dissolved in acetone and applied to the skin on the back of mice. Twenty-four hours later, skin draining lymph nodes were collected for flow detection. The lymph nodes cells were stained for cell surface markers with MHC Class II (I-A/I-E) Monoclonal Antibody, eFluor 450 (clone: M5/114.15.2, eBioscience), CD207 (Langerin) Monoclonal Antibody, PE (clone: eBioL31, eBioscience),, and then the proportion of FITC+LC was observed and calculated.

2.8 Effect of YXL on LC maturation

Epidermal cells were stained with antibodies MHC Class II (I-A/I-E) Monoclonal Antibody, eFluor 450 (clone: M5/114.15.2, eBioscience), CD207 (Langerin) Monoclonal Antibody, PE (clone: eBioL31, eBioscience), BD Pharmingen FITC Hamster Anti-Mouse CD80 (clone: 16-10A1, BD Pharmingen), and BD Pharmingen APC Rat anti-Mouse CD86 (clone:GL1, BD Pharmingen)The ratio of $CD80^+$LC and $CD86^+$LC was detected at 0 h and 72 h after epidermal cells were isolated, and the regulation effect of YXL on LC maturation was investigated.

2.9 Effect of YXL on antigen presentation of LC

One million cells per staining were incubated with 100 μ L 0.5 mg/mL Dextran fluorescein solution. After incubation at 37 for 45min, the antibodies MHC Class II (I-A/I-E) Monoclonal Antibody, eFluor 450 (clone: M5/114.15.2, eBioscience) and BD Pharmingen APC Mouse anti-Mouse CD45.2 (clone: 104, BD Pharmingen) were incubated at 4 in the dark. The ratio of $FITC^+$ LC was detected to determine the antigen presentation effect of LC.

2.10 RNA isolation, reverse transcription and RT-PCR

Total RNA was extracted from skin lesion using a RNAprep Tissue Kit (TianGEN, China) and resolved in RNase-free water. The purity of total RNA was detected by nanodrop 2000 spectrophotometer(Thermo Fisher Scientific, USA). After reverse-transcript into complementary RNA with EvoScript Universal cDNA Master (Roche, USA), Real-time PCR was then performed by using FastStart Universal SYBR Green Master (ROX) (Roche, USA) on ViiA 7 Dx (Applied Biosystems, USA). Primers were shown as: TGFβ1, Forward: 5'-CCA TCG ACA TGG AGC TGG TGA AAG G-3', Reverse: 5'-GCA GTG AGC ACT GAA GCG AAA GCC C-3'; IL-23 p19, Forward: 5'-TGG GCA TCT GTT GGG TCT-3', Reverse: 5'-TGG GCA TCT GTT GGG TCT-3'. GAPDH as the control. All experiments were conducted according to the manufacturer's instructions.

2.11 Culture of langerhans cells (LC) from PBMC

PBMCs were isolated from EDTA-anticoagulated fresh blood by Ficoll density gradient centrifugation (Ficoll, Sigma-Aldrich, German). After several washes with PBS, $1*10^6$ PBMC with culture medium(RPMI-1640 with 10 % FBS) were planted in 24-well plates. Standing at room temperature for 35 min, the supernatant and suspended cells were gently removed. Then cells were cultured in complete medium containing 100ng/ml GM-CSF,20ng/ml rhIL-4,20ng/ml rh TGF-β1 for 6 days. On the 2nd and 4th days, the medium was changed half, and on the 6th day, the cells were collected for flow cytometry. APC Mouse Anti-Human CD1a and PE anti-human CD207 (Langerin) Antibody were used to flow cytometry analysis, and the method was described previously.

2.12 Statistical analyses

The data were represented as mean ± standard deviation(SD), and one-way analysis of variance (ANOVA) was used for statistical analysis. All data were processed by GraphPad prism 8.0 systems. A *p-value* of less than 0.05 was considered statistically significant.

RESUNTS

3.1 Yinxieling(YXL) significantly alleviated Imiquimod induced psoriatic skin inflammation in mice.

After 7 days of continuous administration, the low dose (0.5 g/kg), medium dose (1.0 g/kg) and high (2.0 g/kg) dose of YXL extraction could significantly relieve the skin thickening, redness, scaling, inflammatory cell infiltration and other skin inflammation similar to psoriasis caused by imiquimot in mice, FIGURE.1A. Scores of YXL medium and high dose was significantly lower than that of IMQ group, FIGURE.1B. Further pathological analysis showed that the medium- and high-dose groups reduced the munro's microcysts, cuticle laxity and abscission, epidermal hyperplasia, interspinous cell edema, spinous process prolongation and immune cell exudation caused by skin inflammation similar to psoriatic skin inflammation, FIGURE.1C

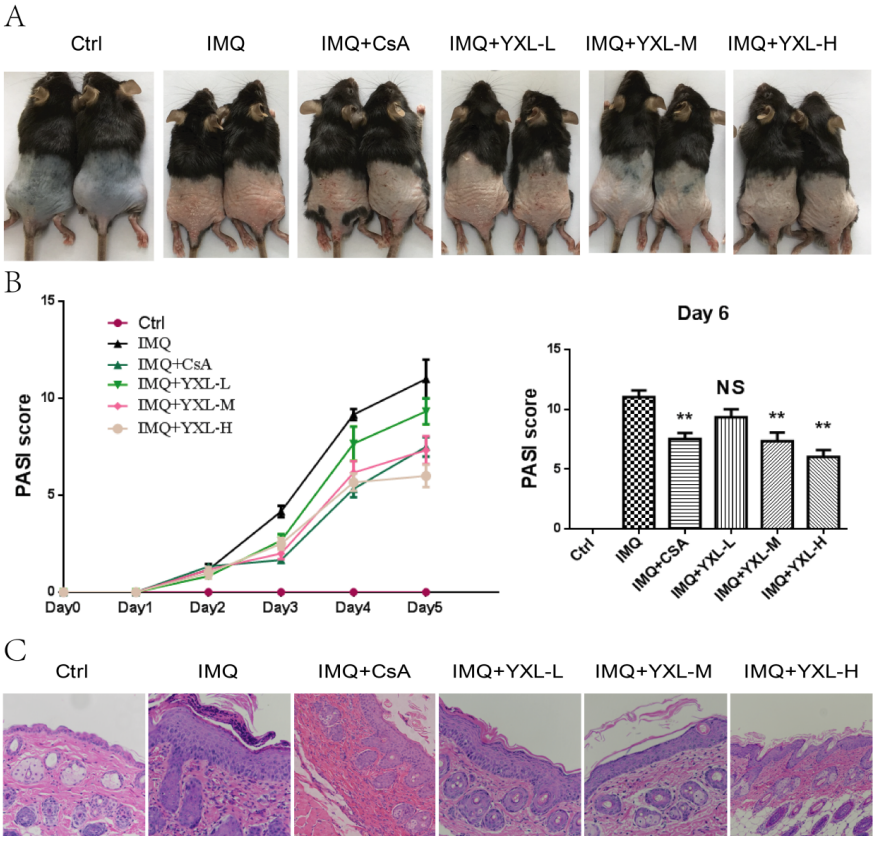


FIGURE 1 YinXieling(YXL) alleviates Imiquimod(IMQ)induced psoriasis-like murine dermatitis. A. Appearance sympotoms of mouse dermatitis; B. The modified psoriasis area and severity index (PASI) scores of the mouse back skin lesions; C. HE staining for murine skin lesions. IMQ: 0.5% Imiquimod cream, 50mg/day/mouse; CSA: cyclosporin A, 25 mg/kg; YXL-L: 0.5g/kg Yingxieling, YXL-M: 1g/kg Yingxieling, YXL-H: 2 g/kg Yingxieling. Data are shown as mean ± SD, n=6, *$p < 0.05$, **$p < 0.01$, comparing to IMQ group.

3.2 Yinxieling(YXL) had no significant effect on dendritic cell subsets in lymph nodes, but inhibited the ratio and number of epidermal langerhans cells (LCs)

The mice were intragastric with Yin Xieling liquid for 7 days, and skin draining lymph nodes were collected for flow analysis. The results showed that the proportion of dendritic cells (DCs) in skin draining lymph nodes of psoriatic mice were not significantly decreased. As well as there were no significant changes in $B220^+$ plasmoid dendritic cells, $CD103^-CD207^+$Langerhans cells, $CD207^+$ and $CD207^-$dermal DCs subsets, as showed in FIGURE 2

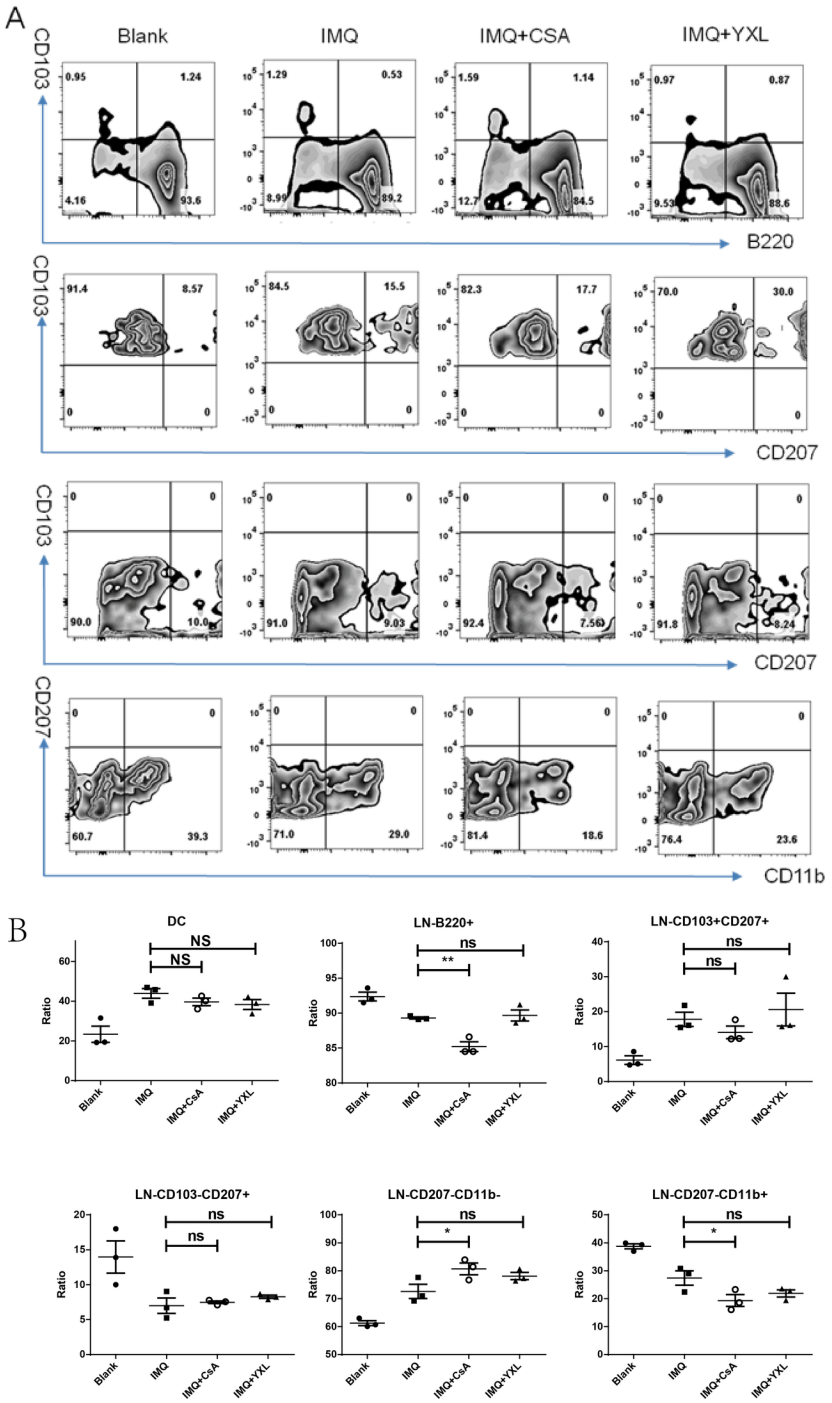


FIGURE 2 Yinxieling(YXL) dosen't have significiant effect on dendritic cells in skin draining lymph nodes. A: Gating strategy and flow cytometry scatter plot to identify total dendritic cells and subsets; B: Frequency of total MHCII^{+}DCs, B220^{+} plasmacytoid DCs, CD103^{-}CD207^{+} LC, CD207^{+} and CD207^{-} dermal DC subsets. n = 3, repreated at least twice with similar results. Comparing to IMQ group, $*p < 0.05$, $**p < 0.01$.

MHCII$^+$CD207$^+$ LCs in epidermis were detected by flow cytometry. The results showed that YXL significantly reduced the proportion and amount of LCs in the epidermis of psoriatic mice (p ¡0.001), as shown in FIGURE.3. It can reduce the inflammation of mouse skin induced by imiquimod and achieve the effect of treating psoriasis.

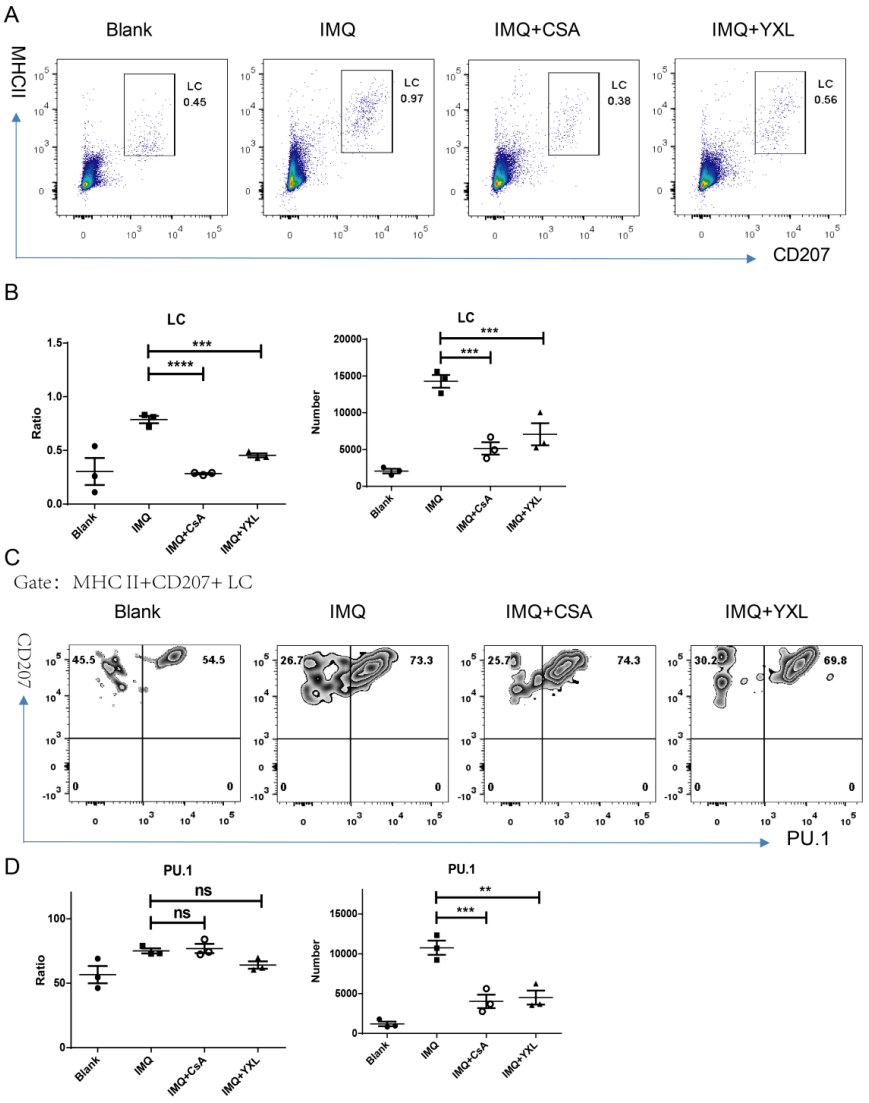


FIGURE 3 Yinxieling(YXL) inhibits the ratio and number of Langerhans cell(LC) and its transcription factor PU.1 in epiderm. A: Gating strategy and flow cytometry scatter plot to identify LCs; B: Frequency of MHCII$^+$CD207$^+$ LCs; C: Gating strategy and flow cytometry scatter plot to identify PU.1; D: Frequency of MHCII$^+$CD207$^+$ PU.1+LCs. n = 3, repreated at least twice with similar results. Comparing to IMQ group, $*p$ <0.05, $**p$ <0.01.

3.3 Yinxieling(YXL) inhibited the IMQ-induced migration of epidermal langerhans cells (LCs)

Langerhans cell migration from the epidermis to the lymph nodes is necessary for specific T lymphocyte responses. In a mouse model of psoriasis-like skin inflammation induced by IMQ, it promoted the migration of epidermal LC to dermal draining lymph nodes. In order to clarify the role of YXL in regulating LC, the migration of LC was studied. Fluorescein isothiocyanate (FITC) was dissolved in acetone and applied to the

back skin of mice. After 24 hours, the skin draining lymph nodes were taken for flow cytometry detection. The results showed that the ratio of FITC$^+$LC was significantly reduced in YXL treated group (p <0.001), indicating that YXL could significantly inhibit the migration of IMQ-induced LC from the epidermis to skin draining lymph nodes, FIGURE 4.

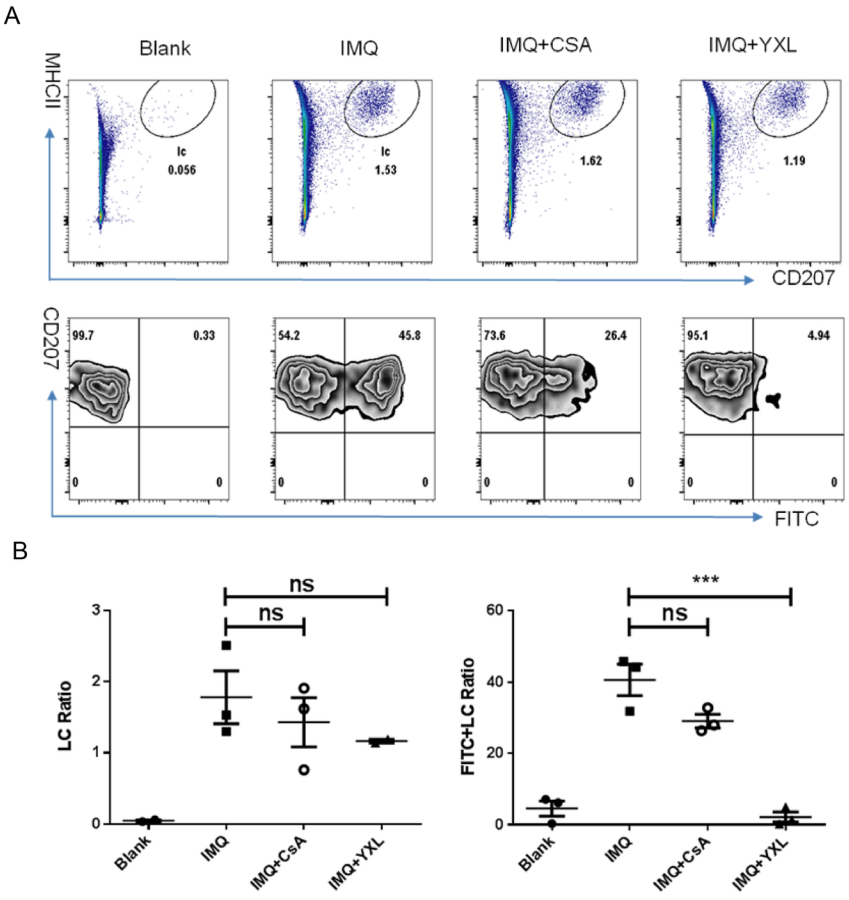


FIGURE 4 Yinxieling(YXL) inhibits the magration of Langerhans cell(LC) from epidermal to skin draining lymph nods. A: Gating strategy and flow cytometry scatter plot to identify LCs and magratory FITC$^+$LCs; B: Frequency of MHCII$^+$CD207$^+$ LCs and magratory FITC$^+$LCs. n = 3, repreated at least twice with similar results. Comparing to IMQ group, $***p$ <0.001.

3.4 Yinxieling(YXL) inhibited the IMQ-induced maturation of langerhans cells (LCs).

Epidermal cells were isolated and the ratio of CD80$^+$LC and CD86$^+$LC was detected at 0h and 72h, respectively, to investigate the regulation effect of YXL on LC maturation. The results showed that after three days of culture, the proportion of mature LC in the YXL group was significantly lower than that in the IMQ group, indicating that YXL could significantly inhibit epidermal LC maturation(p<0.05),as show in FIGURE 5.

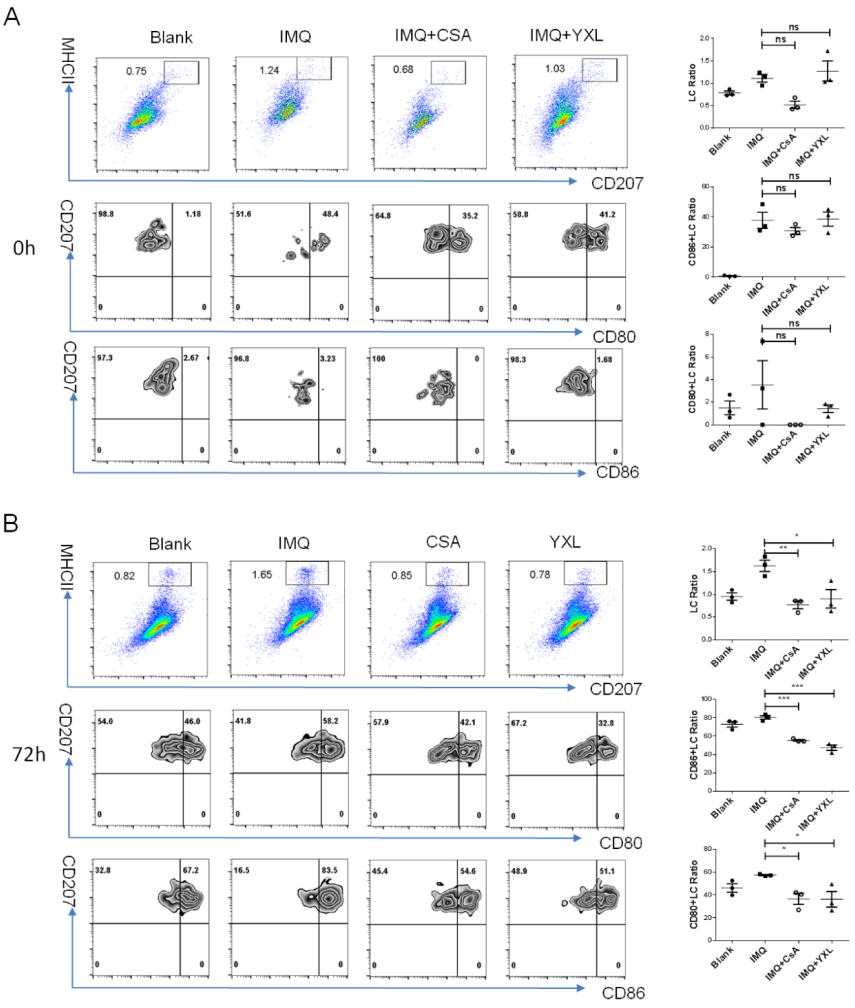


FIGURE 5 Yinxieling(YXL) inhibits the maturation of Langerhans cell(LC). A: Gating strategy, flow cytometry scatter plot to identify MHCII+CD207$^+$LCs, CD80$^+$LCs, CD80+LCs and Frequency of these cells on 0 h; B: Gating strategy, flow cytometry scatter plot to identify MHCII$^+$CD207$^+$LCs, CD80$^+$LCs, CD80$^+$LCs and Frequency of these cells on 72 h. n = 3, repreated at least twice with similar results. Comparing to IMQ group, $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

3.5 Yinxieling(YXL) inhibited antigen presentation of langerhans cells (LCs). Langerhans cells are the most important antigen presenting cells in the skin and play a key role in the pathogenesis of psoriasis. Therefore, flow cytometry was used to detect the antigen presentation effect of LC in the epidermis. As showed in FIGURE.6, the antigen-presenting effect of epidermal LC was significantly inhibited by YXL(p <0.01).

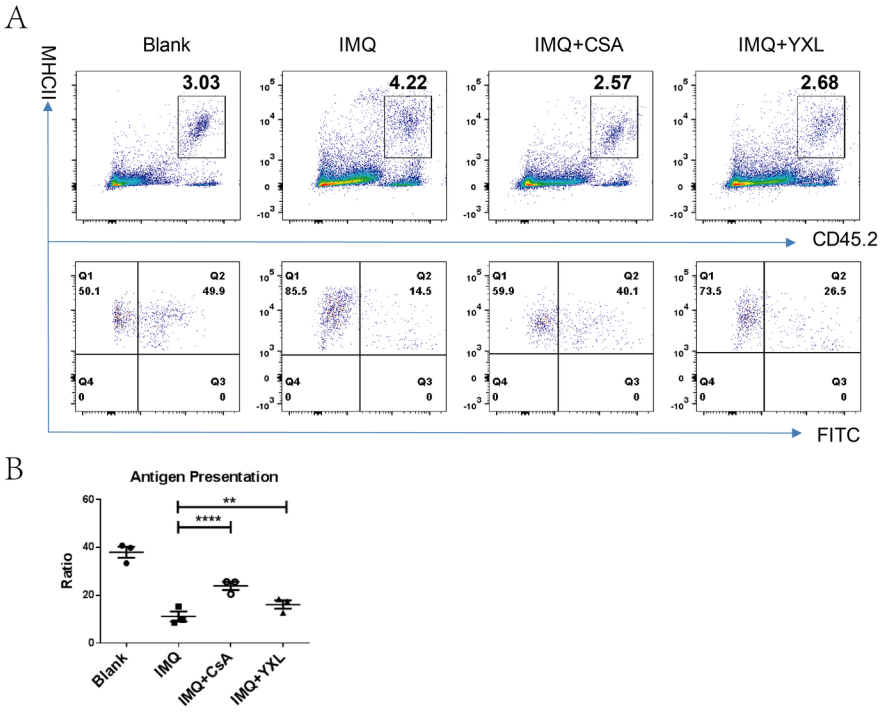


FIGURE 6 Yinxieling(YXL) inhibits the antigen presentation function of Langerhans cell(LC) . A: Gating strategy and flow cytometry scatter plot; B: Frequency of $FITC^{+}$LCs. n = 3, repreated at least twice with similar results. Comparing to IMQ group, ** p <0.01.

3.6 Ψινξιελινγ(ΨΞΛ) ινηιβιτεδ ρελατιε εξπρεσσιον οφ ΤΓΦβ1 μPNA ανδ ΙΛ-23 μPNA.

The results showed that YXL could significantly inhibit the relative expression of TGF-β1 mRNA in the skin lesions of psoriasis mice (p <0.05). LC is the main source of IL-23 in skin, and YXL can significantly inhibit the relative expression of IL-23p19 mRNA in the skin lesions (p <0.05), FIGURE 7.

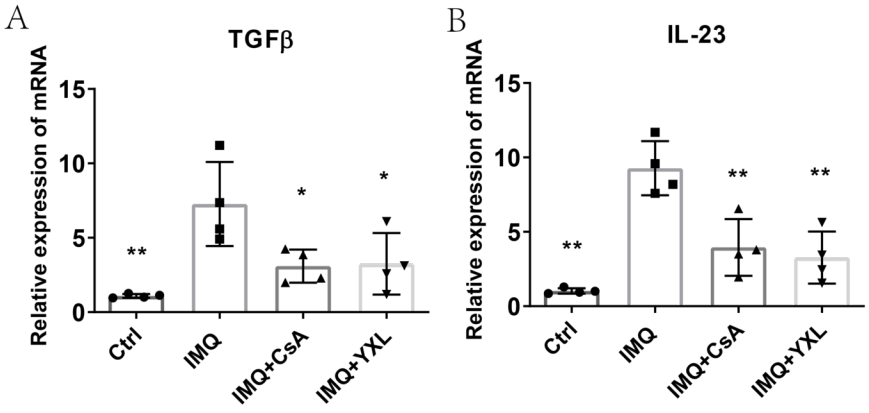
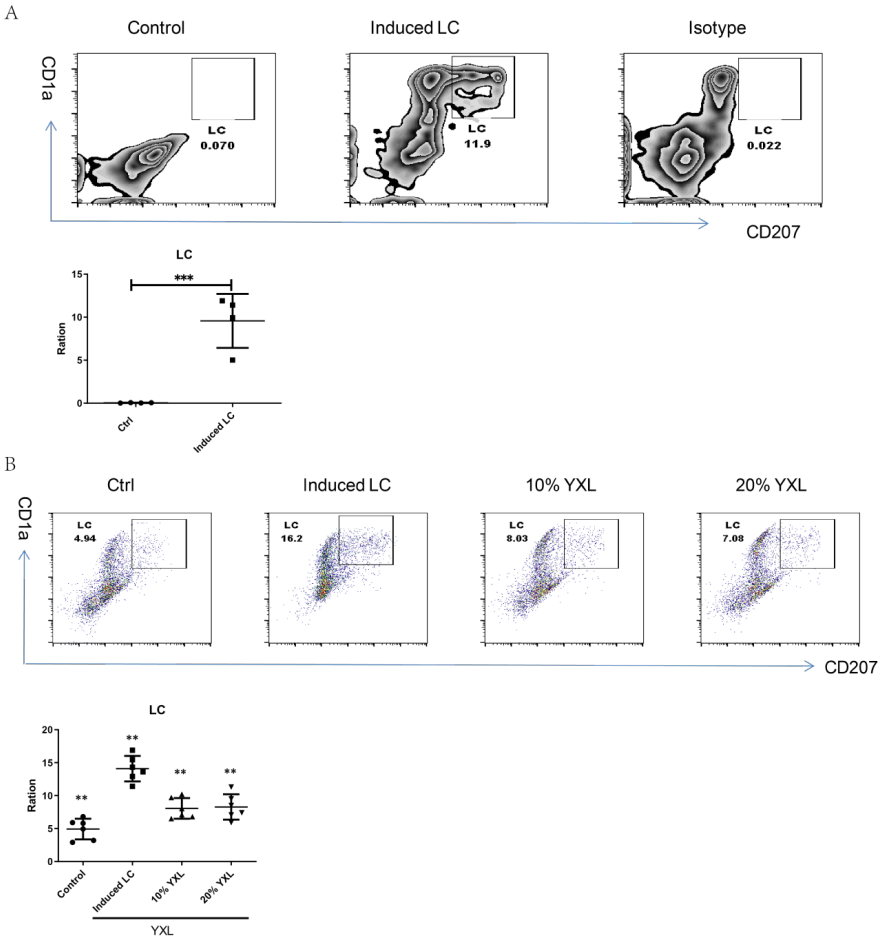


FIGURE 7 Yinxieling(YXL) inhibits the relative expression of TGF-β1 mRNA and IL-23p19 mRNA of skin lesions from psoriasis mice. A: relative expression of TGF-β1 mRNA; B: relative expression of IL-23p19 mRNA. n = 4, repreated at least twice with similar results. Comparing to IMQ group, * p <0.05.

3.7 Yinxieling(YXL) inhibited the differentiation of langerhans cells (LCs) in vitro.

Monocytes in PBMC can be induced to differentiate into LC by cytokines in vitro, and the differentiation ratio is about 10 %, FIGURE 8A. As shown in FIGURE 8B, SD rat serum containing drug for YXL significantly inhibited the differentiation of LC in vitro ($p<0.05$) .



FIGUREure 8. Yinxieling(YXL) inhibits the Langerhans cell(LC) differentiation in vitro . A: Gating strategy, flow cytometry scatter plot and frequency of cytokine induced LC; B: YXL inhibits the Langerhans cell(LC) differentiation in vitro. n = 6, repreated at least twice with similar results. Comparing to induced LC group, * p <0.05.

DISCUSSION

Psoriasis is not just a disFIGUREuring and refractory skin disease, but also a systemic disease associated with metabolic syndrome and cardiovascular diseases [21]. In recent years, the incidence of psoriasis has gradually increased, which has brought great pressure to patients' physical and mental health and economy[22]. In 2013, the annual meeting of the World Psoriasis Federation reached agreement on the new treatment strategy of psoriasis [23]. The main treatment strategies are to control the development, reduce recurrence, prevent complications and improve the quality of life, which is in line with the characteristics of traditional Chinese medicine in the treatment of diseases.

Prescription *Yinxieling* has been used in clinic for more than 20 years with good curative effect and no obvious

adverse reactions. Based on this, this study used IMQ-induced psoriasis-like mouse model to investigate the pharmacological effects of YXL[24]. Light red spots appeared on the second day of modeling. With the continuous effect of modeling drugs, the red patches became darker, the scales increased and the skin thickened, and reached the peak on the fifth day, covering almost all the bare skin. It can be seen from the PASI score curve that both CsA and YXL can effectively improve the clinical symptoms of psoriasis in mice, and the PSAI score is almost lower than that of IMQ group, especially YXL-M. The experiment confirmed that although the peak time of psoriasis-like symptoms in YXL group was basically the same as that in IMQ group, both erythema and scales were relieved in the later stage, indicating that YXL did not delay the inflammatory eruption time of psoriasis-like skin lesions induced by IMQ, but alleviated psoriasis by inhibiting the intensity of inflammatory eruption in this model skin lesions.

DCs stimulate naive T cells through their antigen presentation function to initiate acquired immunity and trigger inflammation. The plasmacytoid dendritic cell $B220^{+}$DCs, when stimulated by bacterial CpG or viral infection, is a potent producer of interferon-α (IFN-α) and interferon-β (IFN-β) [25]. Classical dendritic cells(cDC), otherwise known as myeloid dendritic cells, are the most abundant dendritic cell type in the dermis of healthy mice and are classified by expression of CD11b. Part of the dermal $CD11b^{+}$cDCs in mice express Aldh1a2, the sole rate-limiting factor for retinoic acid production. Retinoic acid is a vitamin A metabolite that promotes the production of Treg cells from primitive $CD4^{+}$T cells. The other part expressed the C-type lectin Langerin (also known as CD207 and CLEC4K), including $CD103^{+}$ and $CD103^{-}$ cells. They developmentally related to $CD8\alpha^{+}$cDCs in secondary lymphoid tissues. When cDCs are activated by proinflammatory cytokines or pathogens, CD103 cDCs mainly secretes IL-12 and CD11b cDCs mainly secretes IL-23. These two cytokines share IL23p40 subunit and are involved in the development of psoriasis [26].

The experimental results showed that CsA had no effect on the total DCs ratio, but reduced the proportion of $B220^{+}$ cells and $CD207^{-}CD11B^{+}$ cells, and promoted the proliferation of $CD207^{-}CD11B^{-}$ cells. However, CsA had no obvious effect on $CD103^{+}CD207^{+}$ and $CD103^{-}CD207^{+}$ subsets. These results indicate that CsA may inhibit the secretion of IL-23 by reducing CD11b+ cells, while YXL has no effect on cDCs in lymph nodes. LC are stellate cells that protrude their dendrites through tight junctions toward the cuticle and thus can detect antigens of the epidermis without disrupting the permeability barrier. After birth, they acquire a typical DC phenotype of histocompatibility complex II (MHC-II) molecule and CD207.

LC is the main source of IL-23 in psoriasis, and the reduction or absence of LC may lead to the attenuation of psoriasis-like lesions[27]. IL-23 /Th17 axis is the main known pathogenesis of psoriasis. IL-23 is a cytokine essential for the expansion and survival of pathogenic Th17 cells. IL-23 is highly expressed in psoriatic skin and mouse models, including models that rely on the local application of TLR7 agonist IMQ [28]. It was also found that the expression of LC in IMQ group was much higher than that in normal group.

LC differentiation is closely related to the expression of the transcription factor PU.1, an ETS family member transcription factor encoded by the Sfpi1 gene, which is an important regulator of many aspects of early hematopoietic and bone marrow cell differentiation [29]. Recently, PU.1 has been shown to control the expression of Flt3 in a dose-dependent manner, promoting the differentiation of cDC and pDC, and synergistically promoting the expression of RUNX3 with TGF-β , which further promotes the differentiation of LC[30]. Therefore, MHC-II and CD207 were used to label LC. The results showed that the ratio and number of LC and PU.1 were highly expressed in psoriatic lesions, while YXL and CsA significantly down-regulated the number and ratio of LC, but only decreased the number of PU.1, indicating that PU.1 was directly proportional to LC. Therefore, YXL may inhibit the expression of LC by reducing PU.1.

LC, as a unique antigen presenting cell in the epidermis, captures exogenous antigens and then passes them to T cells in the skin draining lymph nodes, activating $CD8^{+}$T cells and $CD4^{+}$T cells, further inducing the production of proinflammatory factors and leading to inflammation. The antigen presentation function of LC is powerful, and its ability to transfer from epidermis to local lymph nodes is important for inducing skin immune response [31].

Psoriasis is just an immune-mediated skin disease, one of its pathological features is keratinocyte proliferation[28]. It has been observed that keratinocytes under stress rapidly up-regulate ligands of lymphocyte activating receptor natural killer group 2D (NKG2D), leading to migration of LC populations out of the epidermis and subsequent emergence of $\alpha\beta^{+}$T cells in the epidermis. Enhanced expression of the murine NKG2D ligand Rae-1$\epsilon$was observed in injured skin, while mice lacking NKG2D showed delayed skin wound healing, confirming the importance of keratinocyte and LCs interactions during skin regeneration [32]. The activated LC expresses CCR7 and then travels through dermal lymphatics along the CCL19 and CCL21 chemokine gradients into parcutaneous cortical draining lymph nodes[33]. LC migration is associated with the synergistic effect of inflammatory cytokines IL-1β , IL-18, and TNF, so mice treated with these cytokines or with blocking antibodies against Casp1-/-, IL-1B -/-, and Tnfr2-/- reduced LC migration. Therefore, abnormal proliferation of epidermal keratinocytes in psoriasis may promote LC migration[34].

The LC carrying the neoantigen "descends" into the dermis and, together with the draining lymph, drains into the local lymph node, forming a nest that attracts antigen-specific lymphocytes. These lymphocytes are activated in the lymph nodes, proliferate, and then propagate systemically back primarily to the skin to destroy keratinocytes[35]. The experimental data showed that in IMQ group, the proportion of epidermal LC migrating to lymph nodes increased, and the epidermal LC with antigen-presenting function reduced. LC carrying antigen induced T cell differentiation and produced proinflammatory factors to aggravate psoriasis. After YXL administration, the LC migrating to lymph nodes decreased significantly, while the LC maintaining antigen presentation function in epidermis increased dramatically.

Therefore, YXL may inhibit the differentiation of LC by inhibiting the expression of TGFβ, blocking the migration and maturation of LC and its antigen presentation, thereby inhibiting the release of inflammatory factors IL-23 and alleviating the development of psoriasis.

CONCLUSIONS

Yinxieling significantly alleviates psoriasis-like skin inflammation induced by IMQ in mice. Although it has no obvious effect on DC in skin draining lymph nodes, it can significantly reduce the expression of LC in skin lesions, inhibit the migration and maturation of LC, and prevent its antigen presentation by TGFβ1/PU.1 signaling axis, so as to effectively alleviates the psoriasis-like skin inflammation on mice.

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Author contributions

Dinghong Wu and Chuanjian Lu designed the experiments and guaided all experiments. Ning Li, Dinghong Wu, Jiagu Ke, Qihua Yu, Miaomiao Zhang, Xiaoshu Chai and Qiaoling Wu carried out the experiments. Ning Li and Dinghong Wu wrote and revised the article.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethic approval

All animal experiments were approved by the institutional of Animal Care and Use Committee of the

Guangdong provincial hospital of Chinese Medicine (NO:2018025)and performed in accordance with the National Institutes of Health Guidelines on Laboratory Research.

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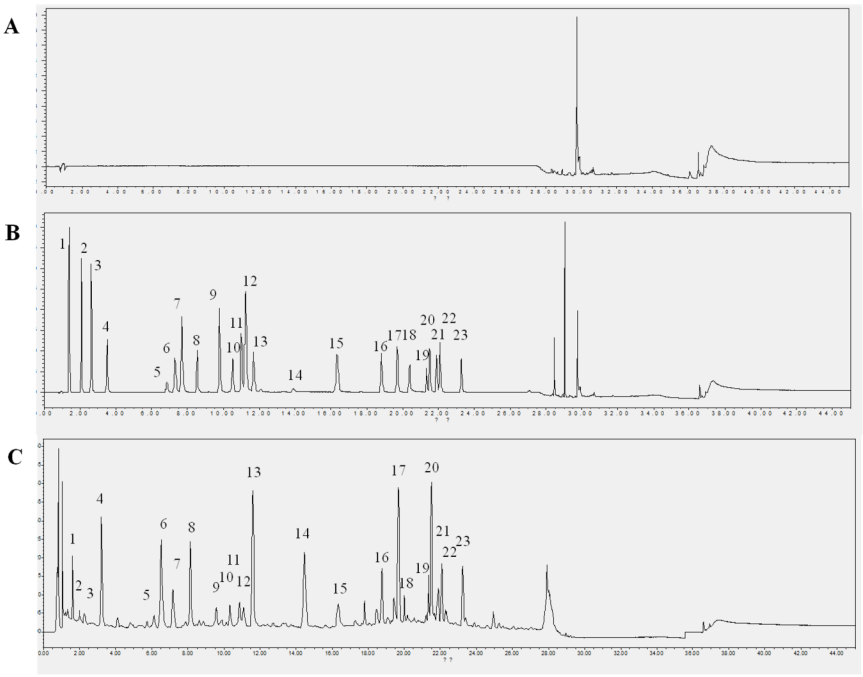

FIGURE S1 UPLC chromatogram of blank sample (A), 26 reference standards (B) and representative sample (C) detected at 290 nm. Small molecules in Yinxieling decoction: 1.Gallic acid; 2.5-Hydroxymethylfurfural; 3.Protocatechuic acid, 4.Neochlorogenic acid; 5.(+)-catechin; 6. Chlorogenic acid; 7. Caffeic acid; 8. Cryptochlorogenic acid; 9. Eleutheroside B1; 10. 3-O-caffeoylshikimic acid ; 11. 4-O-caffeoylshikimic acid; 12.p-coumaric acid; 13.Paeoniflorin; 14.Isofraxidin; 15.Neoastilbin; 16. Galloyl paeoniflorin; 17. Astilbin; 18.Neoisoastilbin; 19.Isoastilbin; 20.Rosmarinic acid; 21.Engeletin; 22.1,7-bis(4-hydroxyphenyl)hepta-4E-6E-dien-3-one; 23. Zederone