LiangxueJiedu Formula improves psoriatic dermatitis by regulating the circadian clock to inhibit IL-17-producing Th17 and γδT cells

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Research Article

Keywords: psoriasis, circadian clock, skin inflammation, Per2/- mice, IL-17, imiquimod, Chinese medicinal herbs

Posted Date: February 21st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2568437/v1

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Abstract

Background

Psoriasis is an immune-mediated inflammatory skin disease. The circadian clock influence immune cells and control the skin's inflammatory response. In this study, we observed the effect of LiangxueJiedu Formula (LXJDF) on imiquimod-induced per2-knockout mice to explore the mechanism of Chinese medicinal herbs in improving skin inflammation via the circadian clock.

Methods

The per2−/− mice were randomly divided into the model group, the LXJDF group, and the positive drug group (dexamethasone). The dorsal skin of mice was smeared with imiquimod at 9:00 AM (ZT1), and the corresponding drugs were given at 10:00AM (ZT2) and 10:00 PM (ZT14), respectively. The wild-type (WT) mice were smeared vaseline as the control group. The skin lesions were observed and PASI was performed for six consecutive days. The pathological morphology of the skin was determined by HE and immunofluorescence (Ki67, loricrin, and IL-17A) staining, and the epidermis thickness was measured. The spleen weight and index were calculated, and the splenocyte subtypes and serum cytokine levels were detected by flow cytometry. The serum melatonin levels were detected by ELISA. The gene expressions of inflammatory cytokines in the skin were determined by qPCR. The gene and protein expressions of circadian clock-related genes (CLOCK, BMAL1, REV-ERBα, NFIL3, and RORγt) in the skin were determined by qPCR and western blot.

Results

LXJDF could significantly improve the psoriasiform skin lesions, including the reduction of PASI, thinning of epidermal thickness, inhibition of keratinocytes proliferation, and parakeratosis at two-time points (ZT2 and ZT14). LXJDF could reduce the spleen weight and index and inhibit the number of Th17 cells, γδT cells, and the serum inflammatory factors levels of IL-17A, IL-17F, TNF-α, IL-22, IL-6. In addition, LXJDF could significantly down-regulate the mRNA expressions of IL-17A, IL-17F, IL-23, and IL-6 in the skin. LXJDF significantly increased the expressions of BMAL1 and REV-ERBα, and decreased NFIL3 and RORγt.

Conclusions

LXJDF ameliorates psoriatic dermatitis by regulating the circadian clock to inhibit IL-17-producing Th17 and γδT cells.

Background

Psoriasis is a chronic inflammatory skin disease characterized by immune cell infiltration, keratinocyte hyperkeratosis, and parakeratosis. Factors such as the environment, seasonal changes, and mental mood can induce or aggravate the disease. For example, most patients with psoriasis recur in spring and autumn, worsen in winter, and relieve in summer [1, 2]. Depression and anxiety can cause or aggravate skin lesions [3]. More than 70% of patients reported more severe itch in the evening or at night. Sleep disturbance caused by itching or pain at night can also aggravate skin lesions [4, 5]. And night shift workers have an increased risk of psoriasis [6]. Psoriasis patients show the characteristics of daily rhythm disturbance, such as abnormal rhythm in patients with heart rate and blood pressure [7]. Melatonin (MT) is a neuroendocrine hormone that exhibits rhythmic changes, and its secretion peaks at night. It regulates seasonal rhythms, circadian rhythms, and immune defense responses. The level of melatonin secretion in patients with psoriasis is abnormal, which is significantly lower than that of the control group at 2:00 AM and considerably higher than average at 6:00, 8:00 AM, and 12:00 PM [8]. Besides, the level of melatonin was negatively correlated with psoriasis area and severity index (PASI), indicating a correlation between
circadian rhythm disorders and the incidence of psoriasis [9]. High-through transcriptome and pathogenesis analysis of clinical psoriasis skin revealed that circadian entrainment is one of the essential pathways closely associated with psoriasis[10].

The circadian clock is a highly conserved transcription-translation feedback loop, like gears in a clock, which maintains the same rhythm in central and peripheral tissues. The molecular mechanism is mainly composed of the core loop and the stable loop. The core loop consists of circadian locomotor output cycles kaput (CLOCK) and brain and muscle-armt-like 1 (BMAL1), and they induce the expression of its inhibitor period (Per) and cryptochrome (Cry). Then the Per-Cry protein complex inhibits the transcription of its genes. The stable loop is an auxiliary feedback loop in which BMAL1 expression is regulated by a subfamily of nuclear receptors, including members REV-ERBs [REV-ERBα (NR1D1), REV-ERBβ (NR1D2)] and retinol retinoic acid receptor-related orphan receptors (RORs) (RORα, RORβ). REV-ERBα/β can inhibit BMAL1 expression, while RORα/β competes with REV-ERBα/β to share a DNA binding site (ROREs) and promote BMAL1 expression [11, 12].

The regulation of the skin's immune system by the circadian clock is an emerging field. Circadian rhythm genes are detected in mouse skin, including immune function-related genes, which suggests that the skin's immune system is regulated by circadian rhythms [13]. Ando et al. applied a circadian clock-related transgenic mice model to confirm the regulatory effect of circadian clock genes on psoriasis[14]. Imiquimod (IMQ), a TLR7/8 agonist, was applied to the mice dorsal skin can induce psoriasis-like skin lesions activated by the interleukin (IL)-23/IL-17 axis[15]. After applying ClockΔ19/Δ19 mice dorsal skin, compared with wild-type (WT) mice, the psoriatic skin lesions were improved, and the inflammatory cytokines IL-17, IL-22, and IL-23p19 in the skin were significantly reduced. Conversely, Per2 gene deletion mice were able to aggravate IMQ-induced inflammatory responses, which may be related to the up-regulation of IL-23R expression in γδT cells.

In recent years, much literature has reported that Traditional Chinese Medicine (TCM) compound has a better curative effect in improving psoriasis skin lesions [16, 17]. Liangxue Jiedu Formula (LXJDF) could effectively improve the symptoms of psoriasis patients by inhibiting inflammation [18]. Therefore, we tried to observe the effect of LXJDF on psoriasis with circadian rhythm disorder by IMQ-induced Per2−/− mice associated with the skin lesions, pathological features, inflammatory factors, and circadian clock related genes.

**Materials And Methods**

**Plant materials and preparation of LXJDF**

LXJDF, which contains nine herbs (Table 1), was provided by TCM Pharmacy of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University and purchased from Beijing Xinglin Pharmaceutical Co., Ltd. The quality of crude drugs was strictly performed according to standard protocols of the Chinese Pharmacopoeia (Version 2020). All voucher specimen were deposited in the specific Herbarium Room of Beijing Xinglin Pharmaceutical Co., Ltd. Total 123 g crude drugs material were soaked for 60 min in 1000 mL purified water (8 times volume) decocted using reflux extraction methods for 40 min. Then filter out drug liquid and decocted in 750 mL purified water (6 times volume) for 20 min. The two drug liquid was combined and concentrated to the experimental dosage. The LXJDF dose was 24.6 g/kg-bw (equal to the human clinical equivalent-effective dose) [19].
<table>
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**UPLC–MS/MS analysis of LXJDF**

An ultra-high performance liquid chromatography 1290 UPLC system with a Waters UPLC BEH C18 column (1.7 µm×2.1mm×100mm) was performed to analyze LXJDF. The flow rate was set at 0.4 mL/min, and the sample injection volume was set at 5 µL. The mobile phase was composed of A (water, and 0.1% formic acid, v/v) and B (acetonitrile) with gradient elution. The multi-step linear elution gradient program was as follows: 0–3.5 min, 95–85% A; 3.5–6 min, 85–70% A; 6–6.5, 70–70% A; 6.5–12 min, 70–30% A; 12.5–12.5 min, 30–30% A; 12.5–18 min, 30–0% A; 18–25min, 0–0% A; 25–26min, 0–95% A; 26–30min, 95–95% A.A Q Exactive Focus mass spectrometer coupled with Xcalibur software was employed to obtain the MS and MS/MS data based on the IDA acquisition mode. During each acquisition cycle, the mass range was from 100 to 1500, and the top three of every cycle were screened, and the corresponding MS/MS data were further acquired. Sheath gas flow rate: 45 Arb, Aux gas flow rate: 15 Arb, Capillary temperature: 400 °C, Full ms resolution: 70000, MS/MS resolution: 17500, Collision energy: 15/30/45 in NCE mode, Spray Voltage: 4.0 kV (positive) or -3.6 kV (negative).

**Animal model and groups**

Male Per2−/− mice and C57BL/6 (WT) mice at 6–8 weeks of age were purchased from Jiangsu GemPharmatech Co. Ltd. All Mice were adaptively reared with free food and water under the conditions with a 12-hour light/dark cycle for 1 week. The lights were turned on at 8:00 AM (Zeitgeber time 0/ZT0) and off at 8:00 PM (= ZT12). Mice dorsal skin were shaved. The next day, all Per2−/− mice were treated with 62.5 mg of 5% imiquimod (IMQ) cream (Sichuan Mingxin Pharmaceutical Company, China) for 6 consecutive days at ZT1 (9:00 AM). The mice were randomly divided into three groups (n = 6): model
group, LXJDF group (24.6g/kg, gavage) and dexamethasone (DEX) group (5mg/kg, gavage). WT mice were treated with vaseline as the control. The corresponding drugs were administered at 10:00 AM (= ZT2) and 10:00 PM (= ZT14), respectively. At the 6th day, mice were sacrificed and samples were taken within 0.5-1 hour after drugs administration, respectively. The experimental procedure was shown in Fig. 1.

All experiments strictly abide by the 'Manual for the Management and Use of Laboratory Animals' issued by the National Institutes of Health (NIH Publication No. 85 – 23, 1996) and follow the request of principles of animal welfare and the ethics of laboratory animals Committee's in Beijing Hospital of Traditional Chinese Medicine, Capital Medical University (License No.2020080201).

PASI

The skin lesions severity in mice was scored by a modified PASI normally used to rank human psoriasis severity [20].

Histopathology and immunofluorescence

Mice skin samples were fixed in 10% formalin, paraffin-embedded, and sectioned. Hematoxylin-eosin (HE) staining was performed for histological examination and evaluation of epidermal thickness. Images were scanned in the Aperio CS2 Leica scanner (Leica, Germany). The epidermal thickness was measured using the software Image Scope™ (Aperio Technologies). For immunofluorescence, sections were incubated with primary antibody including anti-Ki67 (1:400, ab125066, Abcam, USA), and anti-loricrin (1:800, ab155282, Abcam, USA) at 4 °C overnight, then with secondary antibody labeled with Alexa 488 for 1 hr at room time (RT). Nuclei were labeled with DAPI. Images were captured by a laser scanning confocal microscope (Zeiss LSM710, Germany).

Flow cytometry

Mice spleen lymphocyte types were detected by flow cytometry. The spleen lymphocytes were harvested and cultured in RPMI containing 10% FBS, and cells for Th/Th2/Th17 cell types analysis were stimulated with a cell stimulation cocktail (eBioscience, 1:500) for 6hr at 37°C. For surface staining, cells were washed and stained for 20 min at RT with anti-CD3 (17A2) APC-eFluor 780 and anti-CD4 APC-FITC. After intracellular fixation and permeabilization, cells were stained with the following antibodies: anti-IFN gamma (XMG1.2) APC, anti-IL-4 (11B11) PE, and anti-IL-17A (eBio17B7) PerCP-Cy5.5 for 30 min at RT. Cells for Treg and γδT cell types analysis were stained by anti-CD3 (17A2) APC-eFluor 780, anti-CD4 APC-FITC, anti-CD25 (PC61.5) APC, and anti-Gamma Delta TCR(GL3) PE for 20 min at RT. After intracellular fixation and permeabilization by Foxp3/Transcription Factor Staining Kit, cells were stained with anti-Mouse Foxp3 (FJK-16s) PerCP-Cy5.5 for 30 min at 4°C. All the flow cytometry experiment reagents were purchased from eBioscience. Data acquisition was performed on a FACS Diva 6.1 flow cytometer running NovaExpress software.

Enzyme-linked immunosorbent assay (ELISA)

Serum melatonin (MT) concentrations were determined by ELISA (Quantikinemouse Melatonin ELISA MBL Systems, Germany) according to the manufacturer's instructions.

Cytometric bead array (CBA)

The levels of Th17 cytokines (IFN-γ, TNF-α, IL-22, IL-6, IL-10, IL-17A, and IL-17F) (Biolegend, LEGENDplex, 741047) in serum were determined by flow cytometry according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (qPCR)

Mice skin total RNA was isolated using Trizol reagent. cDNA was synthesized using QuantiNov Rev. Transcriptionionkit (Qiagen, Hilden, Germany). qPCR was performed in triplicate with QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany) using 7500 RealTime PCR System (Applied Biosystems, Thermo Fisher, USA). The reaction conditions were started at 95°C for 30s,
followed by 45 cycles at 95°C for 5s and 60°C for 40s. The mRNA expressions were normalized against β-actin using the 2^−ΔΔCt method. The primers are listed in Supplementary Table 1.

Western blot

Total protein was extracted using protein lysis buffer (RIPA:PMSF = 100:1) and quantified using the Pierce™ BCA protein assay kit (Thermo Scientific, USA). Samples with an equal amount of proteins denatured at 95°C for 10 min. Protein bands were separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% milk in TBST, incubated with primary antibodies: CLOCK (Abcam, ab3517, 1:200), BMAL1 (Proteintech, 14268-1-AP, 1:500), REV-ERBα (Abcam, ab174309,1:1000), NFIL3 (CST, 14312,1:1000), and RORγt (Proteintech, 13205-1-AP, 1:500) at 4°C overnight. Then the membranes were incubated with corresponding peroxidase-conjugated IgG antibodies at 37°C for 1hr. The bands were detected using an electrochemiluminescence (ECL) reagent. The intensity of immunoreactive bands was quantified by Image J software. GAPDH antibody was used to confirm the equal amount of protein loading in each lane.

Statistical analysis

The experimental data were analyzed with SPSS 22.0 statistical software (SPSS, Chicago, IL), and quantitative data were expressed as “mean ± SEM”. The difference between the means of the groups was analyzed using a one-way analysis of variance (ANOVA) test evaluated using Tukey multiple comparisons or Kruskal-Wallis’ test for nonparametric values. *P<0.05, **P<0.01 means statistical significance.

Results

Identification of the main compound in LXJDF by UPLC-MS/MS

The main compound of LXJDF was determined by UPLC-MS/MS analysis. The ion chromatograms in positive and negative from UPLC-MS/MS chromatography are shown in Fig. 2. The retention times (tR /min) of ten main compounds in LXJDF, the exact mass number (m/z), and the chemical formula are listed in Table 2. The main components of Fossilia Ossis Mastodi are minerals such as calcium carbonate and calcium phosphate, which have yet to be detected.
Table 2
The main compounds of LXJDF

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<th>Number</th>
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<th>Ion</th>
<th>Exact mass number (m/z)</th>
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<td>2</td>
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Effects of LXJDF on psoriatic skin lesions in IMQ-induced Per2−/− mice
After 6 days of the administration, the dorsal skin lesions of IMQ-induced Per2−/− mice showed infiltration, erythema, and scales at ZT2 and ZT14, respectively. LXJDF and DEX significantly improved the skin lesions (Fig. 3A). Referring to the clinical PASI scoring standard, the skin lesions were scored based on erythema, scales, and infiltration. On the 4th day, under the same IMQ treatment conditions, the total PASI of the model group reached a high value at ZT2, while it was slightly relieved at ZT14. When the mice were sacrificed, the PASI was consistent at two-time points. And LXJDF had statistical differences in erythema, infiltration, and total score, which was no difference at the two-time points (Fig. 3B).

Effects of LXJDF on the pathological characteristics of psoriatic skin lesions in IMQ-induced Per2−/− mice

The dermis of the model group showed inflammatory cell infiltration, and the epidermis appeared scattered with inflammatory cells. The cells in the mitotic phase, especially epidermal cells, proliferated at ZT14. The mice of the LXJDF group had thinner epidermis and less inflammatory infiltration. Compared to the model, LXJDF could significantly reduce the epidermal thickness (P< 0.01). (Figs. 3C and D). The number of Ki67-positive cells in the model group was significantly increased compared to the control group. LXJDF significantly decreased the number of Ki67-positive cells. In the control group, loricrin formed a continuous line in the stratum corneum. The expression of loricrin in the model group was significantly reduced and showed a discontinuous line. LXJDF significantly increased the expression of loricrin, making it a continuous line (Fig. 4). DEX reduced epidermal thickness and improved hyperplasia and parakeratosis.

Effects of LXJDF on the spleen index and splenocyte subtypes in IMQ-induced Per2−/− mice

The results showed that the spleen weight of mice in the model group increased significantly at two-time points. LXJDF showed an inhibition effect on spleen weight at ZT2. LXJDF could significantly inhibit the spleen index (spleen weight/body weight) at ZT2 and ZT14 (Fig. 5A). T lymphocyte subtypes in the spleen were detected by flow cytometry. The results showed that Th17 and γδT cells in the model group were significantly increased, and Treg cells were decreased at ZT2; γδT cells were significantly increased, and there was no significant change in Th17 and Treg cells at ZT14. Th1 and Th2 cell subtypes changed less. LXJDF had a specific inhibitory effect on the increased Th17 and γδT cells at ZT2, while had no significant effect on γδT cells but increased the number of Th17 cells at ZT14 (Fig. 5B and C).

Effects of LXJDF on the serum melatonin levels in IMQ-induced Per2−/− mice

From ZT2 to ZT14, the melatonin level of the control group showed a decrease. On the contrary, the model group showed a noticeable increase. LXJDF and DEX groups had an unwarranted trend. At ZT2, there was no significant difference in melatonin levels among the groups. At ZT14, the model group significantly increased melatonin secretion compared with the control group, and LXJDF had an inhibitory effect on melatonin levels (Fig. 5D).

Effects of LXJDF on the inflammatory cytokines in serum and skin of IMQ-induced Per2−/− mice

At ZT2, the inflammatory factors IFN-γ, TNF-α, IL-22, IL-6, IL-17A, and IL-17F increased, and the level of IL-10 decreased in the model group. LXJDF could significantly reduce the levels of TNF-α, IL-6, IL-17A, and IL-17F and increase the levels of IL-10. At ZT14, the levels of TNF-α, IL-22, IL-17A, and IL-17F in the model group increased to a certain extent, and LXJDF could significantly reduce IL-17F (Fig. 6A). The mRNA expressions of IL-17F, IL-23, and IL-6 in the skin of the model group increased at ZT2. LXJDF could significantly reduce the expressions of IL-23 and IL-6. The mRNA expressions of IL-17A, IL-17F, IL-23, and IL-6 increased significantly at ZT14, and LXJDF showed significant inhibition (Fig. 6B). Immunofluorescence staining detection showed that IL-17A was highly expressed in the dermis of the model group, while LXJDF had a certain inhibitory effect (Fig. 6C).

Effects of LXJDF on the gene expressions of circadian clock in skin of IMQ-induced Per2−/− mice

At ZT2, the gene expressions of CLOCK, BMAL1, and REV-ERBa were decreased in the model group, whereas NFIL3 and RORyt expressions were significantly increased compared to the control group. LXJDF could increase the expressions of BMAL1 and REV-ERBa and significantly reduce NFIL3 and RORyt. All the gene expressions were down-regulated in the control group at
ZT14 compared with ZT2. At ZT14, the gene expressions of CLOCK, BMAL1, NFIL3, and RORγt in the model group were up-regulated compared to the control group. Meanwhile, CLOCK and BMAL1 gene expressions were at the same level as those of ZT2, without reflecting circadian rhythm fluctuation. LXJDF could significantly reduce the expressions of NFIL3 and RORγt and increase REV-ERBα expression (Fig. 7A).

Effects of LXJDF on the protein expressions of circadian clock in skin of IMQ-induced Per2−/− mice

At ZT2, the protein expressions of BMAL1 in the model group were significantly decreased, and the gene expression of NFIL3 was increased compared to the control group. LXJDF could significantly regulate BMAL1 and NFIL3 to normal. At ZT14, compared with the control group, CLOCK, BMAL1, NFIL3, and RORγt protein expressions in the model group were increased, and NFIL3 and RORγt were statistically significant. LXJDF could reduce the expressions of RORγt and NFIL3 (Fig. 7B and C).

Discussion

Many studies have shown that psoriatic dermatitis is regulated by the circadian clock. Per2 is a crucial rhythm gene that directly binds to CLOCK/BMAL1 to reset circadian rhythm and regulate period length [21, 22]. Per2-mutant mice exhibited a shorter cycle than WT mice and non-rhythmic behavioral rhythms [23]. Ando et al. reported that per2-mutant mice exaggerated IMQ-induced dermatitis [14]. Therefore, we applied the imiquimod-induced Per2 knockout mouse model to observe the effect of the circadian clock on skin inflammation and the influence of Chinese herbal compound on psoriasis-like mice with circadian rhythm disorder at two-time points.

The experimental design involved modeling and administration time issues. IMQ immediately caused the circadian clock genes to change. The circadian clock controlling the response IMQ was consistent, whether applied during the day or night. The expression of circadian clock genes reached a stable level after five days of IMQ induction [24]. However, different feeding time can affect inflammation and metabolism by regulating the circadian rhythm. For example, feeding Schedules could alter the skin's Type I IFN response after IMQ. Previously literature reported that the rhythmic skin expression of many immune-associated genes was shifted after daytime-restricted feeding [25]. Therefore, we chose the same time point (ZT1) with IMQ induction to construct a stable and uniform skin inflammatory state and selected two-time points representing day and night (ZT2 and ZT14) for drug intervention in an attempt to observe the effect of LXJDF from the perspective of chronotherapy.

Chronotherapy of psoriasis has been reported. Maxcalcitol, a vitamin D3 analogue used to relieve psoriasis symptoms, is more effective in the active phase of mice with psoriasiform inflammation from early to middle stage than in the rest phase[26]. Glucocorticoids can be used to treat inflammatory diseases, such as psoriasis, which exert multiple effects on the immune system. Endogenous glucocorticoids drive the circadian oscillation of T cell function under physiological conditions. Glucocorticoid receptor (GR) inhibits the expression of REV-ERBα by forming a complex with CLOCK and BMAL1 that binds to the E-box in the REV-ERBα promoter. The activation of GR is an entraining signal for peripheral circadian oscillators that reset the phase of the clock by regulating REV-ERBα and Per1/2 expressions [27, 28]. Therefore, dexamethasone could be used as positive control drug to interfere with circadian clock.

LXJDF could improve IMQ-induced psoriatic skin lesions of Per2−/− mice, reduce scales, erythema and infiltration, and significantly reduced PASI at ZT2 and ZT14. Pathomorphological analysis showed that LXJDF could inhibit mitosis of basal cells in epidermis (Ki67), promote keratosis to improve parakeratosis (loricrin), and reduce epidermal thickness. As a cell proliferation marker, Ki67 has been associated with the severity of psoriatic lesions[29]. The proliferation of keratinocytes is regulated by circadian rhythm due to the bidirectional coupling of cell cycle and circadian rhythm system. Clock genes and the cell cycle regulators can in turn affect the up-regulation of their expression and thus participate in abnormal cell proliferation. After Per2 gene knockout, the expression of CLOCK/BMAL1 gene may contribute to the hyperproliferation of psoriasis[30]. The coordination of the cell cycle in epidermal stem cells by core components of the molecular clock indicating that circadian clock might contribute to the hyperproliferation of keratinocytes in the basal layer of psoriatic plaques [13, 31].
IMQ-induced skin inflammation might override circadian control of the epidermal keratinocyte cycle, thus altering keratinocyte apoptosis and terminal differentiation [24].

LXJDF could regulate melatonin levels so that they tended to be average at ZT14. Melatonin exhibits a daily fluctuating rhythm and regulates the circadian rhythm expression of CLOCK, BMAL1, Per1, Per2, and Cry1 in central and peripheral tissues [32, 33]. Melatonin has anti-inflammatory and antioxidant properties. NF-κB, an important inflammatory pathway factor, can be inhibited by melatonin, reducing pro-inflammatory cytokines [34]. Melatonin levels in C57BL/6 mice showed a peak at 1:00 AM and a trough at 10:00 PM [35]. In our study, the serum melatonin levels in control groups were the same as the above results, which were higher at ZT2 and lower at ZT14. At ZT2, there was no significant difference among the groups. At ZT14, the secretion of melatonin in the model group was significantly increased ZT14, and LXJDF had a certain inhibitory effect on melatonin levels.

The spleen weight and index of IMQ-induced Per2^{−/−} mice were significantly increased, but the rhythmicity was lost. Human and mouse CD4^{+} T lymphocytes are the key cellular mediators of psoriasis, possessing an intrinsic functional circadian oscillator that drives rhythmic responses to activated stimuli such as PMA/ionomycin or via T cell receptors, including changes in cell proliferation and cytokine secretion [36, 37]. There is a direct link between Th17 cell differentiation and the circadian clock at the molecular level [38]. CD4^{+} T lymphocytes in WT mice exhibited different rhythm changes regardless of whether they were stimulated by IMQ, while the rhythms of Per2^{−/−} and IMQ-induced Per2^{−/−} mice disappeared. The γδT cells in IMQ-induced Per2^{−/−} mice significantly increased, especially compared with those in IMQ-induced WT mice, which was consistent with the reports [14]. Non-rhythmic Tregs are driven to rhythmic activity by systemic signals such as glucocorticoid, to confer a circadian signature to chronic inflammatory arthritis [39]. However, we observed that Tregs in WT and Per2^{−/−} mice presented rhythmic oscillation, which disappeared after IMQ stimulation (Supplementary Fig. 1). LXJDF could significantly inhibit the spleen weight and index at two-time points and reduce the numbers of Th17 and γδT cells at ZT2.

Cytokine production exhibits circadian variations, including the crucial cytokines in psoriatic inflammation. Serum levels of TNF-α, IFN-γ, IL-1, IL-2, IL-6, and IL-12 with nocturnal or early morning peaks oscillate in a circadian manner [40]. Some of the cytokine in serum and skin levels are significantly elevated in psoriasis patients. The rhythm of serum cytokines in Per2^{−/−} mice vanished, unlike WT mice that showed diurnal shift. At the same time, the rhythm of the two mice after IMQ-stimulated were less obvious than those of unstimulated (Supplementary Fig. 2). It might be due to the increase of inflammatory factors induced by IMQ that is higher than the amplitude of the rhythm. It is also possible that the time point of detection is not at the peak and valley. LXJDF could significantly inhibit the levels of serum IL-17A, IL-17F, TNF-α, IL-22 and IL-6 at ZT2, and reduce IL-17F level at ZT14. Different from the serum level, the expressions of cytokines in the skin induced by IMQ were up-regulated (Supplementary Fig. 3). LXJDF remarkably inhibited the skin gene expressions of IL-17A, IL-17F, IL-23 and IL-6 at ZT14, but inhibited IL-23 and IL-6 at ZT2. The immunofluorescence detection of IL-17A expression in the skin of mice showed that LXJDF exhibited a certain inhibitory effect. Here, we could see that the expression of circadian clock in different tissues is temporally distinct.

LXJDF could regulate the molecular clock in psoriasiform skin lesionsof IMQ-induced Per2^{+/-} mice. LXJDF significantly increased the expressions of BMAL1 and REV-ERBα, and decreased NFIL3 and RORγt at ZT2 and ZT14 (Fig. 8). The core loop of the circadian CLOCK is a negative feedback loop consisting of CLOCK/BMAL1 and Per/Cry. In the stable loop, the transcription of BMAL1 is repressed by REV-ERBα acting on two DNA response elements located in the BMAL1 promoter [41]. The knock-out of gene Per2 caused the increase of CLOCK/BMAL1. In our study, the gene expressions of CLOCK and BMAL1 in WT mice were higher at ZT2 and lower at ZT14, showing a diurnal difference. IMQ had a significant inhibitory effect on the obvious increase of CLOCK and BMAL1 at ZT2. While the impact of IMQ was unapparent at ZT14, possibly due to the low expressions of CLOCK and BMAL1 (Supplementary Fig. 4). A much larger set of genes becomes dysregulated or rhythm disappeared over five days of IMQ treatment [24]. At ZT2, the gene and protein expressions of CLOCK and BMAL1 in the model group were significantly down-regulated, and LXJDF increased the gene and protein expressions of BMAL1 to a certain extent.
The expression of REV-ERBα in Per2−/− mice was the same as in WT mice, showing a high level at ZT2 and a low at ZT14. The expressions of REV-ERBα in IMQ-treated WT and Per2−/− mice were significantly decreased, in accordance with reports that REV-ERBα downregulation was found in psoriatic lesional and non-lesional skin [10]. It might be result that multiple cell types within the epidermis respond to IMQ by up-regulating the expression of IFN regulatory factor 7 (Irf7) and IFN-sensitive gene (ISG) and down-regulating the expression of REV-ERBα [24]. REV-ERBα has been reported as a key intracellular negative regulator of the proinflammatory immune response in TH17 cells and γδT17 cells. SR9009, a synthetic REV-ERBα agonist, suppressed γδT17 cells in vitro and in vivo. Topical application of SR9009 reduced the inflammatory symptoms of psoriatic dermatitis in mice [42]. NFIL3 has been reported as the intermediate gene between REV-ERBα and RORγt, thus linking the circadian clock to Th17 cell development. NFIL3 can be inhibited by REV-ERBα, and adjust the circadian rhythm of Th17 cells by directly binding and repressing the RORγt promoter [38]. In our experiment, the expression of NFIL3 in IMQ-induced Per2−/− mice upregulated, opposite to REV-ERBα, confirming the inhibitory effect of REV-ERBα on NFIL3. RORγt is an essential nuclear transcription factor for Th17 and γδT cell differentiation. It competes with REV-ERBα at their shared RORE sites, including the IL-17A locus, to regulate IL-17A cytokine expression. However, it contradicts the negative correlation with NFIL3, which is inhibited by REV-ERBα, although we observed the opposite rhythm of RORγt and NFIL3 at two-time points. The network linking REV-ERBα and NFIL3 in Th17 cells is complex and further work is needed to elucidate these transcription factors’ interactions [43]. Therefore, the balance of RORs and REV-ERBs is crucial for the dynamic regulation of their target cells [44]. LXJDF could inhibit Th17 cells and γδT cells differentiation and the secretion inflammatory cytokines IL-17A and IL17F by down-regulating RORγt gene and protein expressions.

The present study has several limitations. The number of Per2−/− transgenic mice was relatively small because we strictly controlled the mice age within two weeks to ensure the experiment’s consistency. A larger sample size may enable the results more precise. The regulation of the circadian clock varies in different tissues or cells. The direct inhibition among REV-ERBα, NFIL3 and RORγt in turn was reported, but there is also reported that a direct relationship REV-ERBα and RORγt in skin. The mechanism is still contradictory and sophisticated. We only selected two-time points of drug intervention which represent day and night. However, for the circadian oscillation of various genes at different times, the detection time point we have chosen might be a different phase, such as at the peak and valley values or in the middle of them. Therefore, if we observed that the value of LXJDF group shows a significant trend from model group to control group, it is considered that the TCM compound has therapeutic effect.

**Conclusion**

In summary, we applied an IMQ-induced Per2−/− mice psoriasiform skin lesion model, and investigated effects of LXJDF on ameliorating psoriatic skin inflammation by suppressing IL-17-producing Th17 and γδT cells through the regulation of circadian clock at two time points. These findings might provide some preliminary implications for chronotherapeutic practice on psoriatic dermatitis or related diseases.

**Abbreviations**

TCM
Traditional Chinese Medicine
CLOCK
circadian locomotor output cycles kaput
BMAL1
brain and muscle-armt-like 1
Per
period
Cry
cryptochrome
RORs
retinol retinoic acid receptor-related orphan receptors
IMQ
imiquimod
MT
melatonin
PASI
psoriasis area and severity index
GR
glucocorticoid receptor
HE
hematoxylin-eosin
ELISA
enzyme-linked immunosorbent assay
CBA
cytometric bead array
qPCR
reverse transcription-polymerase chain reaction
UPLC–MS
ultra-high performance liquid chromatography mass spectrometry.

**Declarations**

**Supplementary information**

The online version contains supplementary material available at

**Acknowledgements**

Not applicable.

**Author contributions**

XX and PL contributed to conception and design of the study. XX, LZ, XL, YL and XH performed the experiments. XX and LZ analyzed the data. XX and PL interpreted the results of the experiments. XX performed the statistical analysis and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (81873119 81603630).

**Availability of data and materials**

All data used/analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The animal experiments in this research were conducted under the request of principles of animal welfare and the ethics of laboratory animals Committee's in Beijing Hospital of Traditional Chinese Medicine, Capital Medical University (License No.2020080201).

**Consent for publication**
Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


Figures

![Figure 1](image)

Experimental procedure of LXJDF effect on IMQ-induced Per2<sup>−/−</sup> mice.
Figure 2

Total ion chromatogram of LXJDF in positive ion mode A and negative ion mode B of UPLC-MS/MS.
Figure 3

Effects of LXJDF on psoriasiform skin lesions in IMQ-induced Per2−/− mice. A Photographs of psoriasiform skin in control, model, LXJDF, and DEX groups (n = 6). B PASI score was monitored to quantify the disease severity by erythema, scales, infiltration, and the total PASI score were calculated. Results are shown as the mean±SEM. *P<0.05, **P<0.01. C Hematoxylin and eosin (H&E) staining of the mice skin. Scale bar=200μm. D Microscopic quantification of epidermal thickness. Results are shown as the mean±SEM. *P<0.05, **P<0.01.
Effects of LXJDF on psoriasiform skin lesions pathological characteristics in IMQ-induced Per2^{-/-} mice. Immunofluorescence stainings for Ki67 and loricrin in the mice skin. Scale bar= 100μm and 20μm (zoom).

Figure 4
Figure 5

Effects of LXJDF on spleen index, splenocyte subtypes and melatonin level in IMQ-induced Per2⁻/⁻ mice. A Spleen weight and spleen index (spleen weight/body weight) in the control, model, LXJDF, and DEX groups (n=6). Results are shown as the mean±SEM. *P<0.05, **P<0.01. B Representative flow cytometry of spleen cell subsets including Th1, Th2, γδT, Th17 cell, and Treg cells. C The cell percentage of Th1, Th2, γδT, Th17 cell, and Treg cells in the control, model, LXJDF, and DEX groups (n =6). Results are shown as the mean±SEM. *P<0.05, **P<0.01. D The change of Melatonin level at ZT2 and ZT14. *P<0.05, **P<0.01.
Figure 6

Effects of LXJDF on inflammatory cytokine levels in serum and skin of IMQ-induced Per2/− mice. **A** CBA analysis of the changes in serum levels of IFN-γ, TNF-α, IL-6, IL-10, IL-17A, IL-17F, and IL-22 in the control, model, LXJDF, and DEX groups (n=6). Results are shown as the mean±SEM. *P<0.05, **P<0.01. **B** Quantitative real-time PCR analysis of the skin mRNA expressions of IL-17A, IL-17F, IL-23, and IL-6 in the control, model, LXJDF, and DEX groups (n=3). Results are shown as the mean±SEM. *P<0.05, **P<0.01. **C** Immunofluorescence stainings for IL-17A in the mice skin. Scale bar= 100μm and 20μm (zoom).
Figure 7

Effects of LXJDF on circadian clock related-gene expressions in IMQ-induced Per2−/− mice skin. A Quantitative real-time PCR analysis of the skin mRNA expressions of CLOCK, BMAL1, REV-ERβα, NFIL3 and RORγt in the control, model, LXJDF, and DEX groups (n=3). Results are shown as the mean±SEM. *P<0.05, **P<0.01. B Representative images of western blot showing CLOCK, BMAL1, REV-ERβα, NFIL3 and RORγt expressions in the skin of control, model, LXJDF, and DEX groups. GAPDH was used to confirm an equal amount of protein. C Quantitation of CLOCK, BMAL1, REV-ERβα, NFIL3 and RORγt expression in different groups (n=3). Results are shown as the mean±SEM. *P<0.05, **P<0.01.
Figure 8

Schematic diagram of LXJDF on the mechanism regulating the circadian clock.

Supplementary Files

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