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Thymic Stromal Lymphopoietin Activates Mouse Dendritic Cells Through the JAK/SYK Pathway in Promoting Th17 Response in Psoriasis

Cuihao Song[1]*, Jie Sun[2]*, Zhenkai Zhao[1], Xu Zhang[3], Xiangyu Ding[1], Xiaoqiang Liang[1], Jia Bai[1], Liyuan Xing[1], Lingling Gong[1], Chengxin Li[1], Biwen Lin[1]

[1]Department of Dermatology, PLA General Hospital, Beijing, China
[2]305 Hospital of People's Liberation Army, Beijing, China
[3]Department of Clinical Laboratory, The First Medical Center of PLA General Hospital, Beijing, China
Contributed equally

Background: Psoriasis is a chronic inflammatory skin disease that has no cure. While the specific cause of psoriasis is unknown, interactions between immune cells and inflammatory cytokines are believed to be important in its pathogenesis. Thymic stromal lymphopoietin (TSLP) is a cytokine produced by epithelial cells that profoundly affects dendritic cells (DCs) and is involved in allergy and inflammatory diseases. In some studies, its expression is higher in the skin of psoriasis patients, whereas it is increased in treated psoriasis patients when compared with untreated patients in others.

Aims: To investigate the role of TSLP in the pathogenesis of psoriasis.

Study Design: In vitro and in vivo study.

Methods: To investigate the effect of TSLP on psoriasis in vivo, a mouse psoriasis model and shRNA targeting TSLP to reduce its expression were used. Mouse primary bone marrow dendritic cells (BMDCs) were cultured in vitro and used to investigate the signaling pathways activated by TSLP.

Results: We found that reducing TSLP expression in psoriasis skin alleviated disease severity. TSLP activated the Janus kinase (JAK)/SYK pathway in psoriatic skin. In vitro studies with BMDCs demonstrated that TSLP increased DC maturation through the JAK/SYK pathway and activated DCs-secreted cytokines that stimulated $CD4^+$ T cells to develop into T helper 17 (Th17) cells by activating STAT3 signaling. The JAK/SYK pathway inhibitor reduced the effect of TSLP on activating BMDCs and promoting Th17 differentiation by $CD4^+$ T cells.

Conclusion: These findings indicated that TSLP exerted its immune-modulating effect in psoriasis through the JAK/SYK pathway.

INTRODUCTION

Psoriasis is a chronic skin disorder characterized by thick, silvery scales and itchy, dry patches on the skin's surface. The World Psoriasis Day consortium estimates that it is a systemic inflammatory disease affecting around 125 million people worldwide. Although psoriasis is primarily a skin disease, it has been shown to influence the mental health impact of those affected.[1-3] Histologically, psoriasis is characterized by epidermal thickness and downward elongation of the rete ridges. The granular layer becomes thinner or disappears, capillaries elongate and dilate, and T cells enter the dermis and epidermis. Neutrophil clusters are occasionally visible on the parakeratotic scale. The disease is caused by complex interactions between genetic, immunological, and environmental factors.[4]

The pathogenesis of psoriasis includes interactions between immune cell types and inflammatory cytokines.[5] T helper cells (Th cells) and a cytokine network of tumor necrosis factor-alpha (TNF-α)/interleukin-23 (IL-23)/IL-17 play crucial roles in the disease. Mature dendritic cells (DCs) produce IL-23 and activate Th17 cells, which promotes IL-17 production.[6-8] IL-17 upregulates the expression of CCL20, a Th17 chemoattractant, creating a positive feedback loop.[9,10] The activity of IL-23 is believed to include the activation of Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2), followed by STAT proteins.[11]

Corresponding author: Chengxin Li/Biwen Lin, Department of Dermatology, PLA General Hospital, Beijing, China

e-mail: chengxinderm@163.com, linbw301@163.com

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ORCID iDs of the authors: C.S. 0009-0009-3621-7483; J.S. 0009-0001-1175-9624; Z.Z. 0009-0003-9906-6658; X.Z. 0009-0000-4244-6498; X.D. 0009-0004-3123-9855; X.L. 0009-0006-1852-0858; J.B. 0009-0005-2887-1507; L.X. 0009-0001-9266-0908; L.G. 0009-0006-0112-3426; C.L. 0009-0000-1882-5121; B.L. 0009-0009-8871-4553.

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STAT3 is a crucial component in Th17 cell differentiation.[12,13] STAT3 directly activates the expression of IL-17A and IL-17F, as well as other genes involved in Th17 cell differentiation, activation, and proliferation, such as RORγT, RORα, BATF, IRF4, AHR, IL-6Rα, and c-Maf.[14]

Thymic stromal lymphopoietin (TSLP) was initially identified as a component that promotes the growth and development of immature B cells.[14,15] During allergic inflammation, TSLP is primarily produced by epithelial cells, keratinocytes, and stromal cells.[16,17] Recent research has revealed that DCs and mast cells secret TSLP.[18,19] TSLP signaling requires binding to its receptor (TSLPR), which forms a heterodimer with the interleukin-7 receptor alpha (IL-7Rα)-chain.[20,21] TSLP stimulation activates JAK1 and JAK2 in primary human DCs and primary human and mouse $CD4^+$ T cells.[22,23] The TSLPR subunit binds and activates JAK2 and IL-7Rα-associated JAK1, leading to activation of several STAT proteins.[22-24] TSLP stimulation activated STATs 1, 3, 4, and 5 and JAKs 1 and 2 in both human peripheral blood-derived $CD11c^+$ DCs and mouse DCs.[22,25]

Studies have shown that TSLP plays a vital role in modulating type 2 immunity at barrier surfaces and is believed to be involved in allergy and inflammatory diseases of the airways, skin, and gut.[22,23,25] Overexpression of TSLP in keratinocytes from patient skin samples provides evidence that it may have a role in the development of psoriasis.[26] The relationship between serum TSLP levels and disease severity shows that TSLP may be involved in disease pathogenesis.[27,28] TSLP has been shown to promote DC maturation and DC-derived IL-23 production, implying that it may impact diseases through the IL-23 pathway.[29-31] One study found that in psoriasis patients, TSLP activates DC and promotes the overexpression of IL-23 by primary blood and skin DCs synergistically with T cell-derived CD40 ligand (CD40L).[26] However, contradictory results also exist. For example, one study found that TSLP mRNA expression levels in the peripheral blood of psoriasis vulgaris patients are lower than in the normal control group and can increase after treatment.[32]

Thus, the exact role of TSLP in the pathogenesis of psoriasis has to be determined. It is yet unknown which signaling pathways TSLP uses to exert its effect or whether modulating TSLP levels in skin lesions directly impacts disease progression. In this study, we used a mouse psoriasis model using shRNA to investigate the role of TSLP in modulating DC activity and disease severity.

MATERIALS AND METHODS

Establishment of imiquimod-induced psoriasis mouse model

BALB/C mice (6-8 weeks old) were acquired from Shanghai Jiesijie Experimental Animal Co., Ltd. [permit number: SCXK (Shanghai) 2018-0004]. The animals were kept in an SPF room with a temperature of 24 °C ± 2 °C and a feeding humidity of 50%-65%. The room maintained a daily light rhythm of 12 h. There was unlimited access to food and water. All animal experiment procedures were approved by the Ethics Committee of the General Hospital of the Chinese People's Liberation Army (approval number: S2023-302-1).

The mice were divided into three groups of eight each. The psoriasis model was constructed as follows: mice were anesthetized with 10% chloral hydrate, and their back hair (20 x 30 mm^2) was removed using a shaver and a hair removal cream. Five percent imiquimod cream (3M Health Care Limited, UK) was applied daily to the back skin of the mice at a dose of 14 mg/day for 7 days to induce psoriasis. In addition to the model group, mice in the treated group were injected with 100 µl of 2.2 x 10^{11}-vg/ml AAV-shTslp-2 subcutaneously before imiquimod induction. The control mice group were shaved only. The psoriasis area severity index was used to monitor and evaluate the severity of skin inflammation in mice. Erythema, induration/thickness, and scaling were scored independently on a scale of 0 to 4 (0 = none, 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe).

Mouse skin tissue staining

Mouse skin tissue samples were fixed in 4% paraformaldehyde for 24 h before being transferred to phosphate-buffered saline (PBS) solution. All staining procedures were performed on 5 µm paraffin-embedded tissue sections. Hematoxylin and eosin (Solarbio, China) and Masson's Trichrome (Solarbio, China) staining were performed according to the manufacturer's instructions.

After antigen retrieval, the tissue section underwent immunohistochemistry staining. The washed and blocked tissue sections were incubated with primary antibodies anti-TSLP (13778-1-AP, Proteintech, China) and anti-KRT6 (A4631, ABclonal, China) overnight at 4 °C. Slides were washed three times with TBST, and the secondary antibody HRP conjugated goat antirabbit immunoglobulin G (IgG) (HA1001, HuaBio, China) was incubated at 37 °C for 1 h. A DBA substrate solution was applied after the slides were washed with TBST three times. Hematoxylin was used to counterstain the specimens.

Immunofluorescence staining was performed on the tissue section after antigen retrieval. The tissue sections were washed and blocked before being incubated with primary antibodies anti-CD11-PE (sc-23951, SCBT, USA), anti-p-JAK1 (Phospho-Tyr1022, #11149, SAB, USA), anti-p-SYK (Phospho-Tyr348, #12293, SAB, USA), anti-TNF receptor-associated factor 6 (anti-TRAF6) (A16991, ABclonal, China), and anti-nuclear factor kappa B (anti-p-NF-κB) p65 (Phospho-Ser536, CST, USA) at a dilution of 1:50 in 1% BSA-PBS solution at 4 °C overnight. Slides were rinsed with TBS, and secondary antibody goat antirabbit IgG conjugated with iFluor™ 488 nm (ab150077, Abcam, UK) was incubated at room temperature for 1 h in the dark. DAPI (C0065, Solarbio, China) staining was performed at room temperature for 20 min in the dark. Slides were sealed in an antifade solution and examined under fluorescence microscopy.

TSLP shRNA construction

shRNA for mouse TSLP was designed using the design tools at https://portals.broadinstitute.org/gpp/public/gene/search using the gene ID NM_021367.2. Three different shTslp sequences for the murine *TSLP* gene were selected. The sequences for cloning into the pAAV-EnCMV-MCS-U6-shRNA plasmid are shown in Table 1.

TABLE 1. shRNA sequences to knockdown mouse TSLP.

shRNA name	Sequence
m-shTslp-1F	5'-GATCCCTTCATGCAATCTCCAGAATGGTACCATTCTGGAGATTGCATGAAGGTTTTTTG-3'
m-shTslp-1R	5'-TCGACAAAAAACCTTCATGCAATCTCCAGAATGGTACCATTCTGGAGATTGCATGAAGG-3'
m-shTslp-2F	5'-GATCGCTACCCTGAAACTGAGAGAAGGTACCTTCTCTCAGTTTCAGGGTAGCTTTTTTG-3'
m-shTslp-2R	5'-TCGACAAAAAAGCTACCCTGAAACTGAGAGAAGGTACCTTCTCTCAGTTTCAGGGTAGC-3'
m-shTslp-3F	5'-GATCCCACTGGTGTTTATTCTTTAAGGTACCTTAAAGAATAAACACCAGTGGTTTTTTG-3'
m-shTslp-3R	5'-TCGACAAAAAACCACTGGTGTTTATTCTTTAAGGTACCTTAAAGAATAAACACCAGTGG-3'

TSLP, thymic stromal lymphopoietin.

The plasmid vectors cloned with each shRNA were confirmed by sequencing. To generate AAV, human embryonic kidney 293T cells were transfected with three plasmids (AAV-shTslp, pAAV-RC, and phelper) at a ratio of 10:5:6 (Miaoling Biology Co., Ltd., China). Viral particles were collected from the culture medium's supernatant and concentrated using PEG8000 precipitation.

Patient skin sample section staining

Human skin tissue samples were obtained from PLA General Hospital with informed consent. A total of 10 samples were collected, and the related patient information is included in the Table S1. Immunohistochemistry staining was performed on tissue slices 5 µm thick and fixed in paraffin. After antigen retrieval, tissue sections were stained with primary antibodies anti-TSLP (13778-1-AP, Proteintech, China) and anti-Phospho-SYK (Y348, #12293, SAB, USA) at 4 °C overnight. Slides were washed three times with TBST, and the secondary antibody HRP conjugated goat antirabbit IgG (HA1001, HuaBio, China) was incubated at 37 °C for 1 h. Following three washes with TBST, the slides were treated with a DBA substrate solution. Hematoxylin was used to counterstain the specimens.

Primary cell culture

The femurs and tibiae of BALB/C mice were rinsed with PBS to collect mouse bone marrow cells. Red blood cells were lysed, washed with RPMI-1640 medium (Gibco, USA), and seeded in 6-well culture dishes (1 x 10^7/well) in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 20 ng/ml IL-4, and 20 ng/ml GM-CSF (Novoprotein, China). Cells were cultured for 8 days to induce DCs. The medium was replenished or refreshed every day. In the first 2 days, most of the cells developed in suspension. Many cells were adherent on the third or fourth day. Unattached monocytes and erythrocytes were removed by replacing the medium with a new induction medium. On the sixth day, several cells resumed their suspended state. Suspended and weakly attached cells were collected as DCs, placed in fresh induction media, and cultured for another 48 h.

Mouse splenocytes were isolated from the spleen. The mouse was sacrificed, and the spleen was separated and homogenized using a cell strainer on a 6-well plate with 5 ml PBS. After the cells were treated with ACK lysis buffer on ice for 5 min to lyse the red blood cells, they were washed twice with PBS. According to the manufacturer's instructions, $CD4^+$ T cells were isolated from the splenocytes using a $CD4^+$ T cell isolation kit (11346D, Thermo Fisher Scientific, USA). The purity of the resulting $CD4^+$ T cells was determined by flow cytometry. In experiments investigating Th17 cell differentiation by $CD4^+$ T cells, purified $CD4^+$ T cells were cultured in regular medium (RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin) with or without the medium used to culture BMDC understated treatment for 72 h at a 4:1 ratio.

Flow cytometry analysis

To determine the purity and maturity of primary bone marrow dendritic cells (BMDCs), mouse bone marrow cells cultured in an induction medium for 8 days were washed and incubated with anti-CD3-FITC, anti-CD11c-PE, anti-CD80-FITC, anti-CD86-FITC, and anti-MHC II-FITC antibodies (Abcam, UK) in PBS with 2% BSA at 4 °C for 30 min. Cells were washed and resuspended in PBS, and flow cytometry analysis was performed using Attune NxT (Thermo Fisher Scientific, USA).

To access the differentiation of Th17 cells, mouse $CD4^+$ T cells were washed and incubated with anti-CD4-Alexa Fluor 488 (BD Pharmingen, USA), anti-CCR6-eFluor 660 (Invitrogen, USA), and anti-IL-17α-Cy5.5 (Invitrogen, USA) in PBS with 2% BSA at 4 °C for 30 min. Cells were washed and resuspended in PBS, and flow cytometry analysis was performed using Attune NxT.

RNA extraction and qPCR

Cells were collected, and RNA extraction was performed using the TRIZOL method according to the manufacturer's instructions. cDNA was synthesized using NovoScript^{RT} reverse transcriptase (E123-01A, NovoProtein Technology Co., Ltd., China). Quantitative PCR was performed using a NovoStart SYBR qPCR SuperMix Plus kit (E166-01A, NovoProtein Protein Technology Co., Ltd., China). Individual gene expression levels were determined relative to the housekeeping genes. The PCR primers are shown in Table 2.

ELISA for TNF-α and IL-23

Cell culture supernatant, serum, and plasma samples were collected and stored at -20 °C before testing. The levels of TNF-α (cat no: EK282, Multisciences, China) and IL-23 (cat no: EK223, Multisciences, China) were measured using an ELISA kit according to the manufacturer's instructions. The plate was immersed in 300 µl wash buffer in each well for 30 s, and the wash buffer was removed. Diluted standard and testing samples were combined

TABLE 2. PCR primers for RT-qPCR analysis.

Gene name	Forward primer sequence	Reverse primer sequence
TSLP	5'-GCAAATCGAGGACTGTGAGAGC-3'	5'-TGAGGGCTTCTCTTGTTCTCCG-3'
TNF-α	5'-GGTGCCTATGTCTCAGCCTCTT-3'	5'-GCCATAGAACTGATGAGAGGGAG-3'
IL-23α	5'-CATGCTAGCCTGGAACGCACAT-3'	5'-ACTGGCTGTTGTCCTTGAGTCC-3'
GAPDH	5'-CATCACTGCCACCCAGAAGACTG-3'	5'-ATGCCAGTGAGCTTCCCGTTCAG-3'
β-actin	5'-CATTGCTGACAGGATGCAGAAGG-3'	5'-TGCTGGAAGGTGGACAGTGAGG-3'

PCR, polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TSLP, thymic stromal lymphopoietin; TNF-α, tumor necrosis factor-alpha; IL-23α, interleukin-23 alpha, GAPDH, glyceraldehyde 3-phosphate dehydrogenase; β, beta.

with the appropriate testing antibody solutions. The plate was gently shaken at room temperature for 2 h. The plate was rinsed with wash buffer, and enzyme-linked biotin solution was added and incubated at room temperature with a gentle shake for 45 min. After plate washing, a substrate solution was added, and the reaction was stopped after 30 min of incubation in the dark. Reading was performed at OD. 450 and OD. 570 using a spectrometer.

Western blot analysis

Skin tissues were collected and stored in liquid nitrogen. The tissues were homogenized with RIPA lysis buffer, and the soluble fraction of each sample was collected. The protein concentration of each sample was measured using the BCA assay (PC0021, Solarbio, China). We used 50 μg of total protein from each sample for Western blot analysis.

Cells were collected after the specified treatment and lysed in RIPA buffer at 4 °C for 10 min. Supernatants were collected, and cell lysates were used for Western blot analysis.

Membranes were incubated with primary antibodies, anti-p-JAK1 (phospho-Y1022), anti-JAK1 (ab138005, Abcam, UK), anti-p-SYK (phospho-Y348, Abcam, UK), anti-SYK (ab40781, Abcam, UK), anti-TRAF6, anti-p-NF-κB p65 (phospho-S536), anti-p65 (ab32536, Abcam, UK), anti-TSLPR (ab224196, Abcam, UK), anti-RORγT (BioLegend, USA), anti-p-STAT3 (phospho-S727, ABclonal, China), anti-β-actin (ab178787, Abcam, UK), and anti-GAPDH (ab8245, Abcam, UK) at 4 °C overnight, whereas secondary antibody incubation was performed at room temperature for 1 h.

Statistical analysis

All data from the present study are presented as the mean ± standard deviation based on three repetitions. The results were analyzed using GraphPad Prism Software (version Prism 8, GraphPad Software, Inc.). The Shapiro-Wilk test was performed to determine whether the samples were normally distributed. The normal distributed parameters were examined using a one-way analysis of variance, followed by Tukey's post-hoc test for statistically significant differences. The skewed distributed parameters were assessed using the Kruskal-Wallis test. A p-value of <0.05 was considered statistically significant.

RESULT

Role of TSLP in the mouse psoriasis model

Three different AAV-shTslp constructs were packed into viral particles separately, and the impact of each shRNA was examined on H22 cells, which had high TSLP expression. Ten microliters of concentrated AAV-shTslp (2×10^{12} vg/ml) were mixed with 5 ml of complete culture medium and added to cultured H22 at 70%-80% confluency. Cells were stored for 8 days after infection before being harvested. The knockdown effect of three different shRNA viral particles was assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot. Figure 1a shows that treatment with all three shTslp-containing viral particles reduced TSLP mRNA and protein levels. The effect of shTslp-2 was the most prominent, with a 76% reduction in mRNA level and a 65% reduction in protein level. Thus, shTslp-2 was used in subsequent experiments.

An animal psoriasis model was established using imiquimod cream on BALB/c mice. To investigate the effect of TSLP in this animal model, mice were subcutaneously injected with AAV-shTslp-2 1 week before imiquimod was administered. The severity of psoriasis on the skin of the mice was assessed and compared. Figure 1b depicts mice treated with imiquimod and AAV-shTslp-2, indicating that imiquimod was able to induce psoriasis in BALB/c mice and pretreatment with AAV-shTslp-2 to reduce TSLP expression reduced the severity of the disease. The shTSLP-treated group had lower evaluation scores for psoriasis lesions in terms of erythema, scaling, and induration/thickness than the non-treated group (Figure 1c).

Immunohistochemistry was used to study the expression of TSLP in these mouse skin tissue sections. Figure 1d shows that TSLP expression was higher in the skin tissue of mice treated with imiquimod cream (model group) than in untreated mice (control group). This increased TSLP expression was not observed in the skin tissue of mice treated with AAV-shTslp-2 before imiquimod administration (model + shTslp-2 group). This result was consistent with our findings in psoriasis patient skin tissue samples, where TSLP expression was higher than in normal people's skin tissue (Figure S1a). The presented images show the staining results from patients 1, 5, and 9, revealing that TSLP levels were increased in the skin samples of moderate and severe patients.

The skin tissue of mice that underwent various treatments was stained multiple times. Figure 1e shows representative images of

hematoxylin and eosin and Masson's Trichrome staining on mouse skin tissue sections. In comparison with the control mice, the skin tissue from psoriasis mice showed epidermal thickening and inward elongation, as evidenced by Masson staining. However, this epidermal thickening was partially reversed in mice treated with AAV-shTslp-2 to reduce TSLP levels.

We then investigated the expression of cytokeratin KRT6, one of the psoriasis indicators, in mouse skin tissue sections. As shown in Figure 1d, psoriasis mice had significantly higher KRT6 expression in their skin tissue than control mice, indicating an increased epidermal keratosis. Pretreatment with AAV-shTslp-2 decreased the increased expression of KRT6 in skin tissue from psoriasis mice.

The activation of the TSLP pathway was analyzed using mouse skin tissues and serum. Western blot analysis of mouse skin tissue (Figure 1f) showed that psoriasis mice had significantly higher levels of TSLPR and IL-7Rα than control mice. The increased expressions of both receptors were reversed in psoriasis mice treated with AAV-shTslp-2. ELISA analysis of TNF-α and IL-23 levels in mouse skin tissue and serum samples (Figure 1g) showed that both cytokines increased in the psoriasis mice compared with the control mice, which were reversed by AAV-shTslp-2 treatment.

Mouse skin tissue sections were used to activate the JAK/SYK pathway via immunofluorescence. As shown in Figure 2a, b, phosphorylated JAK (Y1022) and SYK (Y348) levels in CD11 expression DCs increased in psoriasis mouse skin tissue compared with normal mouse skin tissue, indicating that these two kinases were activated. This activation was not observed in the skin tissue of psoriasis mice treated with AAV-shTslp-2. We found comparable SYK activation in psoriasis patient samples, with higher SYK phosphorylation compared with normal

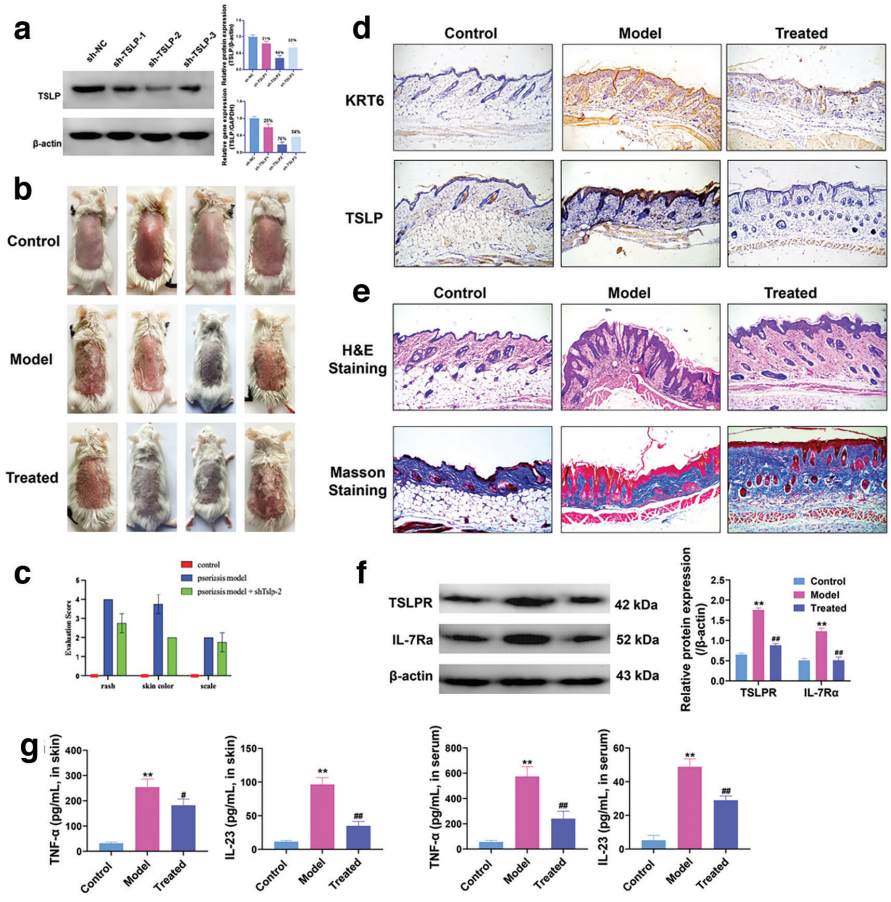

FIG. 1. Effect of shRNA to knockdown TSLP in reducing the severity of the disease on mouse psoriasis model. (a) Efficiency of three different shRNAs on TSLP-RNA and protein (Western) levels in H22 cells. (b) Images of BALB/C mice with imiquimod-induced psoriasis, with and without shTslp-2 treatment. (c) The severity of imiquimod-induced psoriasis in BALB/C mice was assessed using psoriasis area severity index, which includes rash, skin color, and scale. (d) Images of immunohistochemistry staining of mouse skin tissue sections for TSLP and KRT6 expressions. (e) Images of hematoxylin and eosin and Masson's Trichrome staining of the mouse (control, model, and model + shTslp-2 treated) skin tissue sections. (f) Western blot images and quantification of band intensity normalized against β-actin levels for the protein expression of TSLPR and IL-7Rα in mouse skin tissue samples from mice that underwent various treatments (control, model, and model + shTslp-2 treated). (g) ELISA assessment of TNF-α and IL-23 levels in skin tissue and serum from mice that underwent various treatments (control, model, and model + shTslp-2 treated).

TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor; IL-7Rα, interleukin-7 receptor alpha; β, beta; TNF-α, tumor necrosis factor-alpha; IL-23, interleukin-23; H&E, Hematoxylin and eosin.

people's skin tissue samples (Figure S1b). We then investigated the activation of downstream signaling components, as shown in Figure 2c. The protein level of TRAF6 was significantly higher in psoriasis mouse skin tissue than in normal mouse skin tissue. Consequently, NF-κB was activated in psoriasis mouse skin tissue compared with normal mouse skin tissue, as evidenced by higher levels of phosphorylated p65 (S536; Figure 2d). Psoriasis mice treated with AAV-shTslp-2 showed no increase in TRAF6 expression or NF-κB activation in their skin tissue (Figure 2c, d). These results indicated that the JAK/SYK signaling pathway was activated in psoriasis tissue, which might be attributed to increased TSLP expression.

Mechanism of TSLP in activation of mouse dendritic cells

Primary mouse BMDCs were used to investigate the mechanism of TSLP further signaling in vitro.

Mouse bone marrow cells were cultured in the induction medium as described in the material and method sessions for 8 days, and flow cytometry analysis revealed that the cultured cells contained more than 90% DCs after 8 days of growth (Figure 3a).

To investigate the effect of TSLP on DCs, these BMDCs were cultured with TSLP for 48 h, and the expression levels of TNF-α and IL-23α were assessed by RT-qPCR. Figure 3b shows a dose-dependent increase in TNF-α and IL-23α RNA levels compared with GAPDH in response to TSLP in the culture medium. The secreted TNF-α and IL-23 levels in the culture medium supernatants were also increased in a dose-dependent manner, as assessed by ELISA (Figure 3c). Moreover, TSLP appeared to promote the maturation of DC cells. Figure 3d shows that when TSLP was added to the culture medium, more cells expressed higher levels of mature DC markers, such as CD80, CD86, and MHC II. The effect was also dose-dependent.

The effect of TSLP on DCs in activating the JAK-SYK pathway was examined. First, TSLPR expression was significantly increased in BMDCs in response to the presence of TSLP in the culture medium in a dose-dependent manner (Figure 4a, b), indicating that TSLP

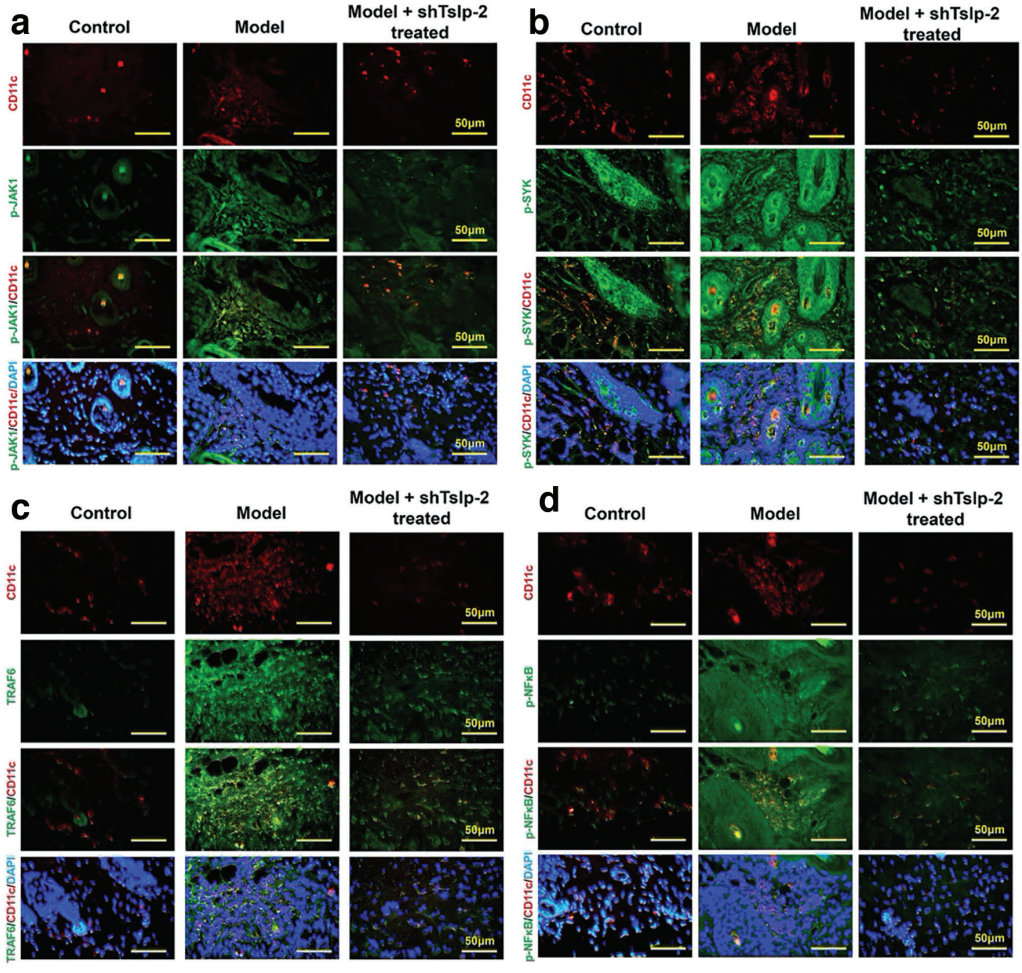

FIG. 2. Activation of the JAK/SYK signaling pathway in mouse skin tissue sections was done by mice that underwent different treatments (control, model, and model + shTslp-2 treated). Representative immunofluorescence images of expression of (a) phosphorylated JAK (Y1002), (b) phosphorylated SYK (Y348), (c) TRAF6, and (d) phosphorylated NF-κB p65 (S536) in mouse dendritic cells (CD11 expression cells).

JAK, Janus kinase; SYK, spleen tyrosine kinase; NF-κB, nuclear factor kappa B; TRAF6, anti-TNF receptor-associated factor 6.

triggered the signaling cascade in BMDCs. Western blot analysis determined if BMDCs cultured with TSLP-activated JAK-SYK pathway components. As shown in Figure 4a, b, TSLP activated both JAK1 and SYK, as phosphorylated JAK1 (Y1022) and SYK (Y348) significantly increased dose-dependent. TSLP stimulation increased protein levels of TRAF6, a key factor in DC maturation via activating the JAK-SYK pathway. Consequently, NF-κB was activated, as shown by increased levels of phosphorylated p65 (S536).

The role of the JAK-SYK pathway in downstream TSLP activation was examined using the SYK inhibitor cerdulatinib (cat no: 1199300-79-6, Shanghai Macklin, Ltd., China) to prevent JAK/SYK pathway activation. BMDCs were treated with TSLP (100 ng/ml) or TSLP (100 ng/ml)/cerdulatinib (30 nm) for 72 h, and the effects of the treatments were assessed. First, we used Western blot analysis to investigate the effect of cerdulatinib on activating the JAK/SYK pathway by TSLP. As shown in Figure 5a, b, TSLPR levels increased in response to TSLP stimulation, as previously demonstrated, but this increase was antagonized by adding the JAK/SYK pathway inhibitor cerdulatinib. As shown in Figure 5a, b, the higher levels of phosphorylated JAK1 and SYK in TSLP-stimulated BMDCs were reduced when cerdulatinib was administered concurrently with TSLP stimulation, indicating that cerdulatinib was able to inhibit JAK1 and SYK activation in the system. Cerdulatinib inhibited the

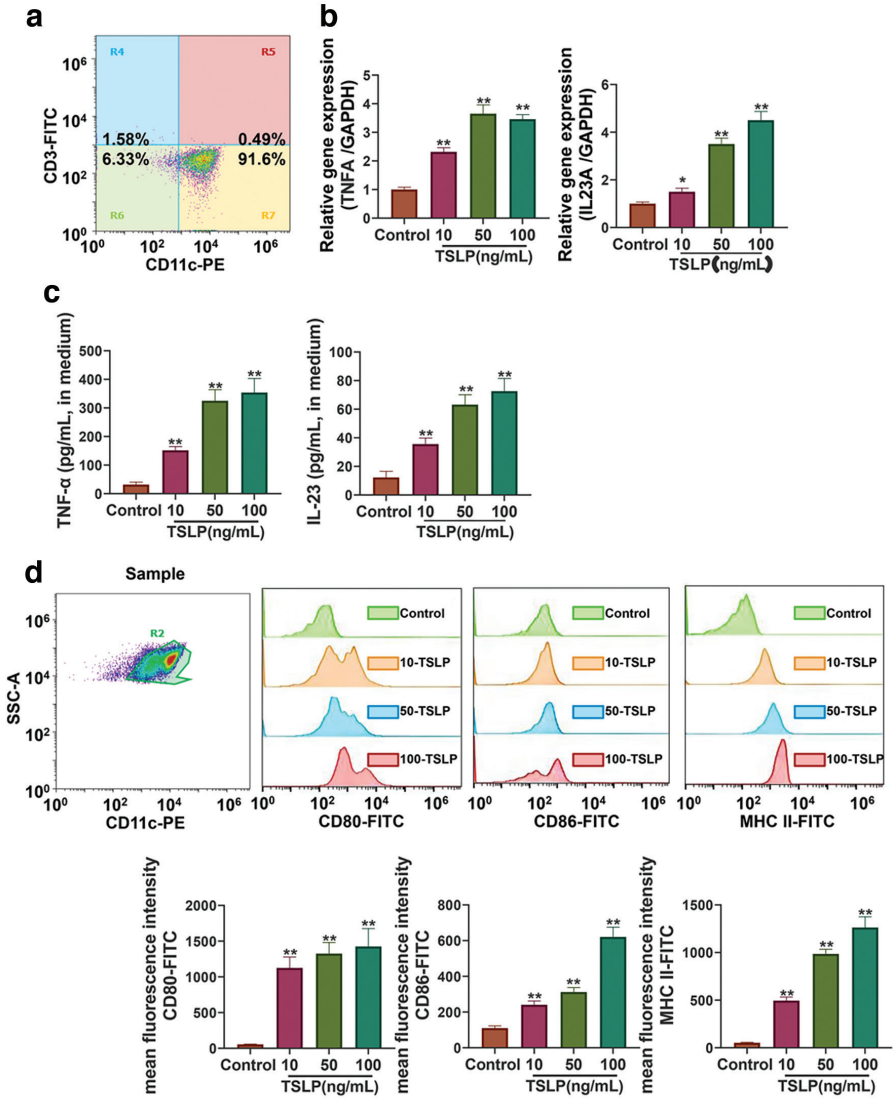

FIG. 3. Effect of TSLP on primary BMDCs in vitro. (a) Flow cytometry analysis of the purity of BMDCs cultured in vitro on day 8. TNF-α and IL-23 expression in BMDCs cultured with various concentrations of TSLP in vitro determined by (b) RT-qPCR and (c) ELISA for culture supernatant. (d) Expression of mature DC markers, CD80, CD86, and MHC II, by CD11-expressing BMDCs cultured with various concentrations of TSLP determined by flow cytometry.

TSLP, thymic stromal lymphopoietin; BMDCs, bone marrow dendritic cells; TNF-α, tumor necrosis factor-alpha; IL-23, interleukin-23; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; DC, dendritic cells.

upregulation of TRAF6 in response to TSLP stimulation. Cerdulatinib, a JAK/SYK pathway inhibitor, effectively inhibited NF-κB activation in response to TSLP stimulation. These results showed that TSLP activated the JAK/SYK pathway in BMDCs, which could be inhibited by cerdulatinib (Figures 5a, b).

The effects of JAK/SYK inhibition on DC maturation were also investigated. As shown in Figure 5c, measured with relative fluorescence intensity by flow cytometry, expression of CD80, CD86, and MCH class II markers by CD11-positive DCs increased in response to TSLP stimulation, which was antagonized by the addition of cerdulatinib during stimulation.

TNF-α and IL-23α of the treated DCs were evaluated using RT-qPCR. Figure 5d shows that TSLP stimulation increased the RNA levels of *TNF-α* and *IL-23α* genes in DCs. Cerdulatinib was administered during stimulation, which prevented these increases.

The effect of inhibiting the JAK/SYK pathway on BMDCs function in terms of inducing Th17 differentiation in response to TSLP stimulation was investigated. Mouse $CD4^+$ T cells isolated from splenocytes were cultured for 72 h in regular medium or regular medium supplemented with the medium used to culture BMDC, with various treatments at a 4:1 ratio. Flow cytometry analysis revealed that when the cell culture medium was supplemented with BMDCs culture medium stimulated with 100 ng/ml TSLP, the $CD4^+CCR6^+IL\text{-}17^+$ Th17 cell population increased significantly among the total $CD4^+$ T cells compared with the one without supplement or one supplemented with BMDCs culture medium without TSLP stimulation (Figure 6a). The Th17 cell population was significantly reduced among the total $CD4^+$ T cells when the supplemented medium was the BMDC culture medium stimulated with TSLP while also containing the JAK/SYK inhibitor cerdulatinib, compared with the supplemented medium without the inhibitor.

Th17 cell differentiation was investigated by assessing IL-17 expression levels in $CD4^+$ T cells and activating the Th17 signaling pathway. As shown in Figure 6b, $CD4^+$ T cells cultured in medium supplemented with BMDC culture medium stimulated with TSLP had higher relative IL-17 mRNA levels (determined by qRT-PCR) and secreted IL-17 in culture medium (determined by ELISA) than cells cultured in regular medium or medium supplemented with BMDC culture medium without TSLP. When $CD4^+$ T cells were cultured in a medium supplemented with BMDC culture medium stimulated with TSLP and cerdulatinib, IL-17 expression decreased. Our results showed that when stimulated with TSLP, BMDC secreted signaling molecules that promote Th17 cell differentiation.

STAT3 plays an essential role in the Th17 T cell differentiation. $CD4^+$ T cells cultured with medium supplemented with BMDC culture medium stimulated with TSLP showed activation of STAT3, as evidenced by an increase in phosphorylated STAT3 (S727) compared with cells cultured with regular medium or medium supplemented with BMDC culture medium without TSLP (Figure 6c, d). Cerdulatinib, a JAK/SYK inhibitor, reduced the level of phosphorylated STAT3 in $CD4^+$ T cells cultured with BMDC culture medium stimulated with

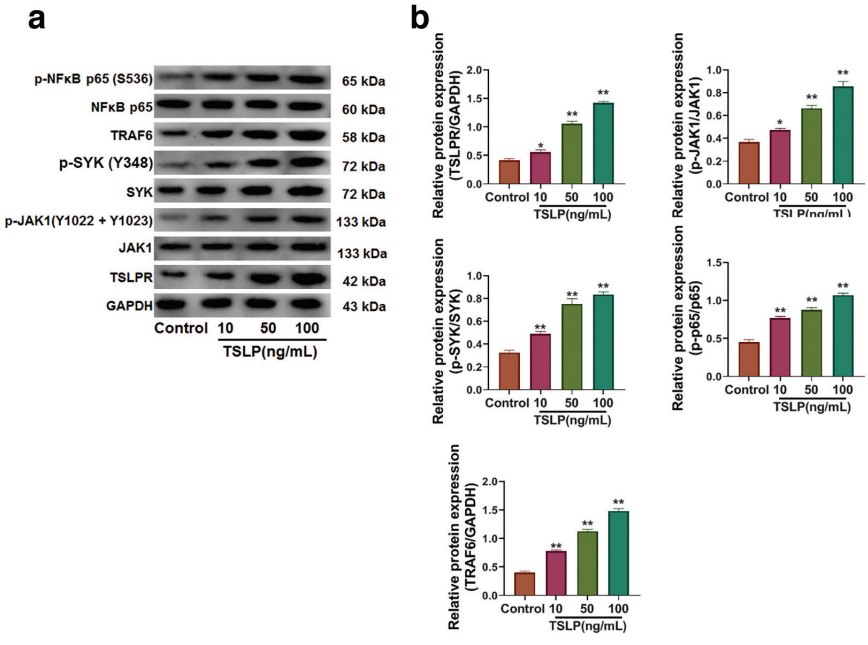

FIG. 4. Activation of the JAK/SYK signaling pathway by TSLP in primary BMDCs in vitro. (a) Representative Western blot images of protein expressions in BMDCs treated with TSLP at various concentrations for TSLPR, phosphorylated JAK1, JAK1, phosphorylated SYK, SYK, phosphorylated NF-κB p65, NF-κB p65, TRAF6, and GAPDH. (b) Quantitative analysis of protein band intensities for TSLPR versus GAPDH, phosphorylated JAK1 versus JAK1, phosphorylated SYK versus SYK, phosphorylated NF-κB p65 versus NF-κB p65, and TRAF6 versus GAPDH.

JAK, Janus kinase; SYK, spleen tyrosine kinase; NF-κB, nuclear factor kappa B; TSLPR, thymic stromal lymphopoietin receptor; TSLP, thymic stromal lymphopoietin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BMDCs, bone marrow dendritic cells; TRAF6, TNF receptor-associated factor 6.

TSLP. We found comparable expression patterns with RORγt protein, a transcription factor for Th17 cells (Figures 6c, d).

DISCUSSION

The role of TSLP in psoriasis was first implied when it was found to be upregulated in psoriatic lesions.[26] In the same study, the authors found that TSLP interacts with CD40Ls to activate DCs and promotes IL-23 production by DCs. Later, it was shown that serum TSLP levels increase significantly in psoriasis patients and are linked to disease severity.[28] Studies have shown that TSLP-DCs stimulate $CD4^+$ T cells to produce Th2 cytokines IL-4, IL-5, and IL-13, but little or no IL-10.[16] Interestingly, $CD4^+$ T cells activated by TSLP-DCs do not produce increased levels of IL-10 or INF-γ, which are known to downregulate Th2 inflammation. TSLP-DCs stimulate $CD4^+$ T cells, resulting in high levels of TNF-α production. Although not a typical Th2 cytokine, TNF-α is closely associated with asthma and allergic inflammation.[33] These findings indicate that TSLP-DCs can produce a distinct type of inflammatory T cells.[34]

This study found that TSLP was upregulated in mouse psoriasis skin tissue, which was associated with higher TSLPR and IL-7Rα levels and increased epidermal keratosis. We showed that using

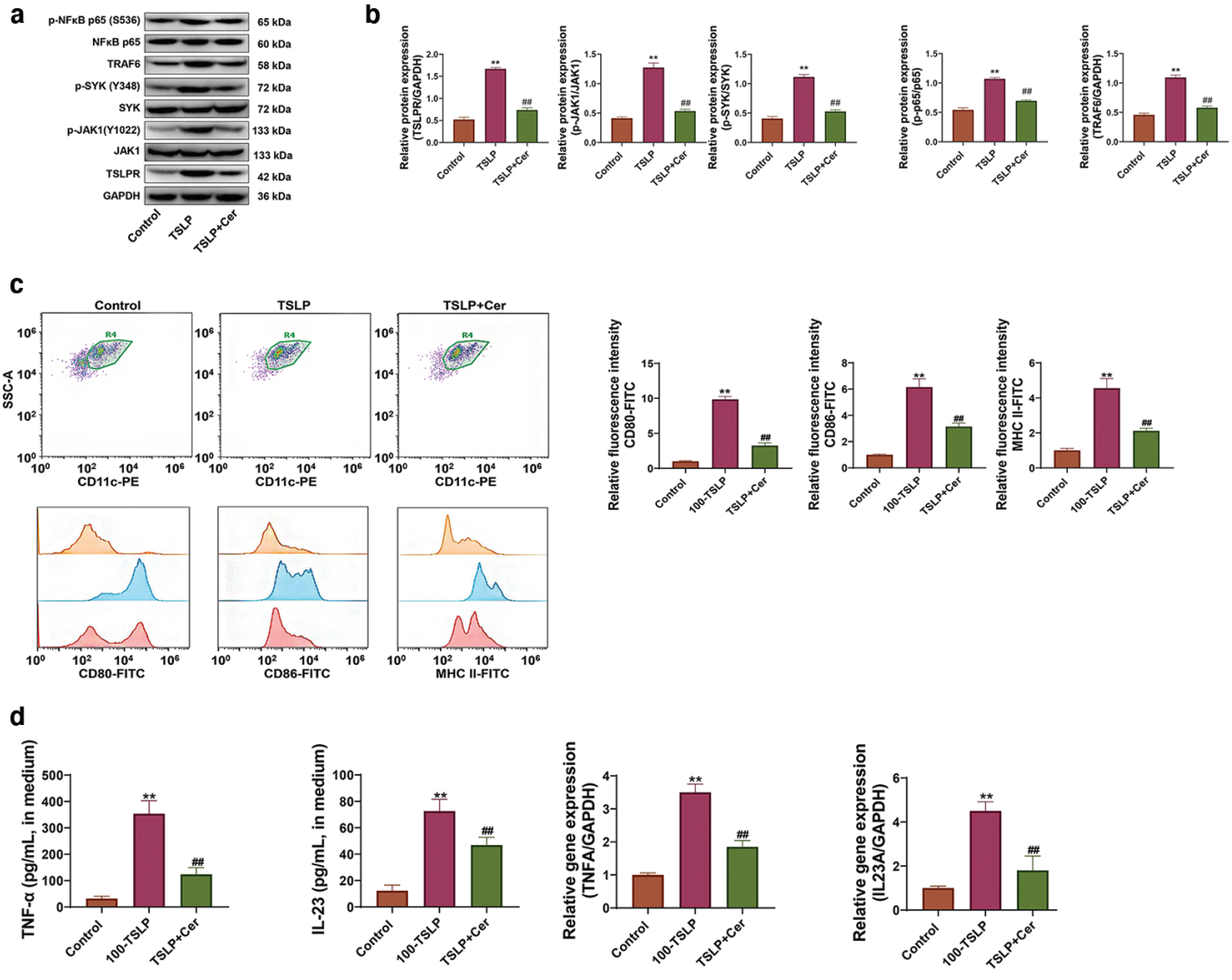

FIG. 5. Effect of cerdulatinib, a dual JAK/SYK inhibitor, on TSLP on BMDCs in vitro. (a) Representative Western blot images of protein expressions in BMDCs treated with or without TSLP or TSLP + cerdulatinib for TSLPR, phosphorylated JAK1, JAK1, phosphorylated SYK, SYK, phosphorylated NF-κB p65, NF-κB p65, TRAF6, and GAPDH. (b) Quantitative analysis of protein band intensities for TSLPR versus GAPDH, phosphorylated JAK1 versus JAK1, phosphorylated SYK versus SYK, phosphorylated NF-κB p65 versus NF-κB p65, and TRAF6 versus GAPDH. (c) Flow cytometry analysis of mature DC marker expression, CD80, CD86, and MHC II, in BMDCs cultured with or without TSLP or TSLP + cerdulatinib. (d) TNF-α and IL-23 production in BMDCs cultured with or without TSLP or TSLP + cerdulatinib determined using RT-qPCR (for RNA levels) and ELISA (for secreted levels).

JAK1, Janus kinase 1; SYK, spleen tyrosine kinase; NF-κB, nuclear factor kappa B; TSLPR, thymic stromal lymphopoietin receptor; TSLP, thymic stromal lymphopoietin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BMDCs, bone marrow dendritic cells; TRAF, TNF receptor-associated factor; DC, dendritic cells.

shRNA to reduce TSLP expression could reverse the histological and pathological changes often associated with psoriasis and reduce TSLPR and IL-7Rα expression levels. Our results indicate comparable observations in this mouse psoriasis model were observed in human psoriasis patients. For the first time, we demonstrated that directly targeting TSLP with shRNA in vivo can reduce the severity of psoriasis. Mouse skin tissue analysis indicated that the JAK/SYK and NF-κB signaling pathways were activated in psoriasis tissue. shRNA targeting TSLP expression might reduce the activation. TSLP activates DCs via JAK1 and JAK2, and the downstream effect requires activation of NF-κB.[22] Serum levels of TNF-α and IL-23 increased in psoriasis mice but reduced when treated with shRNA targeting TSLP. These findings are consistent with the previous findings that TSLP produced by human epithelial cells may activate DCs and increase TNF-α production during allergic inflammation.[16] IL-23 is the critical component for psoriasis. Our findings showed that TSLP was important in inducing IL-23 and contributing to the pathogenesis of psoriasis.

The mechanism of TSLP was further investigated in vitro using mouse primary BMDCs. These studies showed that the addition of TSLP in the culture medium promoted the maturation of BMDCs and activated BMDCs via the JAK/SYK and NF-κB signaling pathways, leading to increased production of TNF-α and IL-23. Cerdulatinib, a JAK/SYK inhibitor, could block the maturation of BMDCs and their production of cytokines, indicating that it activated BMDC through the JAK/SYK pathway. The study also found that DCs can release TNF-α when stimulated by TSLP. Previous studies have shown that TSLP-DCs stimulate $CD4^+$ T cells to produce TNF-α;[34] however, our findings showed that TSLP may directly activate DCs to produce TNF-α. We also found that cytokines secreted by activated BMDCs, presumably IL-23, promoted $CD4^+$ T cell differentiation into Th17 cells by activating the STAT3 signaling pathway. Using the culture supernatant from TSLP-activated DCs to stimulate $CD4^+$ T cells, we showed that the TSLP-DC interaction may directly induce the differentiation of $CD4^+$ T cells into Th17 cells, possibly due to the IL-23 secretion by the activated DCs.

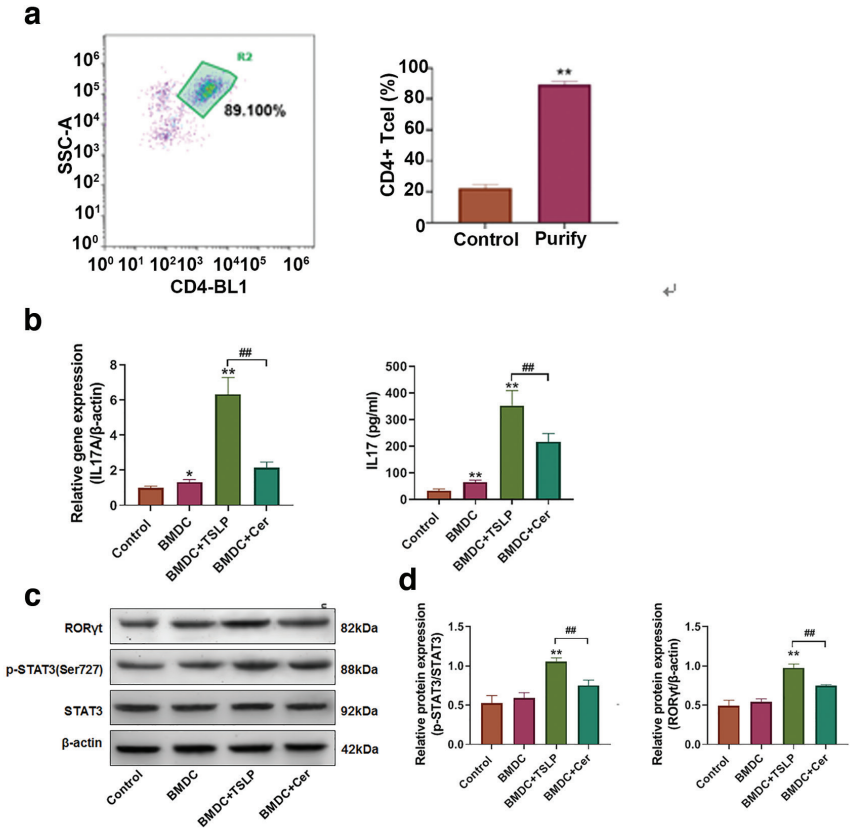

FIG. 6. Effect of secreted signaling components by BMDCs stimulated with TSLP or TSLP + cerdulatinib on the in vitro differentiation of CD4+ T cells. (a) CD4+ T cell differentiation into Th17 cells when cultured with medium supplemented with supernatant from BMDC culture medium stimulated with TSLP with or without cerdulatinib assessed by flow cytometry. (b) IL-17 expression by CD4+ T cells when cultured with medium supplemented with supernatant from BMDC culture medium stimulated with TSLP with or without cerdulatinib assessed using RT-qPCR (for RNA levels in cells) and ELISA (for secreted levels). (c) Representative Western blot images of protein expression in CD4+ T cells when cultured with the medium supplemented with supernatant from BMDC culture medium stimulated with TSLP with or without cerdulatinib for phosphorylated STAT3 (S727), STAT3, RORγt, and β-actin. (d) Quantitative analysis of protein band intensities for phosphorylated STAT3, STAT3, RORγt, and β-actin.

TSLP, thymic stromal lymphopoietin; BMDCs, bone marrow dendritic cells; IL-17, interleukin-17; Th17, T helper 17; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; β, beta.

The presented results identified a TSLP signaling pathway involved in the pathogenesis of psoriasis. TSLP activated DCs through the JAK/SYK pathway, leading to the secretion of inflammatory cytokines TNF-α and IL-23, which activate Th17 cells. Thus, $CD4^+$ T cells were promoted to differentiate into Th17 cells, which contributed to the inflammatory responses observed in psoriasis. These findings indicated that TSLP had a major role in psoriasis.

Fridman et al.[35] found that INCB018424, a potent and selective JAK1 and JAK2 inhibitor, reduces the stimulatory effect of cytokines IL-23, IL-12, and IL-6 on STAT3 activation. This JAK1 inhibitor, when used topically, reduced the inflammation caused by TSLP injected intradermally.[35] In our study, we emphasized the role of TSLP in the pathogenesis of psoriasis. We found that TSLP levels increased in both psoriasis patients' skin samples and established animal models' skin samples. We also found that using shRNA to reduce TSLP levels reduced the severity of the disease. While it effectively alleviates inflammation by targeting JAK1, as indicated by Fridman et al.,[35] our findings showed that TSLP may be an attractive target for psoriasis treatment.

There are several treatments available for psoriasis. Treatment options include topical therapy, phytotherapy, systemic immunosuppressants, and chemical and biological agents, depending on the severity of the disease, the patient's age, and the disease's impact on quality of life.

Several monoclonal antibodies targeting IL-23/IL-17 signaling were approved to treat moderate-to-severe plaque psoriasis in adults. Inhibitors for the components of the signaling cascade were also available.[36-38] JAK inhibitor tofacitinib was approved for chronic plaque psoriasis, whereas more selective TYK2 inhibitors are in trials.[11] In the present study, we demonstrated via mouse model that using a highly effective shRNA in vivo significantly reduced the severity of the disease, and TSLP may be an excellent therapeutic target for treating psoriasis. TSLP-targeting therapeutics have been developed for some inflammatory disorders.[39] Tezepelumab (AMG157/MEDI9929), an anti-TSLP monoclonal antibody, is the most prominent TSLP signaling inhibitor in the treatment of allergic inflammatory diseases and has been approved for severe asthma patients.[40,41] Based on our findings, which show that TSLP plays a crucial role in psoriasis, these therapeutic options targeting TSLP may potentially be appropriate for severe psoriasis.

Ethics Committee Approval: All animal experiment procedures were approved by the Ethics Committee of the General Hospital of the Chinese People's Liberation Army (approval number: S2023-302-1).

Informed Consent: Human skin tissue samples were obtained from PLA General Hospital with informed consent.

Data Sharing Statement: The datasets used and/or analyzed during the current study are included in the main text and supplemental materials.

Authorship Contributions: Concept- C.S., Z.Z., C.L., B.L.; Design- C.S., J.S., J.B., C.L., B.L.; Supervision- C.L., B.L.; Materials- C.S., J.S., Z.Z., X.Z., X.D., X.L., L.X.; Data Collection or Processing- C.S., J.S., Z.Z., X.D., X.L., J.B.; Analysis or Interpretation- C.S., J.S., Z.Z., X.Z., J.B., L.X., L.G.; Literature Search- C.S., J.S., Z.Z., X.D., X.L., C.L., B.L.; Writing- C.S., J.S., L.X., C.L., B.L.; Critical Review- C.S., J.S., Z.Z., X.Z., X.D., X.L., J.B., L.X., L.G., C.L., B.L.

Conflict of Interest: The authors declare that they have no conflict of interest.

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