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## Research Article

# Indirubin attenuates mouse psoriasis-like skin lesion in a CD274-dependent manner: an achievement of RNA sequencing

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It was previously reported that the expression of CD274 was down-regulated in psoriatic epidermis, leading to immune disorders of psoriasis. However, the regulatory mechanisms of CD274 were rarely elucidated. We aimed to explore the regulatory mechanisms of CD274. Skin samples were collected from 18 patients with psoriasis vulgaris and 9 healthy participants for RNA sequencing. Candidate genes were chosen based on degree and k-core difference of genes in the co-expression network. The relations between candidate genes and CD274 were validated by flow cytometry and real-time PCR in primary human epidermal keratinocytes. The therapeutic effect of indirubin was assessed in an imiquimod-treated mouse model. Interferon-$\gamma$ (IFN-$\gamma$), cyclin-dependent kinase (CDK) 1, Toll-like receptor 3 (TLR3), TLR4 and interleukin (IL)-17A were considered as candidate genes. In primary human epidermal keratinocytes, the level of CD274 was obviously increased under the stimulation of IFN-$\gamma$ and CDK1 inhibitor (indirubin), independent of TLR4, TLR3 or IL-17A. Indirubin alleviated the severity of psoriatic mice in a CD274-dependent manner. Co-expression network analysis served as an effective method for the exploration of molecular mechanisms. We demonstrated for the first time that CD274 was the regulator of indirubin-mediated effect on mouse psoriasis-like skin lesion based on co-expression network analysis, contributing to the alleviation of mouse psoriasis-like skin lesion.

## Introduction

Psoriasis vulgaris is one of the most common forms of psoriasis, which is related to T-lymphocyte infiltration in skin [1]. CD274, also known as programmed cell death ligand 1 or B7-H1, was reported to inhibit T-cell differentiation and proliferation thus playing a vital role in the regulation of immune reaction [2,3]. It has been proven that the level of CD274 is decreased in the psoriatic epidermis compared with normal epidermis. Decreased CD274 protein was considered to involve the immune disorders of psoriasis [4]. Furthermore, in the imiquimod (IMQ)-induced psoriatic mouse models, the administration of recombinant CD274 protein decreased psoriatic inflammation and alleviated psoriatic symptoms [5]. Tumor patients were reported to induce psoriasis under the treatment of CD274 antibody [6–8]. These previous studies indicated the vital role of CD274 in psoriasis. Therefore, drugs increasing the level of CD274 in keratinocytes may serve as a potential therapeutic strategy in the treatment of psoriasis. However, the regulatory mechanisms of CD274 in keratinocytes are not fully elucidated and drugs of regulating CD274 have not been found.
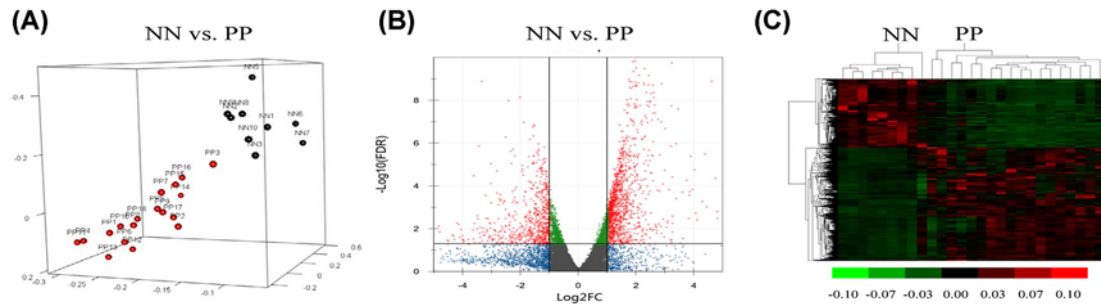
**Figure 1. Genes between the skins from the PP and the NN were differentially expressed**
(**A**) Principal component analysis indicates the whole gene expression pattern of the two groups: PP (red balls) and NN (black balls). (**B**) The volcano plot represents all of the protein-coding genes in the two groups: genes with significantly differential expression (FDR $<0.05$, $-\log_2$FC$->1$) (red dots) and genes without significantly differential expression (other color dots). (**C**) The heat map indicates the differentially expressed genes between the two groups. Each row or column represents one gene or sample, respectively. Green or red bar represents a decreased or increased gene expression, respectively. $-0.1$ represents the lowest expression value of the gene among all the samples and 0.1 represents the highest one.

In the past 10 years, RNA sequencing has been developed as a popular deep-sequencing technology. Compared with other transcriptomics methods, it enables the much more rapid and cost-effective generation of massive amounts of sequences and provides a more precise measurement of levels of transcripts [9]. The technology of RNA sequencing is an effective gene expression profiling method, which has been widely applied in various fields including biological medicine. According to the bioinformatics analysis of differential genes, biomarkers and drug targets have been discovered for the diagnosis and treatment of diseases. Besides, the pathological mechanisms of disease and functions of gene can be evaluated [10–12]. In the field of psoriasis, RNA sequencing has been used to explore many functions of genes, such as defensin-like antimicrobial activity of *LCE* 3 [13] and transcription factor FOXA1 to inhibit the formation of regulatory T-cells subpopulation [14].

In the present study, we conducted RNA sequencing in skin tissues from psoriatic and healthy persons to discuss the CD274-dependent regulatory mechanisms. Cellular and animal experiments were conducted for further detection to ultimately develop CD274-dependent regulatory drugs. We raised a hypothesis that CD274 was the regulator of indirubin-mediated effect on mouse psoriasis-like skin lesion, which contributed to the alleviation of mouse psoriasis-like skin lesion. We believe that the present study might provide a novel insight in the exploration of mechanisms in the pathogenesis of psoriasis vulgaris and development of drugs.

# Materials and methods
## Participants and samples
Normal skins for control were obtained from nine volunteers undergoing surgical operations in the Department of Plastic Surgery, Changhai Hospital, Shanghai, China. Volunteers were observed neither systemic or autoimmune diseases nor relevant family history. Psoriatic skins were obtained from 18 patients with psoriasis vulgaris in the same institution. No patients had received systemic or topical therapies for 4 weeks before the skins were obtained. Ethical approval was obtained from the Ethics Committee of Changhai Hospital and written informed consent was signed by all of the participants in the present study. All of the samples were immediately snap-frozen in liquid nitrogen and stored at $-80°$C for RNA extraction.

## RNA sequencing
Whole skin from psoriatic patients and healthy people was used for total RNA extraction by TRIzol Reagent according to the manufacturer's protocol (Thermo Scientific, Bremen, Germany). RNA concentration and quality were detected by Agilent 2200. Only RNA samples with RNA integrity number more than 6.0 were used to construct a complementary DNA library. The complementary DNA library was prepared using an Ion Total RNA-Seq Kit v2 (Thermo Scientific, Bremen, Germany) according to the manufacturer's protocol. The quantity and quality of obtained complementary DNA libraries were assessed by an Agilent Bioanalyzer 2200 system (Agilent Technologies, Inc., Santa Clara,

CA, U.S.A.). The complementary DNA libraries were then processed for protein sequencing according to commercially available protocol. The sequencing quality was analyzed using RSeQC [15]. Principal component analysis was applied to differentiate if there was a significant distinction between two groups [16].

## Bioinformatics analysis

Differentially expressed genes (DEGs) between psoriatic skins and normal skins were filtered by means of DE-seq. The values of fold change (FC) and statistical significance ($P$) were calculated. False discovery rate (FDR) was detected to correct the $P$ value [17]. After the assessment of FDR and FC, we selected the DEGs with FDR < 0.05 and —$\log_2$FC— > 1 [18]. The function and pathway of the DEGs were analyzed according to the Gene Ontology (GO) database [19,20] and KEGG database, respectively [21]. Fisher's exact test and $\chi^2$ test were applied to classify the GO category and select the significant pathway. According to the significant difference from both GO and pathway ($P < 0.01$), we selected the relevant DEGs and 30 long non-coding RNA with high values of —$\log_2$FC— to build gene co-expression networks. For each pair of genes, we calculated the Pearson Correlation and chose the significant correlation pairs (FDR < 0.01) to construct the network [22]. In the network, the importance of genes was determined by the degree (the line numbers that one gene links to the other) and the k-core (genes that are connected to at least k other genes) [23–26].

## Cell culture and treatment

Primary human epidermal keratinocytes served as the *in vitro* model for the present study. Epidermal keratinocytes were obtained from adult foreskin isolated from patients in the Department of Plastic Surgery, Shanghai Changhai Hospital. Foreskin was rinsed in EpiLife™ medium with 20 μg/ml gentamycin solution and cut into strips (0.5 cm × 1 cm) after removing subcutaneous mucosa. The cut pieces were submerged in the 0.2% dispase II (neutral protease, grade II) solution (Roche, Indianapolis, IN, U.S.A.) and then incubated in a 4°C refrigerator for 16 h. Epidermis was separated from the dermis and incubated in the recombinant trypsin/EDTA solution for 30 min at a 37°C water bath. After neutralized by the defined trypsin inhibitor, the solution was filtered in a 40 μm cell strainer. Keratinocytes were resuspended in EpiLife™ medium with the addition of human keratinocyte growth supplement (Gibco, Carlsbad, CA, U.S.A.) after centrifugation and were seeded in a 25 cm² flask at $5 \times 10^3$ cells/cm². Cells at the passage of 2–3 were used for the experiments. After reaching semi-confluence, cells were stimulated with 25 ng/ml interferon-γ (IFN-γ; PeproTech, Rocky Hill, NJ, U.S.A.), 0.3 μg/ml polyinosinic-polycytidylic acid (poly (I: C); InvivoGen, Toulouse, France), 100 ng/ml IL-17A (PeproTech, Rocky Hill, NJ, U.S.A.), 20 μg/ml indirubin (National Institutes for Food and Drug Control, Beijing, China), 10 μg/ml lipopolysacchride (LPS; Sigma-Aldrich, St. Louis, MO, U.S.A.) as well as relative vehicle reagent (medium or DMSO) for 24 h, respectively. Finally, cells were harvested for flow cytometry or real-time PCR.

## Flow cytometry

Cells were washed three times with cold PBS and surface staining was performed at room temperature for 30 min using anti-human CD274 antibodies or mouse IgG1 kappa isotype control (eBioscience, San Diego, CA, U.S.A.). The results were analyzed by FlowJo software (Tree Star Inc., Ashland, OR, U.S.A.). The levels of CD274 were shown with mean fluorescence intensity (MFI).

## Real-time PCR

Total RNA of cells was extracted by TRIzol Reagent according to the manufacturer's protocol. The concentration and purity of RNA were determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). cDNA was synthesized according to the manufacturer's protocol of PrimeScript™ RT Master Mix (Takara, Dalian, China). Levels of CD274 were assessed using TB Green™ Premix Ex Taq™ II (Takara, Dalian, China) and primers on LightCycler 480 II real-time PCR system (Roche, Mannheim, Germany). The thermal cycles was set at 95°C for 30 s, followed by 40 cycles of 95°C (10 s), 60°C (10 s) and 72°C (10 s). The expression of CD274 was normalized to the internal control by using comparative cycle threshold ($C_T$) method where fold difference $= 2^{-\Delta\Delta C_T}$. GAPDH genes were used as an internal control. GAPDH forward primer: 5′-CAGGAGGCATTGCTGATGAT-3′ and reverse primer: 5′-GAAGGCTGGGGCTCATTT-3′. CD274 forward primer: 5′-GGTGCCGACTACAAGCGAAT-3′ and reverse primer: 5′-GGTGACTGGATCCACAACCAA-3′.
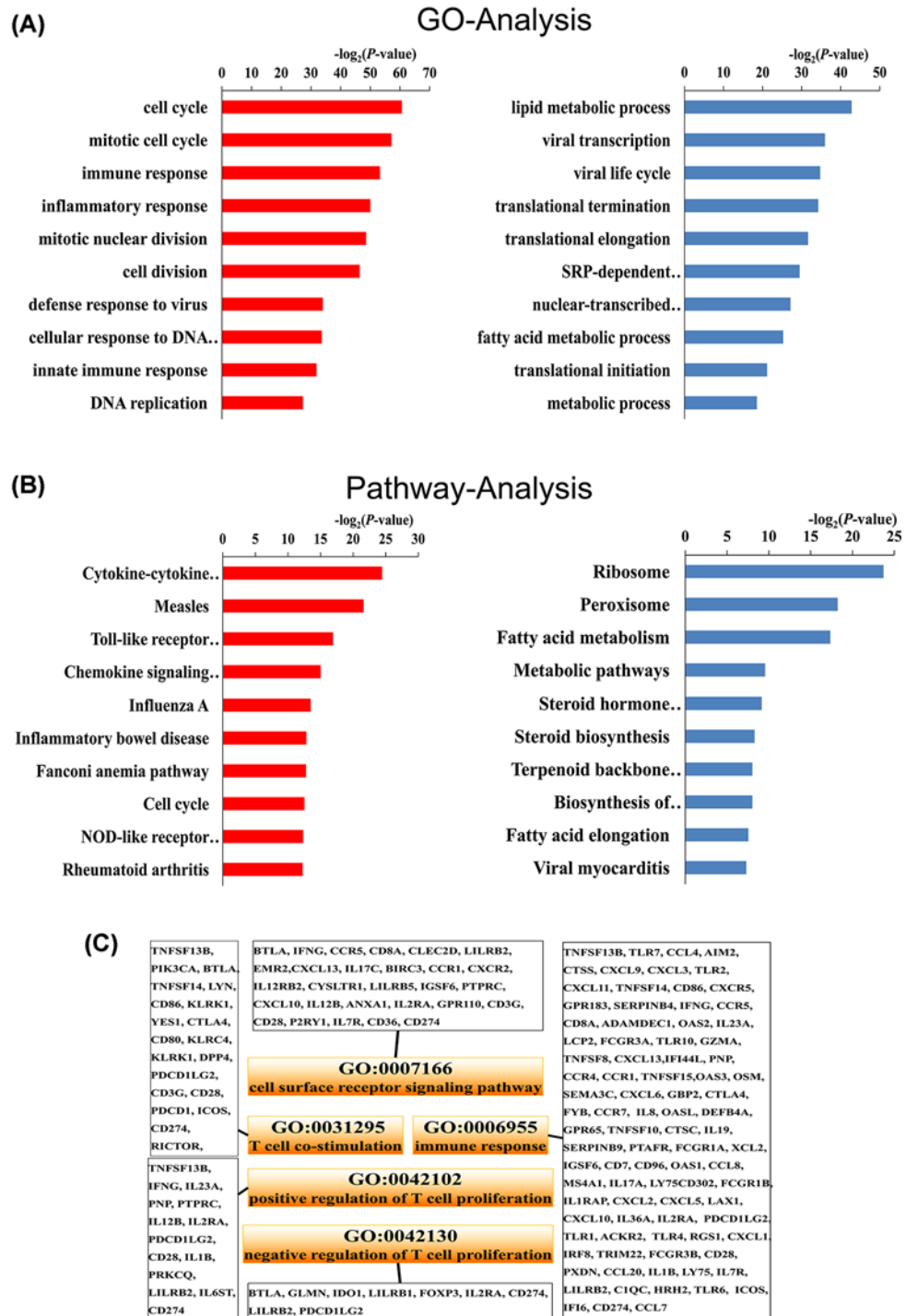
**Figure 2. GO and pathway enrichment analysis of differentially expressed genes**

(**A**) The top 10 GO terms in the biological process clusters. (**B**) The top 10 pathways. The chart with red bars represents up-regulated and blue bars represent down-regulated genes (A and B). (**C**) The five GO terms related to CD274. Charts with orange bars represent the significantly enriched GO terms related to CD274 in the biological process and the corresponding genes are shown in the white boxes.

## Animals

Male BALB/c mice (8 weeks old) were obtained from the experimental animal center of the Second Military Medical University and bred in a specific pathogen-free condition. Mice were maintained on a 12-h light/dark cycle and supplied with food and water *ad libitum*. The animal protocols used in the present study were approved by the experimental animal ethics committee of the Second Military Medical University.

## IMQ-induced psoriasis-like mouse model and *in vivo* treatment

Mice were randomly classified into five groups, including the control, animal model, indirubin, indirubin together with anti-CD274 and CD274-Fc groups. Mice in the model group were treated with 5% IMQ cream (62.5 mg; 3M Health Care Limited, Loughborough, Leicestershire, U.K.) on the shaved dorsal skin for six consecutive days to generate psoriasis-like lesions, while mice in the control group were given vehicle cream. Indirubin (50 mg/kg) or mGoInVivo™ purified anti-mouse CD274 antibody (100 μg; Biolegend, San Diego, CA, U.S.A.) was subcutaneously given every day after IMQ treatment. Recombinant mouse CD274-Fc chimera protein (20 μg; R&D Systems, Minneapolis, MN, U.S.A.) was administrated intradermally onto the back every 12 h after IMQ treatment. Mice were killed on day 7 after the first day of IMQ application.

## Histological analysis

The dorsal skin of mice was fixed overnight in 4% paraformaldehyde solution and embedded in paraffin. The paraffin-embedded specimens were processed for hematoxylin and eosin staining according to the standard procedures.

## Immunofluorescence analysis

Frozen sections of mouse skin tissues were stained overnight with primary antibodies including rabbit anti-mouse cytokeratin (CK)-14 antibody (Abcam, Cambridge, MA, U.S.A.) and rat anti-mouse CD274 antibody (Abcam). After extensive washing, the sections were incubated with secondary antibodies including Alexa Fluo 594-conjugated donkey anti-rat IgG (Life Technologies, Carlsbad, CA, U.S.A.) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies) for 1 h at room temperature. After being washed, slides mounted with sections were stained for 2 min with $4'$-6-diamidino-2-phenylindole (DAPI; Life Technologies) and the colocalization was detected by means of a fluorescence microscope (Olympus, Tokyo, Japan). The average number of fluorescent staining cells from mouse skin was counted from three independent images with a $\times 400$ field.

## Statistical analysis

All data from *in vivo* and *in vitro* studies were expressed as means $\pm$ SD. Data were analyzed by SPSS Statistics 21 software (SPSS Inc., Chicago, IL, U.S.A.). $P$ values were determined by student's unpaired $t$-test or one-way ANOVA followed by Bonferroni's test. $P$ values of less than 0.05 were considered to be statistically significant.

## Ethics Statement

The present study was carried out in accordance with the recommendations of the biomedical research guidelines involving human participants established by the National Health and Family Planning Commission of China. Shanghai Changhai Hospital Ethics Committee approved the present study, and written informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

# Results
## Quality of RNA sequencing and differential genes

The average age of psoriatic patients (PP) was 43.83 $\pm$ 16.29, which was 37.33 $\pm$ 13.73 in the normal controls (NN). No difference in gender or age was observed between the two groups. Detailed characteristics of the participants were listed in Table 1. The RNA quality of all samples conformed to the requirements of the sequencing. The raw sequence data yielded $\sim$2.3 gigabases of data per sample, with an average of 17 million reads (89.0% mapped rate) per sample. The average number of the GC content was $\sim$52% for each sample. Mapping of sequence reads yielded an average of 23,960 genes mapped for each sample. The figure of principal component analysis displayed a sharp separation between the two groups (Figure 1A). Finally, all samples were used to analyze the differential genes. The dataset analyzed during the present study was available in the NCBI Gene Expression Omnibus repository. The accession number for the dataset was GSE114286 ( https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114286).
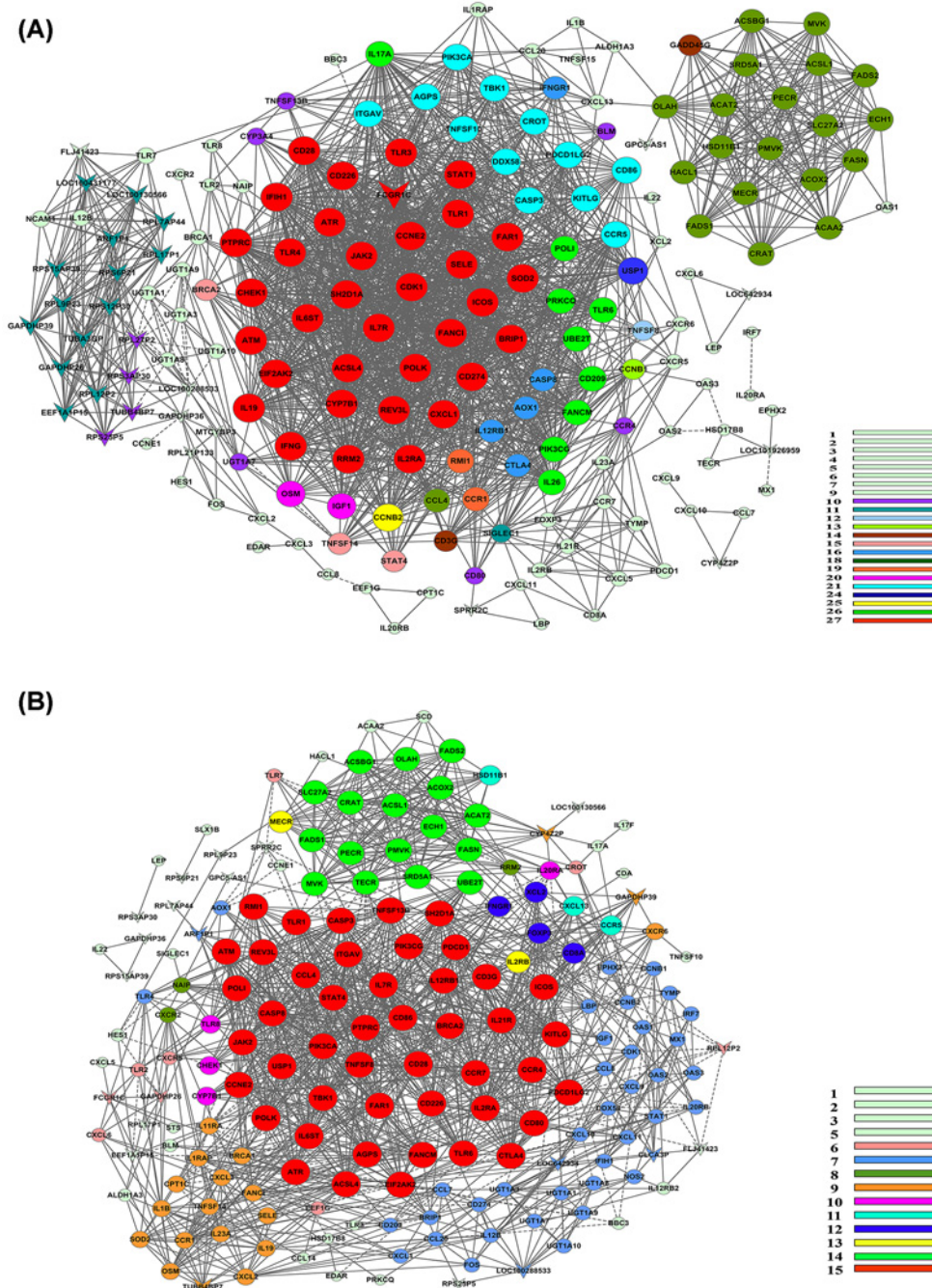
**Figure 3. Gene co-expression networks of normal controls groups and psoriatic patients groups**

(**A** and **B**) Genes are described with nodes in the charts. The vee nodes represent non-coding RNA, and the circle nodes represent protein-coding genes. The k-core values of nodes in the networks are shown in different colors (the color bars are shown on the right bottom corner of each chart), and the volume of the nodes is in proportion to the values of k-core. The lines represent regulatory relations between two genes: positive correlations (solid lines) and negative correlations (long-dashed lines).

We then identified 2597 differential genes including protein-coding genes and un-coding RNA. Among all of the protein-coding genes, 1335 genes were up-regulated in the PP group, while 804 genes were down-regulated. Volcano plot and heat map were applied to describe the differential expression patterns of the protein-coding genes (Figure 1B,C).
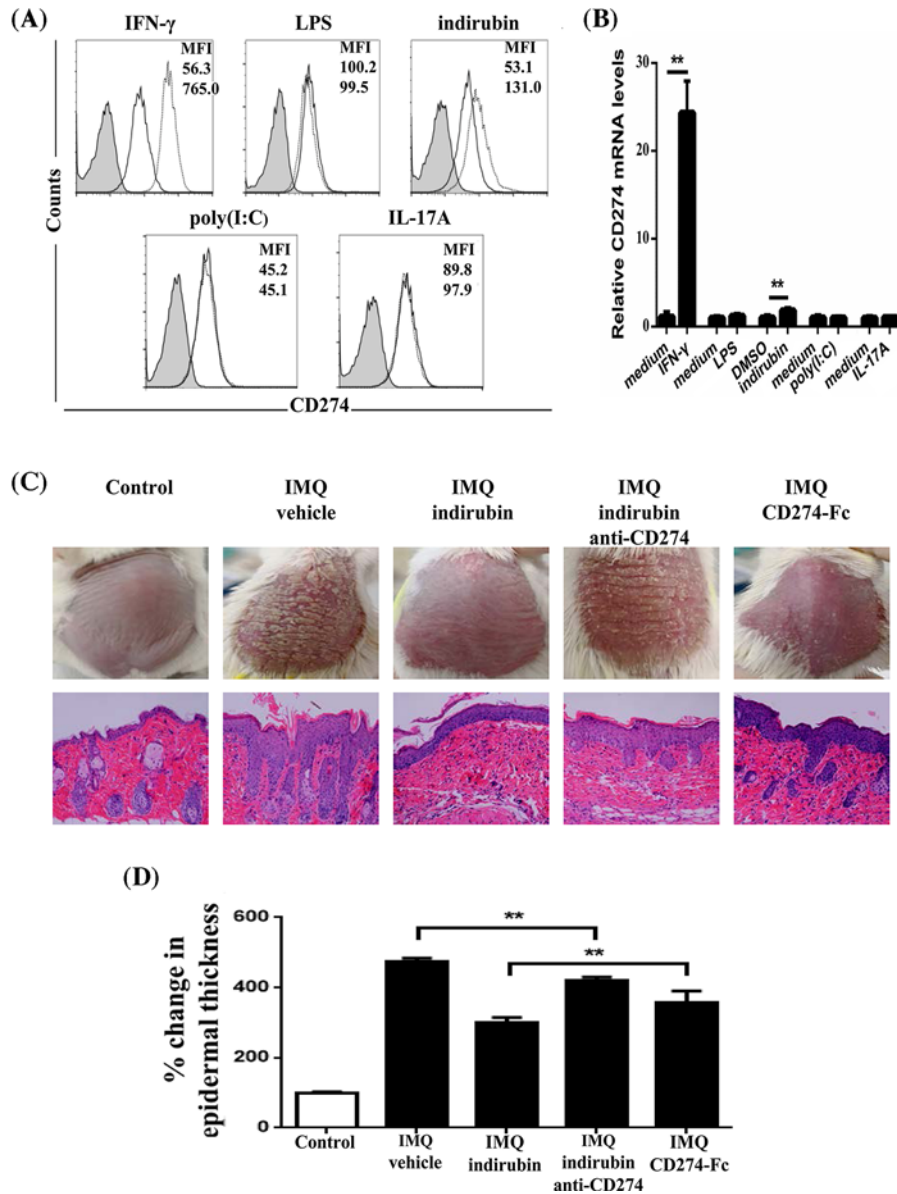
**Figure 4. Expression of CD274 with several stimulations in primary human epidermal keratinocytes and *in vivo* effects of indirubin in an imiquimod (IMQ)-induced mouse model**

(**A** and **B**) Keratinocytes were stimulated with 25 ng/ml IFN-$\gamma$, 10 $\mu$g/ml LPS, 20 $\mu$g/ml indirubin, 0.3 $\mu$g/ml polyinosinic-polycytidylic acid (poly (I: C)), 100 ng/ml IL-17A as well as relative control reagent (medium or DMSO) for 24 h (*n*=6). (**A**) The expression of CD274 in protein was detected by flow cytometry using APC-conjugated antibodies. Representative profiles were shown as histograms, including isotype control antibody (shaded histogram with solid line) and CD274 antibody in the absence (solid line histogram) or presence (dotted line histogram) of stimulations. The levels of CD274 were shown in MFI (B) The mRNA expression of CD274 was analyzed by real-time PCR. (**C** and **D**) Attenuation of psoriasis-like lesion on the back skins of IMQ-induced mice was observed by indirubin, CD274-Fc or anti-CD274 together with indirubin. Repeated IMQ treatment onto the dorsal skins for 6 days resulted in the generation of mouse psoriasis-like lesions. Indirubin, recombinant mouse CD274-Fc chimera protein and anti-mouse CD274 antibody were injected after IMQ treatment. Mice were killed on day 7 after the first day of IMQ application (*n*=6). (**C**) Representative photographs and images of hematoxylin and eosin staining of dorsal skin were shown, bar = 50 $\mu$m. (**D**) Epidermal thickness of the dorsal skin was expressed as the relative percentage over the measurement in untreated mice. Data were presented as mean $\pm$ SD. **$P<0.01$.

**Table 1 Characteristics of psoriatic patients and normal controls**

| | Psoriatic patients (*n*=18) | Normal controls (*n*=9) |
|---|---|---|
| **Age (years)** | | |
| Mean $\pm$ SD | 43.83 $\pm$ 16.29 | 37.33 $\pm$ 13.73 |
| Median | 45.0 (14–68) | 42.0 (20–55) |
| **Gender** | | |
| Male/female | 12/6 | 6/3 |
| **PASI score, 0–72** | | |
| Mean $\pm$ SD | 21.61 $\pm$ 7.99 | |
| Median | 21.5 (8.6–41.3) | |
| **Psoriasis duration (years)** | | |
| Mean $\pm$ SD | 7.61 $\pm$ 8.75 | |
| Median | 4.0 (0–31) | |
| **Age of onset** | | |
| Mean $\pm$ SD | 36.28 $\pm$ 13.51 | |
| Median | 37.0 (13–58) | |

Abbreviation: PASI, psoriasis area and severity index.

## GO and pathway enrichment analysis

In order to learn more about the DEGs, GO analysis and pathway analysis were performed. According to the GO analysis, we found that the up-regulated genes were significantly enriched in 147 GO terms and there were 61 significantly down-regulated GO terms mapped to the GO database. According to the analysis of pathway enrichment, it was observed that there were 11 significantly down-regulated pathways and 33 up-regulated pathways. The top 10 GO terms and pathways were shown in Figure 2A,B, respectively. Additionally, GO terms related to CD274 were listed (Figure 2C). The functions of CD274 were related to the cell surface receptor signaling pathways, T-cell costimulation, immune responses, positive regulation of T-cell proliferation and negative regulation of T-cell proliferation. These results were consistent with the findings from other researches [27].

## Gene co-expression networks

To fully predict the functions of genes, we constructed gene co-expression networks. The networks showed that there were markedly different co-expression patterns in the two groups (Figure 3). In the NN group, the co-expression network generated 188 nodes and 1972 connections. Among the 1972 pairs, 21 pairs presented as negative correlation, while the other pairs presented as positive correlation (Figure 3A). In the PP group, there were a total of 185 network nodes and 1349 connections among all of the genes. Among the 1349 pairs, 105 pairs presented as negative connections, while the other pairs presented as positive connections (Figure 3B). Genes with high k-core values were considered as key genes in the network, such as CD28, TLR3, TLR4, STAT1, JAK2, IFNG, CD274, CDK1, IL-17A and SOD2 in the NN group and JAK2, CD86, STAT4, CD28, IL12RB1, PDCD1, CD80 and PDCD1LG2 in the PP group. Genes related to CD274 in the networks were listed in Table 2. There were 56 genes with a positive correlation with CD274 in the NN group. Except one gene as negative, 16 genes showed a positive correlation with CD274 in the PP group. Based on the principle that genes with high values of differences in the degree (dif-degree) and k-core (dif-kcore) in the networks are considered as core genes in the disease [28], values of dif-degree and dif-kcore of genes listed in Table 2 were calculated and the top 20 genes were listed in Table 3. To detect whether there was connection between those genes and CD274, we further chose genes with values of dif-degree and dif-kcore higher than CD274 and verified in epidermal cells. Five candidate genes including IFN-γ, TLR4, CDK1, IL-17A and TLR3 were ultimately screened.

## Increased expression of CD274 in primary human epidermal keratinocytes stimulated by IFN-γ and indirubin

Since LPS (the activator of TLR4) and IFN-γ was previously reported to increase the expression of CD274 in oral keratinocytes [3,27], we then explored whether similar effects were detected in epidermal keratinocytes. We demonstrated that IFN-γ could significantly increase CD274 both in protein and mRNA levels in primary human epidermal

## Table 2 Genes related to CD274 in the co-expression networks

| Group | Gene | Pearson | *P*-value | FDR | Style |
|-------|------|---------|-----------|-----|-------|
| NN | IL2RA | 0.9732955 | 9.98E-06 | 0.0001717 | Positive |
| | PRKCQ | 0.9687537 | 1.72E-05 | 0.0002295 | Positive |
| | CD28 | 0.9636073 | 2.92E-05 | 0.0003181 | Positive |
| | RRM2 | 0.9579704 | 4.81E-05 | 0.0004146 | Positive |
| | CCR5 | 0.9491639 | 9.28E-05 | 0.0006202 | Positive |
| | SH2D1A | 0.9451843 | 0.0001203 | 0.0007168 | Positive |
| | SOD2 | 0.9420219 | 0.000146 | 0.000804 | Positive |
| | CDK1 | 0.9395096 | 0.0001689 | 0.0008682 | Positive |
| | EIF2AK2 | 0.9374203 | 0.0001898 | 0.0009311 | Positive |
| | STAT1 | 0.935628 | 0.0002092 | 0.0009775 | Positive |
| | IL7R | 0.9274773 | 0.0003149 | 0.0012596 | Positive |
| | PTPRC | 0.9266246 | 0.0003278 | 0.0012927 | Positive |
| | CCNB2 | 0.9249148 | 0.0003547 | 0.0013297 | Positive |
| | TLR4 | 0.9172249 | 0.0004951 | 0.0016521 | Positive |
| | CD86 | 0.9127902 | 0.0005917 | 0.0018396 | Positive |
| | SELE | 0.90586 | 0.0007679 | 0.0021744 | Positive |
| | CCR1 | 0.9043284 | 0.0008113 | 0.0022361 | Positive |
| | ACSL4 | 0.8998301 | 0.0009485 | 0.0024332 | Positive |
| | CHEK1 | 0.897294 | 0.0010326 | 0.0025454 | Positive |
| | IFIH1 | 0.8901775 | 0.0012961 | 0.0029215 | Positive |
| | IL6ST | 0.8842571 | 0.0015483 | 0.0032586 | Positive |
| | CASP8 | 0.8832669 | 0.0015936 | 0.003319 | Positive |
| | TLR3 | 0.8818203 | 0.0016613 | 0.0033809 | Positive |
| | IL19 | 0.8780748 | 0.001846 | 0.0036114 | Positive |
| | FCGR1C | 0.8780748 | 0.001846 | 0.0036114 | Positive |
| | ATR | 0.8765252 | 0.0019264 | 0.0036989 | Positive |
| | JAK2 | 0.8721792 | 0.0021647 | 0.0039831 | Positive |
| | FAR1 | 0.8709298 | 0.0022368 | 0.0040843 | Positive |
| | CCNB1 | 0.8690707 | 0.0023472 | 0.0042117 | Positive |
| | AOX1 | 0.8679496 | 0.0024155 | 0.0042772 | Positive |
| | CTLA4 | 0.8556067 | 0.003261 | 0.0051653 | Positive |
| | TLR6 | 0.854854 | 0.0033184 | 0.0052137 | Positive |
| | IL12RB1 | 0.8535532 | 0.0034191 | 0.0052649 | Positive |
| | PDCD1LG2 | 0.8534694 | 0.0034256 | 0.0052681 | Positive |
| | ATM | 0.8529131 | 0.0034694 | 0.0052953 | Positive |
| | CD3G | 0.8522091 | 0.0035253 | 0.0053394 | Positive |
| | STAT4 | 0.8517455 | 0.0035625 | 0.0053668 | Positive |
| | TNFSF14 | 0.8484067 | 0.0038381 | 0.0056231 | Positive |
| | TLR1 | 0.8479869 | 0.0038737 | 0.0056293 | Positive |
| | UBE2T | 0.8459649 | 0.0040487 | 0.0058049 | Positive |
| | POLK | 0.8386417 | 0.0047275 | 0.0063276 | Positive |
| | BRIP1 | 0.8381076 | 0.0047799 | 0.0063603 | Positive |
| | IL26 | 0.8170625 | 0.0071733 | 0.0081403 | Positive |
| | IL17A | 0.8052876 | 0.0088147 | 0.0092264 | Positive |
| | CD80 | 0.8052343 | 0.0088226 | 0.0092292 | Positive |
| | TNFSF8 | 0.8038217 | 0.0090353 | 0.0093678 | Positive |
| | CCL4 | 0.7989072 | 0.0098021 | 0.0098881 | Positive |
| | CD209 | 0.9464572 | 0.000111 | 0.0006948 | Positive |
| | CD226 | 0.8807744 | 0.0017115 | 0.0034349 | Positive |
| | CXCL1 | 0.8978401 | 0.0010141 | 0.0025218 | Positive |
| | CYP7B1 | 0.9328796 | 0.0002415 | 0.0010628 | Positive |
| | FANCI | 0.9250662 | 0.0003522 | 0.0013281 | Positive |
| | ICOS | 0.9539927 | 6.57E-05 | 0.0004822 | Positive |
| | IFN-γ | 0.8780748 | 0.001846 | 0.0036114 | Positive |
| | PIK3CG | 0.956161 | 5.56E-05 | 0.0004454 | Positive |
| | REV3L | 0.9490468 | 9.35E-05 | 0.0006202 | Positive |

**Table 2 Genes related to CD274 in the co-expression networks (Continued)**

| Group | Gene | Pearson | *P*-value | FDR | Style |
|---|---|---|---|---|---|
| PP | EEF1G | -0.675341 | 0.002101 | 0.0037489 | Negative |
| | IL12B | 0.901116 | 3.36E-07 | 9.64E-06 | Positive |
| | CCL20 | 0.8556256 | 5.97E-06 | 7.67E-05 | Positive |
| | CCL7 | 0.7927733 | 8.72E-05 | 0.0004859 | Positive |
| | DDX58 | 0.7621884 | 0.0002362 | 0.0009399 | Positive |
| | FOS | 0.7618061 | 0.0002389 | 0.0009452 | Positive |
| | IL19 | 0.664267 | 0.0026406 | 0.0043512 | Positive |
| | IL1RAP | 0.6642463 | 0.0026417 | 0.0043512 | Positive |
| | IFIH1 | 0.6626158 | 0.00273 | 0.0044301 | Positive |
| | STAT1 | 0.6597544 | 0.0028909 | 0.0045988 | Positive |
| | CXCL10 | 0.6595464 | 0.0029029 | 0.0046016 | Positive |
| | CXCL11 | 0.6376404 | 0.004416 | 0.0060023 | Positive |
| | NOS2 | 0.628715 | 0.0051931 | 0.0066592 | Positive |
| | CXCL1 | 0.6198472 | 0.006072 | 0.0072939 | Positive |
| | CXCL2 | 0.6630916 | 0.002704 | 0.0044047 | Positive |
| | LOC100288533 | 0.6543663 | 0.003215 | 0.0048891 | Positive |
| | LOC642934 | 0.6395765 | 0.0042606 | 0.0058708 | Positive |

Abbreviations: FDR, false discovery rate; NN, normal controls; PP, psoriatic patients.

**Table 3 Degree and k-core of the top 20 genes**

| GENE | Degree | | K-core | | Dif-Degree | Dif-Kcore |
|---|---|---|---|---|---|---|
| | Case | Control | Case | Control | | |
| IFN-γ | 0 | 41 | 0 | 27 | -41 | -27 |
| IL26 | 0 | 38 | 0 | 26 | -38 | -26 |
| TLR3 | 2 | 51 | 2 | 27 | -49 | -25 |
| PRKCQ | 2 | 37 | 2 | 26 | -35 | -24 |
| IL17A | 4 | 43 | 3 | 26 | -39 | -23 |
| CDK1 | 10 | 53 | 7 | 27 | -43 | -20 |
| TLR4 | 13 | 55 | 7 | 27 | -42 | -20 |
| CD274 | 17 | 56 | 7 | 27 | -39 | -20 |
| CXCL1 | 11 | 44 | 7 | 27 | -33 | -20 |
| BRIP1 | 11 | 38 | 7 | 27 | -27 | -20 |
| TNFSF10 | 1 | 26 | 1 | 21 | -25 | -20 |
| CD209 | 9 | 45 | 7 | 26 | -36 | -19 |
| RRM2 | 13 | 45 | 8 | 27 | -32 | -19 |
| SOD2 | 14 | 55 | 9 | 27 | -41 | -18 |
| FANCI | 17 | 52 | 9 | 27 | -35 | -18 |
| SELE | 11 | 43 | 9 | 27 | -32 | -18 |
| IL19 | 18 | 41 | 9 | 27 | -23 | -18 |
| CCNB2 | 11 | 32 | 7 | 25 | -21 | -18 |
| CYP7B1 | 12 | 57 | 10 | 27 | -45 | -17 |
| CHEK1 | 11 | 52 | 10 | 27 | -41 | -17 |

Abbreviations: Dif-Degree, degree differences; Dif-Kcore, k-core differences.

keratinocytes. However, no such effect was observed under the challenge of LPS (Figure 4A,B). IL-17A, mainly secreted by TH17 and γδT cells, played an important role in psoriasis through acting on keratinocytes to induce a series of changes in epidermis [29]. Furthermore, poly (I: C), a TLR3 agonist, could attenuate the inflammatory reaction in psoriasis-like mouse models [30]. However, neither poly (I: C) nor IL-17A could change the expression of CD274 in epidermal keratinocytes (Figure 4A,B). Indirubin, a CDK1 inhibitor, was the main active ingredient of indigo naturalis, which belonged to a kind of traditional Chinese medicine. It has been demonstrated in clinical studies that external use of indigo naturalis could treat psoriasis mainly through its main active ingredient, indirubin. We further demonstrated that indirubin could increase the level of CD274 in epidermal keratinocytes (Figure 4A,B).
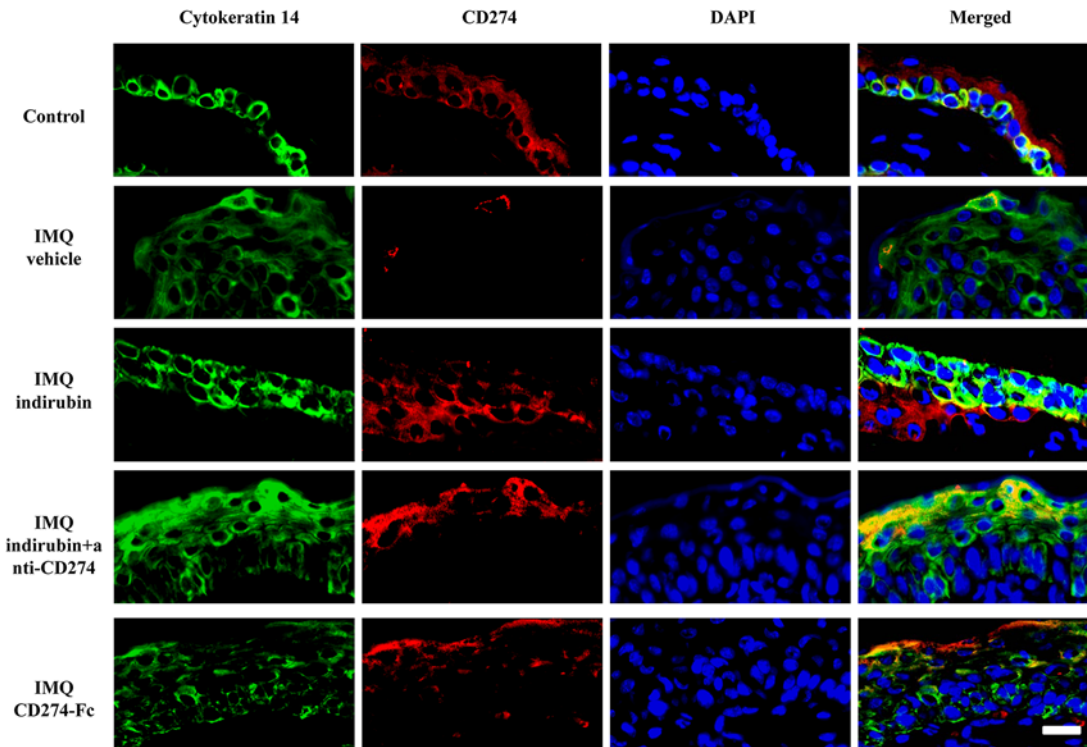
**Figure 5. Expression of CD274 on keratinocytes in imiquimod (IMQ)-induced mouse models**
Mice were divided into five groups, including the control, IMQ, IMQ+indirubin, IMQ+indirubin+anti-CD274 and IMQ+CD274-Fc groups. Daily IMQ treatment onto the dorsal skins for 6 days resulted in the generation of mouse psoriasis-like lesions. For certain groups, indirubin, recombinant mouse CD274-Fc chimera protein or anti-mouse CD274 antibody was administrated after IMQ treatment. Mice were killed on day 7 after the first day of IMQ application. Frozen skin sections were costained with cytokeratin 14 antibody (to mark keratinocytes) and CD274 antibody by means of immunofluorescence. Representative images from the five groups are presented (scale bar = 20 μm).

## Indirubin attenuates the symptoms of mice psoriasis in a CD274-dependent manner

Indirubin has been demonstrated to increase the expression of CD274 in human epidermal keratinocytes, we then detected whether CD274 was involved in the alleviative effect of indirubin on psoriasis. Indirubin significantly alleviated the symptom of imiquimod-induced mice through the observation of reduction of erythema, scaling and epidermal thickness (Figure 4C,D). However, CD274 antibody significantly attenuated the therapeutic effect, indicating the involvement of CD274 in the alleviative effect of indirubin. Furthermore, indirubin showed better therapeutic effect than CD274-Fc, indicating that indirubin was a multi-target agent, with CD274 as one of its effector molecules.

## CD274 expressions on keratinocytes in IMQ-induced mouse models

We finally detected the expression of CD274 on keratinocytes in IMQ-induced mouse models. CK-14 antibody was used to identify skin keratinocytes (Figure 5). We found an extensive expression of CD274 in CK14+ keratinocytes of normal skin, while CD274 was seldom expressed in keratinocytes of IMQ group. After the treatment of indirubin, CD274 expression in keratinocytes was significantly increased in IMQ-induced mouse models, while those effects of indirubin were attenuated by the inhibition of CD274 antibody. Those data indicated the connection between indirubin and CD274 in keratinocytes in psoriasis (Figure 5).

## Discussion

Co-expression analysis has been applied to identify drug acting mechanisms and discover the biomarker of diseases [31,32]. In the present study, we initially performed RNA sequencing on skins from psoriatic patients and healthy persons to construct co-expression networks. By the analysis of 2139 DEGs, 208 significantly differential GO terms and

44 significantly differential pathways were generated. We discovered that the functions of DEGs were mainly related to cell cycle, inflammatory, virus, immune response and metabolic process. The major pathways were cytokine–cytokine receptor interaction, Toll-like receptor signaling pathway, chemokine signaling pathway, cell cycle, metabolic pathways, ribosome, peroxisome, steroid biosynthesis and biosynthesis of unsaturated fatty acids. These results were in line with the previously reported studies [10,32] and indicated that the pathogenesis of psoriasis was related to immunity, inflammation and metabolism. Finally, five candidate genes (IFN-γ, TLR4, CDK1, IL-17A and TLR3) related to CD274 were identified by the co-expression network analysis. Interestingly, the five genes were also reported to play important roles in psoriasis [10].

To our knowledge, this is the first application of co-expression network analysis to evaluate the mechanisms of CD274 in skin. Up-regulation of CD274 in immune cells and several cancer cells was reported to be mostly dependent on TLR or IFN-γ [33]. However, we discovered in the present study that it was IFN-γ but not TLR increased the expression of CD274 in human epidermal keratinocytes. In addition, CDK1 was demonstrated to serve as targets to increase the expression of CD274 by indirubin, an inhibitor of CDK1. Although the other three candidate genes failed to regulate the expression of CD274 in epidermal keratinocytes, yet the three genes were not without connection with CD274. Except for keratinocytes, there were other cells in skins, such as fibroblasts and immune cells. The three genes might produce correlations with CD274 in other skin cells. For instance, TLR3 agonist was reported to up-regulated the expression levels of CD274 on DCs [34]. Here, we mainly focus on the expression of CD274 in keratinocytes.

IFN-γ, mainly produced by type 1 T-helper cells in the skin, was demonstrated to be increased in expression in psoriatic lesions [35]. Similar increases were reported in the expression of IFN-γ-regulated genes [36]. IFN-γ could increase the secretion of inflammatory cytokines in keratinocytes, thus enhancing the inflammatory reaction in skin [37]. In addition, IFN-γ could modulate the immune reaction through the up-regulation of CD274 in endothelial cells, oral keratinocytes and dermal fibroblasts [27,38]. Consistent with those studies, we found in our current study that IFN-γ could increase the level of CD274, thus producing immunosuppressive effect. Those data provide the reason for the failure of IFN-γ antibody in the treatment of psoriasis despite of the pro-inflammatory effect of IFN-γ as shown previously [39].

CDK1 is associated with cell proliferation. In psoriatic skin lesion, the expression level of CDK1 is up-regulated, which is in line with the over proliferation of keratinocytes in the epidermis. Indirubin, an inhibitor of CDK1, has been reported to treat psoriasis effectively in several studies [40,41]. It suppressed the hyper-proliferation of epidermal keratinocytes, which was correlated with CDC25B gene expression [42]. On the other hand, indirubin inhibited EGF-induced EGFR phosphorylation and decreases the expressions of various inflammatory cytokines [42,43]. VEGFR-mediated JAK/STAT3 signaling was blocked by indirubin in endothelial cells [44]. In addition, indirubin could decrease the number of CD4$^+$T cells and enhance the percentage of regulatory T cells in mice [45]. In our study, we also found the multi-target feature of indirubin, with the function related to CD274. Indirubin could increase the level of CD274 in epidermal keratinocytes and alleviate the symptom of psoriasis-like mice depending on CD274, indicating the value of indirubin in the treatment of psoriasis. The function of indirubin related to CD274 displayed its important role in immunoregulation.

In conclusion, in the present study, we have screened five candidate genes through RNA sequencing and found that IFN-γ and CDK1 were associated with CD274 through *in vitro* experiments. Further animal experiments demonstrated that CD274 could serve as one of the effector molecules of indirubin, a CDK1 inhibitor, against psoriasis. Although we have preliminarily uncovered the mechanisms of CD274 in epidermal keratinocytes through sequencing, the specific mechanisms in which indirubin and IFN-γ effect CD274 remain unclear. Studies are demanded for further exploration.

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## Author Contribution

Jinhong Hu designed the study protocol. Xiaochun Xue and Jianhua Wu wrote the manuscript. Xiaochun Xue, Jianhua Wu, Jianguo Xu, Junhui Li, Haiying Dai, Congshan Tao and Chao Li performed the experiments. Xiaochun Xue analyzed the collected data. Chao Li revised the manuscript.

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## Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Funding

## Abbreviations

CDK, cyclin-dependent kinase; DEG, differentially expressed gene; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FC, fold change; FDR, false discovery rate; IFN-$\gamma$, interferon-$\gamma$; IL, interleukin; IMQ, imiquimod; JAK/STAT3, janus kinase/signal transducers and activators of transcription 3; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; TLR, Toll-like receptor; VEGFR, vascular endothelial growth factor receptor.

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