

Downregulation of *RUNX3* moderates the frequency of Th17 and Th22 cells in patients with psoriasis

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Abstract. Psoriasis is a common chronic inflammatory and T cell-mediated skin disease. Runt-related transcription factor 3 (*RUNX3*), one of the runt-domain family of transcription factors, has been reported to be a susceptibility gene for psoriasis. The present study was designed to delineate the role and underlying mechanism of *RUNX3* involved in the differentiation of T helper (Th) 17 and Th22 cells in psoriasis. The results of the present study demonstrated that the expression of *RUNX3* increased significantly in CD4-positive (CD4⁺) T cells from patients with psoriasis, compared with healthy controls. In addition, increased levels of interleukin (IL)-6, IL-20 and IL-22, and increased frequencies of Th17 and Th22 cells were found in the patients with psoriasis patients, compared with the healthy controls. It was also found that the overexpression of *RUNX3* increased the levels of Th17- and Th22-associated cytokines in the CD4⁺ T cells from the healthy controls. However, the inhibition of *RUNX3* reduced the levels of the associated cytokines and decreased the frequency of Th17 and Th22 cells in the CD4⁺ T cells from the patients with psoriasis. Taken together, the present study suggested that *RUNX3* regulated the differentiation of Th17 and Th22 cells in psoriasis, providing a promising therapeutic strategy for the treatment of psoriasis.

Introduction

Psoriasis is a common chronic inflammatory skin disease with a prevalence of 0.2-3%, depending on the population of origin (1). It is well known that psoriasis is a T-cell mediated disease, with a disturbed balance in the interplay

of keratinocytes and immune cells (2). The pathogenesis of CD4-positive (CD4⁺) T helper (Th) lymphocytes is important in psoriasis progression. It has been reported that interleukin (IL-22), as well as IL-17, are involved in the pathogenesis of psoriasis (3,4). These two cytokines can be secreted by Th17 cells. In addition, a previous study identified T cells, which produced IL-22 alone, without IL-17 or interferon γ (IFN γ), and these were subsequently termed as Th22 cells (5). Th17 and Th22 cells are affected by the cytokine, IL-23, which is also involved in psoriasis (6). A further study demonstrated that the frequencies of Th17 and Th22 cells were significantly elevated in patients with psoriasis. These studies demonstrated that Th17 and Th22 cells are important in the pathogenesis of psoriasis (7).

An increasing number of studies have demonstrated the vital role of genetics in the development of psoriasis, and >30 loci have been identified as susceptibility genes in psoriasis (8,9). In a genome-wide association study, runt-related transcription factor 3 (*RUNX3*) was identified as a genetic risk factor for psoriasis (10). In addition, a previous study demonstrated that the expression of *RUNX3* was increased in patients with psoriasis patients, confirming this result (11). *RUNX3* is one of the runt-domain family of transcription factors, which has been reported to be a tumor suppressor gene, and regulates cell proliferation and apoptosis in several types of human tumor (12). *RUNX3* is commonly expressed in the spleen, peripheral blood, spinal cord cells, bone marrow, B cells and T cells (13). The inactivation of *RUNX3* is considered to be associated with the occurrence and development of various human diseases, including gastric and colon cancer (14,15) and other epithelial diseases (16). *RUNX3* is important in the function of the immune system. Studies have reported that *RUNX3*-deficient mice spontaneously develop two immunological abnormalities: Airway hypersensitivity and colitis (17,18). Following *RUNX3* knockdown in T cells, the target mice spontaneously developed asthma-like features, including elevated levels of IgA, IgE and IgG1, and the infiltration of eosinophils and lymphocytes in the lung (19). Furthermore, *RUNX3* is important in the differentiation of T cells (20). However, its role and underlying mechanism in the differentiation of Th17 and Th22 cells in psoriasis have not been investigated in detail.

In the present study, the expression level of *RUNX3* and the frequencies of Th17 and Th22 cells in psoriasis were determined. In particular, the role of *RUNX3* in regulating the

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Abbreviations: Ps, psoriasis; Th, T helper; IL, interleukin; IFN γ , interferon γ ; *RUNX3*, runt-related transcription factor 3

Key words: psoriasis, runt-related transcription factor 3, T helper 17, T helper 22

differentiation of CD4⁺T cells and the underlying mechanism in psoriasis were investigated. By the forced overexpression and knockdown of RUNX3, the present study determined the importance of RUNX3 in psoriasis through examining its effect on regulating the levels of Th17 and Th22 cells. Taken together, the results of the present study may identify a novel therapeutic target and provide a foundation for gene therapy in psoriasis.

Materials and methods

Patients. The study cohort in the present study comprised 32 patients with psoriasis (20 males and 12 females), as well as 30 healthy control individuals (18 males and 12 females). The age of the patients with psoriasis was between 20 and 48 years the healthy controls, between 22 and 50 years old. The patients were recruited from the Dermatological department of the First Affiliated Hospital, Xinxiang Medical University (Weihui, China) from July 2013 to June 2014. All patients were diagnosed by clinical features and disease activity was assessed by Psoriasis Area and Severity Index (21), patients were excluded if they had any of the following conditions: Diabetes mellitus; renal failure; history of neoplasm; major cardiovascular and cerebrovascular disease. The healthy controls were recruited as volunteers at the First Affiliated Hospital and Xinxiang Medical University (Weihui, China. The study was approved by the ethics committee of Xinxiang Medical University (approval no. 2014055) and written informed consent was provided by all participants.

Specimen collection. Fasting venous peripheral blood samples (10 ml) were collected from the patients with psoriasis and the healthy control individuals. Following centrifugation for 15 min at 335.4 x g and 4°C, the serum samples were stored at -80°C until further use. CD4⁺ T cells were isolated from each blood sample using a human CD4⁺ positive selection magnetic column (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The absolute counts of the CD4⁺ T cells were measured using flow cytometry. The purity of the CD4⁺ T cells was typically >90, according to the protocols, and the cells were cultured in human T cell culture AIM V Medium CTS at a density of 10⁵ at 5% CO₂ and 37°C (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were grown to 80% confluence.

RNA extraction and reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR). Total RNA was extracted from the cultured CD4⁺ T cells using TRIzol reagent and chloroform (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols and the following temperature protocol: 30°C for 10 min and 42°C for 30 min. The concentration and purity of the total RNA were detected using an ultraviolet (UV) spectrophotometer (BioSpectrometer; Eppendorf AG, Hamburg, Germany). cDNA was synthesized using M-MLV reverse transcriptase (Clontech Laboratories, Palo Alto, CA, USA). The mRNA was analyzed using an RT-qPCR mixture system containing cDNA templates, primers synthesized by Shanghai Sangon Biological Engineering and Technology & Services Co., Ltd.

(Shanghai, China), and Absolute SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Inc.). The PCR primers (Shanghai Sangon Biological Engineering and Technology & Services Co., Ltd., Shanghai, China) used were as follows: RUNX3 F 5'-ACCTGTCAACGGCCAGAAC-3', R 5'-TTC CAGTGAGGACAGGCCAAG-3'; β-actin F 5'-GGCGGC ACCACCATGTACCCT-3', R 5'-AGGGGCCGGACTCGT CATACT-3'. PCR cycling conditions were as follows: Enzyme activation 95°C 15 min per cycle, denaturation 95°C at 15 sec per 40 cycles and annealing/extension at 60°C for 60 sec using a LightCycler sequence detection system (480; Roche Diagnostics, Indianapolis, IN, USA). The gene mRNA expression levels were normalized to those of β-actin. Data were analyzed using the 2^{-ΔΔC_q} method (22). This experiment was performed three times.

Western blot analysis. Total protein was extracted from the CD4⁺ T cells using a radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) and quantified using a UV spectrophotometer. A total of 25 μg protein per lane separated by 10% sodium dodecyl sulfate-polyacrylamide gel (Thermo Fisher Scientific, Inc.) electrophoresis. Following being transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.) and blocked in Tris-buffered saline-Tween buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 5% nonfat dry milk for 30 min, the membranes were incubated for 16 h at 4°C with polyclonal rabbit anti-RUNX3 (cat. no. sc-30197; 1:1,000) or polyclonal rabbit anti-β-actin antibodies (cat. no. sc-7210; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by incubation with the corresponding horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (cat. no. sc-2004; 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature and washed twice with phosphate-buffered saline. A fluorescent Western blotting detection system (Thermo Fisher Scientific, Inc.) was used. Densitometry analysis was performed using MultiGauge 3.1 software (Thermo Fisher Scientific, Inc.). The protein expression levels were normalized to those of β-actin.

Flow cytometric analysis. The percentages of Th17 and Th22 in CD4⁺ T cells were detected using flow cytometric analysis, according to the manufacturer's protocols. Briefly, the cells at a density of 10⁵ were incubated with phorbol ester, ionomycin and monensin (all Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C. The cells were then stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD4 (cat. no. 550628; 1:100; BD Biosciences, San Diego, CA, USA), phycoerythrin (PE)-labeled mouse monoclonal anti-IL-17 (cat. no. 12-7178; 1:100; eBioscience, Inc., San Diego, CA, USA), and PE-labeled monoclonal mouse anti-IL-22 (cat. no. 12-7229-41; 1:100; eBioscience, Inc.), respectively, for 30 min at room temperature. The results were analyzed using CellQuest Pro 5.1 software (BD Biosciences, Franklin Lakes, NJ, USA).

CD4⁺ T cell transfection. For the transfection of the CD4⁺ T cells, RUNX3 small interfering (si)RNA was purchased from GenePharma (Shanghai, China), and lentiviral vectors containing the RUNX3 gene were constructed (GeneChem Co., Ltd., Shanghai, China). The CD4⁺ T cells were seeded at 10⁶ cells/well transfected with the RUNX3 lentiviral plasmid,

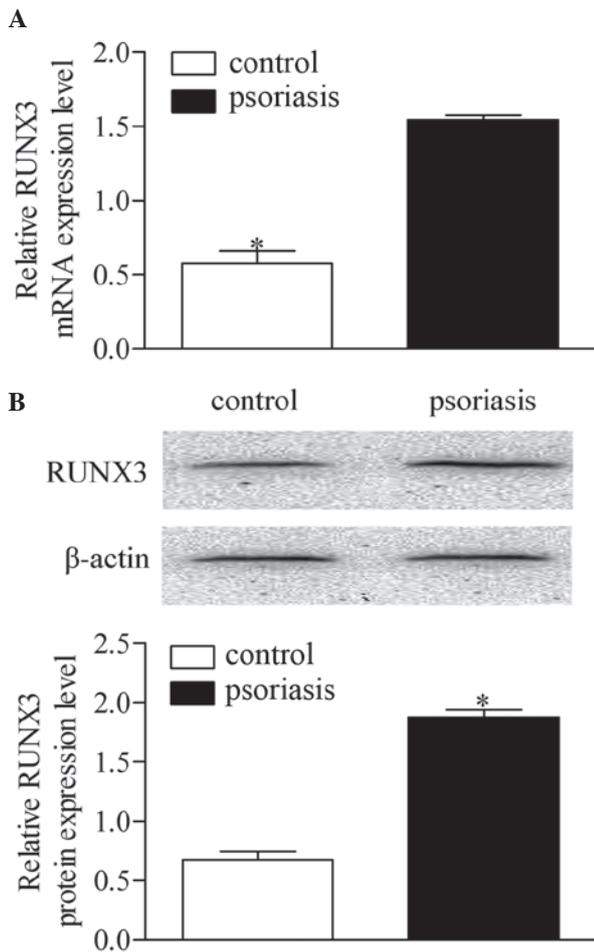


Figure 1. Expression of *RUNX3* in CD4⁺ T cells from patients with psoriasis and healthy controls. (A) mRNA expression levels of *RUNX3* were measured using reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression levels of *RUNX3* were measured using Western blot analysis. Data are presented as the mean \pm standard deviation of three experiments. * $P < 0.05$, vs. control group. *RUNX3*, runt-related transcription factor 3.

empty lentiviral vector, *RUNX3* siRNA and siRNA control, respectively, according to the instructions provided with the Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection at room temperature, the cells were collected for further analysis.

Enzyme-linked immunosorbent assay (ELISA). The cytokine concentrations were measured using corresponding quantification ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocols. Optical density values (OD values) were read at 450 nm using a microplate reader (SynergyH1; BioTek, Winooski, VT, USA) following the assay. The concentrations of cytokines were calculated, according to the corresponding OD value and concentration of the standard substance.

Statistical analysis. All data were processed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean \pm standard deviation. Statistical analysis was calculated using one-way analysis of variance followed by a Bonferroni test. $P \leq 0.05$ were considered to indicate a statistically significant difference.

Results

Expression of RUNX3 is increased in CD4⁺ T cells from patients with psoriasis. To determine the expression level of *RUNX3*, CD4⁺ T cells were isolated from blood samples from each patient with psoriasis and control subject. The mRNA expression of *RUNX3* was measured using RT-qPCR. As shown in Fig. 1A, the mRNA expression level of *RUNX3* was significantly increased in the patients with psoriasis, compared with the healthy controls. Western blot analysis showed that the protein level of *RUNX3* was also significantly elevated in the patients with psoriasis, compared with the healthy controls (Fig. 1B). These results revealed a notable upregulation of *RUNX3* in CD4⁺ T cells from patients with psoriasis, which is relevant to psoriasis development.

Th17 and Th22 cells are dysregulated in patients with psoriasis. To investigate the roles of Th17 and Th22 cells, the present study detected the serum levels of IL-6, IL-17, IL-20, IL-22 and IL-23 in the supernatant of CD4 T cells of patients with psoriasis. The statistical analyses revealed that the protein expression levels of IL-6, IL-20 and IL-22 were significantly higher in the patients with psoriasis, compared with the healthy controls. However, no significant differences were found in the concentrations of IL-17 and IL-23 between the psoriatic patients and control group (Fig. 2A). To further confirm the dysregulation of Th17 and Th22 cells, the present study evaluated the frequency of Th17 and Th22 cells using flow cytometric analysis (Fig. 2B and C). The results showed that the frequencies of the Th17 and Th22 cells increased significantly in the patients with psoriasis, compared with the healthy controls.

RUNX3 overexpression increases concentrations of cytokines associated with Th17 and Th22 in normal CD4⁺ T cells. The *RUNX3* lentiviral plasmid and empty lentiviral vector were transfected into CD4⁺ T cells from healthy controls to investigate the effect of *RUNX3* overexpression in normal CD4⁺ T cells. Following 48 h of transfection, the expression level of *RUNX3* was detected using Western blot analysis. The results showed that the expression of *RUNX3* increased significantly in the CD4⁺ T cells transfected with the *RUNX3* lentiviral plasmid, compared with the empty lentiviral vector-transfected control group (Fig. 3A). In addition, the concentrations of IL-6, IL-17, IL-20 and IL-22 increased significantly with *RUNX3* overexpression, compared with the control group. No significant differences were observed in the concentrations of IL-23 between groups (Fig. 3B).

RUNX3 knockdown inhibits the production of cytokines associated with Th17 and Th22 in CD4⁺ T cells from patients with psoriasis. The *RUNX3* siRNA and siRNA control were transfected into CD4⁺ T cells from patients with psoriasis to detect whether the inhibition of *RUNX3* can reverse immune dysfunction. The results of the Western blot analysis showed that the expression of *RUNX3* decreased significantly in the CD4⁺ T cells transfected with *RUNX3* siRNA, compared with the control group (Fig. 4A). Additionally, the concentrations of IL-6, IL-20 and IL-22 decreased significantly following *RUNX3* siRNA transfection, compared with the control group.

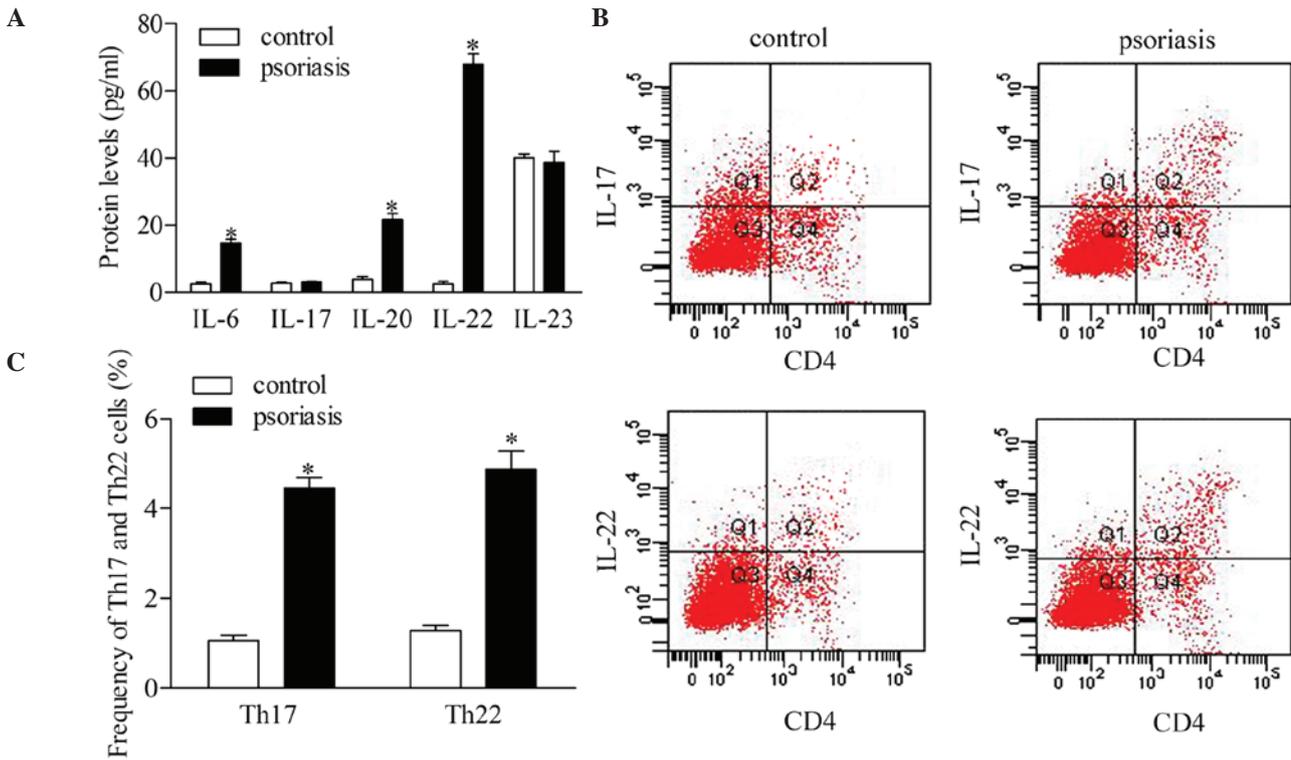


Figure 2. Comparison of Th17 and Th22 in CD4⁺ T cells from patients with psoriasis and healthy controls. (A) Expression levels of Th17 and Th22-associated cytokines were measured using an enzyme-linked immunosorbent assay. (B) Frequencies of Th17 and Th22 cells were detected using flow cytometry. (C) Percentages of Th17 and Th22 cells in patients with psoriasis and healthy controls. Data are presented as the mean ± standard deviation of three experiments. *P<0.05, vs. control group. Th, T helper; IL, interleukin.

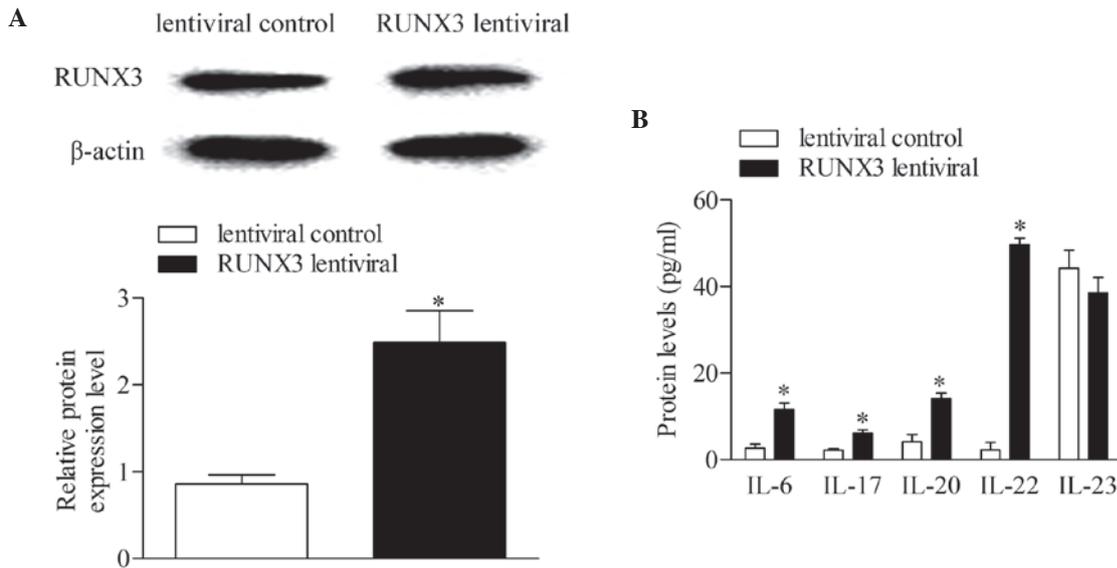


Figure 3. Overexpression of *RUNX3* regulates cytokines associated with Th17 and Th22 in CD4⁺ T cells from healthy controls. (A) Expression levels of *RUNX3* were measured using Western blot analysis. (B) Expression levels of Th17 and Th22-associated cytokines was measured using an enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation of three experiments. *P<0.05, vs. control group. *RUNX3*, runt-related transcription factor 3; Th, T helper; IL, interleukin.

No significant differences were observed in the concentrations of IL-17 and IL-23 between groups (Fig. 4B).

RUNX3 knockdown represses the frequency of Th17 and Th22 cells in CD4⁺ T cells from patients with psoriasis. To further investigate the effect of *RUNX3* inhibition on Th17 and Th22

cells in CD4⁺ T cells, the present study detected the frequencies of Th17 and Th22 cells from patients with psoriasis transfected with *RUNX3* siRNA and the siRNA control using flow cytometry. The results showed that the frequencies of Th17 and Th22 cells in the *RUNX3* siRNA transfection group were decreased significantly, compared with those in the control group (Fig. 5).

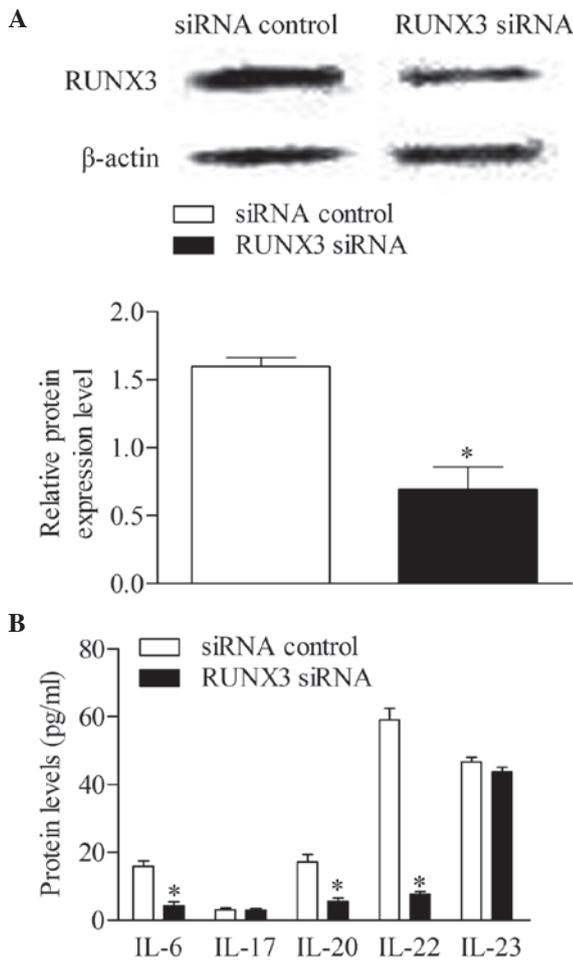


Figure 4. Inhibition of *RUNX3* regulates cytokines associated with Th17 and Th22 in CD4⁺ T cells from patients with psoriasis. (A) Expression levels of *RUNX3* were measured using Western blot analysis. (B) Expression levels of Th17 and Th22-associated cytokines were measured using an enzyme-linked immunosorbent assay. Data are presented as the mean \pm standard deviation of three experiments. * $P < 0.05$, vs. control group. *RUNX3*, runt-related transcription factor 3; Th, T helper; IL, interleukin; siRNA small interfering RNA.

Discussion

Psoriasis is well known as a T cell-dependent autoimmune disease of the joints and skin (23). Studies have suggested that there is a genetic contribution, and >30 risk loci for psoriasis have been identified (9). Genetic factors explain 68% of the variation in the susceptibility to psoriasis (24). Tsoi *et al* demonstrated that *RUNX3* may be a susceptibility gene for psoriasis (10). In addition, Apel *et al* further confirmed that *RUNX3* is a candidate for involvement in psoriasis and other T cell-mediated diseases (11). The present study showed that the expression level of *RUNX3* increased significantly in CD4⁺ T cells from patients with psoriasis, compared with the healthy controls, which confirmed that *RUNX3* is a susceptibility gene for psoriasis and was consistent with the previous reports.

CD4⁺ T cells are the most important element of adaptive systems and immune homeostasis. According to the local cytokine environment and the nature of the encountered stimulus itself, naïve CD4⁺ T cells are differentiated into various subsets of Th or regulatory T cells (25). One of the T cell

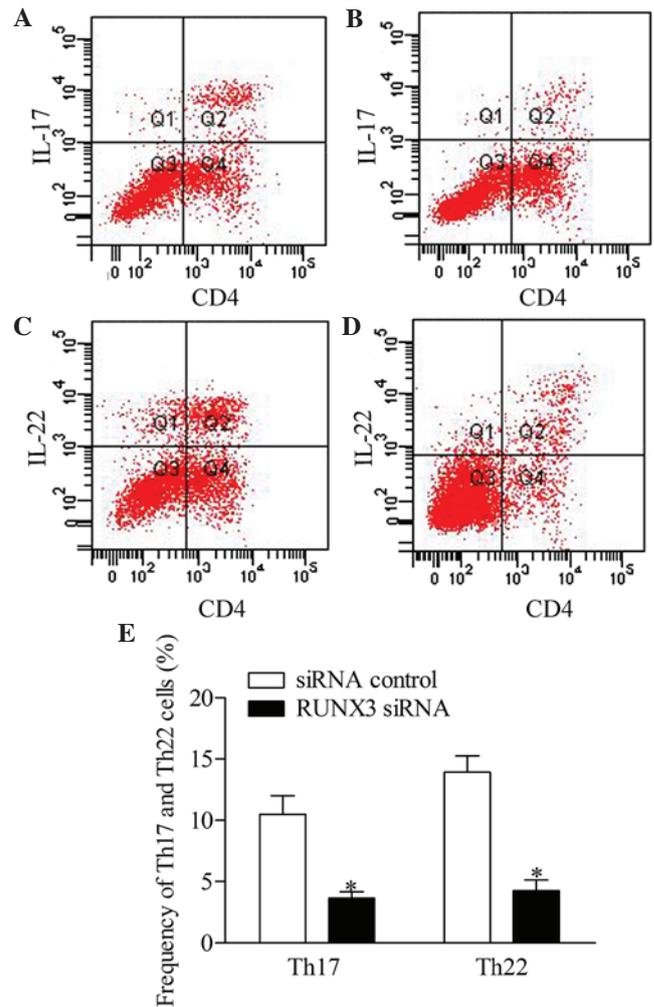


Figure 5. Inhibition of *RUNX3* regulates the frequencies of Th17 and Th22 in CD4⁺ T cells from patients with psoriasis. The frequencies of Th17 and Th22 cells in CD4⁺ T cells from patients with psoriasis transfected with *RUNX3* siRNA or an siRNA control was detected using flow cytometry. (A and B) Flow cytometric analysis of Th17 in CD4⁺ T cells from patients with psoriasis. The percentages of cells in the Q2 region represent the percentage of Th17 cells. (C and D) Flow cytometric analysis of Th22 in CD4⁺ T cells from patients with psoriasis. The percentages of cells in the Q2 region represent the percentage of Th22 cells. (E) Percentages of Th17 and Th22 cells in CD4⁺ T cells from patients with psoriasis transfected with *RUNX3* siRNA or an siRNA control. Data are presented as the mean \pm standard deviation of three experiments. * $P < 0.05$, vs. control group. *RUNX3*, runt-related transcription factor 3; Th, T helper; IL, interleukin; siRNA, small interfering RNA.

subsets, Th17 cells, is known for producing IL-17, IL-6, IL-21, IL-22 and tumor necrosis factor (TNF) (26). Th17 cells are differentiated from naïve CD4⁺ T cells under the effect of the transforming growth factor β (TGF- β), IL-6 and IL-1, whereas their expansion and survival are controlled predominantly by IL-23 (27,28). Previous investigation has shown that Th17 cells can be generated by IL-6, IL-23 and IL-1 β in the absence of TGF- β (29). It has been reported that cytokines produced by Th17 cells initiate parakeratosis and acanthosis hyperkeratosis (30). Th17 cells have been demonstrated to be involved in the development of T cell migration and activation, neutrophil and monocyte chemotaxis, and neovascularization (31).

Th17 cells were originally considered to be the predominant source in the production of the IL-22 cytokine, however studies have shown that certain CD4⁺ T cells secrete IL-22

alone, without IL-17, and these have subsequently been termed Th22 cells (32). Th22 cells also produce cytokines, including IL-26 and IL-13. Studies have demonstrated that TNF- α and IL-6 can promote the Th22 phenotype, with the assistance of plasmacytoid dendritic cells (33,34). The levels of Th17 and Th22 cells have been shown to be abnormal in patients with psoriasis (7). In the present study, it was found that the serum levels of IL-6, IL-20 and IL-22 were elevated in patients with psoriasis patients, compared with healthy control individuals. It is widely known that IL-6 contributes to the inflammatory and autoimmune processes of Th17 cells (31), and elevated serum levels of IL-6 have been observed in patients with psoriasis in numerous studies (35,36). IL-20 is produced by keratinocytes in the presence of IL-22, IL-17 and TNF- α , but not IFN- γ or IL-20 itself (37). The effect of IL-20 often appears in the later phases of psoriasis pathogenesis (38), and increased levels of IL-20 have been noted in lesional skin, as well as in the blood, in patients with psoriasis (39). In addition, the present study found that the frequencies of Th17 and Th22 cells were elevated in the patients with psoriasis, compared with the healthy controls. No significant differences were found in the concentrations of IL-17 and IL-23 between the patients with psoriasis and control group. This confirmed the involvement of Th17 and Th22 cells in the pathogenesis of psoriasis, and the increased level of IL-22 without elevated IL-17 level suggested that Th22 cells are more important in the inflammatory process.

As has been previously reported, *RUNX3* is important in the differentiation of T cells (20). Thus, the present study investigated the potential role and mechanism of *RUNX3* in regulating Th17 and Th22 cells in psoriasis. The effect of *RUNX3* overexpression on the expression levels of Th17 and Th22 cell-associated cytokines was compared with normal CD4⁺ T cells. The results showed that the levels of IL-6, IL-17, IL-20 and IL-22 increased significantly due to the overexpression of *RUNX3*, similar to the physiological features in psoriasis. Currently, the *RUNX3* gene has become important in potential therapeutic targets for psoriasis. Liang *et al.* (40) demonstrated that signaling transducer and activator of transcriptions 4, which is a central mediators in the generation of inflammation during protective immune responses and immune-mediated diseases, may be an effective therapeutic target for autoimmune diseases, including psoriasis (40). It has been reported that *RUNX3* is a therapeutic target for gastric cancer and can be involved in a various human diseases, including psoriasis, according to its function in immune dysfunction (41). In the present study, the effect of *RUNX3* knockdown was detected in CD4⁺ T cells from patients with psoriasis. The results showed that the inhibition of *RUNX3* suppressed the production of IL-6, IL-17, IL-20 and IL-22. In addition, the frequencies of Th17 and Th22 cells decreased significantly following transfection with *RUNX3* siRNA. These results suggested that the inhibition of *RUNX3* regulated Th17 and Th22 cells, leading to an improvement in immune dysfunction in psoriasis.

In conclusion, the present study demonstrated that *RUNX3* was upregulated in CD4⁺ T cells from patients with psoriasis. In addition, the importance of Th17 and Th22 cells in psoriasis was confirmed. Notably, the present study found that the inhibition of *RUNX3* affected the differentiation of CD4⁺ T cells,

which downregulated the frequencies of Th17 and Th22 cells in psoriasis. Consequently, targeting *RUNX3* may support a promising therapeutic strategy for the treatment of psoriasis.

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