'Psoriasis 1' reduces T-lymphocyte-mediated inflammation in patients with psoriasis by inhibiting vitamin D receptor-mediated STAT4 inactivation

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Abstract. Psoriasis is an immune-mediated dermatosis characterized by T-lymphocyte-mediated epidermal hyperplasia, for which there are currently no effective clinical treatments. 'Psoriasis 1' is a Chinese herbal medicine formulation that has been recently used extensively in China for treating patients with psoriasis. However, the molecular mechanism of action of this potent formulation has not yet been fully elucidated. In the present study, the effects of 'Psoriasis 1' on T ymphocytes in patients with psoriasis were investigated and the underlying molecular mechanism was discussed. Blood samples were collected from 40 patients with psoriasis. ELISA was employed to assess the levels of tumour necrosis factor-α, interferon-γ, interleukin (IL)-2, IL-6, transforming growth factor-β, IL-4, IL-12, IL-23 and vitamin D (VD). Western blot and quantitative PCR analyses were used to investigate the expression levels of VD receptor (VDR) and signal transducer and activator of transcription (STAT)4 in T lymphocytes. 'Psoriasis 1' was observed to significantly increase $CD4^+$ T cells. It also notably upregulated the mRNA and protein expression of VDR, and downregulated the mRNA and protein expression of STAT4. Moreover, the suppression of VDR was found to aggravate the inflammatory response, which was reversed by 'Psoriasis 1.' Thus, this formulation reportedly decreased the inflammation mediated by T lymphocytes in patients with psoriasis through inhibiting VDR-mediated STAT4 inactivation.

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Introduction

Psoriasis is a recalcitrant disease characterized by immune-mediated skin inflammation, manifesting clinically as scaly, itchy, well-defined red patches on the skin (1). Psoriasis is known to affect ~2% of the population worldwide (at least 200 million patients) and considered to be the most common autoimmune skin disease in adults (2). Several risk factors, such as age, bacterial infection, family history of psoriasis, smoking, alcohol consumption and physical inactivity, are known to trigger psoriasis (3-8). Psoriasis may cause severe disruption to the daily lives of the patients (9) and it is considered to be a serious global health concern.

Abnormal keratinocyte proliferation and inflammatory infiltration of the skin lesions are the major characteristics of psoriasis (10). Several studies have indicated that the imbalance between pro-inflammatory mediators, such as tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2 and IL-6, and anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-4, plays an important role in the underlying disease aetiology (11). Furthermore, extensive experimental and clinical evidence indicates that the accumulation of immune cells, particularly a large number of activated T cells, plays a key role at the initial stages of psoriasis (12,13). The initial T cells differentiate into Th17 cells ($CD4^+$ T cells), which in turn produce several cytokines, forming a large network headed by Th1- and Th17-type cytokines (14). The formation pathway of Th17 cells is also known as the IL-23/IL-17 inflammatory reaction axis (10,15), the activation of which can quickly involve the neutrophils and lead to the production of inflammatory mediators.

Signal transducer and activator of transcription (STAT) is a family of primary effectors that can generate numerous pro-inflammatory cytokines that are involved

in the development and progression of psoriasis (16). The STAT member STAT4 transports signals from IL-12, IL-23 and IFN-α/β to the nucleus, resulting in the activation of dendritic cells, differentiation of Th17 cells and production of IFN-γ (17). Moreover, the vitamin D receptor (VDR) is a ligand-activated transcription factor that belongs to the steroid/retinoid/thyroid hormone receptor superfamily (18,19). Vitamin D3 [1,25(OH)2D3], a metabolite of vitamin D (VD), is a ligand that binds to VDR and exerts biological activity (19). VDR is expressed in activated T lymphocytes and is involved in anti-proliferative and pro-differentiation pathways in monocytes/macrophages (20). The lack of VDR causes activation of the STAT3 signalling pathway and inhibition of the VDR to regulate high-level glucose-induced retinal ganglion cell damage through induction of the STAT3 pathway (21,22). Therefore, the VDR/STAT4 signalling pathway may be of value as a target for psoriasis treatment.

Although patients with psoriasis are prescribed various chemical formulations for treatment, the prevalence of this disease continues to increase. Although systemic biological drugs that successfully target specific inflammatory cytokines have been developed, they are not recommendable for long-term treatment due to their high cost (23). As compared with Western medicines, Chinese medicines and their active extracts are more cost-effective and low-toxicity, and may they therefore be more suitable and effective for psoriasis treatment (24,25). Triptolide is one of the effective components of *Tripterygium wilfordii* Hook. F., a traditional Chinese herb that exerts anticancer and immunosuppressive effects by suppressing the function of T cells, as well as the secretion of IL-1, IL-6, IL-8, TNF-α and prostaglandin E2 by human peripheral blood monocytes. 'Psoriasis 1' is composed of 13 Chinese herbs, and it has been successfully used for treating patients with psoriasis in China over the last few decades. Following treatment with 'Psoriasis 1,' several patients observe an improvement in skin lesions, and our earlier studies have also demonstrated that 'Psoriasis 1' plays a protective role in patients with psoriasis by inhibiting T-lymphocyte-mediated inflammation (26,27). However, the molecular mechanism of action of 'Psoriasis 1' has yet to be fully elucidated. Consequently, in the present study, T lymphocytes were collected from the peripheral blood of patients with psoriasis and the effects of 'Psoriasis 1' and underlying molecular mechanisms were investigated.

Materials and methods

Reagents and materials. Ficoll-Hypaque solution was supplied by Becton, Dickinson and Company. CD4 beads were purchased from Miltenyi Biotec GmbH. PE anti-human CD3 (cat. no. 300307-1), APC anti-human CD4 (cat. no. 317415-1), and FITC anti-human CD8 (cat. no. 344703-1) were obtained from BioLegend, Inc. RPMI-1640 medium and FBS were obtained from Invitrogen; Thermo Fisher Scientific, Inc. Anti-human TNF-α (cat. no. STA00D), IFN-γ (cat. no. SIF50), IL-2 (cat. no. S2050), IL-6 (cat. no. S6050), TGF-β (cat. no. SB100B), IL-4 (cat. no. S4050), IL-12 (cat. no. 10018-IL), IL-23 (cat. no. S2300B) and VD (cat. no. DVDBP0B) ELISA kits were supplied by R&D Systems, Inc. The bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Institute of Biotechnology. RNAiso Plus reagent, PrimeScript® RT reagent and SYBR® PremixEx Taq™ II (TliRNaseH Plus) were purchased from TaKaRa Biotechnology Co., Ltd. Protein extraction kit was supplied by KeyGen Biotech. Co., Ltd. Rabbit anti-VDR (cat. no. ab3508), anti-STAT4 (cat. no. ab235946), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. no. ab6721) were supplied by Abcam. NC-siRNAs (cat. no. SIC001) and VDR-siRNAs (cat. no. EHU010441) were purchased from RiboBio Co., Ltd. VD3 was purchased from Sigma-Aldrich; Merck KGaA.

Components of the 'Psoriasis 1' formulation. 'Psoriasis 1' was supplied by the First Affiliated Hospital of Guangzhou University (Guangzhou, China). Its components include *Rhizoma smilacis glabrae* (30 g), *Folium isatidis* (30 g), *Radix isatidis* (15 g), *Angelica sinensis* (15 g), *Hedyotis diffusa* (15 g), Szechuan lovage rhizome (12 g), plantain herb (12 g), *Fructus kochiae* (12 g), *Lobelia chinensis* (15 g), *Nidus vespae* (12 g), *Rhizoma alismatis* (12 g), *Cortex dictamni* (12 g) and *Radix glycyrrhizae* (6 g) (28).

Subjects and blood sampling schedule. A total of 40 patients with psoriasis (15 men and 25 women) and 40 healthy individuals (18 men and 22 women) were enrolled in the present study. The mean age of the patients was 43.2±11.6 years, and the mean disease duration was 5.8±1.3 years. The mean age of the healthy individuals was 41.6±12.7 years. Serum samples from the patients and healthy subjects were collected between March 2018 and October 2019. The baseline characteristics of the patients with psoriasis are summarized in Table I.

The inclusion criteria were as follows: i) Diagnostic criteria of psoriasis vulgaris and patients with typical lesions; ii) men and women aged between 18 and 60 years; iii) patients without serious heart, liver, kidney, and other chronic systemic or autoimmune diseases; iv) patients who had not used immunosuppressants, corticosteroids, vitamin A, or any other anti-psoriasis drugs within the last 4 weeks; v) patients who signed the informed consent form.

The exclusion criteria included the following: i) Patients with erythrodermic psoriasis, psoriasis affecting the joints, and pustular psoriasis; ii) patients with skin diseases other than psoriasis vulgaris; and iii) pregnant and lactating women.

All investigational procedures were approved by the Institutional Review Board of the First Affiliated Hospital of Guangzhou University [ZYYECK(2017)020 and ZYYECK(2019)030], and written informed consent was obtained from all the participants.

Preparation of peripheral blood mononuclear cells (PBMCs). Peripheral blood samples from each patient were obtained by venipuncture and collected in heparin tubes BD Vacutainer® CPT™ (BD Biosciences). Subsequently, PBMCs were isolated from the whole blood by Ficoll-Hypaque density gradient centrifugation as per the manufacturer's instructions. The PBMCs were carefully collected from the thin interface layer between the plasma and red blood cells, and then rinsed to remove the platelets. Finally, they were suspended in 1ml of PBS solution and the number of cells was counted. The viable

Table I. Baseline characteristics of patients with psoriasis vulgaris.

No.	Sex	Age (years)	PASI	Familiarity for psoriasis	Comorbidities
1	F	59	8.5	No	None
2	M	42	13.5	No	Hypercholesterolaemia
3	F	24	6.5	No	None
4	M	60	20.5	No	None
5	F	48	9	No	None
6	M	40	7.2	No	None
7	F	35	12.9	Aunts	None
8	F	42	4.2	No	None
9	F	32	14	No	None
10	F	49	10.5	No	Enteritis
11	M	22	18.4	No	None
12	M	23	15	No	None
13	F	25	7	No	None
14	M	58	5.5	No	Arterial hypertension
15	F	30	19	Grandmother	None
16	F	19	8	No	None
17	F	28	8.2	No	None
18	F	21	12.5	No	Arterial hypertension
19	M	52	11	No	None
20	M	30	10.5	No	None
21	F	26	10.3	No	None
22	F	30	12.7	No	None
23	F	35	18	No	None
24	M	37	8	No	None
25	M	45	9	No	Nephritis
26	F	28	15	No	Anaemia
27	F	49	18.2	No	None
28	F	41	12.4	No	None
29	M	52	18	No	None
30	F	53	20.6	No	Coronary heart disease
31	F	42	16.3	No	None
32	F	33	13.4	Both parents	None
33	M	25	12.8	No	None
34	M	24	11	No	None
35	M	29	10.9	No	None
36	M	55	11.7	No	Arterial hypertension
37	F	43	15.8	No	None
38	F	57	15	No	None
39	F	29	10.3	Aunts	None
40	F	37	10.8	No	None

F, female; M, male; PASI, Psoriasis Area and Severity Index.

and dead cells were distinguished by staining with 0.1% trypan blue at room temperature for 5 min.

T lymphocyte isolation, culture and identification. Under sterile conditions, T lymphocytes were segregated using MACS prep HLA T Cell Isolation kit (Milteny Biotec GmbH, 130-110-128) following the manufacturer's instructions. A total of $1x10^7$ cells were centrifuged at 300 x g for 10 min at 4˚C and suspended in 80 μl buffer. CD3 microbeads were added to the cells and incubated at 4˚C for 15 min. The suspension was centrifuged (800 x g for 5 min at 4˚C), cells were added to an MS column, and collected for $CD3^+$ T-cell identification. The purity of T lymphocytes was identified using PE-conjugated CD3 antibody (Thermo Fisher Scientific, Inc., cat. no. 17-0032-82) for 30 min by flow cytometry (FCM). $CD3^+$ T cells with a purity of >95% were

Table II. Primer sequences used for quantitative PCR assay.

ID	Sequence (5'-3')	Product length (bp)
GAPDH	Forward: TGTTCGTCATGGGTGTGAAC Reverse: ATGGCATGGACTGTGGTCAT	154
STAT4	Forward: TGTTGGCCCAATGGATTGAAA Reverse: GGAAACACGACCTAACTGTTCAT	119
IL-17A	Forward: TCACAATCCCACGAAATCCAG Reverse: GTGAGGTGGATCGGTTGTAG	144
TNF-α	Forward: CCTTCATCCACTCTCCCAC Reverse: CACATCTTTCACCCATCCCA	76
IL-22	Forward: CTGTGAGCTCTTTCCTTATGGG Reverse: GGTGCGGTTGGTGATATAGG	149
VDR	Forward: GTGGACATCGGCATGATGAAG Reverse: GGTCGTAGGTCTTATGGTGGG	181

STAT, signal transducer and activator of transcription; IL interleukin; TNF, tumour necrosis factor; VDR, vitamin D receptor.

cultured in RPMI-1640 medium with 10% FBS at 37˚C and 5% CO_2.

Cell treatment. i) To investigate the effects of 'Psoriasis 1' on T lymphocytes of patients with psoriasis, the T lymphocytes were divided into the following five groups: Control; low dose of 'Psoriasis 1' (1 mg/ml); medium dose of 'Psoriasis 1' (2 mg/ml); high dose of 'Psoriasis 1' (4 mg/ml), and positive control (10 μM triptolide; Cayman Chemical Company) groups. Various concentrations of 'Psoriasis 1' were prepared by dissolving in sterile saline and used to pretreat T lymphocytes for 24 h (29-32); the control group was pretreated with sterile saline for 24 h.

ii) The role of the VDR/STAT4 signaling pathway in the anti-psoriasis action of 'Psoriasis 1' was investigated. T lymphocytes were therefore divided into six groups: Normal control siRNA (NC-siRNA); VDR-siRNA; NC-siRNA plus 'Psoriasis 1' (4 mg/ml); VDR-siRNA plus 'Psoriasis 1' (4 mg/ml); NC-siRNA plus triptolide (10 μM); and VDR-siRNA plus triptolide (10 μM) groups.

iii) Furthermore, T cells were treated with VD3 (1 nM), high dose of 'Psoriasis 1' (4 mg/ml), or VD3 (1 nM) plus 'Psoriasis 1' (4 mg/ml) for 48 h.

siRNA transfection. T lymphocytes from patients with psoriasis were collected, and T lymphocytes ($1.8x10^6$ cells/well) were seeded onto 6-well plates. After 24 h, 10 μM NC-siRNA (Sigma-Aldrich; Merck KGaA, cat. no. SIC001) and 10 μM VDR-siRNA (Sigma-Aldrich; Merck KGaA, cat. no. EHU010441) were dissolved separately in Opti-MEM and then mixed gently with Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min for siRNA liposome formation. The mixture was added to the cells. After 48 h of incubation at 37˚C, subsequent experimentation was conducted.

FCM analyses. The changes in $CD4^+$ and $CD8^+$ T cells were investigated using FCM assay to detect the effects of 'Psoriasis 1' on T lymphocyte subsets. The cells ($1x10^5$) were incubated with fluorochrome-conjugated antibodies directed at the CD markers: PE anti-human CD3 and APC anti-human CD4 (or FITC anti-human CD8). Gated CD3-positive events were investigated for $CD4^+$ and $CD8^+$ T cells. FCM analyses were conducted using FACSCalibur (Becton, Dickinson and Company).

ELISA. The supernatant from T lymphocytes was collected, and the TNF-α, IFN-γ, IL-2, IL-6, TGF-β, IL-4, IL-12, IL-23 and VD levels were evaluated using ELISA kits as per the manufacturer's instructions (R&D Systems, Inc.). Undiluted supernatant medium was incubated for 2 h at room temperature in a 96-well plate. Then, diluted biotin-labelled antibody mixture (100 μl) was added into each well and incubated for 1 h. The diluted streptavidin-HRP-conjugated secondary antibodies were added and incubated for 60 min. Finally, 100 μl of substrate solution was added into each well. A microplate reader (Thermo Fisher Scientific, Inc.) was used to read the plate.

Reverse transcription-quantitative PCR (RT-qPCR) assay. To extract the total RNA from T lymphocytes ($1x10^6$), RNAiso Plus reagent was used as per the manufacturer's protocol (TaKaRa Biotechnology Co., Ltd.). To synthesize cDNA, the PrimeScript® RT reagent kit was used as per the manufacturer's instructions (TaKaRa Biotechnology Co., Ltd.) in a TC-512 PCR system (Techne Ltd.). TransScript® Top Green qPCR SuperMix in an ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform qPCR, using GAPDH for normalization. The thermocycling conditions were as follows: 94˚C for 34 sec followed by 40 cycles of 94˚C for 5 sec, 60˚C for 15 sec and 72˚C for 10 sec. The pre-primers and post-primers of VDR and STAT4 are shown in Table II. GAPDH was used as an internal control, and the expression of mRNA was calculated using a 7900 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and quantified using the $2^{-\Delta\Delta Cq}$ method (24).

Western blot assay. Ice-cold RIPA buffer (Sigma-Aldrich; Merck KGaA, cat. no. R0278) supplemented with 1 mM PMSF was used to extract total protein (20 μg) from T lymphocytes. A BCA protein assay kit was used for measuring the protein concentration. Samples were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 10% (w/v) non-fat dried milk and incubated overnight at 4˚C using primary antibodies against VDR (1:1,000) and STAT4 (1:1,000). After washing with PBS for 3 times, the membranes were incubated with a secondary antibody (1:2,000, cat. no. ab6721; Abcam) at room temperature for 1 h. The expressions of VDR and STAT4 were visualized using ECL Plus chemiluminescence reagent kit (Beyotime Institute of Biotechnology). Finally, a ChemiDoc XRS bioimaging system (Bio-Rad Laboratories, Inc.) was used for imaging the protein bands, and the protein expressions were normalized using GAPDH.

Cell counting kit-8 (CCK-8) assay. The treated cells ($1x10^4$) were seeded onto a 96-well plate. Then, 10 μl of CCK-8 was added to each well according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.) following incubation for 1 h.

High-performance liquid chromatography (HPLC). A C18 reverse-phase column (5 μm, Waters Corporation) was used for the investigation. The mobile phase was composed of acetonitrile and 0.1% trifluoroacetic solution, and HPLC was conducted at room temperature at a flow rate of 1.0 ml/min. The detection was performed at 280 nm. The HPLC was performed using RSLCnano system (Thermo Fisher Scientific, Inc.).

Statistical analysis. SPSS 18.0 statistical software (SPSS, Inc.) was used to perform statistical analysis. All values are presented as mean ± standard deviation. An unpaired Student's t-test was used for comparisons between two groups. One-way analysis of variance followed by Tukey's post hoc test were used to evaluate the differences between multiple independent groups, followed by the least significance difference test. $P<0.05$ or $P<0.01$ was considered to indicate statistically significant differences.

Results

'Psoriasis 1' increases CD4$^+$ T lymphocyte subsets. First, FCM analysis was used to detect the CD4$^+$ T and CD8$^+$ T cells in healthy individuals and patients with psoriasis. The numbers of CD4$^+$ T cells were found to be significantly lower in the peripheral blood of psoriatic patients compared with those in healthy controls, whereas the numbers of CD8$^+$ T cells exhibited no obvious changes (Fig. 1A). Next, the psoriatic patients were treated with different doses of 'Psoriasis 1' to investigate its effects on the distribution of T lymphocyte subsets. As shown in Fig. 1B and C, 'Psoriasis 1' treatment significantly increased the percentage of CD4$^+$ T cells compared with the control group, but no notable changes were observed in CD8$^+$ T cells. The numbers of Th1, Th2 and regulatory T cells (Tregs) were also assessed, and the percentages of Th2 and Tregs were also recorded corresponding to those of CD4$^+$ T cells (Fig. S1). Moreover, a high dose of 'Psoriasis 1' exerted similar effects as those of triptolide on the distribution of T lymphocyte subsets (Fig. 1B and C). CCK-8 assay was also performed to examine the viability of cells on treatment with 'Psoriasis 1' or triptolide, and it was observed that a high concentration of 'Psoriasis 1' or triptolide reduced cell proliferation (Fig. S2A). These results indicate that 'Psoriasis 1' affected the distribution of T lymphocyte subsets in patients with psoriasis.

'Psoriasis 1' inhibits the inflammatory response of T lymphocytes. ELISA was used to examine the inflammation mediated by T lymphocytes. A comparison between the control and 'Psoriasis 1' or triptolide groups is shown in Fig. 2A-F. The levels of inflammatory factors, such as TNF-α, IFN-γ, IL-2 and IL-6, were found to be significantly decreased following 'Psoriasis 1' treatment, and those of anti-inflammatory mediators, such as TGF-β and IL-4, were recorded to be notably higher in the 'Psoriasis 1' and triptolide groups. Moreover, both 'Psoriasis 1' and triptolide were found to significantly reduce the expression of IL-23 and IL-17, indicating that 'Psoriasis 1' and triptolide inhibit the activation of the IL-23/IL-17 axis (Fig. 2G and H). Moreover, 'Psoriasis 1' and triptolide reduced the expression of VD (Fig. 2I).

'Psoriasis 1' suppresses VDR-mediated STAT4 signalling. As VD functions are primarily activated by its nuclear receptor, VDR (33), the effects of 'Psoriasis 1' on the mRNA and protein levels of VDR and STAT4 were investigated. The findings indicated that 'Psoriasis 1' and triptolide notably upregulated the VDR mRNA and protein expression (Fig. 3A and B). Moreover, 'Psoriasis 1' and triptolide markedly downregulated the STAT4 mRNA and protein expression (Fig. 3A and B). Furthermore, a high dose of 'Psoriasis 1' exerted a similar therapeutic effect as that of triptolide. These findings suggest that 'Psoriasis 1' and triptolide suppress VDR-mediated STAT4 signalling. The STAT3 level was also measured, and no notable difference among groups was observed (data not shown). Furthermore, ELISA results indicated that the IL-17A, TNF-α and IL-22 levels decreased with 'Psoriasis 1' treatment in a dose-dependent manner (Fig. 3C).

VDR mediates 'Psoriasis 1'-induced regulation of CD4$^+$ T lymphocyte subsets in patients with psoriasis. To investigate the role of VDR on the effects of 'Psoriasis 1' against psoriasis, VDR-siRNA was used to reduce VDR expression. Western blot assay was also employed to confirm the transfection efficacy of VDR siRNAs, and we found that VDR expression was significantly reduced in the VDR siRNA-2 and VDR siRNA-3 groups compared to that in the NC-siRNA group, particularly the VDR siRNA-3 group (Fig. 4A). Therefore, VDR siRNA-3 was selected as it had the strongest knockdown effect. Next, compared with NC-siRNA, the percentage of CD4$^+$ T cells was found to be significantly reduced by VDR-siRNA. Both 'Psoriasis 1' and triptolide were observed to notably upregulate the percentage of CD4$^+$ T cells in the VDR-siRNA group compared with the NC-siRNA group (Fig. 4B). However, the VDR suppression by siRNA markedly reversed the effects of 'Psoriasis 1' or triptolide on CD4$^+$

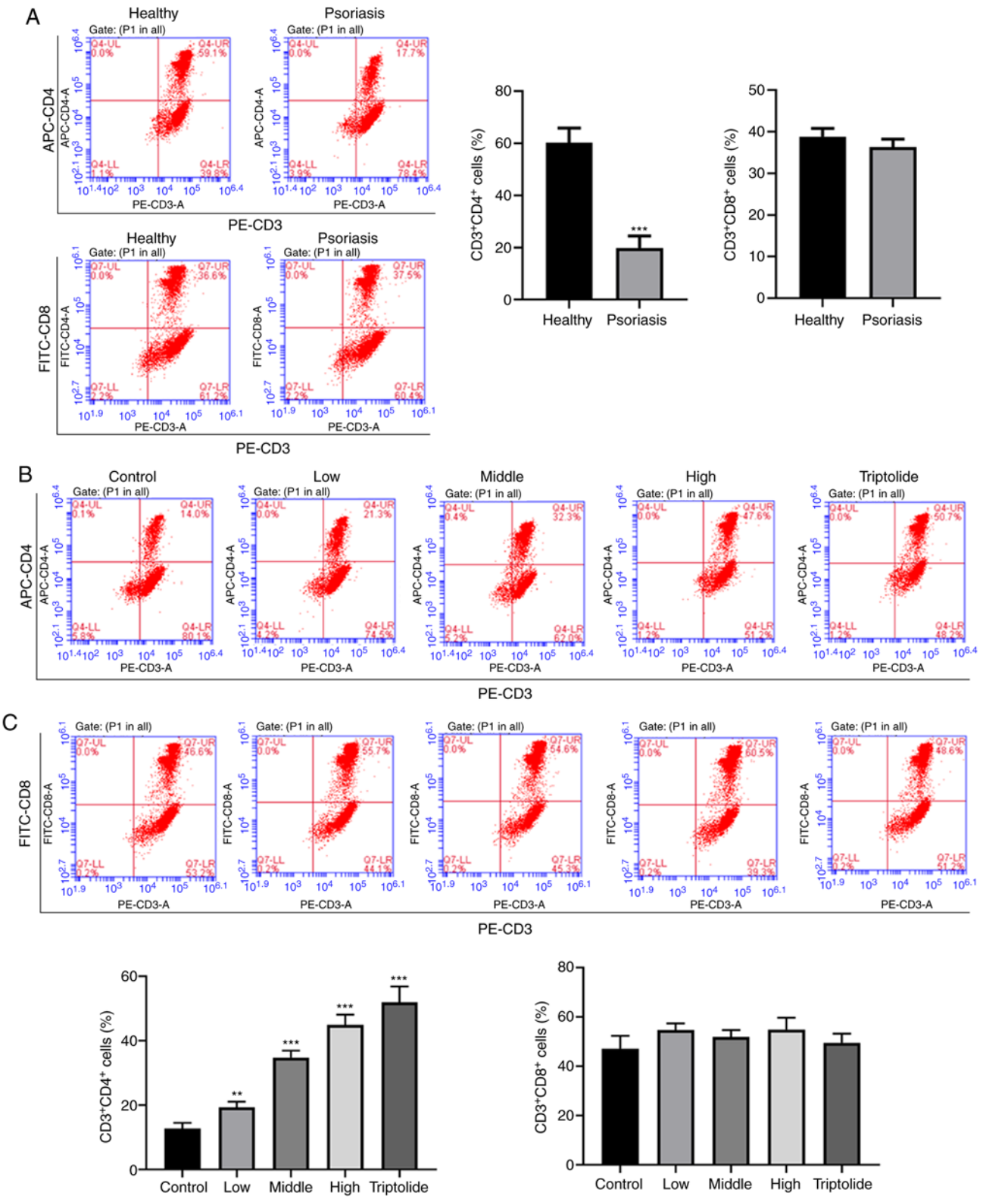

Figure 1. 'Psoriasis 1' affects the distribution of T-lymphocyte subsets. (A) T lymphocytes were isolated from healthy subjects and patients with psoriasis and flow cytometry was performed to identify the subsets of T lymphocytes. The percentages of $CD4^+$ and $CD8^+$ T cells in healthy individuals and patients with psoriasis are shown. $^{***}P<0.001$ vs. healthy. n=5. (B and C) Effect of 'Psoriasis 1' on the percentage of $CD4^+$ and $CD8^+$ T cells. $^{**}P<0.01$ and $^{***}P<0.001$ vs. control group. n=5. Data are presented as mean ± standard deviation.

T cells. Moreover, the percentage of $CD8^+$ T cells did not differ significantly among the groups (Fig. 4C). Cell viability was investigated after 'Psoriasis 1' or VDR siRNA treatment, and the inhibition of VDR was found to reverse the decrease in cell proliferation induced by high 'Psoriasis 1' concentration (Fig. S2B). Therefore, these outcomes indicate that the VDR signalling pathway is involved in the regulatory effect of 'Psoriasis 1' on T lymphocyte subsets.

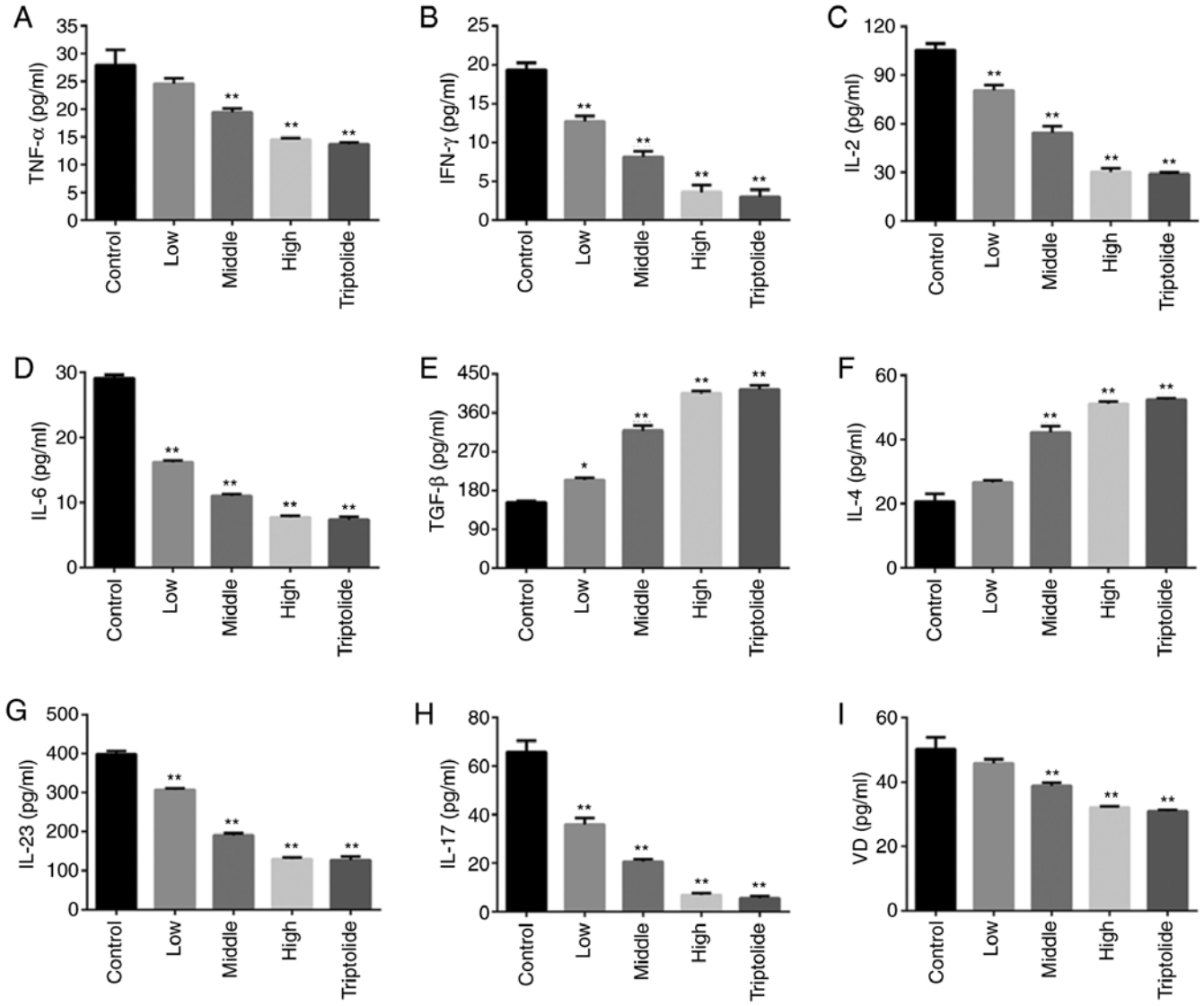

Figure 2. 'Psoriasis 1' inhibits inflammatory response in T lymphocytes. Effects of 'Psoriasis 1' at different doses and triptolide on the levels of (A) TNF-α, (B) IFN-γ, (C) IL-2, (D) IL-6, (E) TGF-β, (F) IL-4, (G) IL-23, (H) IL-17 and (I) VD in T lymphocytes. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 vs. control group. n=4. TNF, tumour necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; VD, vitamin D.

VDR regulates 'Psoriasis 1'-induced T-lymphocyte mediated inflammation. To assess the effects of VD and VDR on T lymphocyte inflammation, VD3 alone or in combination with 'Psoriasis 1' was added and cytokine expression was measured. The effects of VD3 alone were similar to those of 'Psoriasis 1' and are shown in Fig. S3; a combination of the two exerted no synergistic effect, indicating that 'Psoriasis 1' affected VDR expression, resulting in modified cytokine expression. Moreover, the inflammation mediated by T lymphocytes was examined following inhibition of VDR; compared with NC-siRNA, VDR-siRNA was found to significantly increase the levels of inflammatory factors, including TNF-α, IFN-γ, IL-2 and IL-6, which were notably reduced by a high dose of 'Psoriasis 1' or triptolide, as shown in Fig. 5A-D. VDR-siRNA markedly reduced the levels of anti-inflammatory mediators, including TGF-β and IL-4, which were augmented by a high dose of 'Psoriasis 1' or triptolide (Fig. 5E and F). In addition, the effects of 'Psoriasis 1' on the IL-23/IL-17 axis and VD level after VDR-siRNA transfection were examined using ELISA, and both 'Psoriasis 1' and triptolide were found to reverse the promoting effects of VDR siRNA on IL-23, IL-17 and VD levels (Fig. 5G-I). These findings indicated that 'Psoriasis 1' may inhibit the inflammatory response by way of the VDR signalling pathway. Furthermore, to identify the ingredient of 'Psoriasis 1' that exerted the most prominent anti-inflammatory effect, HPLC was performed and revealed that four components of 'Psoriasis 1,' namely caffeic acid, liquiritin, quercetin and flavone, may be the active components that possess the most prominent therapeutic properties (Fig. S4).

'Psoriasis 1' inhibits the VDR/STAT4 signalling pathway following VDR knockdown. As shown in Fig. 6A, compared with the NC-siRNA group, VDR-siRNA was observed to significantly downregulate the mRNA and protein levels of VDR; However, high doses of 'Psoriasis 1' and triptolide significantly upregulated the mRNA and protein expressions

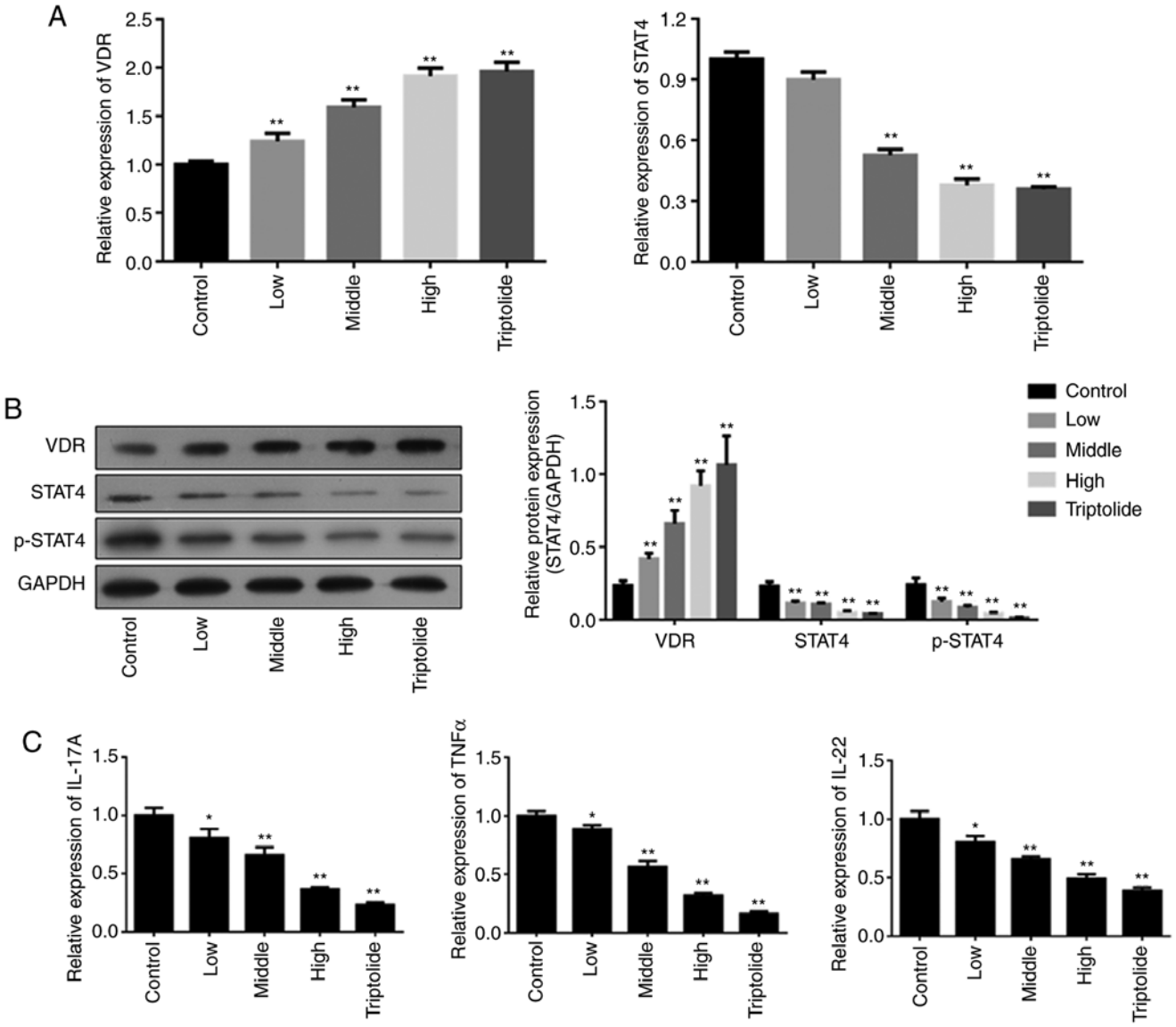

Figure 3. 'Psoriasis 1' inhibits VDR-mediated STAT4 inactivation. (A and B) Effects of different doses of 'Psoriasis 1' and triptolide on the mRNA and protein expressions of VDR and STAT4 in T lymphocytes. (C) ELISA was performed to confirm the levels of IL-17A, TNF-α and IL-22. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 vs. control group. n=5. VDR, vitamin D receptor; STAT, signal transducer and activator of transcription; IL interleukin; TNF, tumour necrosis factor.

of VDR. Furthermore, compared with the NC-siRNA group, VDR-siRNA markedly upregulated the mRNA and protein levels of STAT4, which were reversed by high dose of 'Psoriasis 1' or triptolide (Fig. 6B).

Discussion

Psoriasis, an immune-mediated disease, is characterized by T-lymphocyte-driven epidermal hyperplasia, which is easily recurrent and refractory to treatment, severely affecting the physical and mental health of the patients (10,34). Earlier epidemiological studies have reported an increased risk of certain diseases among patients with psoriasis, such as stroke, thromboembolism, coronary heart disease and certain types of cancer. However, a significant number of patients do not respond satisfactorily to the currently available clinical treatments (24). 'Psoriasis 1' was recently demonstrated to be successful in treating patients with psoriasis in China (28). Therefore, the effects of 'Psoriasis 1' on T lymphocytes in patients with psoriasis and the possible underlying molecular mechanism were investigated in the present study.

A negative correlation between the percentage of $CD4^+$ T cells and the severity of psoriasis has been recorded in earlier studies (35,36). The percentage of $CD4^+$ T cells was observed to be significantly lower in psoriatic patients compared with that in healthy controls and 'Psoriasis 1' was reported to markedly increase the percentage of $CD4^+$ T cells, indicating that 'Psoriasis 1' may alleviate psoriasis. HPLC demonstrated that four components of 'Psoriasis 1' may be the active components possessing the most prominent therapeutic properties.

The typical characteristics of psoriasis are abnormal keratinocyte proliferation and inflammatory cell infiltration (10,15). The effects of 'Psoriasis 1' on the inflammatory response in

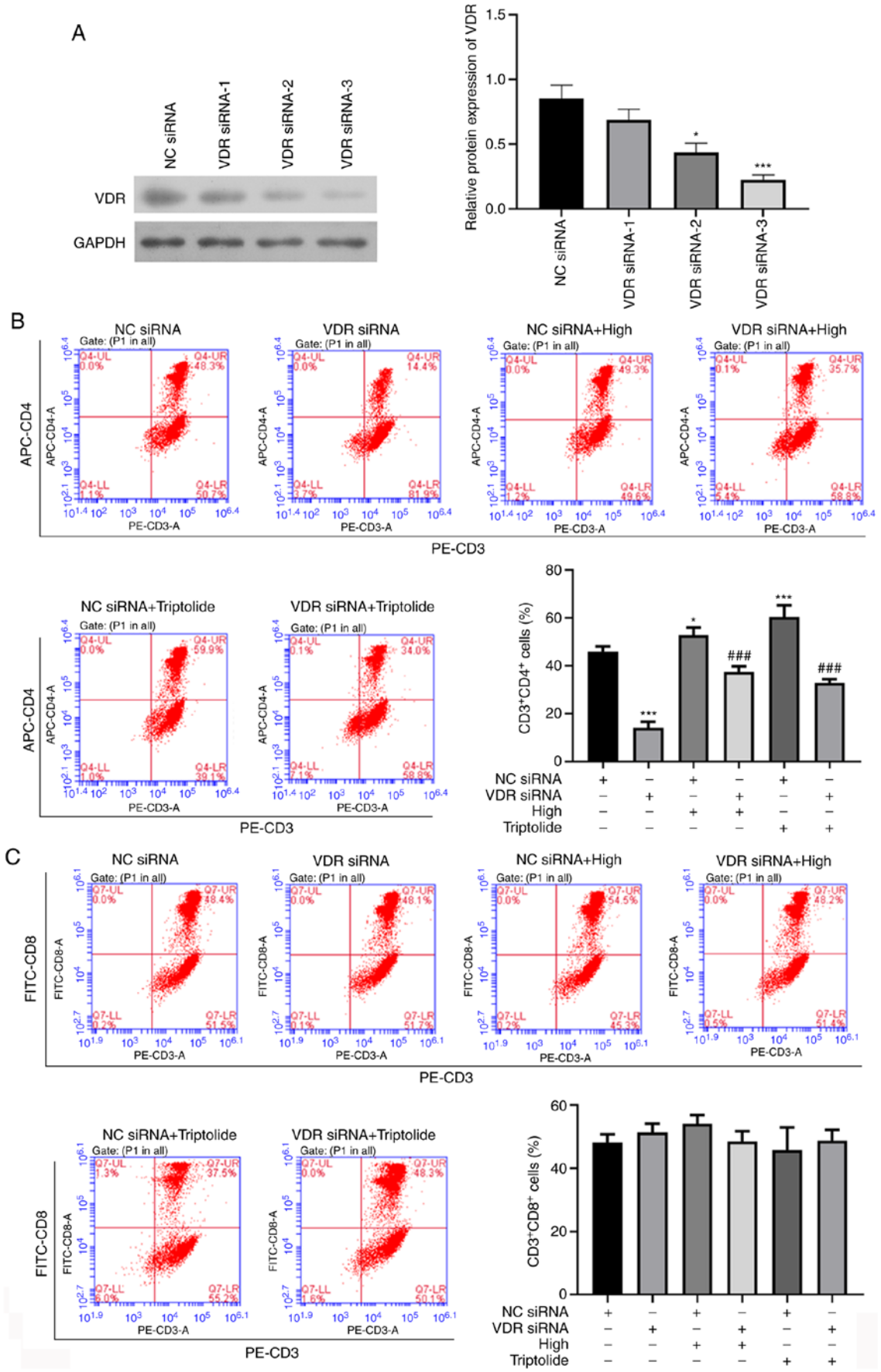

Figure 4. 'Psoriasis 1' affected the T lymphocyte subsets following VDR-siRNA transfection. T lymphocytes were isolated from patients with psoriasis and transfected with NC or VDR siRNA. (A) The transfection efficacy of VDR by siRNAs was identified through western blot assay. (B) Effect of high-dose 'Psoriasis 1' and triptolide on the ratio of $CD4^+$ T cells after VDR-siRNA transfection. (C) Effect of high-dose 'Psoriasis 1' and triptolide on the ratio of $CD8^+$ T cells after VDR-siRNA transfection. Data are presented as mean ± standard deviation. *P<0.05 and ***P<0.001 vs. NC-siRNA group; $^{###}$P<0.001 vs. VDR-siRNA group. n=5. VDR, vitamin D receptor.

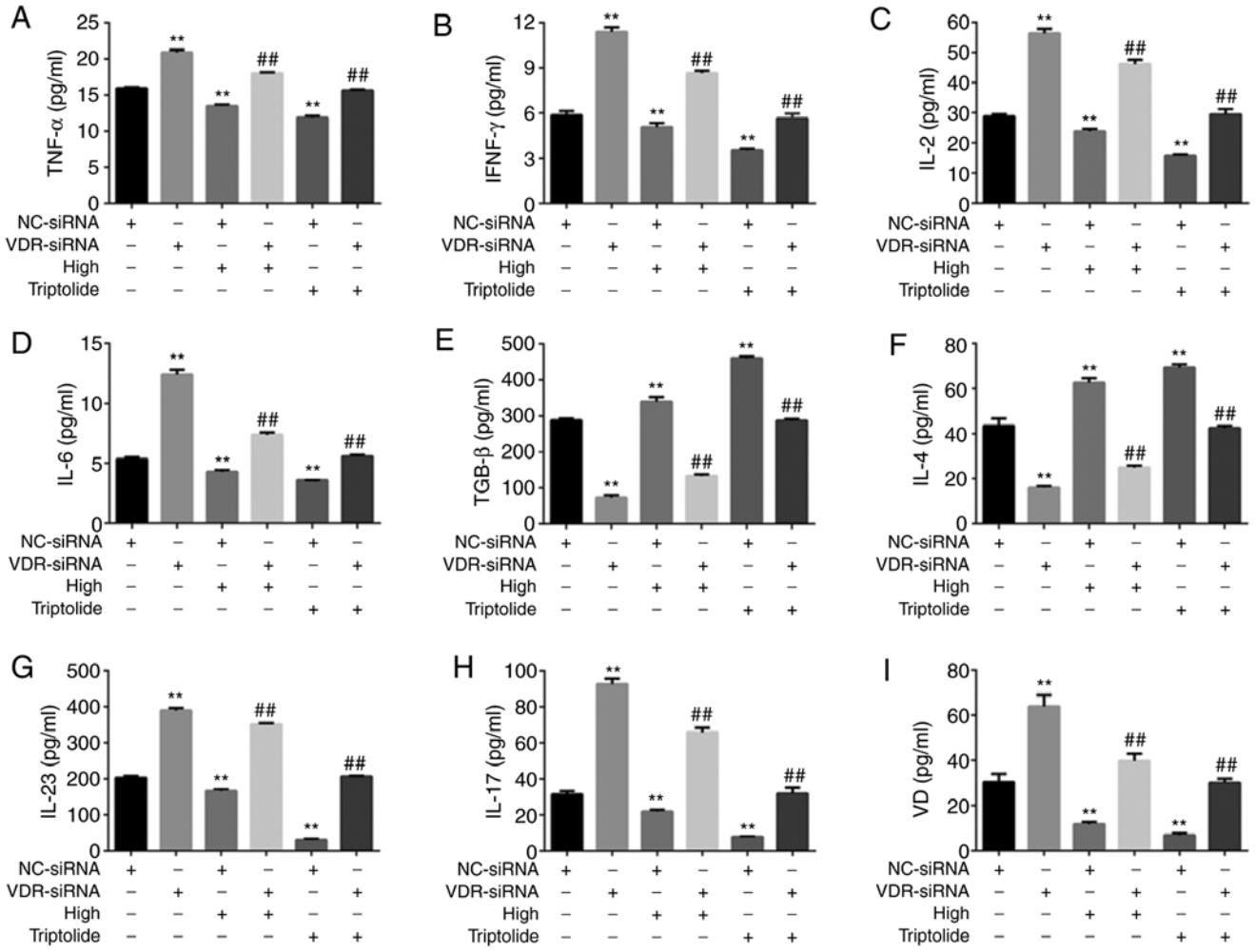

Figure 5. 'Psoriasis 1' inhibited inflammatory response following VDR-siRNA transfection. T lymphocytes were isolated from patients with psoriasis and transfected with NC or VDR siRNA. Effects of high-dose 'Psoriasis 1' and triptolide on the levels of (A) TNF-α, (B) IFN-γ, (C) IL-2, (D) IL-6, (E) TGF-β, (F) IL-4, (G) IL-23, (H) IL-17 and (I) VD in T lymphocytes after VDR-siRNA transfection. Data are presented as mean ± standard deviation. **P<0.01 vs. NC-siRNA group; $^{\#\#}$P<0.01 vs. VDR-siRNA group. n=5. VD, vitamin D; VDR, VD receptor; IL interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor.

T lymphocytes were examined, and 'Psoriasis 1' was observed to significantly decrease the levels of pro-inflammatory mediators, such as TNF-α, IFN-γ, IL-2 and IL-6, and increase the levels of anti-inflammatory cytokines, such as TGF-β and IL-4. Moreover, IL-23 promotes the proliferation of Th17 cells and regulation of immune response (37), whereas IL-17, a pro-inflammatory cytokine released by activated T cells, triggers the inflammation cascade (38). The IL-23/IL-17 axis participates in autoimmune disorders, which indicates a new direction for psoriasis treatment (12). In the present study, 'Psoriasis 1' was shown to inhibit the activation of the IL-23/IL-17 axis by downregulating the expression of IL-17A and IL-23. 'Psoriasis 1' was also found to reduce the VD level. These findings indicate that 'Psoriasis 1' inhibits inflammatory response in T lymphocytes.

VDR has been reported to inhibit psoriasis-like skin inflammation by suppressing the STAT signalling pathways (39). 'Psoriasis 1' has been reported to reduce psoriasis-like skin inflammation by inhibiting VDR-mediated nuclear NF-κB and STAT signalling pathways, including the downregulation of STAT4 and pSTAT4 (40). Similar to previous reports, the findings of the present study also revealed that the expression of STAT4 and pSTAT4 decreased in a dose-dependent manner with 'Psoriasis 1' treatment. The effects of the STAT4/VDR signalling pathway on the actions of 'Psoriasis 1' against T-lymphocyte-mediated inflammation were further evaluated by VDR-siRNA experiments. The inhibition of the VDR protein and activation of the STAT4 protein achieved by VDR-siRNA markedly reduced the percentage of $CD4^+$ T cells and further intensified the inflammatory response in T lymphocytes. In addition, VDR silencing inhibited 'Psoriasis 1' that increased the percentage of $CD4^+$ T cells, induced T-lymphocyte mediated inflammation and suppressed the activation of STAT4. Therefore, these outcomes demonstrated that 'Psoriasis 1' inhibited inflammatory responses in T lymphocytes via the VDR/STAT4 signalling pathway. The mRNA expression of VDR was highly dependent on cell differentiation state, while VDR expression was found to be regulated by Erk and PI3K signalling in a myeloid leukaemia cell line wherein p38 activity appeared irrelevant (41,42), suggesting that, in addition to various intracellular signalling pathways cooperating to regulate the expression of VDR, the

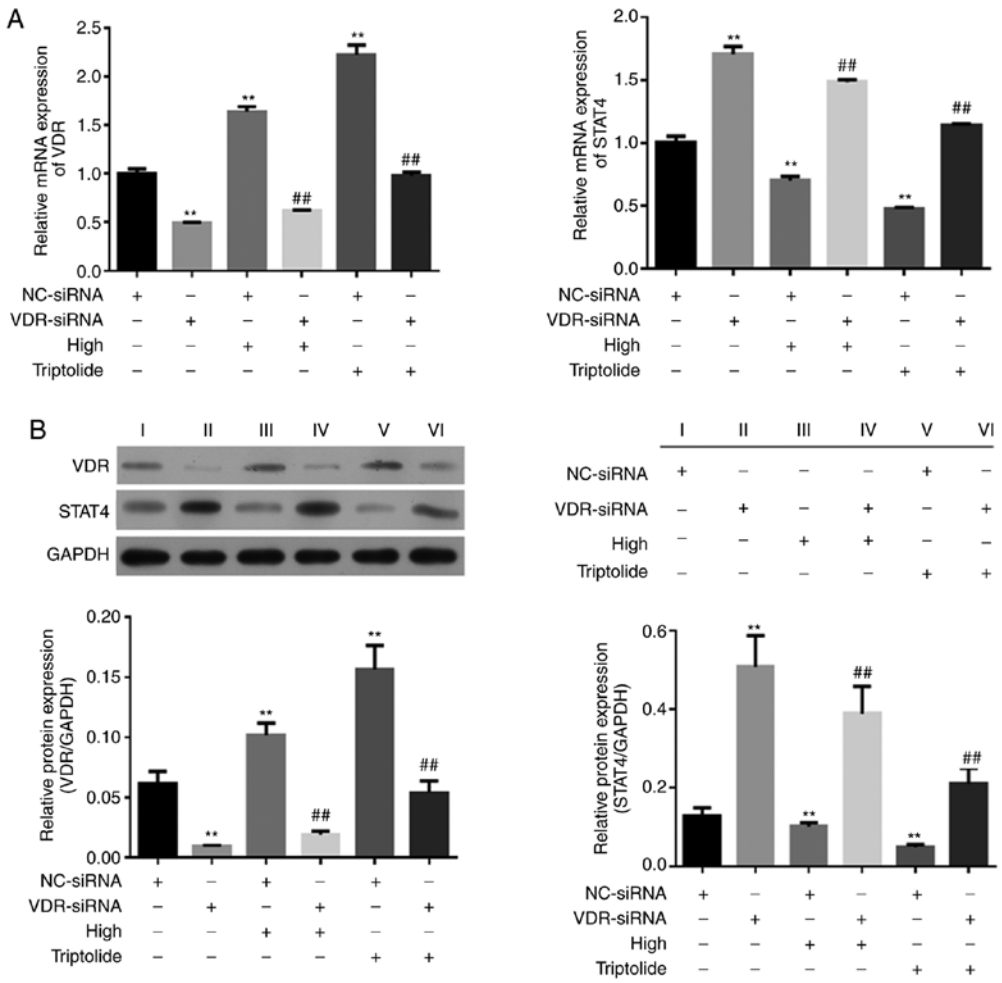

Figure 6. 'Psoriasis 1' inhibited the VDR/STAT4 signalling pathway after VDR-siRNA transfection. T lymphocytes were isolated from patients with psoriasis and transfected with NC or VDR siRNA. Effects of high-dose 'Psoriasis 1' and triptolide on the (A) mRNA and (B) protein levels of VDR and STAT4 in T lymphocytes following VDR-siRNA transfection. Protein expressions were quantified by grey value analysis. Data are presented as mean ± standard deviation. **P<0.01 vs. NC-siRNA group; $^{\#\#}$P<0.01 vs. VDR-siRNA group. n=5. VDR, vitamin D receptor; STAT, signal transducer and activator of transcription.

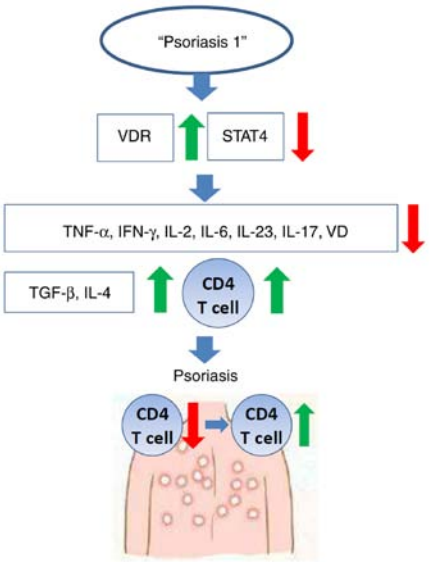

Figure 7. Working model. 'Psoriasis 1' decreases the inflammatory responses in T lymphocytes and increases the percentage of CD4$^+$ T cells in patients with psoriasis by inhibiting the VDR-mediated STAT4 signalling pathway. TNF, tumour necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; VDR, vitamin D receptor; STAT, signal transducer and activator of transcription.

implicated signalling events differ among various cell types and varying cell differentiation states. Therefore, further investigation of the mechanism through which VDR regulates alternative signalling pathways is required. Moreover, it was recently discovered that the silencing of STAT4 led to a reduction in the serum levels of IFN-γ and IL-2, with an elevation in the serum levels of IL-6 and IL-10 (43). IFN-γ production was lowered in STAT4 KO-derived splenocytes, but no significant differences in the levels of IL-12 and TNF-α were found when compared to those in WT mice (44). The effect of STAT4 reduction on VDR expression should be further elucidated in future studies.

In conclusion, 'Psoriasis 1' was found to decrease the inflammatory response in T lymphocytes and increase the percentage of CD4$^+$ T cells in patients with psoriasis through inhibiting VDR-mediated STAT4 signalling (Fig. 7). The findings of the present study highlight the clinical relevance of VDR and may enable researchers to further investigate its therapeutic potential.

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Availability of data and materials

All the datasets generated and analysed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

YG and WS designed the study, performed the experiments and conducted the statistical analysis. YG wrote the manuscript. XC and HW revised the manuscript and procured the funding. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All investigational procedures were approved by the Institutional Review Board of the First Affiliated Hospital of Guangzhou University [ZYYECK(2017)020 and ZYYECK(2019)030]. Written informed consent was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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