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Original Article

Interleukin-21 is associated with the severity of psoriasis vulgaris through promoting CD4+ T cells to differentiate into Th17 cells

Ying Wang[1,2], Li-Li Wang[1,2], Hao-Yu Yang[2,3], Fei-Fei Wang[2,3], Xue-Xiu Zhang[4], Yan-Ping Bai[1,2]

[1]Clinical Institute of China-Japan Friendship Hospital, Graduate School of Peking Union Medical College, Beijing, China; [2]Department of Dermatology & Venerology, China-Japan Friendship Hospital, Beijing, China; [3]Beijing University of Chinese Medicine, Beijing, China; [4]Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

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Abstract: Interleukin-21 (IL-21) and T helper 17 (Th17) cells are known to be involved in the pathogenesis of psoriasis, but little is known about their relationship in psoriasis. Herein, we investigated whether IL-21 could regulate Th17 cell induction in patients with psoriasis vulgaris. 32 patients with psoriasis vulgaris and 13 healthy controls were recruited. Flow cytometry was used to detect the frequencies of cells mainly secreting IL-21 (including IL-21+CD4+ T and IL-21+ Th17 cells) and Th17 cells. An enzyme-linked immunosorbent assay (ELISA) was used to determine the serum content of IL-21. Severity of the psoriasis was evaluated by a Psoriasis Area and Severity Index (PASI) score. In addition, the differentiation of CD4+ T cells with IL-21 and the different frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were assessed, as were serum levels of IL-21 in patients with moderate to severe psoriasis before and after treatment. Our results showed that the levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were significantly increased in patients and positively associated with PASI score ($P < 0.01$). Moreover, the levels of IL-21, IL-21+CD4+ T cells and IL-21+ Th17 cells were positively correlated with the frequency of Th17 cells ($P < 0.01$). In vitro experiments demonstrated that IL-21 could promote CD4+ T cells to differentiate into Th17 cells. After a 4-week treatment of acitretin and a topical therapy, all the immune markers observed in patients decreased significantly ($P < 0.01$), but the levels remained higher than those in healthy controls ($P < 0.01$). These findings indicate that IL-21 might promote Th17 cell induction in psoriasis and might be a potential immune marker for targeting this disease.

Keywords: Psoriasis, interleukin-21, Th17 cells, acitretin

Introduction

Psoriasis is a common, multifactorial, chronic inflammatory disease that occurs in 2-3% of the global population [1, 2]. Psoriasis vulgaris is the most common form of the disease and is characterized by well-demarcated, erythematous, scaly papules and plaques [3]. Although the pathogenesis of psoriasis has not been fully understood, it is currently widely considered an immune-mediated disease [4]. The pathologic hallmarks of psoriatic plaque include hyper-proliferation of keratinocytes in the epidermis and infiltration of T lymphocytes, dendritic cells, neutrophils, and other inflammatory cells [5]. Laboratory and clinical investigations have indicated that T helper 17 (Th17) cells, characterized by the production of Interleukin-17 (IL-17), play an essential role in the pathogenesis of psoriasis [6]. These findings have enriched our understanding of the mechanisms underlying the disease. Thereafter, biological drugs that target IL-17 signalling, including brodalumab, ixekizumab, and secukinumab, have been tested for the treatment of psoriasis, and their therapeutic value has been confirmed [7].

Interleukin-21 (IL-21) is a pleiotropic cytokine that is composed of four α-helical bundles and produced predominantly by CD4+ T cells [8], especially Th17 cells and T follicular helper (Tfh) cells [9, 10]. IL-21 is unique among many cytokines as it affects differentiation, prolifera-

Table 1. Characteristics of the participants

Group	PV† n = 32	HC‡ n = 13	*P*
Age (mean ± SD, years)	47.22 ± 10.35	42.47 ± 11.21	> 0.05
Gender (F/M§)	19/17	9/8	> 0.05
PASI¶ score (mean ± SD)	16.33 ± 8.06	-	-
Mild (n = 8)	7.17 ± 2.09	-	-
Moderate (n = 15)	14.80 ± 2.75	-	-
Severe (n = 9)	25.32 ± 6.61	-	-

†: Psoriasis vulgaris; ‡: Healthy controls; §: Female and Male; ¶: Psoriasis area and severity index.

tion and multiple functions of both immune cells and non-immune cells [11, 12]. IL-21 mainly affects B cells by inducing proliferation, class switching or death, in mature B cells [13]. It has been reported that IL-21 can induce the apoptosis of dendritic cells by a signal transducer and activator of transcription 3 (STAT3) in a Bim-dependent manner [14]. In addition, IL-21 plays an important role in regulating CD4+ T cell differentiation and fate. It can contribute to the generation of Th17 cells while impeding the development of regulatory T cells (Tregs) [9, 15]. Furthermore, several studies have demonstrated that IL-21 participates in the process of many autoimmune diseases, such as rheumatoid arthritis [16] and systemic sclerosis [17], by regulating Th17 cell differentiation. However, the relationship between IL-21 and Th17 cells in patients with psoriasis vulgaris is less clear.

In this study, we aim to address the function of IL-21 on Th17 cells in psoriasis. First, the serum level of IL-21 and the frequencies of IL-21+CD4+ T cells, Th17 cells, and IL-21+ Th17 cells were determined in the peripheral blood of patients with psoriasis and healthy controls, and their associations with Psoriasis Area and Severity Index (PASI) score were analysed. Second, the correlations between the serum level of IL-21, the frequency of IL-21+CD4+ T cells, the frequency of IL-21+ Th17 cells, and the frequency of Th17 cells were evaluated. Then, the promotional effect of IL-21 upon the differentiation of CD4+ T cells into Th17 cells was assessed in vitro. Finally, the characteristics of IL-21 and Th17 cells in psoriatic patients were observed before and after treatment with acitretin and a topical therapy. Our findings might have important implications for our understanding of the immunopathogenesis of psoriasis.

Materials and methods

Patients

Thirty-two patients with psoriasis vulgaris and thirteen healthy controls were enrolled for this study in our hospital. There were no significant differences between groups in age or gender. The diagnoses of psoriasis vulgaris were made according to the diagnostic criteria of Nestle [18]. The disease activity of patients with psoriasis was assessed using a PASI score at the time of blood collection and categorized as mild (0-10), moderate (11-20), or severe (>20) [19]. There were no other autoimmune diseases, systemic diseases, or active infections in any of the participants. No patients had received any systemic therapy for at least one month before enrolment. Of the 32 patients, there were 24 patients with moderate to severe psoriasis receiving 20 mg of oral acitretin (Fangxi, Chongqing Huapont Pharm. Co., Ltd.) once daily and a topical therapy of calcipotriol ointment (Daivonex, LEO Laboratories Limited, Ireland) and mometasone furoate cream (Eloson, Shanghai Xianlingbaoya Pharm. Co., Ltd.) once daily for 4 weeks. An efficacy index, ((PASI score before treatment-PASI score after treatment)/PASI score before treatment) × 100%, was used to estimate the clinical efficacy of the treatment for psoriasis. The definitions of clinical efficacy included cured (efficacy index ≥ 90%), excellent (efficacy index < 90% and ≥ 60%), effective (efficacy index < 60% and ≥ 20%) and invalid (efficacy index < 20%). The treatment efficacy rate was calculated as ((cured number + excellent number + effective number)/total number) × 100%. The blood samples were collected from the healthy controls and patients before and after treatment. The study was approved by the Ethics Committee of China-Japan Friendship Hospital. Written informed consent was obtained from each subject. The clinical background of the patients is shown in **Table 1**.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-21 from healthy donors and patients with psoriasis before and after treatment were determined using an ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

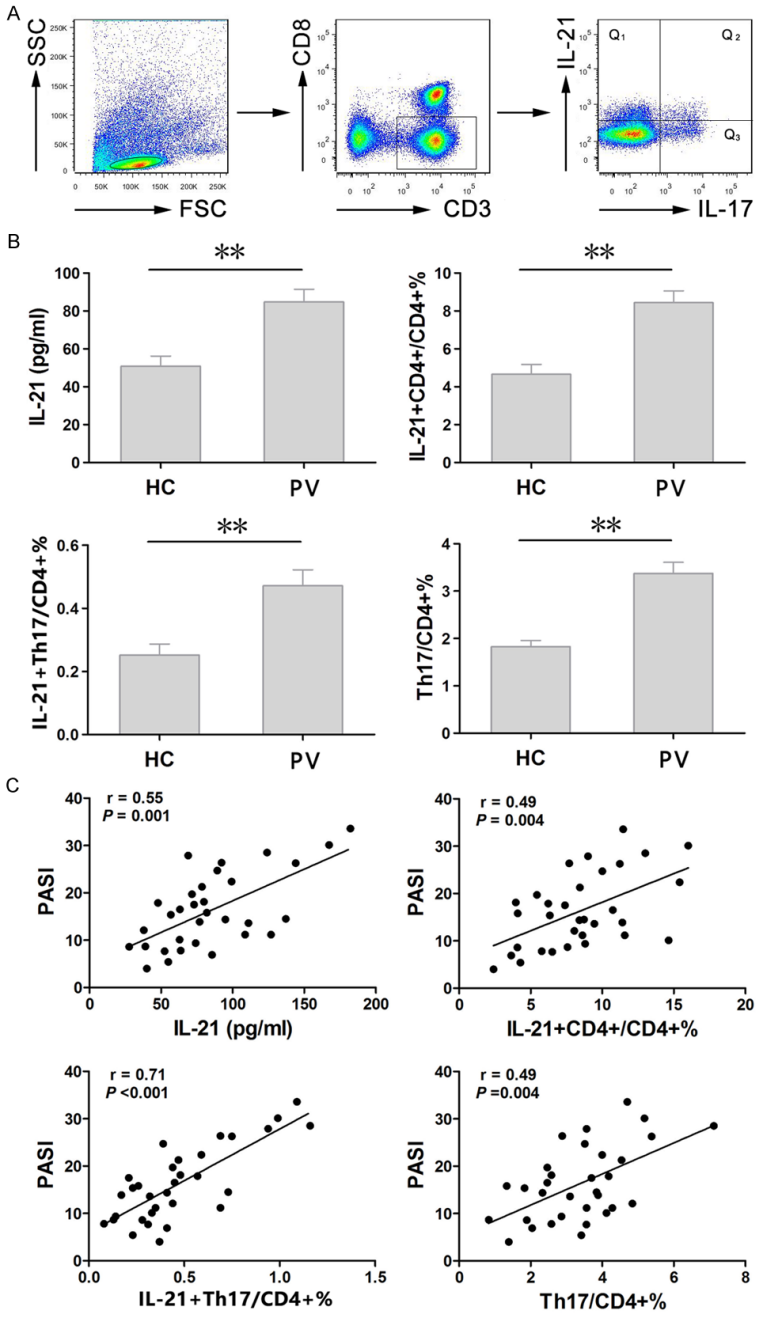

Figure 1. The levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells are significantly increased and positively correlated with PASI scores in psoriasis. A. A fluorescence-activated cell sorter (FACS) was used to detect the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and T helper 17 (Th17) cells in peripheral blood samples. B. The level of Interleukin-21 (IL-21) was measured by Enzyme-linked immunosorbent assay (ELISA). Compared with the healthy control samples, the levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells in the psoriasis patient samples were significantly increased. C. We analysed the correlation between these immune markers and the severity of disease (PASI score) in 32 patients with psoriasis vulgaris. Positive correlations were found between the levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells, and Th17 cells and PASI score. $^{**}P < 0.01$.

Flow cytometric analysis

Phycoerythrin (PE)-conjugated anti-IL-21 was purchased from BD PharMingen (San Diego, CA, USA), and all other antibodies used in flow cytometry were from eBioscience (San Diego, CA, USA). One sample of freshly heparinized peripheral blood (200 μL) was incubated for 6 hours with phorbol-12-myristate-13-acetate (PMA, 300 ng/mL, Sigma-Aldrich, St. Louis, MO) and ionomycin (1 μL /mL, Sigma-Aldrich) in 800 μL of RPMI 1640 medium supplemented with 10% foetal calf serum. Monensin (0.4 μM, BD PharMingen) was added during the first hour of incubation. Then, a Cytofix/Cytoperm kit (BD PharMingen), anti-CD3, anti-CD8, anti-IL-17, and anti-IL-21 antibodies were used in one sample according to the manufacturers' protocols. The stained cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analysed using FlowJo software (Tritar, USA).

CD4+ T cell purification and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples freshly obtained from 13 healthy controls or from 15 patients with moderate psoriasis by Ficoll-Hypaque density gradient centrifugation and used to purify CD4+ T cells. The total cell number was counted in the presence of trypan blue dye to evaluate cell viability. For positive selection of CD4+ T cells, MACS CD4 microbeads (Miltenyi Biotec, Auburn, CA, USA) were incubated with the PBMCs and applied to a MidiMACS separation column

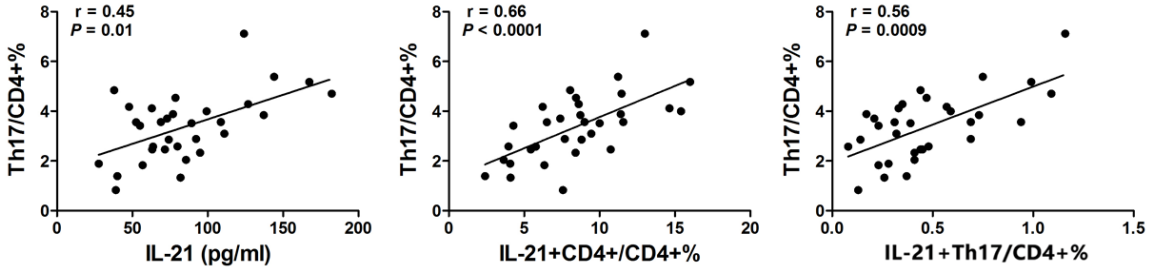

Figure 2. The levels of IL-21, IL-21+CD4+ T cells and IL-21+ Th17 cells are positively associated with Th17 cells. To evaluate the relationship between IL-21 and Th17 cells in patients with psoriasis vulgaris, we investigated the correlations between the serum content of IL-21, the peripheral frequencies of IL-21+CD4+ T cells, the percentage of IL-21+ Th17 cells and the circulating Th17 cells. All the immune markers were positively correlated with the frequency of Th17 cells.

(Miltenyi Biotec). The purity of the CD4+ T cells was determined by flow cytometry (> 95%) for each population. Purified CD4+ T cells were seeded onto U-bottom 96-well plates at 5×10^5 cells/well in RPMI 1640 medium containing 10% foetal calf serum (Life Technologies, Carlsbad, CA, USA) with 1 μg/ml plate-bound anti-CD3 and 1 μg/ml anti-CD28 (eBioscience, San Diego, CA, USA) with or without 50 ng/ml IL-21 (eBioscience, San Diego, CA, USA) for 5 days at 37°C in 5% CO_2. After incubation, the cells were collected and stained with surface antibodies for flow cytometry.

Statistical analysis

SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. The data are presented as the mean ± standard deviation. The Mann-Whitney U-test was applied for the between-group comparisons. The Wilcoxon signed-rank test was used to compare data from the same individual before and after treatment. Spearman's correlation was used to analyse the association between PASI score and the immune markers detected as well as the correlation between the frequency of Th17 cells and the immune markers detected. The Chi-square test was used to assess difference among the clinical data. For all tests, $P < 0.05$ was considered statistically significant.

Results

High levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were positively correlated with PASI scores in psoriasis

To understand the potential role of IL-21 and Th17 cells in psoriasis, we detected the serum IL-21 levels and the frequencies of peripheral IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells in 32 patients with psoriasis vulgaris (PV) and 13 healthy controls (HC). As shown in **Figure 1A**, the serum IL-21 levels in the patients were higher than those in the healthy controls (84.84 ± 37.40 pg/mL vs 50.93 ± 19.18 pg/mL, $P < 0.01$), and significant differences in the frequencies of IL-21+CD4+ T cells (8.45 ± 3.49% vs 4.67 ± 1.82%, $P < 0.01$), IL-21+ Th17 cells (0.47 ± 0.28% vs 0.25 ± 0.12%, $P < 0.01$) and Th17 cells (3.37 ± 1.34% vs 1.83 ± 0.46%, $P < 0.01$) in peripheral blood were found between the patients with psoriasis vulgaris and the healthy donors. In addition, to investigate whether those markers had relationships with psoriasis severity, we analysed the correlations of the levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells, and Th17 cells with PASI score. Interestingly, they positively correlated with PASI score (**Figure 1B**, r = 0.55, $P = 0.001$; r = 0.49, $P = 0.004$; r = 0.71, $P < 0.001$; r = 0.49, $P = 0.004$).

The levels of IL-21, IL-21+CD4+ T cells and IL-21+ Th17 cells have positive correlations with Th17 cells

To gain insight into the relationship between IL-21 on Th17 cells in psoriasis, we first analysed the association between the serum level of IL-21 and the frequency of Th17 cells in psoriasis, and then investigated the association between circulating IL-21-secreting cells, including IL-21+CD4+ T cells and IL-21+ Th17 cells, with Th17 cells. As shown in **Figure 2**, the serum level of IL-21 was positively correlated with the frequency of Th17 cells (r = 0.45, $P = 0.01$). Similar relationships were also found between both IL-21+CD4+ T cells and IL-21+

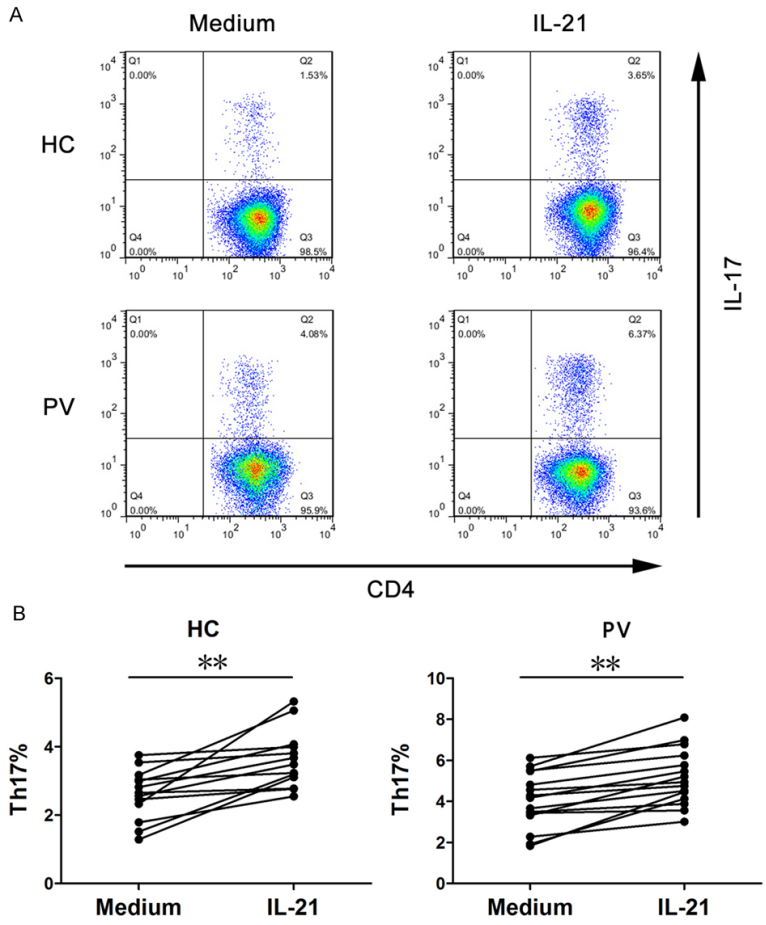

Figure 3. IL-21 promotes Th17 cell induction. A. Peripheral CD4+ T cells from healthy controls and patients with psoriasis vulgaris were cultured in the indicated conditions with anti-CD3 and anti-CD28 for 5 days. Th17 cells were identified by intracellular staining. Representative FACS scatter plots of IL-17+CD4+ T cells are shown. B. In healthy controls, stimulation with IL-21 could dramatically increase the number of Th17 cells induced from CD4+ T cells. In addition, there was a similar response of CD4+ T cells to IL-21 in patients with psoriasis vulgaris. $^{**}P < 0.01$.

Th17 cells and Th17 cells (r = 0.66, $P < 0.0001$; r = 0.56, $P = 0.0009$).

IL-21 induced CD4+ T cell differentiation into Th17 cells

We cultured purified CD4+ T cells from patients with psoriasis vulgaris and healthy controls with or without IL-21. The cells were collected on day 5 for intracellular staining. Our data showed that stimulation with IL-21 significantly increased the frequency of Th17 cells among CD4+ T cells (**Figure 3A** and **3B**, $P = 0.0002$; $P < 0.001$), indicating that IL-21 can promote CD4+ T cells to differentiate into Th17 cells in psoriasis and furthering our understanding of the pathogenesis of psoriasis.

The serum level of IL-21 and the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells before and after treatment

To assess intraindividual variations of the serum level of IL-21, circulating IL-21+ CD4+ T cells, IL-21+ Th17 cells and Th17 cells during treatment, 24 patients with moderate to severe psoriasis received acitretin and a topical therapy for 4 weeks, and these immune markers were detected before and after treatment. As shown in **Table 2**, the treatment efficacy rate was 87.5%. The mean PASI scores before and after treatment were 19.30 ± 6.73 and 6.53 ± 5.07, which were significantly different ($P < 0.01$). In addition, we found that the serum level of IL-21 and the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were all significantly decreased after treatment (**Figure 4A**, $P < 0.01$; $P < 0.01$; $P < 0.01$; $P < 0.01$). However, compared with those of the healthy controls, the levels of immune markers detected after treatment remained significantly higher (**Figure 4B**, $P < 0.0001$; $P = 0.0049$; $P = 0.0417$; $P = 0.0293$).

Discussion

In the present study, we evaluated the influence of IL-21 on Th17 cells in psoriasis. We found that the serum level of IL-21 was dramatically increased in patients with psoriasis and significantly correlated with PASI score and the frequency of Th17 cells. Moreover, stimulation with IL-21 increased the expression of Th17 cells in CD4+ T cells purified from psoriasis patients. There were also significant reductions of the serum level of IL-21 and the

Table 2. The clinical efficacy of patients with psoriasis after treatment

	Total	Cured	Excellent	Effective	Invalid	Clinical efficacy rate
No. (%)	24	4 (16.67)	10 (41.67)	7 (29.17)	3 (11.11)	21 (87.5)

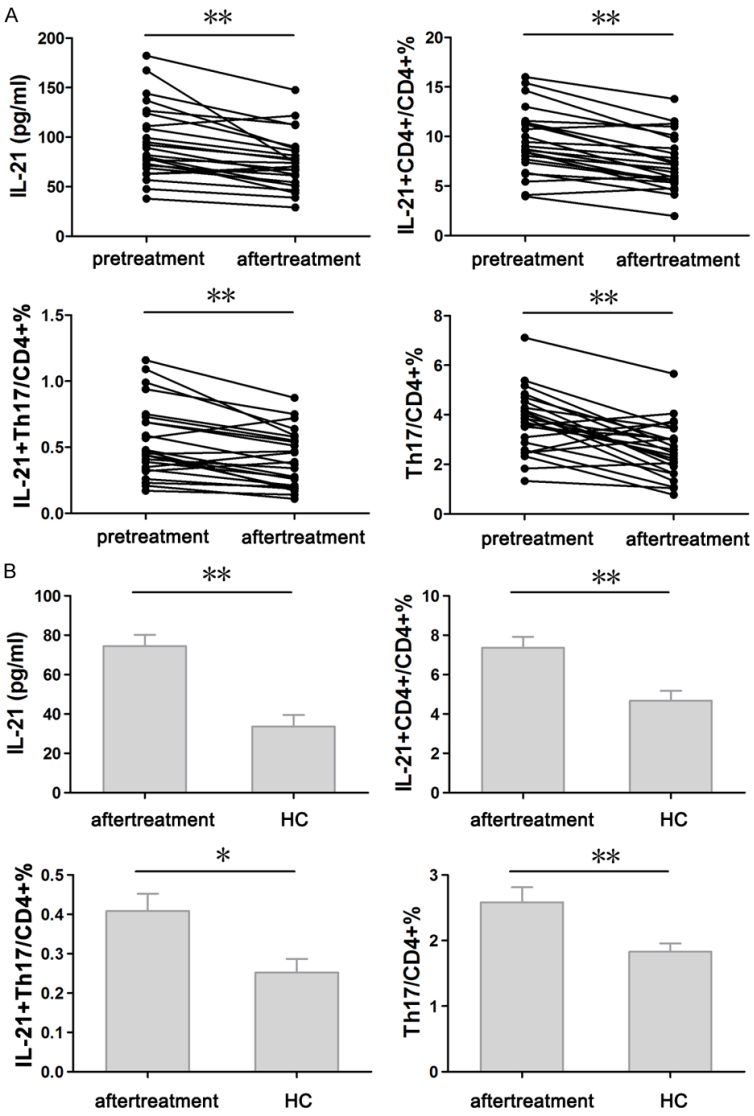

Figure 4. Acitretin influenced IL-21 and Th17 cells in psoriasis. A. After a 4-week systemic treatment of acitretin, the serum level of IL-21 and the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were significantly decreased. B. To further understand the immune state of the patients after treatment, we investigated the differences between these immune markers in psoriatic patients after treatment and healthy controls. Though the levels of IL-21, IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were dramatically decreased after a 4-week treatment, they were still higher than were those found in healthy individuals. *$P < 0.05$. **$P < 0.01$.

frequency of Th17 cells in patients after treatment. To our knowledge, this is the first study to investigate the characteristics of IL-21+ Th17 cells in psoriasis and analyse the relationship between IL-21 and Th17 cells.

IL-21 plays an important role in regulating the development and function of immune cells. Binding to IL-21 receptor (IL-21R), IL-21 can influence the fate specification of CD4+ T cells, enhance natural killer (NK) cell cytotoxicity against tumour cells, and induce B cells to robustly differentiate into memory and plasma cells [8]. To investigate the characteristics of IL-21 in patients with psoriasis vulgaris, we detected the serum content of IL-21 using ELISA. Our data showed that the serum level of IL-21 was significantly increased in psoriatic patients and positively correlated with PASI score and the frequency of Th17 cells. In addition, we first analysed the characteristics of immune cells mainly secreting IL-21 in psoriasis, including IL-21+CD4+ T cells and IL-21+ Th17 cells. The frequencies of IL-21+CD4+ T cells and IL-21+ Th17 cells were obviously higher in the psoriatic patients than that in the healthy controls and were also associated with PASI score and Th17 cell frequency. These results suggested that IL-21 might play an important role in the pathogenesis of psoriasis in vivo and be involved in the disease status of patients with psoriasis vulgaris. He [20] and Niu [21] have also confirmed the high serum level of IL-21 and its positive relationship

with PASI score in psoriatic patients. In addition, high IL-21 protein and mRNA levels were observed in all samples taken from lesional psoriatic skin compared with those from nonlesional skin of the same individuals and from normal controls, and IL-21-expressing circulating CD4+ T cells were more common in individuals with psoriasis than in control individuals [22]. Caruso [22] also found that epidermal thickness was decreased via neutralizing IL-21 in a human psoriasis xenograft severe combined immunodeficiency (SCID) model, supporting the pathogenic role of IL-21 in psoriasis. However, Oliveira [23] showed that IL-21 levels were numerically higher in healthy controls than in Brazilian patients with psoriasis, but the difference was not statistically significant. The reasons for such differences are unclear, and we suppose that differences might arise due to race, experimental design or experimental conditions.

The roles of Th17 cells in the pathogenesis of most common autoimmune diseases have been well described [24, 25]. The differentiation of naïve CD4+ T cells into Th17 cells is induced in the presence of many cytokines, such as transforming growth factor beta (TGF-β), IL-6, IL-21 and IL-23. A series of studies have demonstrated that IL-21 is an essential autocrine amplification factor for the induction of Th17 cells [26]. It has been demonstrated that IL-21 can overturn cell differentiation from the regulatory T cell pathway to the Th17 cell pathway [27] and that the secretion of IL-21 by the Th17 cells can further expand and stabilize this population of cells [28]. Moreover, the role of IL-21 on CD4+ T cells has been shown to be important for the induction of the IL-23 receptor (IL-23R), and numerous studies have observed that IL-23R expression on effector T cells is critical for evoking the pathogenic potential of Th17 cells [29-31]. Lee [32] showed that IL-21R deletion in a transgenic mouse model reduced the incidence and severity of spontaneous experimental autoimmune encephalomyelitis (EAE), which was associated with a defect in Th17 cell generation. Lei [17] showed that IL-21 contributed to bleomycin-induced fibrosis in mice via stimulating the generation of Th17 cells. We found that there were similar CD4+ T cells responses to IL-21 in samples from psoriatic patients and healthy controls in vitro. Stimulation with IL-21 could dramatically increase the percentage of Th17 cells in purified CD4+ T cells from patients with psoriasis vulgaris, suggesting that IL-21 is one of the primary drivers in the pro-inflammatory milieu in psoriasis.

Finally, to better understand the dynamic changes of these immune markers in psoriatic patients, we detected the serum levels of IL-21 and the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells before and after treatment with acitretin and topical drugs. After treatment, the PASI scores, the serum levels of IL-21 and the frequencies of IL-21+CD4+ T cells, IL-21+ Th17 cells and Th17 cells were significantly decreased. The clinical efficacy rate of acitretin was 87.5%. Our findings indicated that acitretin could inhibit IL-21 and Th17 cells in psoriasis vulgaris. Numerous studies have demonstrated that retinoids could suppress Th17 in autoimmune diseases, such as autoimmune myositis, allergic asthma and multiple sclerosis [33-35]. Niu [36] found that Th17 cells were significantly decreased in lesions of patients with psoriasis after acitretin treatment. Recently, Wu [37] reported that acitretin could improve the clinical symptoms of psoriasis via reducing Th17 cell differentiation. However, Caproni [38] reported that acitretin did not affect IL-17 serum levels in psoriatic patients. Therefore, further research is needed to explore the relationship between acitretin and Th17 cells. We also found that the levels of these markers were significantly higher than those in healthy controls, suggesting that the immune state of the psoriatic patients after a 4-week treatment remained activated and that the patients required continued therapy.

Conclusions

In conclusion, our findings demonstrate that IL-21 is closely related to the severity of psoriasis vulgaris. The effects of elevated IL-21 may be involved in elevating the level of Th17 cells in psoriasis vulgaris. These findings highlight the role of IL-21 in the pathogenesis of psoriasis, identifying a potential therapeutic target for the treatment of psoriasis.

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Disclosure of conflict of interest

None.

Address correspondence to: Yan-Ping Bai, Department of Dermatology & Venerology, China-Japan Friendship Hospital, No. 2, Yinghua East Street, Chaoyang District, Beijing, 100029, China. Tel: 86-13910758509; Fax: 86-10-64289840; E-mail: zhi@tsinghua.edu.cn

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