Original Research

IL-22$^+$CD4$^+$ T-cells in patients with active systemic lupus erythematosus

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Abstract

Interleukin-22 (IL-22) is a regulator of autoimmune diseases. However, the role of IL-22$^+$CD4$^+$ T-cells in the pathogenesis of systemic lupus erythematosus (SLE) remains unclear. This study is aimed at elucidating the potential role of IL-22 and IL-22$^+$CD4$^+$ T-cells in patients with SLE. A total of 22 patients with freshly diagnosed SLE and 18 age-/gender-matched healthy controls ($n = 18$) were evaluated for the frequency of different types of IL-22-producing CD4$^+$ T-cells by flow cytometry analysis following *in vitro* stimulation. The concentrations of plasma IL-22, IL-17A, IFNγ, serum complement factors (C3, C4), C-reactive protein (CRP), antidouble-stranded (ds) DNA and anti-Smith (Sm) antibodies were measured. The potential association among these measures was analyzed. The percentages of IL-22$^+$IL-17$^-$IFNγ^-, IL-22$^+$IL-17A$^-$IFN-γ^+, IL-22$^-$IL-17$^+$IFN-γ^- and IL-22$^+$IL-17$^+$IFN-γ^- CD4$^+$ T-cells and the levels of plasma IL-22 and IL-17A in the patients were significantly higher than that in the healthy controls ($P < 0.01$). The frequency of Th22 cells was correlated positively with that of Th17 and IL-22$^+$IL-17$^+$ CD4$^+$ T-cells, and the frequency of Th17 and IL-22$^+$CD4$^+$ T-cells was correlated positively with the values of SLE disease activity index (SLEDAI), but not with the values of CRP, ERS and C3 in SLE patients. Our data suggest that both Th17 and IL-22$^+$CD4$^+$ T-cells may participate in the pathogenesis of SLE.

Keywords: SLE, Th22, IL-22, Th17, Th1

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, and its etiology remains unclear. SLE is characterized by the imbalance of immune regulation. Various types of autoantibodies are detected in patients with SLE and contribute to the pathogenesis of SLE.[1] In addition, recent studies have suggested that T-cells and their inflammatory cytokines play an important role in the pathogenesis of SLE.[2,3]

Interleukin (IL)-22, a cytokine, is originally described as an IL-9-induced cytokine and is termed as an IL-10-related T-cell-derived-inducible factor.[4] IL-22 shares the IL-10R2 receptor for signaling with other members in the IL-10 cytokine family. IL-22 is produced by CD4$^+$ T-cells, natural killer (NK) cells, NKT-cells and activated dendritic cells.[5–9] IL-22 can be produced by Th22 cells and also by Th17 cells, along with IL-17.[10] IL-22 can enhance innate immunity against infection in the mucosal membrane and promote epithelial cell proliferation and tissue regeneration.[10] On the other hand, IL-22 is a critical regulator of the pathogenesis of psoriasis, Crohn's disease and rheumatoid arthritis (RA). IL-22 mediates IL-23-induced acanthosis and dermal inflammation, and stimulates pro-inflammatory cytokine production by intestinal epithelial cells.[11,12] IL-22 promotes osteoclastogenesis and induces chemokine production in an animal model of RA.[13] In addition, IL-22 can synergistically, with tumor necrosis factor α (TNF-α), promote pro-inflammatory responses.[14] However, the importance of IL-22 in the pathogenesis of SLE is in debate. While previous studies have shown significantly lower levels of IL-22 in SLE patients[7,15,16] a recent study reveals significantly higher percentages of IL-22$^+$CD4$^+$ T-cells in SLE patients, which are correlated positively with disease activities.[7]

Th22 cells are CCR6$^+$CCR4$^+$CCR10$^+$ and depend on aryl hydrocarbon receptor, a key transcription factor, for their differentiation.[8,9,14] Th22 cells have unique signature and profile of gene expression, becoming an independent subset of CD4$^+$ T-cells.[14] The development of Th22 cells

is regulated positively by IL-6 and TNF-α as well as plasmacytoid dendritic cells.[17] Previous studies have shown that a high frequency of Th22 cells is present within psoriatic lesions and in the peripheral blood of patients with psoriasis and RA.[18,19] However, little is known about the role of Th22 in the pathogenic process of SLE in humans.

Th17 cells are $CCR4^+CCR6^+IL\text{-}23R^+CXCR3^-$ inflammatory $CD4^+$ T-cells and can produce IL-17A, but not interferon γ (IFN-γ).[20,21] Th17 cells are also capable of producing TNFα, IL-6, IL-21, IL-22, and IL-26. Previous studies have shown that a high frequency of Th17 cells and high levels of IL-17 display in patients with SLE and are correlated positively with the severity of SLE.[2,22] In addition, IL-22 can also be produced by IFNγ-secreting Th1 cells. Given that Th1 and Th17 cells have been associated with the development and progression of SLE,[3,23,24] how these T-cells interact with Th22 cells in patients with SLE has not been clarified.

In this study, we characterized the frequency of peripheral blood Th1, Th17 and Th22 cells by flow cytometry analysis and measured the concentrations of plasma IFNγ, IL-17 and IL-22 by enzyme-linked immunosorbent assay (ELISA) in Chinese patients with SLE and healthy subjects. Furthermore, we analyzed the potential correlation of the frequency of Th22 and Th17 with the clinical parameters of SLE in these patients.

Table 1 Demographic and clinical characteristics of the participants

Characteristics	Healthy controls (n = 18)	SLE patients (n = 22)
Male/female	2/16	3/19
Age (year), median (range)	25 (17–30)	26 (18–32)
Disease duration	ND	22 (100)[†]
SLEDAI, median (range)	ND	13 (2–38)
Positive anti-dsDNA	ND	18 (83%)
Positive anti-Sm	ND	5 (23%)
Positive antinuclear antibodies	ND	20 (91%)
CRP (mg/L), median (range)	7.2 (0–15)	24.45 (2.99–202)*
ESR (mm/h), median (range)	3 (0–5)	39 (4–120)*
C3 (units/mL)	1.12 (0.9–1.8)	0.46 (0.36–0.86)*
C4 (units/mL)	0.26 (0.1–0.4)	0.07 (0.02–0.14)*
WBC ($\times 10^9$/L) median (range)	6.31 (4.09–9.13)	6.28 (3.68–9.18)

The normal range of CRP and ESR was 0–15 mg/L and 0–5 mm/h, respectively
The normal range of C3 and C4 was 0.9–1.8 and 0.1–0.4 units/mL, respectively
SLEDAI, SLE disease activity index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ND, not determined
*$P < 0.05$ versus the controls
[†]All of the patients had clinical symptoms for less than three months

Materials and methods

Study subjects

Twenty-two patients with new onset SLE were recruited from the inpatient service of the First Hospital of Jilin University, Changchun, China between March and October 2011. Eighteen gender- and age-matched healthy volunteers were recruited from the outpatient service of the same hospital. All patients fulfilled the ACR Classification for SLE.[25] The degrees of disease activity in those patients were assessed using the SLE disease activity index (SLEDAI), and a score $\geq$6 was defined as active disease. Subjects were excluded if she/he had a history of myositis, systemic sclerosis or other autoimmune diseases, a recent infection or had received immunosuppressive or glucocorticoid therapy within the past six months. Written informed consent was obtained from individual participants. The experimental protocol was established according to the guidelines of the Declaration of Helsinki and approved by the Human Ethics Committee of Jilin University.

Data collection

The baseline demographic and clinical data of individual participants were collected from hospital records and reviewed by experienced physicians. Their demographic and clinical characteristics are summarized in Table 1. Blood samples were obtained for routine laboratory tests of full blood cell counts and the concentrations of serum C-reactive protein (CRP), complement C3 and C4, anti-Sm, and anti-dsDNA antibodies. The levels of serum anti-dsDNA and anti-Sm were determined by indirect immunofluorescence using special kits, according to the manufacturers' instruction (Oumeng, Beijing, China). The concentrations of serum C3, C4 and CRP were determined by scatter turbidimetry on a Siemens special protein analysis instrument (Siemens Healthcare Diagnostics Products, GmbH, Germany).

Isolation and stimulation of peripheral blood mononuclear cells

Peripheral venous blood samples were collected from individual participants who had fasted overnight, and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK). PBMCs at 10^6/mL were stimulated in duplicate with phorbol 12-myristate 13-acetate (PMA, 1 μg/mL) and ionomycin (50 μg/mL; Sigma, St Louis, MO, USA) in 10% human AB type of serum in RPMI 1640 medium at 37°C in a humidified incubator with 95% air and 5% carbon dioxide for four hours and cultured for another two hours in the presence of brefeldin A (0.5 μg/mL; Sigma). The same cells were cultured in medium alone and used as negative controls.

Flow cytometry analysis

The stimulated PBMCs were harvested and stained with PerCP-anti-CD4 (Becton Dickinson, San Diego, CA, USA), followed by being fixed with 4% paraformaldehyde (30 min/RT) and then permeabilized with 0.5% saponin in 10% FBS in PBS (30 min/RT). After being washed,

the cells were stained with FITC-anti-IFN-γ, Alexa-Flour-anti-IL-17 (Becton Dickinson) and PE-anti-IL-22 (R&D Systems, Minneapolis, MN, USA), respectively. The frequency of cytokine-specific T-cells was determined by flow cytometry analysis on a BD FACSCalibur (Becton Dickinson) using FlowJo 7.6.2 software.

Enzyme-linked immunosorbent assay

The concentrations of plasma IFN-γ, IL-17, and IL-22 in individual participants were determined by ELISA using specific cytokine kits, according to the manufacturers' instruction (R&D Systems).

Statistical analysis

All data are expressed as individual values, median and range of each group of subjects. The difference between groups was analyzed using the Mann-Whitney *U* test. The potential correlation between variables was analyzed by the Spearman rank correlation test. All statistical tests were performed using SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA). A two-sided *P* value of <0.05 was considered statistically significant.

Results

A higher frequency of IL-22$^+$CD4$^+$ T-cells in patients with SLE

To determine the potential role of IL-22$^+$CD4$^+$ T-cells in the pathogenesis of SLE, a total of 22 patients with newly diagnosed SLE and 18 gender- and age-matched healthy subjects were recruited. As expected, there was no significant difference in the distribution of gender and age, but the levels of serum ESR and CRP in patients with SLE were significantly higher than that in the controls (Table 1). Conversely, the levels of serum C3 and C4 in the patients were significantly lower than that in the controls. Furthermore, patients had varying values of SLEDAI and some patients were positive for anti-dsDNA and anti-Sm antibodies.

To determine the frequency of different types of IL-22$^+$CD4$^+$ T-cells, PBMC were isolated from individual participants and stimulated with PMA and ionomycin *in vitro*. Subsequently, the cells were stained intracellularly with antibodies against different cytokines and the percentages of IL-22$^+$IL-17A$^-$, IL-22$^-$IL-17A$^+$, IL-22$^+$IL-17A$^+$IFNγ^-, and IL-22$^+$IL-17A$^-$IFNγ^+ T-cells in CD4$^+$ T-cells were determined by flow cytometry analysis (Figure 1). The frequency of IL-22$^+$IL-17A$^-$ CD4$^+$ and IL-22$^-$IL-17A$^+$ CD4$^+$ T-cells in SLE patients was significantly higher than that in the controls ($P < 0.0001$ for both). Further analysis indicated that the percentages of IL-22$^+$IL-17A$^+$IFNγ^- and IL-22$^+$IL-17A$^-$IFNγ^+ CD4$^+$ T-cells in the patients was also significantly greater than that in the controls ($P = 0.001$ and 0.005, respectively). Together, these data indicated a high frequency of IL-22$^+$CD4$^+$ T-cells in patients with active SLE.

The percentages of Th22 cells are correlated positively with the percentages of Th17 or IL-22$^+$IFNγ^-IL-17A$^+$ CD4$^+$ T-cells in SLE patients

Next, we analyzed the relationship between different types of CD4$^+$ T-cells in SLE patients and found that the percentages of CD4$^+$IL-22$^+$IL-17A$^-$IFNγ^- Th22 cells in SLE patients were correlated positively with the percentages of IL-22$^-$IL-17A$^+$IFNγ^- CD4$^+$ Th17 cells ($R = 0.22170$, $P = 0.0132$, Figure 2a). Similarly, the percentages of Th22 cells were also correlated significantly with the percentages of IL-22$^+$IL-17A$^+$IFNγ^- CD4$^+$ T-cells in this population ($R = 0.2214$, $P = 0.0133$, Figure 2b). However, the frequency of different types of IL-22$^+$ cells was not significantly associated with the frequency of IL-22$^-$IL-17A$^-$IFN-γ^+ CD4$^+$ T-cells in the patients with SLE (data not shown). Therefore, these different types of IL-22$^+$CD4$^+$ T-cells were closely associated with Th17 cells, but not with Th1 cells in SLE patients.

High levels of plasma IL-22 and IL-17 are detected in patients with SLE

To determine the function of different subsets of IL-22- and IL-17A-producing CD4$^+$ T-cells, we measured the concentrations of plasma IFN-γ, IL-17A and IL-22 in the patients and controls. We found that the concentrations of plasma IL-22 in the patients with SLE (36.06 ± 6.18 pg/mL) were significantly higher than that in the controls (31.69 ± 2.625 pg/mL, $P = 0.0039$, Figure 3a). Similarly, the levels of plasma IL-17A in the patients (62.67 ± 6.445 pg/mL) were also significantly higher than that in the controls (60.06 ± 3.715 pg/mL, $P = 0.0369$, Figure 3b). However, there was no significant difference in the levels of plasma IFN-γ between the patients and controls ($P > 0.05$, data not shown). Further analysis indicated that the percentages of Th22 cells were correlated positively with the concentrations of plasma IL-22 in SLE patients ($R = 0.9005$, $P < 0.0001$, Figure 3c; Figure 4).

The frequency of IL-22$^+$CD4$^+$ T-cells and Th17 cells is correlated positively with SLEDAI in SLE patients

Finally, we examined the potential association between IL-22-producing, Th17 and Th1 cells with the severity of disease in this population. We found that the percentages of IL-22$^+$CD4$^+$ ($P = 0.0003$, $R = 0.5497$, Figure 3a) and Th17 T-cells ($P = 0.0296$, $R = 0.2490$; Figure 3b) were correlated positively with the values of SLEDAI in SLE patients. However, there was no statistically significant association between the SLEDAI scores and the percentages of Th1 cells in this population ($P > 0.05$, data not shown). Furthermore, there was no significant correlation between the frequency of different subsets of functional CD4$^+$ T-cells and the values of ESR, the levels of serum CRP, C3, and C4 in SLE patients ($P > 0.05$).

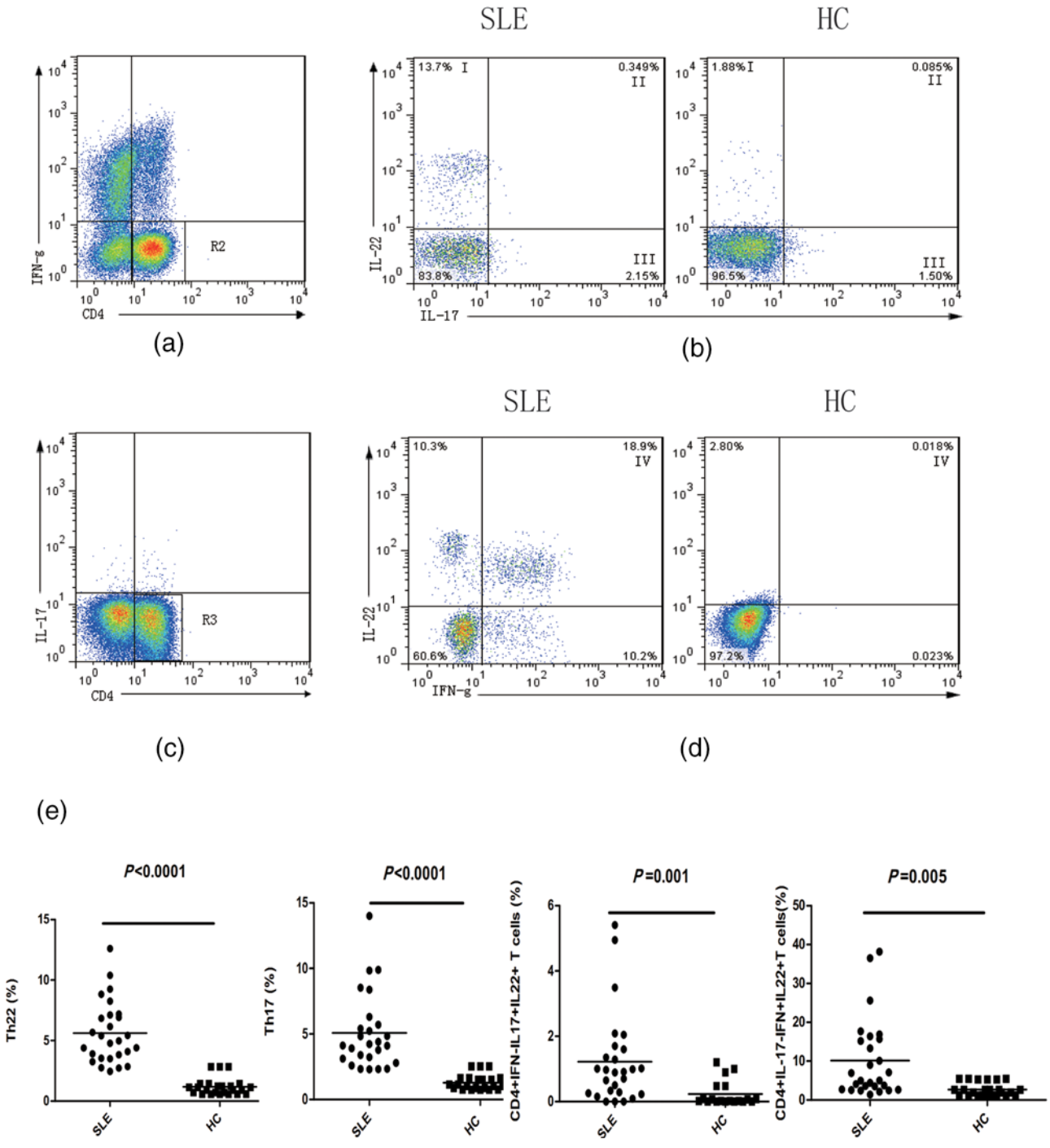

Figure 1 Flow cytometry analysis of the frequency of IL-22$^+$CD4$^+$ T-cells in patients with systemic lupus erythematosus (SLE) and controls. Peripheral blood mononuclear cells from SLE patients and healthy controls were stimulated *ex vivo* with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for six hours. The cells were stained with PerCP-anti-CD4. After being fixed and permeabilized, the cells were stained with FITC-anti-IFN-γ, Alexa-Flour-anti-IL-17 and PE-anti-IL-22 and analyzed by flow cytometry. The cells were first gated on IFNγ^-CD4$^+$ T-cells (R2) to characterize the frequency of Th22 (IL-22$^+$IL-17$^-$ IFNγ^-CD4$^+$), IL-22$^+$IL-17$^+$IFNγ^-CD4$^+$ or Th17 (IL-22$^-$IL-17$^+$IFNγ^-CD4$^+$) cells or on IL-17$^-$CD4$^+$ T-cells (R3) to analyze the frequency of IL-22$^+$IL-17$^-$IFNγ^+CD4$^+$ T-cells, respectively. Data are representative charts or expressed as individual values of 22 patients and 18 controls. (a) Flow cytometry analysis of the frequency of cells gated on PerCP-anti-CD4$^+$ and FITC-anti-IFN-γ^-; (b) flow cytometry analysis of the frequency of Th22(I), IL-22$^+$IL-17$^+$ IFNγ^-CD4$^+$ (II) or Th17 (III) cells; (c) flow cytometry analysis of the frequency of cells gated on PerCP-anti-CD4$^+$ and Alexa-Flour-anti-IL-17$^-$; (d) flow cytometry analysis of the frequency of IL-22$^+$IFNγ^+IL-17$^-$CD4$^+$ T-cells(IV); and (e) quantitative analysis. (A color version of this figure is available in the online journal)

Discussion

In this study, we found that the percentages of IL-22$^+$IL-17A$^-$IFNγ^-, IL-22$^+$IL-17A$^+$IFNγ^-, IL-22$^-$IL-17A$^+$IFNγ^- and IL-22$^+$IL-17A$^-$IFNγ^+ CD4$^+$T-cells in patients with SLE were significantly higher than that in the controls. Furthermore, we found that the percentages of IL-22$^+$ CD4$^+$ T-cells and Th17 cells were correlated positively with the values of SLEDAI in SLE patients. These data were consistent with a recent report[7] and extended previous

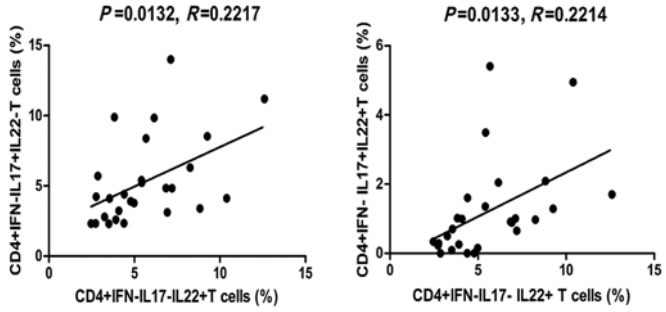

Figure 2 The percentages of IL-22$^+$CD4$^+$ T-cells are correlated with the frequency of IL-17$^+$CD4$^+$ T-cells. The potential association between the frequency of IL-22$^+$CD4$^+$ T-cells and IL-17$^+$CD4$^+$ T-cells in patients with systemic lupus erythematosus was analyzed by the Spearman rank correlation test. Data are expressed as individual values of the percentages of IL-22$^+$IL-17$^-$IFNγ^- CD4$^+$ T-cells against the frequency of CD4$^+$IL-22$^+$IL-17$^+$ IFNγ^- or CD4$^+$IL-22$^-$IL-17$^+$IFNγ^- T-cells in CD4$^+$ T-cells. There was no significant association between the frequency of IL-22$^+$CD4$^+$ T-cells and IFNγ^+CD4$^+$ T-cells (data not shown)

findings of a high frequency of IL-22$^+$CD4$^+$ T-cells in patients with ankylosing spondylitis (AS), RA, and SLE.[7,26] Our findings further support the notion that IL-22$^+$CD4$^+$ T and Th17 cells contribute to the pathogenesis of SLE in humans. Our novel data also suggest that the frequency of IL-22$^+$CD4$^+$ or Th17 cells may be a biomarker for the evaluation of disease severity in SLE patients. Our findings may provide new insights into understanding the pathogenesis of SLE in humans.

IL-22 can be secreted by different types of memory and effector CD4$^+$ T-cells.[27] We found that the frequency of IL-22$^+$IL17A$^-$IFN-γ^- and IL-22$^-$IL-17A$^+$IFN-γ^- or IL-22$^+$IL-17A$^+$IFN-γ^- and IL-22$^+$IL-17A$^-$IFN-γ^+ CD4$^+$ T-cells was closely associated in SLE patients. Our data suggest that these different types of IL-22$^+$CD4$^+$ T-cells may collaborate and contribute to the pathogenesis of SLE in humans. Although a previous study suggests that IL-22$^+$CD4$^+$ T-cells may be different from Th17 and Th1 cells,[28] we found that the frequency of IL-22$^+$IL-17A$^-$IFN-γ^+ CD4$^+$ T-cells was near two-fold higher than other subsets tested in SLE patients. It is possible that activation of CD4$^+$ T-cells may begin to differentiate into IL-22$^+$IFNγ^+ T-cells and then switch to a direction towards IL-17$^+$ T-cells during the pathogenic process of SLE in humans. Hence, the environmental cytokine profiles may be crucial in determining the ontogenic plasticity of CD4$^+$ T-cell progenitors. We are interested in further investigating the ontogenic plasticity of CD4$^+$ T-cell progenitors during the pathogenic process of SLE in humans.

We found the levels of plasma IL-22 and serum CRP in SLE patients were significantly higher than that in the controls. However, we recognized that our findings were in disagreement with previous studies that have shown significantly lower concentrations of plasma IL-22 and CRP in SLE patients than that in healthy controls.[7,15,16] The difference in the levels of plasma IL-22 and CRP between our study and those of others may stem from the different populations of patients studied, varying disease severities, and durations as well as therapeutic status. All of the patients were recruited from the inpatient service in our study, and they had more severely clinical symptoms. Furthermore, our patients had clinical symptoms within three months, while another study had some patients with standard treatment and a much longer duration.[7] Indeed, treatment with glucocorticoid dexamethasone (DEX) reduces the frequency of peripheral blood IL-22$^+$ T-cells and the levels of plasma IL-22 in patients with acute bacterial infection.[29] In the other two studies, there was no clear information about the disease duration.[15,16] In addition, although we have excluded individuals with fever and obvious infection, we cannot completely exclude the possibility of some patients with atypical infection, which may increase the levels of plasma IL-22 and CRP. Alternatively, it is possible that there are dynamic changes in the levels of plasma IL-22 and CRP during the pathogenic process of SLE. The levels of plasma IL-22 and CRP in SLE patients may start at a high level and decline with the disease progression or after standard treatment. We are interested in further investigating the dynamic changes in the levels of plasma IL-22 in patients with different stages and activities of SLE.

IFN-γ and IL-17 are important for the pathogenesis of SLE.[22,24] In this study, we detected a higher frequency of IL-17$^+$ T-cells in SLE patients, which was positively

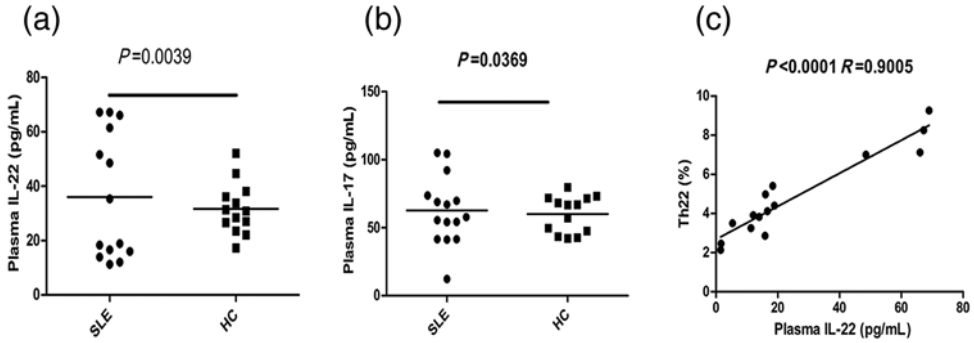

Figure 3 The levels of plasma interleukin (IL)-17 and IL-22 levels and their relationships with IL-22$^+$CD4$^+$ T-cells in participants. The levels of plasma IL-17 and IL-22 in individual participants were determined by enzyme-linked immunosorbent assay and the potential association between the levels of plasma IL-17 and IL-22 and the frequency of IL-22$^+$CD4$^+$ T-cells in CD4$^+$ T-cells in patients was analyzed by the Spearman rank correlation test. Data shown are mean values of individual participants from three separate experiments. (a) The levels of plasma IL-22; (b) the levels of plasma IL-17 and (c) the correlation analysis ($n = 15$). There was no significant association between the levels of plasma IL-22 and the frequency of IL-17$^+$CD4$^+$ T-cells in CD4$^+$ T-cells and between the levels of plasma IL-17 and the frequency of IL-22$^+$CD4$^+$ T-cells in CD4$^+$ T-cells in patients with systemic lupus erythematosus (data not shown)

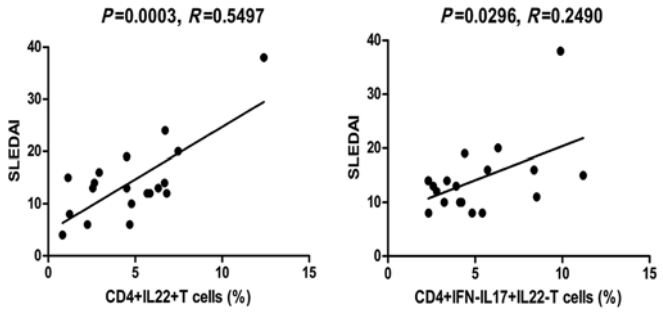

Figure 4 The percentages of Th17 and IL-22$^+$CD4$^+$ T-cells are correlated with the severity of the disease in patients with systemic lupus erythematosus (SLE). The potential association of the percentages of Th17 and IL-22$^+$CD4$^+$ T-cells in CD4$^+$ T-cells with the values of SLEDAI in patients with SLE was analyzed by the Spearman rank correlation test. Data shown are the values of SLEDAI against the percentages of Th17 and IL-22$^+$CD4$^+$ T-cells in CD4$^+$ T-cells in patients with SLE ($n = 16–18$)

correlated with the severity of SLE and higher levels of plasma IL-17A in SLE patients. Our data were consistent with previous reports and support the generally accepted view that Th17 cells are crucial for the pathogenesis of SLE in humans.[2,22] While higher levels of IFN-γ mRNA transcripts are detected in patients with SLE,[3,23] another study shows that lower levels of serum IFN-γ are observed in patients with SLE.[24] We observed a similar frequency of IL-22$^-$IL-17A$^-$IFN-γ^+ CD4$^+$Th1 cells and similar levels of plasma IFNγ in both SLE patients and healthy controls. Given that IFN-γ can be secreted by activated CD4$^+$, CD8$^+$ T-cells, macrophages and NK cells, it is possible that other types of cells secrete more IFN-γ while a low frequency of Th1 cells secrete low levels of IFN-γ, balancing the levels of plasma IFN-γ in SLE patients.

Conclusion

In summary, our data indicated a higher frequency of IL-22$^+$CD4$^+$ T-cells in SLE patients, which was correlated positively with the percentages of Th17 cells and the values of SLEDAI. Our findings suggest that IL-22$^+$CD4$^+$ T-cells may contribute to the pathogenesis of SLE and that the frequency of IL-22$^+$CD4$^+$ may be valuable for the evaluation of disease severity in SLE patients. We recognized that our study had limitations of small sample size and testing at single time point as well as the lack of antigen specificity. We are interested in further investigating the role of individual types of IL-22$^+$CD4$^+$ T-cells in the pathogenesis of SLE and the dynamic changes in the frequency of different types of IL-22$^+$CD4$^+$ T-cells during the pathogenic process of SLE in humans.

Author contributions: All authors participated in designing, analyzing results and writing the manuscript.

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