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ORIGINAL ARTICLE

Upregulated expression of STAT3/IL-17 in patients with systemic lupus erythematosus

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Abstract

Elevated IL-17 levels with higher Th17 numbers are identified in systemic lupus erythematosus (SLE). STAT3 signaling plays a crucial role in the Th17 generation, and SOCS3 negatively regulates their formation. We investigated IL-17, STAT3, and SOCS3 expression, and analyzed their correlations to elucidate the regulatory mechanisms of IL-17 production in SLE. This study enrolled 32 patients, and venous mononuclear cells (MNCs) were isolated with further purification of CD4-positive T cells. IL-17 and SOCS3 levels were measured by real-time quantitative PCR, and pSTAT3/STAT3 expression was analyzed by immunoblot. Elevated IL-17 and SOCS3 levels were identified in lupus patients. There were higher IL-17 levels in lupus nephritis (class IV) than in SLE without renal involvement. Positive correlations were found between IL-17 levels and SOCS3 expression, lupus activity (SLEDAI-2K), or daily proteinuria. There were higher intensities of pSTAT3/β-actin and STAT3/β-actin in SLE, and a positive correlation between IL-17 expression and pSTAT3/β-actin or STAT3/β-actin intensity. Lupus nephritis (class IV) had higher STAT3/β-actin intensity than SLE without renal involvement. These results suggest upregulated STAT3/IL-17 expression in lupus patients. Such findings might facilitate the development of novel compounds and the application of existing therapeutics targeting the STAT3/IL-17 signal in SLE.

Keywords IL-17 · Lupus nephritis · STAT3 · Systemic lupus erythematosus

Introduction

In systemic lupus erythematosus (SLE), there is a persistently activated immune system with the production of humoral mediators, and elevated IL-17 levels with higher Th17 numbers have been identified in blood samples and renal tissues, raising a therapeutic potential targeting IL-17 or related signaling components in lupus patients [1]. Notably, the suppressor of cytokine signaling (SOCS) family containing SOCS1 to 7 and cytokine-inducible SH2 protein functions as an E3 ubiquitin ligase to mediate the degradation of associated proteins through their N-terminal and SH2 regions [2, 3]. These members are regarded as critical participants of the negative feedback loop regulating the quality and intensity of the cytokine signaling. SOCS1 and SOCS3 are potent inhibitors of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway through directly binding with JAK to inhibit its kinase activity via their KIR domains [3]. Increasing evidence indicates that SOCS1 and SOCS3 are dysregulated in human autoimmune disorders like SLE [4], and extensive investigations have shown impaired production of SOCS with hyper-activated type I interferon/JAK/STAT signaling pathway in SLE, suggesting the clinical intervention to upregulate SOCS expression as a therapeutic strategy [4, 5]. Furthermore, SOCS3 has been shown to negatively regulate the Th17 formation by using gene-targeted mice to study their formation [6], and previous studies have demonstrated a crucial role of STAT3 signaling in the human Th17 generation [7].

In this study, we investigated the expression of IL-17, STAT3, and SOCS3 in venous mononuclear cells (MNCs) with further purification of CD4-positive T cells to analyze their correlations to elucidate the regulatory mechanisms of IL-17 production in lupus patients including the lupus nephritis (LN) victims.

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Materials and methods

Study patients

Thirty-two patients fulfilling the American College of Rheumatology revised Criteria for SLE [8], 30 females and 2 males with ages from 19 to 45 years (32.3 ± 1.4), were enrolled into this study. Another 20 age- and sex-matched healthy individuals were served as a control group. The Institutional Review Board approved this study with the informed consent obtained from each participant. Medical records of these patients were reviewed for demographic, clinical, and laboratory data. Their disease activities were assessed by the SLEDAI-2K [9], and laboratory parameters were analyzed, including daily proteinuria amounts, anti-DNA levels, C3 and C4 concentrations, and peripheral blood cell counts (red blood cells, platelets, total leukocytes, and lymphocytes). The diagnosis of LN was based on a histopathological evaluation of renal biopsy, and/or long-term serial follow-up of blood and urine examinations [10]. Twenty patients in this study had LN according to the diagnosis criteria used in this report.

MNCs preparation and CD4-positive T cells purifications

MNCs were isolated from heparinized venous blood samples by Ficoll-Paque PLUS (GE Healthcare) in enrolled lupus patients and healthy volunteers. CD4-positive T cells were purified from MNCs through a positive selection column of magnetic cell sorter (Miltenyi Biotec), as previously described [11].

Real-time quantitative polymerase chain reaction

After RNA was isolated from MNCs with further synthesis into cDNA, the real-time quantitative PCR was performed to quantify IL-17, SOCS3, and GAPDH levels by using SYBR Premix Ex Taq (TaKaRa) in the SmartCycler (Cepheid), as previously described [12]. The primer sequences were as follows. Forward 5′-GCAATGAGGACCCTGAGAGA-3′/reverse 5′-CCCACGGACACCAGTATCTT-3′, forward 5′-GCCACCTACTGAACCCTCCT-3′/reverse 5′-ACGGTCTTCCGACAGAGATG-3′, and forward 5′-CTCATGACCACAGTCCATGCCATC-3′/ reverse 5′-CGTTCAGCTCTGGGATGACCTTG-3′. The comparative Ct method was used to calculate the relative abundance of IL-17 and SOCS3 as compared with the expression of GAPDH.

Immunoblot assay

The RIPA lysis buffer-treated MNCs lysates were subjected to the immunoblot analysis with antibodies against tyrosine 705 phosphorylated STAT3 (pSTAT3, Santa Cruz Biotechnology), STAT3 (Santa Cruz Biotechnology), SOCS3 (Cell Signaling), or β-actin (Sigma-Aldrich) in combination with a horseradish peroxidase-labeled secondary antibody (Jackson ImmunoResearch), developed with an ECL plus system (Amersham), and analyzed by a Biospectrum imaging system (UVP) for the chemiluminescence detection, as previously described [12]. The signal intensities were quantitated by the Image J software (NIH).

Statistical analysis

Data were represented as mean ± standard error of the mean (SEM). Mann-Whitney rank sum test was used to compare relative mRNA expression levels of IL-17 or SOCS3 and intensity ratios of pSTAT3/β-actin or STAT3/β-actin in immunoblot between different groups. The Pearson correlation coefficient test with linear regression analysis was performed to correlate between relative mRNA expression levels, intensity ratios in immunoblot, SLEDAI-2K scores and/or miscellaneous laboratory parameters. P value less than 0.05 was considered statistically significant in this study.

Results

The MNCs samples were examined for IL-17 mRNA expression. Significantly higher IL-17 levels were found in lupus patients in comparison with healthy controls (Fig. 1a, 93.66 ± 30.12 versus 1.59 ± 0.27, $P = 0.036$). Furthermore, there was a significant positive correlation between IL-17 levels and SLEDAI-2K scores (Fig. 1b, $r = 0.388$, $P = 0.028$) or daily proteinuria amounts (Fig. 1c, $r = 0.360$, $P = 0.043$). In addition, significant positive correlations existed between IL-17 levels and proteinuria more than 1 g/day ($n = 18$, $r = 0.586$, $P < 0.01$) or proteinuria in patients with renal involvement ($n = 20$, $r = 0.588$, $P < 0.01$). Patients with LN had higher IL-17 levels than those without the renal involvement (106.50 ± 43.43 versus 72.26 ± 36.33). In 8 patients with class IV LN, there were significantly higher IL-17 levels than in 12 patients without renal involvement (Fig. 1d, 263.20 ± 83.38 versus 72.26 ± 36.33, $P = 0.019$). No significant correlations were found in other laboratory parameters including anti-DNA levels, C3 and C4 concentrations, and blood cell counts.

Next, we examined the SOCS3 mRNA expression in MNCs. There were higher levels in lupus patients in comparison with healthy controls (Fig. 2a, 1.61 ± 0.60 versus 0.98 ± 0.11). The correlation coefficients were calculated between the SOCS3 expression and IL-17 levels, disease activity scores, or miscellaneous laboratory data. There was a significant positive correlation between SOCS3 and IL-17 expression levels in lupus patients (Fig. 2b, $r = 0.406$, $P = 0.021$). No correlations were found in SLEDAI-2K scores, daily

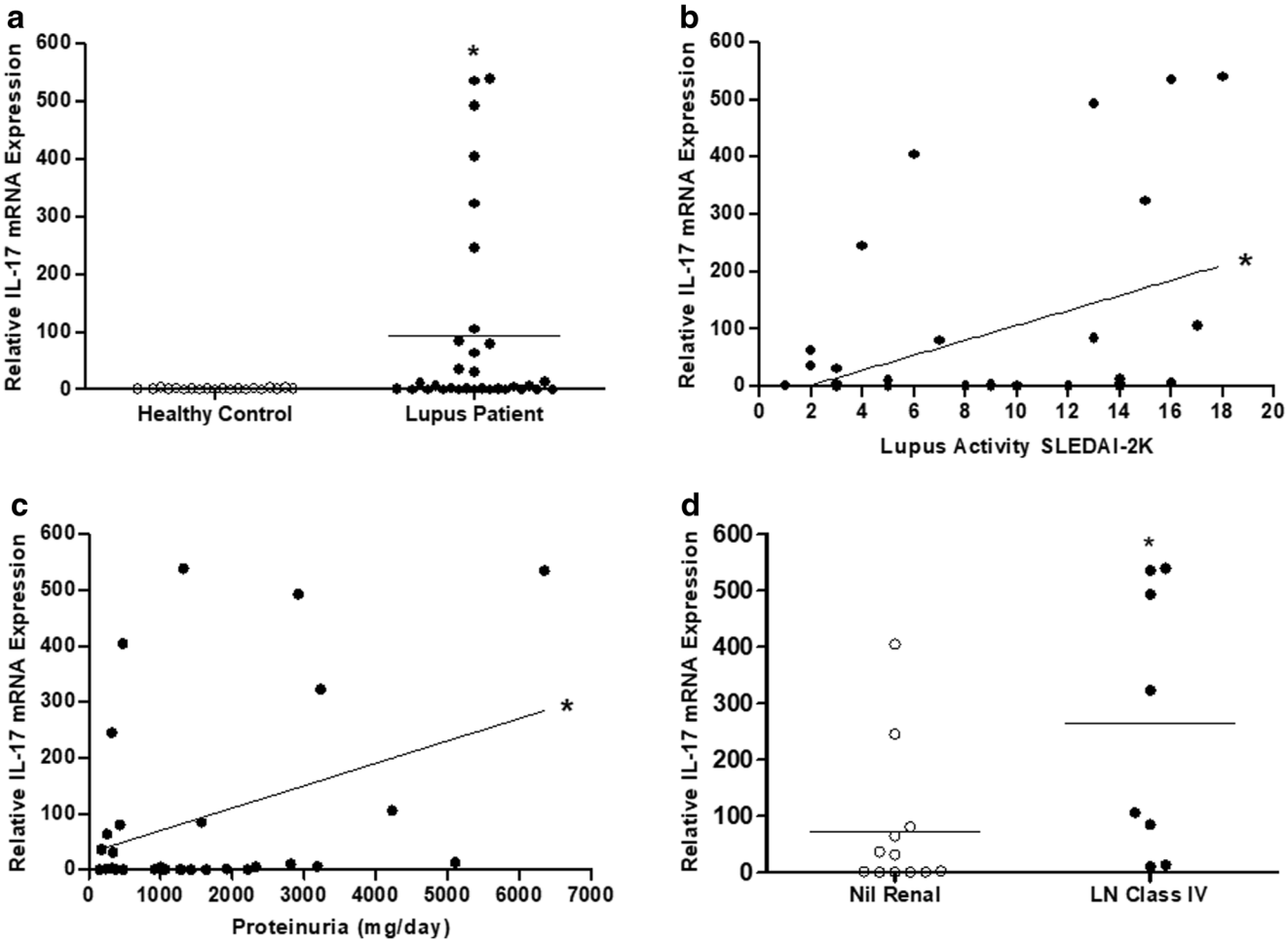

Fig. 1 IL-17 expression in MNCs from lupus patients and healthy controls. **a** Significantly higher IL-17 levels in lupus patients than healthy controls. **b** A significant positive correlation between IL-17 expression levels and SLEDAI-2K scores. **c** A significant positive correlation between IL-17 expression levels and daily proteinuria amounts. **d** Significantly higher IL-17 expression levels in LN class IV patients than SLE without renal involvement. $n = 32$ for a lupus patient, $n = 20$ for healthy control, $n = 12$ for nil renal, and $n = 8$ for LN class IV. LN, lupus nephritis; $^*P < 0.05$

proteinuria amounts, and other laboratory parameters. In addition, we examined the expression of SOCS3 protein in MNCs from selected lupus patients and healthy subjects. Similar to the expression levels of mRNA levels, there were

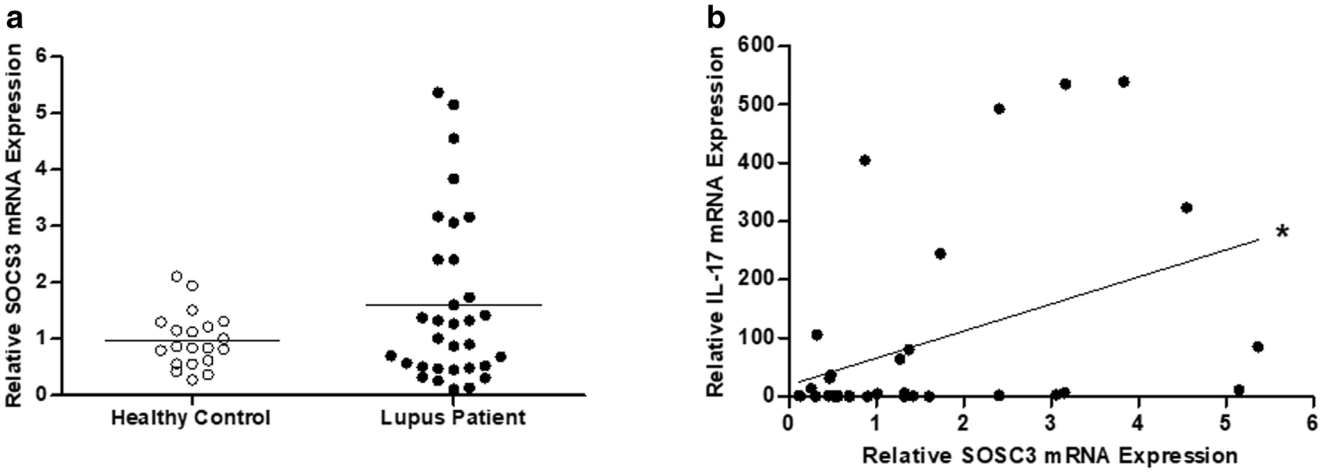

Fig. 2 SOCS3 expression in MNCs from lupus patients and healthy controls. **a** Higher SOCS3 levels in lupus patients than healthy controls. **b** A significant positive correlation between SOCS3 and IL-17 expression levels. $n = 32$ for lupus patient and $n = 20$ for healthy control; $^*P < 0.05$

higher levels of SOCS3/β-actin density ratios in lupus patients as compared with healthy controls (data not shown).

We further investigated the expression of pSTAT3 and STAT3 in MNCs. Figure 3a demonstrates the representative immunoblot graphs with pSTAT3, STAT3, and β-actin expression. In Fig. 3b, there were significantly higher intensity ratios of pSTAT3/β-actin (0.288 ± 0.078 versus 0.206 ± 0.041, $P < 0.01$) and STAT3/β-actin (0.465 ± 0.191 versus 0.325 ± 0.068, $P < 0.01$) in lupus patients as compared with healthy controls. For IL-17 expression levels in SLE, significant positive correlations were identified in pSTAT3/β-actin ($r = 0.387$, $P = 0.029$) and STAT3/β-actin intensity ratios (Fig. 3c, $r = 0.417$, $P = 0.018$). LN patients had higher pSTAT3/β-actin (0.296 ± 0.081 versus 0.274 ± 0.073) and STAT3/β-actin intensity ratios (0.482 ± 0.213 versus 0.438 ± 0.152) than those without the renal involvement. In 8 patients with class IV LN, there were higher pSTAT3/β-actin (0.347 ± 0.082 versus 0.274 ± 0.073) and significantly higher STAT3/β-actin intensity ratios (Fig. 3d, 0.666 ± 0.232 versus 0.438 ± 0.152, $P = 0.015$) than 12 patients without renal involvement.

Finally, we purified CD4-positive T cells from MNCs. In Fig. 4a and b, the IL-17 and SOCS3 levels were significantly higher in lupus patients as compared with age- and sex-matched healthy individuals (For IL-17, 62.55 ± 25.71 versus 2.71 ± 0.62, $P < 0.01$; for SOCS3, 1.855 ± 0.384 versus 1.018 ± 0.174, $P = 0.032$). There were significantly higher levels of pSTAT3/β-actin and STAT3/β-actin intensity ratios from lupus patients in comparison with healthy controls (Fig. 4c, pSTAT3/β-actin, 0.424 ± 0.033 versus 0.359 ± 0.042, $P = 0.047$; STAT3/β-actin, 0.617 ± 0.093 versus 0.472 ± 0.037, $P < 0.01$).

Discussion

IL-17 plays an important role in the SLE pathogenesis, especially with renal involvement [1, 13]. Increased circulating Th17 cell frequencies from lupus patients were demonstrated by our previous experiments [14], and elevated IL-17 levels in SLE were identified in this study, both with a positive

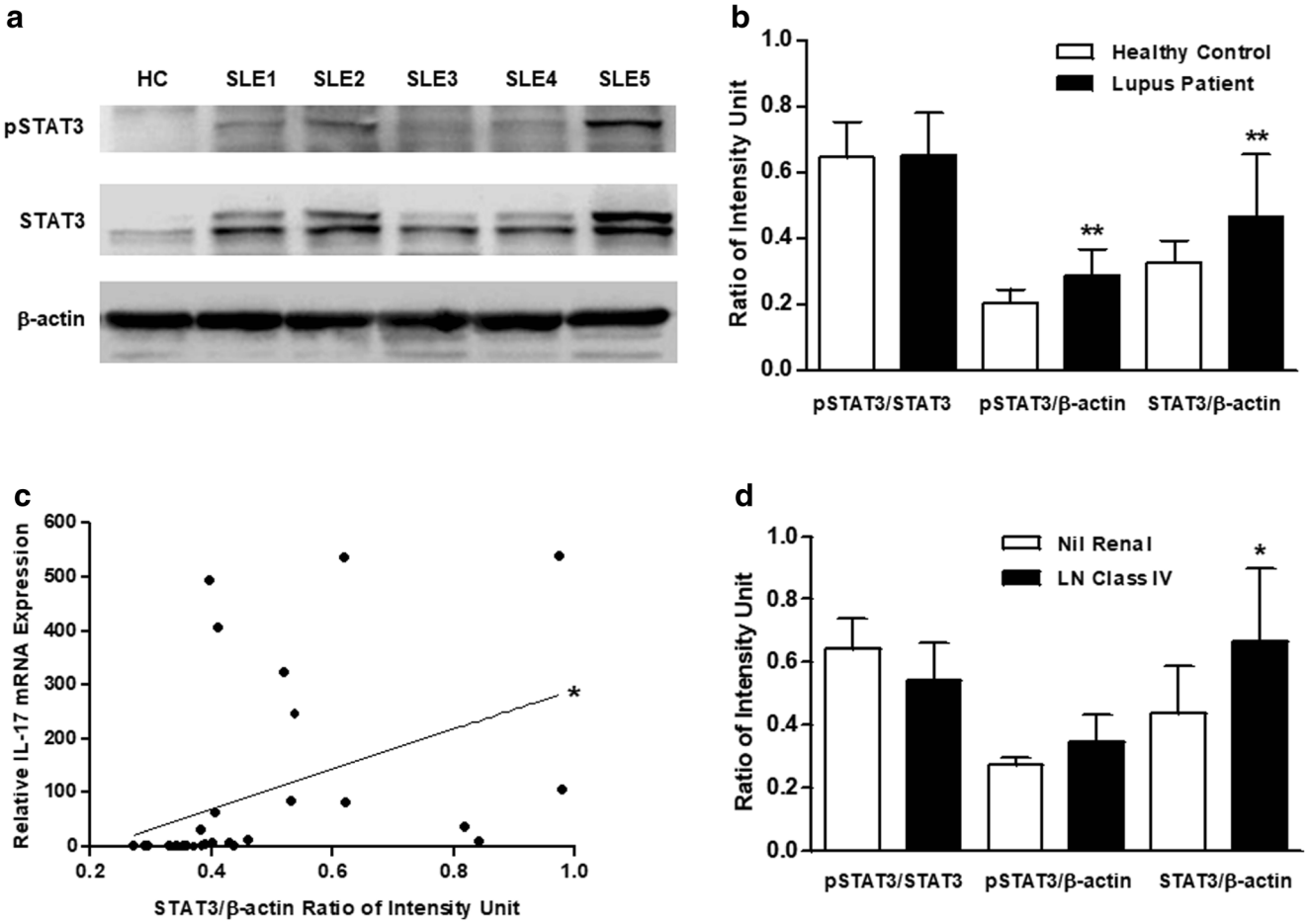

Fig. 3 pSTAT3 and STAT3 expression in MNCs from lupus patients and healthy controls. **a** Representative immunoblot graphs with pSTAT3, STAT3, and β-actin expression from lupus patients and healthy control. **b** Significantly higher intensity ratios of pSTAT3/β-actin and STAT3/β-actin in lupus patients than healthy controls. **c** A significant positive correlation between STAT3/β-actin intensity ratios and IL-17 expression levels. **d** Significantly higher IL-17 intensity ratios of STAT3/β-actin in LN class IV patients than in SLE without renal involvement. $n = 32$ for lupus patient, $n = 20$ for healthy control, $n = 12$ for nil renal, and $n = 8$ for LN class IV. HC, healthy control; LN, lupus nephritis; $^*P < 0.05$, $^{**}P < 0.01$

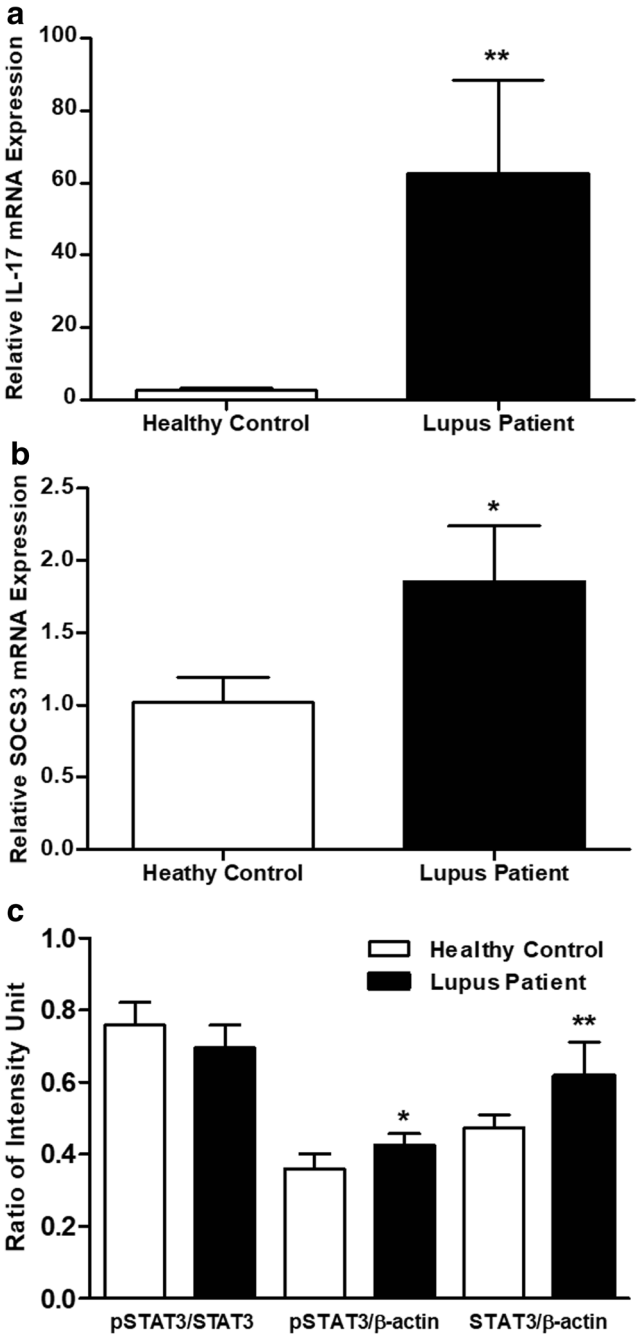

Fig. 4 IL-17 and pSTAT3/STAT3 expression in CD4-positive T cells from lupus patients and healthy controls. **a** Significantly higher IL-17 levels in lupus patients than healthy controls. **b** Significantly higher SOCS3 levels in lupus patients than healthy controls. **c** Significantly higher intensity ratios of pSTAT3/β-actin and STAT3/β-actin in lupus patients than in healthy controls. $n = 5$ for lupus patient and $n = 5$ for healthy control; $^{*}P < 0.05$, $^{**}P < 0.01$

correlation between disease activity scores or daily proteinuria amounts. Furthermore, dysregulated T cell signaling transduction promoting the IL-17 production and Th17 migration has been reported to be involved in the LN pathogenesis [1]. Despite the accumulation of IL-17-expressing cells in the kidney of MRL/lpr and NZB/W lupus mice, deficiency or neutralization of IL-17 failed to affect the disease severity in these models [13]. Notably, an anti-IL17 monoclonal antibody with the FDA-indication for psoriasis/psoriatic arthritis [15] has been prescribed in a patient with both psoriasis and LN, resulting in an improvement in renal involvement [16]. Such contradictory results in targeting IL-17 pathway probably reflect the differences of Th17/IL-17 response in LN between murine models and human patients. Continuous efforts to establish the aberrant Th17/IL-17 signaling dysfunction in SLE might initiate clinical trials with the existing medications and/or preclinical compounds, further contributing to the expansion of novel therapeutics in lupus patients.

Elucidating the JAK/STAT signal transduction has brought insights into disease mechanisms and provided the basis for the pharmacological development [17]. Notably, aberrant STAT signal has been identified in lupus patients with activated STAT3 in T cells [18, 19]. Intra-peritoneal injection of a small-molecule STAT3 inhibitor could delay the onset of proteinuria and lower autoantibodies titers in the lupus-prone MRL/lpr mice [20]. In this study, in addition to increased IL-17 production, there were higher expression levels of pSTAT3 and STAT3 in purified CD4 T cells from lupus patients, consistent with the previous findings of activated STAT3 status in SLE T cells and monocytes [18, 19, 21]. By using the gene knockout mice model, STAT3 has been demonstrated to directly bind the IL-17 promoter of Th17 cells [6], and T cell-specific deletion of STAT3 can significantly affect the expression of IL-17 [22]. In the studies related to human Th17 formation, the STAT3 signaling has been shown to play a critical role [7]. Indeed, our results revealed higher ratios of pSTAT3/β-actin and STAT3/β-actin with a positive correlation between IL-17 expression and pSTAT3 or STAT3 intensity in a lupus patient. Taken together, these findings implicated STAT3 as an upstream signal responsible for the IL-17 production in SLE.

The cytokine signaling can regulate immune responses and coordinate various pathogenic processes in human disorders, and such signals join in activating the JAK/STAT pathway, tightly regulated by the miscellaneous SOCS molecules [3, 23]. Nevertheless, despite a more than 3-fold upregulation of SOCS3 mRNA levels in lymphocytes from a lupus disease model *Sle1ab* mice [24], there were inconsistent findings in the SOCS3 expression by examining MNCs from lupus patients [5, 18]. Interestingly, higher SOCS3 levels have been identified in MNCs from other autoimmune disorders like rheumatoid arthritis as compared with healthy controls [25]. We identified elevated levels of SOCS3 and a positive correlation between the SOCS3 and IL17 expression in lupus patients, indicating a lack of negative regulation from SOCS3 on IL-17 expression in SLE. Our results suggest a parallel upregulated SOCS3 signaling following the overacted STAT3/IL-17 expression, leading to the flare-up of disease activity in lupus patients without the inhibitory efficacy.

In conclusion, we demonstrate the upregulation of STAT3/IL-17 expression in lupus patients. Such findings might facilitate the development of novel compounds and the application of existing therapeutics targeting the STAT3/IL-17 signal in SLE.

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Author contributions Author contributions are as follows. MFL created the concept. CRW designed the study. SYC and PYK performed the experiments. SYC, MFL, and CRW analyzed the data. CRW wrote the manuscript.

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Compliance with ethical standards

Disclosures None.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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