Glucocorticoids enhance Th17/Th1 imbalance and signal transducer and activator of transcription 3 expression in systemic lupus erythematosus patients

Catuxa Prado, Banesa de Paz, Jesús Gómez, Patricia López, Javier Rodríguez-Carrio and Ana Suárez

Abstract

Objective. To investigate the relative amounts of Th17 and Th1 cells present in SLE patients and the possible effects of treatments or disease features on these populations.

Methods. Peripheral blood mononuclear cells were collected from 75 SLE patients and 19 healthy controls and the proportion of Th17 and Th1 populations were assessed by flow cytometry measuring the amount of IL-17 and IFN-γ-producing cells. Gene expression of signal transducers and activators of transcription 3 (STAT3), STAT4, IL-6R and IL-12R were determined in 30 patients and 8 healthy individuals by real-time RT-PCR. Data were related to clinical and immunological parameters and to the treatment followed during the past 3 months.

Results. Th17 cells and the Th17/Th1 ratio were significantly increased in SLE patients treated with glucocorticoids compared with healthy individuals, untreated patients or those under other treatments. No association was detected with clinical parameters, but patients with anti-ENA antibodies also displayed increased Th17 responses. Disease activity (SLEDAI) is associated with the Th17/Th1 index only in glucocorticoid-treated patients. In line with these results, gene expression of STAT3 and IL-6R was up-regulated in patients taking these drugs. Accordingly, we found a positive correlation between the Th17/Th1 ratio and STAT3 levels.

Conclusions. The present work provides the first evidence that aberrant Th17/Th1 balance in SLE is linked to the use of glucocorticoids and suggests that the up-regulatory effect of these drugs on the Th17 population could be associated with their ability to increase STAT3 and IL-6R expression. Additionally, anti-ENA positivity could represent a potential biomarker for Th17 bias.

Key words: Systemic lupus erythematosus, Th17, Glucocorticoids, Signal transducers and activators of transcription 3.

Introduction

SLE is an autoimmune disorder in which chronic stimulation of the immune system results in inflammation that affects many organs. SLE patients are often treated with strong immunosuppressive regimens, including glucocorticoids, cytotoxic drugs and anti-malarial compounds. Due to its anti-inflammatory effects, glucocorticoids are widely used to treat patients with autoimmune or inflammatory diseases; however, regimens used to treat many other autoimmune conditions cannot maintain disease control in most SLE patients. Therefore more aggressive approaches may be employed to provide transient reductions in disease activity, although they often do not induce remission or prevent end-organ damage. The reason why treatment of some SLE patients requires high glucocorticoid doses or other strong immunosuppressive regimens is not clear.

Glucocorticoids mainly act by binding to the intracellular glucocorticoid receptor. The complex then migrates to the nucleus, where it can bind to cognate DNA.
sequences [glucocorticoid responding elements (GREs)], present in the promoter of several immune and non-immune genes, modulating their transcription. In addition, glucocorticoids can function by interacting with other transcription factors and interfering with their actions. Actually, inhibition of nuclear factor kappa B (NF-kB) activity is thought to be the most important mechanism by which glucocorticoids exert their anti-inflammatory effects [1]. Moreover, in the past few years, several functional interactions between glucocorticoids and signal transducers and activators of transcription (STATs), which play a critical role in mediating signalling downstream of cytokine receptors, have been described, suggesting the existence of relevant biological implications [2].

It is known that glucocorticoid treatment is able to modulate the production of a variety of cytokines, the resulting cytokine milieu being critical to the development of distinct Th lineages. Linkage of certain cytokines to their receptors on the surface of naïve CD4+ T cells leads to the activation of specific STATs, which translocate into the nucleus and bind to target sequences in the promoter of various genes, thus resulting in the differentiation towards determinate Th lineage. In fact, STAT4, an IL-12 signal transducer, is required for Th1 generation [3], whereas STAT3 is the key signal transducer for most of the cytokines involved in Th17 generation, such as IL-6 [4, 5], IL-23 [5, 6] and IL-21 [7].

Production of IL-17 has been reported to be augmented in SLE patients [8-12], suggesting that this cytokine may have a potential role in the pathogenesis of the disease. A previous work concerning IL-17 and IFN-γ-producing cells (Th17 and Th1, respectively) has suggested the existence of a Th17/Th1 imbalance in lupus patients [13]. Several hypotheses have been proposed to explain the alterations found in these populations; however, it remains unknown why IL-17 is increased in lupus and the possible effects of treatments on Th17 cells.

In the present work, we investigated the Th17/Th1 balance in SLE patients, analysing the amount of IL-17 and IFN-γ-producing cells and the gene expression of STAT3, STAT4 and the specific receptors for IL-6 and IL-12, demonstrating a relevant effect of glucocorticoids on the deviation towards a Th17 response observed in lupus patients.

**Materials and methods**

**Subjects**

Seventy-five patients with a diagnosis of SLE based on the ACR criteria were selected from the Asturian Register of Lupus [14, 15]. Nineteen sex- and age-matched healthy donors were used as controls. Information on clinical and immunological manifestations was obtained by reviewing clinical histories. At the time of sampling, disease activity was scored based on the SLEDAI and patients were also asked questions about the treatment they received over the previous 3 months (Table 1). This study has been performed in compliance with the Declaration of Helsinki.

**Table 1** Characteristics and disease parameters of SLE patients

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>No treatment</th>
<th>Without corticoids</th>
<th>With corticoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE patients (n)</td>
<td>12</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>Gender: women/men</td>
<td>12/0</td>
<td>30/2</td>
<td>30/1</td>
</tr>
<tr>
<td>Age at diagnosis, mean (s.d.), years</td>
<td>34.25 (14.21)</td>
<td>37.41 (15.14)</td>
<td>33.52 (13.01)</td>
</tr>
<tr>
<td>Disease duration, mean (s.d.), years</td>
<td>8.36 (4.27)</td>
<td>13.99 (9.22)</td>
<td>11.71 (9.23)</td>
</tr>
<tr>
<td>Clinical manifestations, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malar rash</td>
<td>5 (41.7)</td>
<td>17 (53.1)</td>
<td>19 (61.3)</td>
</tr>
<tr>
<td>Discoid lesions</td>
<td>0 (0.0)</td>
<td>6 (18.8)</td>
<td>4 (12.9)</td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>7 (58.3)</td>
<td>22 (68.8)</td>
<td>16 (51.6)</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>6 (50.0)</td>
<td>14 (43.8)</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td>Arthritis</td>
<td>8 (66.7)</td>
<td>23 (71.9)</td>
<td>25 (80.6)</td>
</tr>
<tr>
<td>Serositis</td>
<td>3 (25.0)</td>
<td>4 (12.5)</td>
<td>6 (19.4)</td>
</tr>
<tr>
<td>Renal disorder</td>
<td>2 (16.7)</td>
<td>13 (40.6)</td>
<td>11 (35.5)</td>
</tr>
<tr>
<td>Neurological disorder</td>
<td>1 (8.3)</td>
<td>2 (6.3)</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>Haematological disorder</td>
<td>5 (41.7)</td>
<td>23 (71.9)</td>
<td>23 (74.2)</td>
</tr>
<tr>
<td>Presence of anti-dsDNA</td>
<td>7 (58.3)</td>
<td>24 (75.0)</td>
<td>24 (77.4)</td>
</tr>
<tr>
<td>Anti-dsDNA levels, mean (s.d.), U/ml</td>
<td>24.37 (30.79)</td>
<td>22.01 (36.07)</td>
<td>39.29 (85.32)</td>
</tr>
<tr>
<td>Presence of anti-ENA</td>
<td>3 (25)</td>
<td>20 (62.5)</td>
<td>13 (41.9)</td>
</tr>
<tr>
<td>Anti-SSA+</td>
<td>3 (25.0)</td>
<td>16 (50.0)</td>
<td>10 (32.3)</td>
</tr>
<tr>
<td>Anti-SSB+</td>
<td>1 (8.3)</td>
<td>8 (25.0)</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>Anti-RNP+</td>
<td>0 (0.0)</td>
<td>6 (18.8)</td>
<td>6 (19.4)</td>
</tr>
<tr>
<td>Anti-Sm+</td>
<td>0 (0.0)</td>
<td>2 (6.3)</td>
<td>7 (22.6)</td>
</tr>
<tr>
<td>Other treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-malarials</td>
<td>-</td>
<td>32 (100)</td>
<td>22 (71.0)</td>
</tr>
<tr>
<td>Immunosuppressants</td>
<td>-</td>
<td>3 (9.4)</td>
<td>12 (38.7)</td>
</tr>
</tbody>
</table>

aAZA, CSA, mycophenolate mophetil or CYC.
and was granted ethical approval by the Regional Ethics Committee for Clinical Investigation. All participants provided written informed consent.

Production of intracellular cytokines
Peripheral blood mononuclear cells (PBMCs) were isolated from patients’ and controls’ blood samples by Ficoll-Hypaque density centrifugation. The 2 × 10^6 cells/ml were cultured in RPMI 1640 (Invitrogen, UK) supplemented with 10% of heat-inactivated foetal calf serum (FCS) (Hyclone, USA) and stimulated with phorbol-12-myristate-13-acetate (PMA) (20 ng/ml) and ionomycin (500 ng/ml) in the presence of 2 μM of monensin (all from Sigma Aldrich, Germany) at 37 °C in 5% CO₂. After 5 h of culture, cells were extracellularly stained with anti-CD4 APC, CD45RO FITC (BD Pharmingen) and then fixed, permeabilized (Cytofix/Cytoperm, BD Pharmingen) and intracellularly stained with anti-IFN-γ PerCP-Cy5.5 (eBioscience) and anti-IL-17 PE (eBioscience). Appropriate isotype controls (from BD Pharmingen) were used in every sample. Analysis was carried out after acquiring 20,000 CD4+ lymphocytes on a BD FACS Canto II flow cytometer (BD) with FACSDiva Software 6.1.2 (BD).

RT–PCR analysis of gene expression
mRNA was isolated from whole-blood samples using the mRNA Isolation Kit for Blood/Bone Marrow (Roche Diagnostics, Germany). Reverse transcription was carried out using an RT² First Strand Kit (SABiosciences) and cDNA was amplified by the RT² SYBR® Green qPCR Master Mix (SABiosciences) in an ABI7900HT instrument. The 2^(-∆∆Ct) method was used to calculate the fold induction relative to expression levels of CD4. The primer pairs used were designed and purchased from SABiosciences.

Statistical analysis
Results are expressed as median and interquartile range (IR). Differences between groups were evaluated by the Mann–Whitney U test. Correlations were carried out using Spearman’s rank correlation test. All statistical analyses were performed using SPSS software (version 15.0). Graphs were drawn with GraphPad Prism software (version 4.0).

Results
SLE patients under glucocorticoid treatment have an impaired balance of Th17 and Th1 responses
A prior report has suggested that an imbalance between Th17 and Th1 responses could be a typical feature of lupus [13]. In an attempt to quantify these two subsets, we wanted to determine the amount of effector Th cell-expressing IL-17, IFN-γ or both cytokines in PBMCs from 75 lupus patients and 19 healthy controls. Thus we analysed by flow cytometry the production of these cytokines in the CD4+ population after 5 h of stimulation with ionomycin plus PMA. As expected, the majority of IL-17+ CD4+ and IFN-γ+ CD4+ T cells expressed CD45RO both in patients and healthy controls (see supplementary figure 1, available as supplementary data at Rheumatology Online); thus we analysed CD45RO+ effector Th cells expressing these cytokines. We noticed that the IL-17 mean fluorescence intensity (MFI) values were significantly higher in lupus patients compared with controls, whereas the IFN-γ MFI was slightly lower (Fig. 1A). No significant differences were observed in the frequency of IL-17+IFN-γ- (Th17) or IL-17+IFN-γ+ (Th1) CD4+CD45RO+ cells between patients and controls, although the Th17/Th1 ratio was significantly increased in SLE patients. Nevertheless, this possible Th17 bias could be related to specific treatment or disease characteristics rather than represent a typical feature of SLE. In fact, Fig. 1B indicates that the Th17/Th1 ratio was strikingly increased in patients under glucocorticoid treatment [mean (s.d.) dose 7.56 (4.74) mg prednisone/day], whereas there were no significant differences between controls, untreated patients and those without glucocorticoid therapy. Accordingly, the frequency of Th17 cells and IL-17 MFI levels were increased and the Th1 subset and IFN-γ MFI were decreased in the group of patients taking corticoids, while double-positive IL-17+IFN-γ+ cells were not altered (Fig. 1B and C). Moreover, Th17 bias seemed to be related to the dose of glucocorticoids because patients treated with <5 mg/day (n=9) did not present this effect.

Finally, we did not find a significant correlation between the Th17/Th1 index and SLEDAI score (r=0.205, P=0.094) or anti-dsDNA titre (r=0.072; P=0.557), suggesting that increased Th17 responses are independent of disease activity. In fact, untreated patients presented high SLEDAI scores (Fig. 2A). But, curiously, the SLEDAI score correlated positively with the Th17/Th1 ratio in corticoid-treated patients but not in other patient groups (Fig. 2B).

Increased Th17 responses in anti-ENA-positive patients
Then we assessed the possible association between a Th17/Th1 imbalance and particular clinical or immunological features. The analysis of clinical characteristics of SLE patients did not show significant differences in Th17 cells or the Th17/Th1 index. The presence of autoantibodies, however, seemed to be associated with Th17 responses. In fact, patients with antibodies against ENA (SSA, SSB, Sm or RNP) presented with a higher percentage of Th17 cells than anti-ENA-negative patients, whereas no differences were detected related to the presence of anti-dsDNA antibodies (Fig. 3A). Anti-ENA- and anti-DNA-positive and -negative patients showed similar levels of Th1 cells, except those anti-Sm-positive patients, who displayed lower percentages of Th1 cells (Fig. 3B).

Given that both anti-ENA-positive and glucocorticoid-treated patients presented with a higher percentage of Th17 cells, we analysed both effects together (Fig. 3C). All patients treated with glucocorticoids presented high Th17 levels and differences between those positive and negative for anti-ENA antibodies did not reach statistical significance [2.33 (1.60) vs 1.76 (1.24), P=0.258]. However, patients presenting anti-ENA antibodies who
**Fig. 1** Th17 bias in SLE patients under glucocorticoid treatment. Th17, Th1 and IL17+IFNγ+CD4+CD45RO+ cells were determined as described in the ‘Materials and methods’ section. (A) MFI values of IL-17 and IFN-γ, percentage of Th17 and Th1 and Th17/Th1 ratio from HCs and SLE patients. (B) Th17/Th1 ratio, Th17, Th1, IL17+IFNγ+CD4+CD45RO+ cells and MFI values in HCs (n=19) and SLE patients classified according to treatment: untreated (n=12), taking <5 mg prednisone/day (GC < 5, n=9), at least 5 mg prednisone/day (GC ≥ 5, n=22) and under other treatment (no GC, n=32). (C) Representative plots. Bars represent median (IR). *P < 0.05 compared with HC. HC: healthy controls; GC: glucocorticoids. n.t.: untreated patients.

**Fig. 2** Relation between Th17/Th1 index and SLEDAI score in patients under corticoid treatment. (A) SLEDAI score in SLE patients organized by treatment. Bars represent median (IR). (B) Correlations between Th17/Th1 index and SLEDAI in patients taking glucocorticoids and those under other treatments. n.t.: untreated patients.
were users of another treatment did not show an elevated percentage of Th17 cells compared with healthy individuals [1.47 (0.98) vs 1.37 (0.88)].

**STAT3 and IL-6R are up-regulated in SLE patients taking glucocorticoids**

The differentiation of CD4+ T lymphocytes towards different Th lineages is mediated by cytokine binding to specific receptors, which leads to the activation of different STATs. Thus we sought to assess the effect of glucocorticoid treatment on CD4+ T cells from SLE patients quantifying gene expression of IL-6R and STAT3, involved in Th17 responses, and IL-12R and STAT4, required for Th1 generation. To this end we analysed the expression of these molecules in 30 SLE patients (non-treated: n = 6; without glucocorticoids: n = 10; with glucocorticoids: n = 14) and 8 healthy controls at the time of the quantification of Th1 and Th17 cells by real-time RT-PCR. Figure 4A shows that patients receiving glucocorticoids [mean (s.o.) dose: 10.16 (7.58) mg/day] presented higher STAT3 levels than other patients and healthy controls, whereas no significant differences were detected between controls, untreated patients and those with other treatments. STAT4 levels, however, did not show significant differences. In a similar way, IL-6R expression was significantly increased in corticoid-treated patients, whereas IL-12R levels (β1 and β2 chains) were similar in all the groups tested. As expected, a strong relationship was detected between STAT3 and IL-6R expression (r = 0.899, P < 0.001) (Fig. 4B). Moreover, we found a positive correlation in all tested individuals between the Th17/Th1 ratio and gene expression of STAT3 (r = 0.461, P = 0.004) and IL-6R (r = 0.333, P = 0.041), thus supporting the relevance of these molecules for Th17 polarization.

**Discussion**

In this work, we show a link between the use of corticoids and alteration of the balance between Th17 and Th1
populations observed in SLE patients. An increased proportion of Th17 cells [8–10] and a dysregulated Th17/Th1 index [13] have been previously described in SLE patients, suggesting a detrimental role of IL-17 production in the disease. In fact, several works have shown increased amounts of Th17 cells in patients with high SLEDAI scores [8, 10], and a positive correlation has been described between this population and disease activity [8, 10, 13]. However, to the best of our knowledge, this is the first study showing an enrichment of the Th17 population associated with the use of steroid treatment. Nevertheless, the majority of SLE patients with active disease included in previous reports were on glucocorticoid treatment, whereas inactive patients were not. In the present work, however, we studied a comparatively large number of SLE patients (n = 75), which included patients treated with corticoids and with other therapies, as well as untreated patients with high SLEDAI scores, thus avoiding this problem.

In our work, Th17 cells did not correlate with disease activity or anti-dsDNA titre, but, interestingly, they were increased in patients with antibodies against ENA antigens. This result supports the different origin of autoantibodies in SLE. In fact, development of antibodies against SSA and SSB, two apoptosis-related molecules [16], are favoured by a pro-inflammatory environment [17, 18], whereas the presence of anti-dsDNA is associated with humoral response and high IL-10 levels [19, 20]. Therefore anti-ENA positivity at the onset of the disease could represent a potential biomarker for high Th17 bias, especially during the use of glucocorticoids, thus hampering the control of the disease under this treatment. Therefore, in patients presenting these autoantibodies, it would be advisable to use non-steroid therapies or low doses of steroids. However, a prospective longitudinal study must be performed to confirm this result.

In an attempt to determine a possible origin for the dysregulated Th17/Th1 balance in steroid-treated patients, we quantified the gene expression of STAT3 and STAT4, two signal transducers related to the development of Th17 [21] and Th1 [22] cells, respectively. In the same way, IL-6 is critical for the polarization of human

**Fig. 4** STAT3 and IL-6R gene expression is up-regulated in SLE patients taking glucocorticoids. (A) mRNA relative expression of STAT3, STAT4, IL-6R, IL-12Rβ1 and IL-12Rβ2 were assessed using real-time RT-PCR as described in the ‘Materials and methods’ section. STAT3 and IL-6R are up-regulated in patients on GCs (n = 14) as compared with HCs (n = 8), n.t. (n = 6) and those on other treatments (no GC, n = 10). No differences were found on STAT4, IL-12Rβ1 and IL-12Rβ2 mRNA relative expression. Bars represent median (IR). (B) Correlations between STAT3 levels, IL-6R expression and Th17/Th1 ratio. GC: glucocorticoids; HC: healthy controls; n.t.: untreated patients.
CD4+ T cells towards Th17 populations, probably because IL-6R signal through STAT3 [4]. In fact, our data showed a strong relationship between IL-6R and STAT3 expression. We found that SLE patients treated with glucocorticoids presented increased levels of STAT3 and IL-6R, in accord with the data of Th17/Th1 imbalance. Increased levels of STAT3, associated with high transcriptional activity of STAT3-inducible genes, have been previously reported in SLE patients [23]. Likewise, it has been suggested that the up-regulated IL-6 production reported in lupus patients [24–26] may play a role in the development of the disease. Actually, IL-6R-blocking therapies, used in RA [27] and other autoimmune diseases [28] can improve lupus symptoms [29], especially nephritis.

The fact that glucocorticoids increment IL-17 production may seem surprising, but is not contradictory with current knowledge. It has been shown that these drugs synergize with several cytokines, such as IL-6 or IL-10, rather than inhibit their activities [30–32]. Different levels of interactions have been reported in the synergism with IL-6. First studies showed that glucocorticoids were able to induce the expression of IL-6R [32]. More recently, a physical interaction has been discovered between glucocorticoids and IL-6-induced STAT3 [33]. Formation of the glucocorticoid complex may explain the reason why STAT3 target genes can be superinduced in the presence of glucocorticoids, and also the fact that STAT3 could be a co-activator of GRE-mediated transcription. A similar synergism has been reported with IL-10 [34]. Glucocorticoids can increase IL-10 expression in vivo and in vitro [30, 31, 35] and it has been demonstrated that they induce the binding of STAT3 to the STAT motif of the IL-10 promoter [34]. Interestingly, a number of Th17-related cytokines signal through STAT3, so they could establish an analogous synergism with glucocorticoids. Thus the up-regulatory effect of glucocorticoids on IL-17 production in SLE patients could be a result of a superinduction of IL-17 gene mediated by IL-6R signalling through STAT3, despite the lack of identifiable GREs on IL-17 promoter. However, other direct or indirect mechanisms could be involved in the effect of glucocorticoids on Th17/Th1 balance.

Rheumatology key messages

- SLE patients treated with glucocorticoids display an alteration in the Th17/Th1 balance.
- Expression of STAT3 and IL-6R, involved in Th17 differentiation, are up-regulated in steroid-treated patients.
- Anti-ENA positivity could be used as a potential biomarker for Th17 bias.

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Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology Online.

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