Abstract

Background – Aim: Loss of tolerance to self-antigens represents the critical factor for pathogenesis initiation in systemic lupus erythematosus (SLE) and lupus nephritis (LN). T regulatory cells (Tregs) and related cytokines (IL-6, IL-10, TGF-β1) are the main mediators of this mechanism. The aim of this study was the evaluation of Tregs and IL-6, IL-10 and TGF-β1 in the course of LN patients.

Patients – Methods: Twenty LN patients (18 female, 2 male, mean age 33.8±8.8 years, mean disease duration 87.2±63.4 months) were included; twelve with a histological diagnosis (10 class IV+V, 2 class V). CD4+CD25highFOXP3+ Tregs were analyzed in 61 samples (44 active, 17 quiescent disease). IL-6, IL-10 and TGF-β1 were evaluated in 18 patients (10 active, 8 inactive LN). Disease activity was evaluated with SLE Disease Activity Index (SLEDAI). Other parameters included C3/C4d complement fragments, anti-dsDNA antibodies and proteinuria. Analysis was performed with Kruskal-Wallis test; p<0.05 was considered significant.

Results: Tregs were significantly lower in active LN (0.71±0.29% vs. 1.14±0.19% of the CD4+ T cells, p<0.001). IL-6 and IL-10 were significantly higher (IL-6: 6.25±2.38 vs. 1.62±1.66pg/ml, p<0.001, IL-10: 5.8±3.8 vs. 1.7±2.6pg/ml, p=0.025), whereas TGF-β1 was lower (16529±7962 vs. 25957±6776pg/ml, p=0.034) in active LN. C3 and C4d were significantly lower in active disease (C3: 82.1±22.3 vs. 152±29.9mg/dl, p<0.001 and C4d: 8.7±4.3 vs. 21±7.7mg/dl, p<0.001).

Conclusions: Tregs and TGF-β1 were significantly lower in active LN, while IL-6 and IL-10 were detected in higher levels. Impairment of IL-6/TGF-β1 axis seems to drive Tregs reduction in active LN; on the contrary, IL-10, despite its known regulatory profile, seems to exert mainly proinflammatory functions in LN.

Key words: immune regulation, interleukin-6, interleukin-10, lupus nephritis, T regulatory cells, transforming growth factor-β1.

Introduction

Renal involvement in systemic lupus erythematosus (SLE)
represents a decisive prognostic factor for such patients\textsuperscript{1}. Disease pathogenesis remains largely unknown; however, reactive lymphocytes, auto-
antibodies targeting nuclear antigens and immune complexes have been implicated\textsuperscript{2}. Central immune
tolerance to certain nuclear antigens is incomplete
even in healthy individuals and results in reactive peripheral lymphocytes, identified in both B and T
cell compartments\textsuperscript{3,4}. Thus, a further failure in the
mechanisms of peripheral tolerance is critical in
initiating the pathogenetic process in lupus nephritis
(LN). In this context, T regulatory cells (Tregs),
which represent the main mediators for suppressing
reactive lymphocytes in the periphery, are believed
to play a determinant role\textsuperscript{5}.

Following early experiments in lupus-prone mice,
several investigators demonstrated quantitative and/or
qualitative defects of these cells in human SLE\textsuperscript{6-8}.
However, significant differences between human and
mice Tregs do exist; for instance, in humans, only
Tregs with the highest CD25 surface expression exert
suppressive capacity and this is strongly correlated
with the intracellular expression of Foxp3, the master
gene for Tregs’ differentiation\textsuperscript{9}. Consequently, this
particular immunophenotype (CD4\textsuperscript{+}CD25\textsuperscript{high}
FOX3P3+) has been successfully employed to
quantify their frequency in peripheral blood.

Recent studies have shown that Tregs differ-
entiate and function in a sensitive equilibrium
with their effector counterparts in LN, mainly
the Th17 cells\textsuperscript{10,11}. Soluble mediators, such as IL-6 and
TGF-β, are of paramount importance; IL-6 drives
the differentiation of naïve CD4\textsuperscript{+} T cells towards
Th17, while transforming growth factor beta 1
(TGF-β1) predominance is responsible for the ex-
pansion of Tregs\textsuperscript{12}. Their mechanisms of action
involve both cell-to-cell contact and cytokines, such
as IL-10 and TGF-β1 and are able to target virtually
all effector immune cells\textsuperscript{13,14}.

The exact role of these cells and their related
cytokines in LN has not been fully elucidated. Aim
of the present study was the assessment of peripheral Tregs and certain cytokines (IL-6, IL-10,
TGF-β1) in LN patients in regard to disease activity
and response to administered therapy.

**Patients – Methods**

Twenty LN patients (18 females, 2 males, mean
age 33.8±8.8 years, mean disease duration 87.2±63.4
months) were included in the study from January
2008 until December 2011. Twelve of them had a his-
tological diagnosis; 10 with class IV +V LN and 2
with pure class V LN, according to the current patho-
logic classification\textsuperscript{15}. The remaining eight patients
were diagnosed with SLE, according to the American
College of Rheumatology revised criteria\textsuperscript{16,17}. LN di-
agnosis in these patients was based on the presence
of proteinuria (>500mg/24h) and/or hematuria
and/or urinary casts, according to the recent recom-
mandations from European League Against Rheumatism (EULAR)\textsuperscript{18}. One female patient had
already developed end stage renal disease (requiring
dialysis) at the time of diagnosis. Twenty age- and
sex-matched healthy individuals served as controls in
order to quantify the “normal” numbers of Tregs.

The Human Ethics Review Committee of the
Medical School of Aristotle University of Thes-
saloniki approved the study protocol and a signed in-
formed consent was obtained from each subject.

**Laboratory Methods and Study Design**

Tregs (phenotype CD4\textsuperscript{+}CD25\textsuperscript{high}FOX3P3+)
were assessed by triple-colour flow cytometry
(Fluorescence Activated Cell Sorter, FACS, EPICS
COULTER XL\textsuperscript{®}), in whole blood samples, shortly
after venepuncture. Cells were stained with anti-
CD4 (13B8.2, Immunotech) FITC (Fluoroscein
Isothiocyanate), anti-CD25 (B1.49.9, Immunotech)
ECD (Phycoerythrin-Texas-Red-X) and anti-
FOX3 (PCH101, e-Bioscience) PE (Phyco ery-
thrin). Intraprep TM solutions (Beckman-Coulter)
were used to increase leukocyte membrane perme-
ability and induce red blood cell lysis. A representa-
tive FACS figure is presented in Figure 1.

Cytokine assessment was performed in stored
(-76 °C) serum samples by enzyme-linked im-
munosorbent assay (ELISA) with the following
commercially available kits: Human IL-6
BMS213HS, Human IL-10 BMS215HS and high
sensitivity Human TGF-β1 BMS249/3CE, all from
Bender MedSystems® GmbH, Vienna, Austria.
Normal range for each cytokine was considered the
one reported by the manufacturer.

Additional laboratory parameters included full
blood count, erythrocyte sedimentation rate (ESR),
serum creatinine and proteinuria (24h urine collec-
tion), C3/C4d complement components (nephelom-
etry, Dade Behring, Newark, DE, USA) and anti-
dsDNA antibodies (immunofluorescence assay, IFA, on a *Crithidia luciliae* substrate).

Clinical assessment was made using the SLE Disease Activity Index (SLEDAI), according to its initial description. Active LN was defined by the presence of significant proteinuria (≥500mg/24h) and/or hematuria and/or urinary casts in the absence of urinary infection (based on negative urine culture). The SLICC/ACR (Systemic Lupus International Collaborating Clinics) index was used for assessment of cumulative damage.

Patients were divided into group A (active unstable LN, n=8) and group B (inactive quiescent LN, n=12). CD4+CD25<sup>high</sup>FOXP3+ Tregs were assessed in 61 samples (44 in group A and 17 in group B) in total. Six out of eight active patients were administered intravenous cyclophosphamide (6 monthly pulses of 500mg/m<sup>2</sup>/month) plus oral methylprednisolone (8-32mg/day, slow tapering) for remission induction; in these patients, Tregs were assessed monthly. In the other two active patients, mycophenolate mofetil (720-1080mg/day) in the other 6 patients. IL-6, IL-10 and TGF-β1 were evaluated in 18 patients (10 active, 8 inactive LN) once in total; all assessments were made in parallel with Tregs’ evaluation.

**Statistical analysis**

Analysis was performed using the non-parametric Kruskal-Wallis test for independent variables and Monte Carlo simulation. All *p* values were two-tailed and *p*<0.05 was considered to be statistically significant. Correlations were made with Pearson’s correlation co-efficient. The SPSS software package (version 20.0) was used for data analysis.
Results

Patients with unstable active LN (n=8) were younger than patients with quiescent disease (34.8±8.3 vs 51±12.8 years, p<0.001) whereas they had lower disease duration (92±55.3 vs 179.8±118 months, p=0.018), Table 1. White blood cells were not significantly different between the two groups (5948±3848/μl vs 6089±1349/μl, p=0.863), while lymphocytes were lower in active disease (1059±557/μl vs 1506±606/μl, p=0.021), based on first assessment before treatment adjustment (during disease relapse).

Tregs were significantly lower in patients with active LN in comparison to inactive patients and healthy controls (0.71±0.29% of the CD4+ T cells vs 1.14±0.19% vs 1.49±0.19% respectively, p<0.001, absolute numbers 4±2.3 vs 8.9±4.4 vs 13.2±3.1 cells/mm3, p<0.001), (Figure 2). Tregs’ numbers for group A patients represent values before treatment adjustment. In addition, they were inversely correlated to disease activity (r correlation coefficient to SLEDAI -0.596), (Figure 3). After remission induction (achieved in all active patients), Tregs’ numbers were restored in the levels of inactive patients (1.24±0.29% of CD4+ T cells, absolute numbers 10.7±4.6 cells/mm3, p=0.215, after 6 months), data not shown.

IL-6 levels were significantly elevated in active disease in comparison to inactive LN (6.25±2.38 vs 1.62±1.66pg/ml, p<0.001, normal range 1.45±1.14 pg/ml). Likewise, IL-10 was found in higher levels in these patients (5.8±3.8 vs 1.7±2.6pg/ml, p=0.025, normal range 2.57±2.7pg/ml). On the contrary, TGF-β1 was marginally lower in active LN (16529±7962 vs 25957±6776pg/ml, p=0.034, normal range 25500 ±8400 pg/ml), (Figure 4).

Table 1. Demographic characteristics and parameters studied comparatively in active and inactive lupus nephritis (LN). SLEDAI: Systemic Lupus Erythematosus Disease Activity Index, SLICC/ACR: Systemic Lupus International Collaborating Clinics/American College of Rheumatology.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Active LN</th>
<th>Inactive LN</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.8±8.3</td>
<td>51±12.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>92±55.3</td>
<td>179.8±118</td>
<td>0.018</td>
</tr>
<tr>
<td>WBC (/μl)</td>
<td>5948±3848</td>
<td>6089±1349</td>
<td>0.863</td>
</tr>
<tr>
<td>Lymphocytes (/μl)</td>
<td>1059±557</td>
<td>1506±606</td>
<td>0.021</td>
</tr>
<tr>
<td>Tregs (%CD4+)</td>
<td>0.71±0.29%</td>
<td>1.14±0.19%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tregs (cells/mm3)</td>
<td>4±2.3</td>
<td>8.9±4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>6.25±2.38</td>
<td>1.62±1.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>5.8±3.8</td>
<td>1.7±2.6</td>
<td>0.025</td>
</tr>
<tr>
<td>TGF-β1 (pg/ml)</td>
<td>16529±7962</td>
<td>25957±6776</td>
<td>0.034</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>45.7±23.9</td>
<td>39.1±16.7</td>
<td>0.366</td>
</tr>
<tr>
<td>C3 (mg/dl)</td>
<td>82.1±22.3</td>
<td>152±29.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C4d (mg/dl)</td>
<td>8.7±4.3</td>
<td>21±7.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>1445±2348</td>
<td>197±111</td>
<td>0.010</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>11.7±5.9</td>
<td>2.2±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SLICC/ACR</td>
<td>1.6±1.22</td>
<td>2.93±0.73</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fig. 2. Comparative Tregs variations in active lupus nephritis (LN), inactive disease and healthy controls. Left bars: Tregs as %CD4+ T cells. Right bars: Tregs absolute numbers.
Concerning simultaneous measurements of these parameters (Tregs, IL-6, IL-10, TGF-β1), correlation analysis showed that IL-6 and IL-10 were inversely related to Tregs (r=-0.446 and -0.321), whereas TGF-β1 was positively related to these cells (r=0.1).

ESR was not significantly differentiated (45.7±23.9 vs 39.1±16.7 mm/h, p=0.366). On the contrary, C3 and C4d complement fragments were significantly lower in patients with active disease (C3: 82.1±22.3 vs 152±29.9 mg/dl, p<0.001 and C4d: 8.7±4.3 vs 21±7.7 mg/dl, p<0.001). Anti-dsDNA were positive in 6/8 active patients (75%) and in 5/12 inactive patients (41.7%).

Serum creatinine was not affected in neither patients’ group, except for one patient in group A (where serum creatinine raised 1.5x upper limit of normal transiently) and one patient in group B (who was on dialysis since LN diagnosis). Proteinuria was more severe in active LN (1445±2348 vs 197±111 mg/24h, p=0.010). All the aforementioned parameters refer to values before remission induction treatment; after remission, proteinuria in active patients was similar to inactive individuals (234±86 mg/24h, p=0.34).

SLEDAI was significantly higher in active disease (before treatment adjustment 11.7±5.9 vs 2.2±1.8, p<0.001), while the Damage Index SLICC/ACR was significantly higher in inactive patients (2.93±0.73 vs 1.6±1.22, p<0.001).

Discussion

The role of Tregs in human LN pathogenesis and outcome has not been extensively investigated thus far; only 5 relevant studies have been published in human subjects. In the present study, Tregs, either as a proportion of CD4+ T cells or as absolute numbers, were found in significantly lower numbers in active disease. This finding comes in agreement with most studies in the field, although there is one study where no significant differences were demonstrated. It should be mentioned that in the present study, a comparatively larger number of samples were evaluated, whereas a significant proportion of the patients were re-assessed over time in different phases of disease activity. Furthermore, Foxp3 mRNA, the critical factor for Tregs’ differentiation, has been reported to be significantly up-regulated in the urine of active LN patients and predicted a poor therapeutic response. The controversy between defective Tregs and overexpressed Foxp3 in active SLE may be explained by the Foxp3 expression in other T cells and/or the destruction of Tregs by their effector counterparts. In terms of their functional status, experimental studies described intact suppressive activity of these cells in murine lupus, a finding that was also confirmed in human LN.

It was recently stressed that Tregs decrement in active LN was accompanied by a compensatory expansion of the Th17 lymphocytes, along with an increment of related cytokines IL-17 and IL-23. In addition, several investigators have shown that other Th17-specific cytokines, particularly IL-6, was significantly higher in active LN and may represent...
a promising biomarker for assessing the activity of renal involvement in SLE\textsuperscript{26-28}. Furthermore, it was recently shown that urinary levels of IL-6 are higher in active LN in comparison to patients with no renal involvement\textsuperscript{26,29}. In a mechanistic basis, it was shown that dendritic cell derived IL-6 is able to inhibit the function of Tregs in lupus-prone mice\textsuperscript{30}.

On the other hand, serum TGF-β1 has been repeatedly found in lower levels in such patients, while its urinary levels were reportedly higher in comparison with inactive LN\textsuperscript{11,31,32}. Our findings are in line with these observations, further reinforcing the hypothesis that disturbances in the IL-6/TGF-β1 axis may further promote Tregs/Th17 equilibrium towards the pro-inflammatory Th17 phenotype.

Successful remission induction was accompanied by Tregs restoration in all LN patients in the present study. Administered regimens, mainly methylprednisolone and cyclophosphamide, are considered nonspecific; consequently, Tregs' increment probably represents an indirect result. Other investigators demonstrated that rituximab, a B cell targeted agent, is able to lead to a significant increment of Tregs, which is strongly related to clinical remission\textsuperscript{21,22}. The underlying mechanism is not known, however, it can be hypothesized that B cell depletion would result in immune complexes reduction, decreased dendritic cells' stimulation and less IFN-α production. This would further lead to the over-expression of regulatory cytokines, such as TGF-β1, and Tregs expansion.

Concerning IL-10, its serum levels were found significantly higher in active LN. This finding has been reported recently, while its levels returned to normal after remission induction\textsuperscript{33}. Increased IL-10 levels are not paradoxically observed in SLE. This cytokine displays multiple roles in disease pathogenesis, either as a promoter of Th2 differentiation and inhibitor of Th17 expansion (by reducing IL-23) or by being the critical factor for B cell maturation\textsuperscript{35-37}. In this context, IL-10 reflects disease activity rather than being a suppressive/regulatory cytokine.

Limitations to be considered in the present study include the small number of patients, which does not allow for definite conclusions, and the lack of functional assays for more precise Tregs characterization. Furthermore, it would be helpful to assess the urinary levels of the studied cytokines and/or Foxp3 mRNA, in parallel to serum levels, in order to clarify their importance in LN activity and response to treatment. In addition, the simultaneous study of the Th17 cell population would further elucidate disease pathogenesis.

In conclusion, Tregs were found significantly lower in active LN, while their numbers were restored after remission induction. Serum levels of IL-6 and IL-10 were higher in active disease, while TGF-β1 was decreased in the same patients. Impairment of IL-6/TGF-β1 axis seems to drive Tregs reduction in active LN.
χαμηλότερα σε ενεργό ΝΛ (0.71±0.29% έναντι 1.14±0.19% των CD4+ Τ κυττάρων, p<0.001). Οι IL-6 και IL-10 ήταν χαμηλότερος (16529±7962 έναντι 25957 ± 6776 pg/ml, p=0.025), ενώ ο TGF- β ήταν χασκίτικος -β, νεφρίτιδα λύκου, Τ ρυθμιστικά κύτταρα . Τη διαταραχή του άξονα IL-6/TGF- β φαίνεται να σχετίζεται με την ασκίτικη ρύθμιση, ιντερλευκίνη -10, μεταμορφωτικό αυξητικό παράγοντας -β, νεφρίτιδα λύκου, τ φυσιολογικά κύτταρα.

**Δήλωση σύγκρουσης συμφερόντων**

Δεν αναφέρεται σύγκρουση συμφερόντων

Conflict of interest statement

None declared

**References**


*Received for publication 11/12/2013
Accepted in revised form 14/02/2014

**Παρελήφθη στις 11/12/2013
Έγινε αποδεκτή μετά από τροποποιήσεις στις 14/02/2014

---

**Corresponding Author**
Konstantinos Tselios
Clinical Immunology Unit
2nd Department of Internal Medicine,
Hippokration General Hospital
Aristotle University of Thessaloniki,
Konstantinoupoleos St. 49, 546 42
Thessaloniki, Greece
Tel.: 0032 310 892239
Fax: 0032 310 992794
e-mail: tselioskostas2@gmail.com