Deficient or abundant but unable to fight? Estimation of circulating FoxP3⁺ T regulatory cells and their counteracting FoxP3⁻ in rheumatoid arthritis and correlation with disease activity

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Abstract  Aim of the work: Persistent inflammation and recurring activity in rheumatoid arthritis (RA) during a quarterback of T regulatory cells (Tregs) intrigued rheumatologists. Tregs' most specific marker is the forkhead box P3 (FoxP3) denoting FoxP3⁺ cells as suppressors whereas FoxP3⁻ as effectors. This study evaluates subset distribution of peripheral blood (PB) CD4⁺ CD25⁺ Tregs according to FoxP3 expression in RA to better understand its role in pathogenesis.

Patients and methods: In our observational cross-sectional study PB Tregs from 40 RA patients and 20 age and sex matched healthy controls (HC) were characterized and quantified by flow cytometry. Disease activity was evaluated by DAS28. Patients were divided into: active RA group (ARA) and remission RA group (RRA).

Results: Significantly higher CD4⁺ CD25⁻ FoxP3⁺ Tregs were found on comparing RRA, ARA patients and HC (mean 153.25 ± 6.29, 136.3 ± 3.27 and 97.25 ± 6.25, respectively) with a statistically highly significant difference (F = 553.13, p < 0.001). CD4⁺ CD25⁻ FoxP3⁺ increased 3-folds in RRA and 4-folds in ARA compared to HC and CD4⁺ CD25⁺ FoxP3⁻ increased 1.6-folds in RRA and 1.5-folds in ARA. Thus the ratio of FoxP3⁻/FoxP3⁺ cells is altered from 1:3 in HC to...
1. Introduction

The regulatory T cells (Tregs) CD4+CD25+ which specifically express the forkhead family transcription factor (FoxP3) are essential for the maintenance of immunological self-tolerance and homeostasis. Tregs suppress the activation, proliferation, and effector function of other lymphocytes in physiological and pathological immune responses. Therefore, control of the development, survival, and function of Tregs is instrumental for effective controlled immune responses [1].

FoxP3 is specifically expressed in CD4+CD25+ Tregs which is crucial for their development and is needed to maintain the suppressive function of the mature peripheral Tregs [2]. Decreased FoxP3 expression causes immune disease by subverting the suppressive function of Tregs and converting them into effector cells [3].

Tregs have a central role in protecting an individual from autoimmunity. This role was first identified in mice in which the absence or depletion of these cells resulted in the development of autoimmune disorders [4,5].

Rheumatoid arthritis (RA) is a common chronic autoimmune inflammatory disease characterized by destruction of the synovial joints, leading to progressive disability, increased morbidity and premature mortality [6,7]. The genetic predisposition to RA is dominated by the major histocompatibility complex HLA-DR and because the function of HLA-DR is to present peptides to CD4+ T cells, a role for antigen-activated T cells in at least the initiation of RA is implied [8].

Data regarding the number of circulating Tregs in RA patients compared to healthy individuals are inconclusive and contradictory. Some studies showed a decrease of circulating Tregs in RA patients compared to healthy individuals [9] whereas others showed no difference [10] and some indicated an increase [11]. While accumulating evidences support that CD4+CD25+ Tregs play an essential role in controlling and preventing autoimmunity [12], several reports have described increased numbers of CD4+CD25+ T cells also in the synovial fluid of RA patients [11,13]. Interestingly, many studies have reached the seemingly paradoxical conclusion that autoimmune arthritis can develop despite the presence of CD4+CD25+ Tregs. These observations raise the question of whether these cells in arthritic patients are perhaps dysfunctional, or functional but unable to prevent disease, or are modifying it in some manner [14].

Tregs’ role in other rheumatic diseases such as systemic lupus erythematosus (SLE) is suggested. Characterization of circulating Tregs in SLE patients has been analyzed based on expression of intracellular FoxP3 transcription factor [15]. Some reports have shown a decrease of CD4+CD25+ FoxP3+ Tregs in SLE patients compared to healthy individuals with inverse correlation with activity [16,17] and without this correlation [18]. However, other studies revealed normal levels [19-21]. Other reports showed an increase of circulating CD4+CD25+ FoxP3+ Tregs that correlates with disease activity [22-24].

Hence, CD4+CD25+ Tregs can play a critical role in the prevention of autoimmunity. At present, however, how and whether they participate in the development of RA remains unclear [14].

In this study, we aimed at clarifying the subset distribution of CD4+CD25+ Tregs according to FoxP3 (whether a lack of suppressors FoxP3+ or excess of effectors FoxP3−) in RA (both in remission and activity) for a better understanding of rheumatoid arthritis pathogenesis.

2. Patients and methods

The present study is an observational cross-sectional one that included 40 patients with RA fulfilling the new EULAR/ACR criteria of 2010 [25]. Twenty age and sex matched healthy subjects were also included in the study and served as control group. All patients were seropositive for rheumatoid factor (RF) and/or anticitrullinated protein (AntiCCP) antibodies. All the patients were on methotrexate (MTX) and folic acid. Written consent was obtained from all patients and controls after a full explanation of the study which was approved by the local ethics committee.

2.1. Exclusion criteria

Patients with acute infection, other autoimmune disorders or those receiving any other disease-(modifying antirheumatic drug(s)) DMARDS other than MTX were excluded from the study.

2.2. Clinical assessment

- Full history taking including disease duration and drug intake.
- Thorough clinical assessment of the patients which included the following:

  * Disease activity was evaluated using 28 tender and swollen joint count disease activity score (DAS28) [26] [high disease activity >5.1, moderate disease activity <5.1 and >3.2, low disease activity <3.2, remission <2.6]. Patients were divided into two groups: (1) those with active RA (ARA group) (n = 20) (2) those whose disease was in clinical remission (RRA group) (n = 20). We intended to recruit equal number of patients in each group. All patients in the group with active RA and the remission group were on stable therapy MTX and folic acid.
2.3. Laboratory assessment

- Complete blood count (Cell Dyne-2700, Abott Lab., USA).
- Erythrocyte sedimentation rate (ESR) using Westergren method.
- CRP by latex agglutination using AVITEX CRP supplied by Omega Diagnostics Ltd.
- Detection of anti CCP antibodies: serum anti CCP levels were assessed by an ELISA methodology using QUANTA Lite TM CCP3 IgG semiquantitative ELISA kit, INOVA Diagnostics, San Diego, CA USA.
- RF was detected by the kit supplied by Biotec Laboratories based on agglutination test using particles sensitized with human IgG.
- Liver function tests and kidney function tests using Synchron CX9 (Beckman instrument Inc. Brea, California, USA).
- Flow cytometry.

Two milliliters of the peripheral venous blood was collected from each subject using vacutainer containing anticoagulant potassium ethylene diamine tetra acetate (EDTA) in a final concentration of 1.5 mg/ml.

Fifty microliters of whole anticoagulated blood was lysed using 1 ml IQ test lysing reagent (Beckman coulter, Miami, USA) followed by washing with phosphate buffer saline (PBS) (Oxoid, Hampshire, UK). After that, the cells were stained with combinations of the following antibodies (5 ul each): anti-CD-25-PE, anti-CD4-FITC and isotypes controls (FITC and PE) (Beckman, Coulter). The test tubes were then incubated in the dark for 20 min followed by washing with PBS. Intracellular staining FoxP3-PE-Cy5 (eBioscience, California, USA) was as follows: anticoagulated whole blood was fixed and permeabilized using FoxP3 staining buffer set (eBioscience) according to the manufacturer’s instructions with certain modifications in brief.

After washing, the cell pellet was resuspended in 0.5 ml of freshly prepared fixation/permeabilization working solution and incubated for 30 min at 4 °C in the dark. This was followed by washing once with PBS followed by washing once again with 1 ml of 1x permeabilization buffer. Ten microliters of FoxP3-PE-Cy5 was added and incubated for 30 min at 4 °C in the dark. Lastly washing with PBS followed by resuspension in PBS for analysis was done.

Data acquisition and analysis were performed on EP-ICS XL flow cytometry using SYSTEM II version three software with a standard three color filter configuration.

Lymphocytes were gated via their forward and side scatter light properties. Dot blot was set as FL1 and FL2 markers which represent CD4+(FITC) and CD25+(PE), respectively. T regulatory lymphocytes were identified as cells double positive for FITC and PE fluorescence.

The statistical analysis was performed by IBM SPSS statistics (V. 19.0, IBM Corp., USA, 2010) used for data analysis. Data were expressed as Mean ± SD for quantitative measures. The following tests were done:

1. Comparison of two independent mean groups for parametric data using Student’s t test.
2. Comparison between two independent groups for non-parametric data using Wilcoxon Rank Sum test (Mann–Whitney–Wilcoxon test).
3. Comparison between three independent groups for parametric data using analysis of variance (ANOVA) and further by analysis of covariance (ANCOVA) and Tukey’s test.
4. Ranked Spearman correlation test to study the possible association between each two variables among each group. Also partial correlation was performed to measure the degree of association between two variables, with the effect of a controlling variable removed.

3. Results

### 3.1. Clinical and laboratory data of RA patients

This study included 40 patients with RA, 20 in the activity ARA group (18 females and 2 males), 20 in the remission RRA group (17 females and 3 males) and 20 in the healthy control (HC) group (17 females and 3 males); their ages ranged from 29 to 56 years in the ARA group (mean 43.50 ± 6.86), from 28 to 55 years in the RRA group (mean 41.30 ± 7.81) and from 27 to 55 years in the HC group (mean

| Table 1 Clinical and laboratory data of the RA patients. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| RA patient groups | | | |
| ARA group | RRA group | T-test or Mann–Whitney | |
| Mean ± SD | Mean ± SD | T or Z | p value |
| Disease duration (years) | 6.80 ± 4.59 | 5.40 ± 3.22 | 1.107 | 0.275 |
| DAS28 | 5.18 ± 0.95 | 1.87 ± 0.52 | 13.598 | <0.001* |
| TJC | 7.00 ± 3.21 | 0.35 ± 0.48 | 5.525 | <0.001* |
| SJC | 2.75 ± 0.96 | 0.20 ± 0.41 | 5.489 | <0.001* |
| ESR (mm/hour) | 51.75 ± 18.66 | 9.65 ± 2.70 | 9.981 | <0.001* |
| CRP (mg/dl) | 24.00 ± 9.76 | 4.40 ± 1.31 | 8.896 | <0.001* |
| PGH | 4.25 ± 1.77 | 0.45 ± 0.64 | 5.367 | <0.001* |
| MTX (mg/week) | 17.25 ± 2.91 | 13.37 ± 1.22 | 5.48 | <0.001* |

ARA = active rheumatoid arthritis, RRA = remission rheumatoid arthritis, DAS = disease activity score, TJC = tender joint count, SJC = swollen joint count, ESR = erythrocyte sedimentation rate, CRP = C reactive protein, PGH = patient global health, MTX = methotrexate.
41.15 ± 7.82). There was no significant difference between the three groups as regards age or sex.

The disease duration ranged from 3 to 18 years in the ARA group (mean 6.80 ± 4.59) and from 2 to 15 years in the RRA group (mean 5.40 ± 3.29) with no significant difference between the two groups ($t = 1.107, p = 0.275$). Patients were on MTX ranging from 12.5–25 mg/week (mean 17.25 ± 2.9 in ARA group and 13.73 ± 1.22 in RRA group) as well as one tablet of folic acid (5 mg) only on the following day.

Evaluation of disease activity by DAS28 in the ARA group ranged from 3.01 to 6.41 (mean 5.18 ± 0.95) and in the RRA group from 0.97 to 2.58 (mean 1.87 ± 0.52). The active group had a higher ESR, CRP, tender joint count (TJC), swollen joint count (SJC) and patient global health (PGH) values compared to those of the inactive group (Table 1).

### 3.2. Comparison between T regulatory cells in RA patients and healthy controls

Comparison between patients and controls as regards T regulatory cells showed that there was a statistically highly significant difference ($p < 0.001$) as regards CD4$^+$ T cells, CD4$^+$CD25$^+$ T cells as well as CD4$^+$CD25$^+$FoxP3$^+$ and CD4$^+$CD25$^+$FoxP3$^-$ subsets (in terms of cell number/µl blood) being all increased in patients (Table 2) and (Figs. 1–3). Also an increase of statistically highly significant difference ($p < 0.001$) found in the relative percentage of CD4$^+$CD25$^+$FoxP3$^+$ out of the CD4$^+$CD25$^+$ T cell population was observed in patients (Table 2). Further, on performing the Tukey’s test after ANOVA test there was also a statistically highly significant difference between each two groups of the three groups ($p < 0.001$) in all studied T cell subsets. The T cells were higher in patients in comparison to controls and higher in ARA in comparison to RRA except CD4$^+$CD25$^+$FoxP3$^-$ that was higher in RRA than in the ARA group. Putting MTX as a covariant and performing analysis of covariant ANCOVA test to eliminate its effect on the difference between the RA groups as regards CD4$^+$CD25$^+$FoxP3$^-$, a statistically non-significant value was found for MTX on this comparison ($F = 0.114, p = 0.738$), while still a statistically highly significant difference was found between the two groups ($F = 253.168, p < 0.001$).

### 3.3. Comparison between active and inactive RA patients and controls as regards folds of increase of CD4CD25 cell subsets

Increase of CD4$^+$CD25$^+$FoxP3$^-$ from 35.10 ± 5.33 in controls to 107.05 ± 7.74 in RRA and 139.95 ± 5.17 in ARA being 3-folds in the RRA group and 4-folds in the ARA group.

### Table 2: Comparison between RA patients and control as regards T regulatory cell number.

<table>
<thead>
<tr>
<th>Cell population (cell number/µl blood)</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4$^+$</td>
<td>ARA</td>
<td>1402.10 ± 29.68</td>
<td>$F = 2515.94$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>1369.45 ± 22.18</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>917.80 ± 18.58</td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$</td>
<td>ARA</td>
<td>276.25 ± 7.66</td>
<td>$F = 2728.518$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>260.80 ± 7.33</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>132.35 ± 4.97</td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$/CD4</td>
<td>ARA</td>
<td>19.70 ± 0.57</td>
<td>$F = 536.03$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>19.04 ± 0.53</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14.42 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$FoxP3$^+$</td>
<td>ARA</td>
<td>136.30 ± 3.28</td>
<td>$F = 553.13$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>153.25 ± 6.29</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>97.25 ± 6.25</td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$FoxP3$^-$</td>
<td>ARA</td>
<td>139.95 ± 5.17</td>
<td>$F = 1499.75$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>107.05 ± 7.74</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>35.10 ± 5.33</td>
<td></td>
</tr>
<tr>
<td>Relative %</td>
<td>ARA</td>
<td>49.34 ± 0.72</td>
<td>$F = 414.84$</td>
</tr>
<tr>
<td></td>
<td>RRA</td>
<td>58.78 ± 2.46</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>73.48 ± 3.84</td>
<td></td>
</tr>
</tbody>
</table>

ARA = active rheumatoid arthritis, RRA = remission rheumatoid arthritis, CD = cluster differentiation, Relative % = percentage of CD4$^+$CD25$^+$FoxP3$^-$ out of CD4$^+$CD25$^+$ T cells.
Figure 2  Flow cytometric analysis of CD4+CD25+ T Cells in HC group (a) RRA group (b) and in ARA group (c) HC = healthy control, ARA = active rheumatoid arthritis, RRA = remission rheumatoid arthritis.

Figure 3  Examples of flow cytometric results of FoxP3+ T cells in (a) HC group (b) RRA group (c) ARA group. HC = healthy control, ARA = active rheumatoid arthritis, RRA = remission rheumatoid arthritis.
Increase of CD4+CD25+FoxP3+ from 97.25 ± 6.25 in control group to 153.25 ± 6.29 in RRA and 136.30 ± 3.28 in ARA being 1.6-folds in RRA and 1.5-folds in ARA. Thus the ratio of FoxP3/C0 cells is altered from 1:3 in the control group to 2:3 in the RA group and 1:1 in ARA.

3.4. Correlations between CD4+CD25+FoxP3+ T cells and disease activity and various studied variables in RA patients

Correlation studies (Table 3) between CD4+CD25+FoxP3+ T cells and clinical data of RA patients showed a negative statistically highly significant correlation \( (p < 0.001) \) between CD4+CD25+FoxP3+ and DAS28 \( (r = -0.861) \) (Fig. 4a), TJC \( (r = -0.764) \), SJC \( (r = -0.796) \) and PGH \( (r = -0.757) \). As regards laboratory data, a negative highly significant correlation between CD4+CD25+FoxP3+ and ESR and CRP was found \( (r = -0.759 \) and \(-0.753, \) respectively). On the other hand there was non-significant correlations between CD4+CD25+FoxP3+ and both age and disease duration.

3.5. Correlations between CD4+CD25+FoxP3+ and therapy

A negative correlation of statistically high significance was found between CD4+CD25+FoxP3+ and MTX dose in RA patients \( (r = -0.596, \) \( p < 0.001) \). On further eliminating the effect of this correlation and studying partial correlation between CD4+CD25+FoxP3+ and DAS28 there was still a negative statistically highly significant correlation \( (r = -0.84, \) \( p < 0.001) \).

3.6. Correlations between various T cell subsets and DAS28

See Table 4.

4. Discussion

Despite the presence of an increase in immunoregulatory cells in RA, chronic inflammation persists and activity recurs. This finding has been intriguing rheumatologist and immunologist trying to justify the presence of activity during this quarterback of cells usually aiming to fight inflammatory process.

The main problem hindering the study of Tregs is the lack of a single marker that defines all cells with regulatory activity. CD4+CD25+ T cells have been identified as the best-characterized and apparently most important Treg cell population [27]. The forkhead box P3 (FoxP3) transcription factor is now considered the most specific marker, as it is essential for both Treg cell development and function [28].

Over-expression of FoxP3 by in vitro transfection induces T cells to exert a complete or partial suppressive activity [29]. On the contrary, decreased FoxP3 expression enhances autoimmune responses by subverting the suppressive function of Treg cells and converting Treg cells into effector cells [3].

In this observational cross-sectional study, RA patients showed higher levels of CD4+CD25+ Tregs in the peripheral blood as compared to healthy controls. This is in accordance with Han et al., 2008 who studied CD4+CD25+ T cell numbers

Table 3 Correlations between CD4+CD25+FoxP3+ T cells and disease activity and various studied variables in RA patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>CD4+CD25+FoxP3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>(-0.084, 0.606)</td>
</tr>
<tr>
<td>DD (years)</td>
<td>(-0.196, 0.225)</td>
</tr>
<tr>
<td>DAS28</td>
<td>(-0.861, &lt;0.001)</td>
</tr>
<tr>
<td>TJC</td>
<td>(-0.764, &lt;0.001)</td>
</tr>
<tr>
<td>SJC</td>
<td>(-0.796, &lt;0.001)</td>
</tr>
<tr>
<td>ESR (mm/hour)</td>
<td>(-0.759, &lt;0.001)</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>(-0.753, &lt;0.001)</td>
</tr>
<tr>
<td>PGH</td>
<td>(-0.757, &lt;0.001)</td>
</tr>
</tbody>
</table>

DD = disease duration, DAS = disease activity score, TJC = tender joint count, SJC = swollen joint count, ESR = erythrocyte sedimentation rate, CRP = C reactive protein, PGH = patient global health.

Table 4 Correlations between T cells and disease activity in RA patients.

<table>
<thead>
<tr>
<th>T cells (cell number/µl blood)</th>
<th>DAS28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>0.542</td>
</tr>
<tr>
<td>CD4+CD25+</td>
<td>0.762</td>
</tr>
<tr>
<td>CD4+CD25+/CD4+</td>
<td>0.559</td>
</tr>
<tr>
<td>CD4+CD25+FoxP3+</td>
<td>-0.861</td>
</tr>
<tr>
<td>CD4+CD25+FoxP3-</td>
<td>0.945</td>
</tr>
<tr>
<td>Relative %</td>
<td>-0.948</td>
</tr>
</tbody>
</table>

DAS = disease activity score, CD = cluster differentiation, Relative% = percentage of CD4+CD25+FoxP3+ out of CD4+CD25+ T cells.

Figure 4 Correlation between DAS28 and (a) CD4+CD25+FoxP3+ T cells (b) CD4+CD25+ T cells (c) CD4+CD25+FoxP3- T cells.
in 99 RA patients and 44 control subjects and demonstrated it to be higher in RA patients [11]. They further questioned the paradox that since a main purpose of CD4⁺CD25⁺ Treg cells is to control autoimmunity disease, whether this increased number in the peripheral blood is present but unable to ameliorate disease activity or present in order to ameliorate it. Also in consistency with Van Ameloot et al., who stated the same finding but viewed it as a trial to suppress the disease [13].

Our work found that RA patients showed higher levels of PB CD4⁺CD25⁺FoxP3⁺ cells as compared to HC. This is in accordance with Ryder et al., 2010 who found FoxP3 expression to be higher in RA and further studied CTLA4 to find no concomitant increase indicating that the Tregs were not functioning in an optimal fashion despite of their increased number [30]. Our observation is also concomitant with a previous study which stated that both CD4⁺CD25⁺ T cell subtypes (those expressing FoxP3 and those not expressing it) contributed to the total increase of these cells in RA compared to HC but clarifying that though CD4⁺CD25⁺FoxP3⁺ cells were increased the increase of CD4⁺CD25⁺FoxP3⁻ cells was even more as compared to HC [11]. On the other hand, Suzuki et al., 2011 demonstrated significantly lower FoxP3 levels in PB upon studying 23 RA patients and 25 SLE comparing them to 8 HC. They stated that this decreased expression in RA and SLE may reflect Treg cell abnormalities in these autoimmune diseases [31]. While back in 2007, Lin et al. demonstrated that FoxP3 expression in RA was of no significant difference in comparison to healthy control subjects [32].

Upon those contradictory findings; increased, decreased or no different than HC, the FoxP3 expression stood yet unexplained in relation to RA pathogenesis. Hence, our study was interested to verify in RA patients the FoxP3⁺ cells’ state as an absolute number as well as when studied in terms of percentage of CD4 T cells and in relation to the opposing force of FoxP3⁻ as regards the folds of increase of each. Moreover, we aimed to verify whether this was related to disease activity.

Therefore, in this work the percentage of FoxP3⁺ cells among CD4⁺CD25⁺ T cells in PB of RA was compared to HC; it was found to be significantly lower than HC. This finding is suggestive of weakened suppressive response of Tregs in RA in comparison to HC: although the FoxP3⁺ cells were found to be increased as an absolute number compared to HC, their percentage out of the CD4⁺CD25⁺ T cell population was not even equal to that of healthy individuals but lower. This is in accordance with Han et al., 2008 [11]. Also in consistency with Sempere-Ortells et al., 2009 who studied the different phenotypes of regulatory T cells in 60 RA patients and 40 HC laying stress on FoxP3 considering it the most specific marker for Tregs as it is essential for both their development and function. They found a significant decrease in the percentage of this subset in RA compared to HC [33].

As regards the comparison between FoxP3⁺ and opposing FoxP3⁻ cells, our study found that both are increased in RA but with an imbalance favoring FoxP3⁻ increase. The FoxP3⁺ increase in RA was almost one and a half fold the level in HC while the FoxP3⁻ increase was about 3-folds the level in HC and even 4-folds in patients with activity. So both CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁻FoxP3⁻ are increased in RA patients but it is their balance that is more important; the abundance of FoxP3⁻ cells gave the effector function the upper hand over the suppressive function represented in FoxP3⁺ cells that also increased in number but to the same extent as FoxP3⁻ cells.

In our study, the methotrexate (MTX) dose was found to be in a negative correlation with the CD4⁺CD25⁺ FoxP3⁺ cells, this is in accordance with Ryder et al., 2010 who noted a negative correlation between FoxP3 mRNA and the dose of MTX in a study performed on 50 RA patients and 10 healthy controls [30]. On the other hand, Oh et al., 2013 studied the effect of various disease-modifying anti-rheumatic drugs (DMARDs) on the suppressive function of CD4⁺CD25⁺ regulatory T cells and stated that each DMARD had a different effect on Treg function demonstrating that sulfasalazine and lefunomide inhibited the anti-proliferative function of Tregs on cocultured T effector cells (Teffs) and reduced Treg expression of FoxP3 mRNA, whereas MTX and infliximab did not [34]. Our results could be explained by the hindering effect of MTX on folate as reported by Kinoshita et al., 2012 that blockade of folate receptor four and treatment with methotrexate, which inhibits folate metabolic pathways, decreased colonic FoxP3⁺ Tregs [35]. We studied the partial correlation between FoxP3⁺ Tregs and DAS28, hence eliminating MTX effect and still the negative correlation between them was strongly revealed. Further studies especially long term observational ones are needed to specify the MTX impact on those cells. Better folic acid supplementation in terms of dose or close interval to MTX administration might mend the suggested inhibitory effect on FoxP3⁺ cells if proven to be true.

In conclusion, CD4⁺CD25⁺FoxP3⁺ and CD4⁺CD25⁺ FoxP3⁻ both contribute to the Tregs’ increase in RA. Their ratio might be the key to understand the inability of Tregs to control disease; the effector function of FoxP3⁻ cells is not adequately met by a sufficient suppressive function by FoxP3⁺ cells. This imbalance is related to the presence of RA and to its activity. Restoring this imbalance by future targeting therapeutics might be of great benefit in RA patients.

5. Conflict of interest

There is no conflict of interest of the authors.

References


