LEVELS OF REGULATORY AND EFFECTOR T CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS.

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Abstract
CD4⁺CD25⁺ regulatory T cells and CD4⁺CD25⁻ effector T cells are crucial in maintaining immune system homeostasis and are thus potential therapeutic targets for autoimmune disease, such as rheumatoid arthritis (RA). Forkhead box p3 (Foxp3), which is required for regulatory T (T_{reg}) cell development and its suppressive function, is also critical in sustaining normal immune function. In the present study, the characteristics of regulatory T cells, effector T cells, and Foxp3 levels were explored in patients with RA and compared with healthy controls. Flow cytometry of peripheral blood was used to determine the level of expression of these cell populations in 70 RA patients from the Hospital of the University of the Andes and 32 healthy individuals in Mérida, Venezuela. No significant differences in the frequencies of T_{reg} cells, effector T cells, or Foxp3 were observed in peripheral blood when RA patients were compared with healthy controls. Despite previous studies that have revealed local synovial differences in T_{reg} cells, effector T cells, and Foxp3 expression in RA patients when compared with healthy controls, the findings of the present study suggest that there are no significant peripheral differences in this patient population. Keywords: Regulatory T cells, effector T cells, Foxp3, rheumatoid arthritis, autoimmune disease.

INTRODUCTION.
The phenomenon of autoimmune suppression by T cells was first described by Nishizuka and Sakakura (1969) when they found that thymectomy of neonatal mice resulted in organ-specific autoimmune disease which could be prevented by thymus grafting. CD4⁺CD25⁺ regulatory T (T_{reg}) cells have now been shown to be essential in maintaining immune homeostasis and preventing autoimmune disease via immunosuppression (Morgan et al. 2003). Likely originating in the thymus, T_{reg} cells comprise only a minority of CD4⁺ T cells, approximately 5-10% (Maloy & Powrie 2001).

Upon T cell receptor stimulation, this subpopulation of T cells results in immunosuppression by inhibiting interleukin (IL)-2 transcription, decreasing the subsequent proliferation of responding CD4 and CD8 T cells, and by inhibiting upregulation of IL-2 receptors via cell-cell contact mechanisms (McHugh & Shevach 2002). Suppression of the immune response is further accomplished by the direct regulation of antigen-presenting cell (APC) and B cell function (Bystry et al. 2001). The occurrence of a

process termed ‘infectious tolerance’ has been demonstrated in some studies in which T_{reg} cells induce naïve CD4^+ T cells to differentiate into additional T_{reg} cells. This multiplication of suppressive effect is at least partially mediated via soluble inhibitory cytokines such as IL-10 or transforming growth factor (TGF)-β (Cobbold & Waldmann 1998). It has been further postulated that there are subpopulations of T_{reg} cells with varying immunosuppressive activity, termed CD4^+CD25^{high/bright} and CD4^+CD25^{intermediate/dark}. Studies have indicated that CD4^+CD25^{high/bright} cells may exhibit the majority of suppressive function of T_{reg} cells and that CD4^+CD25^{intermediate/dark} cells may represent recently activated cells (Cao et al. 2003, Kunitasu et al. 2000).

The transcription factor Forkhead box p3 (Foxp3) has been shown to be critical for the development and suppressive function of CD4^+CD25^{T cells (Fontenot et al. 2003). Individuals with mutations in the Foxp3 gene have been found to exhibit a disorder termed IPEX (immune deregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, characterized by overwhelming systemic autoimmunity. Although some studies suggests that Foxp3 is specific for CD4^+CD25^{+ regulatory T cells (Hori et al. 2003), other research has shown that Foxp3 expression can also be induced in CD4^+CD25^{T cells (Walker et al. 2003). Nonetheless, Foxp3 is currently the most commonly used marker for T_{reg} cells.

Rheumatoid arthritis (RA) is a chronic multisystem inflammatory disease presumed to be associated with autoimmunity. The hallmark of the disease is persistent inflammatory synovitis, primarily in peripheral joints and in a symmetric pattern, with the potential for destruction of both cartilage and bone (Miller-Blair et al. 1996). Subsequent deterioration of joint integrity leads to significant morbidity and mortality. Various cells (i.e. CD4^+ T cells, B cells, mononuclear phagocytes, fibroblasts, osteoclasts, and neutrophils) and their pro-inflammatory cytokines (i.e.: tumor necrosis factor (TNF)-α and IL-1β) are involved in the pathologic processes leading to RA. The chronic nature of the disease process suggests immune deregulation, either caused by excessive inflammatory response, lack of suppression of inflammation, or a combination of both mechanisms (Van Amelsfort et al. 2004).

In spite of increasing understanding of the immunoregulatory properties of this subpopulation of cells, the role of CD4^+CD25^{regulatory T cells in the pathogenesis of autoimmune disease is still poorly understood. Recent investigations in T_{reg} cells and autoimmune disorders, such as systemic lupus erythematosus (SLE) and multiple sclerosis (MS), have found decreased regulatory T cells in patients when compared with healthy controls (Lyssuk et al. 2007, Parietti et al. 2007, Viglietta et al. 2004). Similarly, a study revealed decreased percentages of T_{reg} cells in the blood of patients with type 1 diabetes, another autoimmune disorder (Kukreja et al. 2002).

Recent analysis of regulatory T cells and RA is conflicting. While some studies show comparable numbers of CD4^+CD25^{cells in peripheral blood from RA patients when compared to controls (Liu et al. 2005, Möttönen et al. 2005, Van Amelsfort et al. 2004), other studies have revealed significantly decreased frequency of peripheral CD4^+CD25^{high cells in RA patients (Cao et al. 2004). However, the finding of significantly increased numbers of synovial regulatory T cells with intact immunosuppressive activity in RA patients has been confirmed by numerous studies (Cao et al. 2003, Möttönen et al. 2005, Van Amelsfort et al. 2004). These findings provide evidence of quantitative differences in T_{reg} cells localized in the synovial joints of patients with RA, with the need to further characterize the pattern of T_{reg} cells in the peripheral blood.

In the present study, our aim was to compare the frequency and phenotype of CD4^+CD25^{regulatory T cells, CD4^+CD25^{effector T cells, and Foxp3 in the peripheral blood of patients with RA and healthy individuals to determine any difference in these cells group.

**METHODOLOGY.**

**Patients.**

A total of 70 RA patients (67 women, 3 men) and 32 healthy controls (28 women, 4 men) were enrolled in this study. Patients were randomly selected from the Rheumatology Service of the Hospital of the University of the Andes in Mérida, Venezuela. Disease status was determined by the American College of Rheumatology (ACR) global functional status classification (Hochberg et al. 1992). The characteristics of the patients are shown in table 1 (age, male/female, & ACR status). There was no significant difference in average age between the RA and control groups (P > 0.05). All of the patients were receiving treatment with corticosteroids, disease-modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, or a combination of the previous medications. Control peripheral blood was obtained randomly from 32 healthy volunteers in the city of Mérida. Informed consent was obtained and approval
for this study was acquired from the institutional medical ethics review board.

Table 1. RA and control subject characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RA group</th>
<th>Control group</th>
</tr>
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<tbody>
<tr>
<td>Age, mean ± SD yrs</td>
<td>50.50 ± 11.80</td>
<td>52.70 ± 10.43</td>
</tr>
<tr>
<td>No. female/No. male</td>
<td>67/3</td>
<td>28/4</td>
</tr>
<tr>
<td>ACR Status-Functional Classification (I-IV)</td>
<td>I- 48 patients (68.5%)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>II- 18 patients (25.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III- 4 patients (5.7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV- 0 patients (0%)</td>
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</table>

Phenotype analysis.

All reagents were purchased from Dako in Glostrup, Denmark. The following monoclonal antibodies (mAb) were used: anti-CD4 conjugated with fluorescein isothiocyanate isomer 1 (FITC), anti-CD3 conjugated with R-phycocerythrin (RPE)/cyanin 5 (Cy5), anti-CD25 conjugated with RPE and anti-Foxp3 conjugated with PE. After drawing 100 µl of peripheral blood, 10 µl each of anti-CD3/RPE-Cy5, anti-CD4/FITC and anti-CD25/RPE, respectively, were added and mixed in the test tubes. The samples were incubated in the dark at room temperature (18-22°C) for 15-30 minutes. Erythrocyte lysing procedure was performed according to the reagent manufacturer’s recommended procedure. The cells were centrifuged at 300 x g for 5 minutes at 18-22°C, the supernatant was aspirated, and 3 ml of phosphate buffer solution (PBS) was added. This step was repeated and the fluid resuspended in ~0.5mL PBS. Viable lymphocytes were gated based on their forward/CD3 scatter profile and isotype control staining. CD3+ cells were then gated for CD4, CD25, and Foxp3 expression. Analyses were immediately performed on a FACSort flow cytometer. Using CellQuest software (Becton Dickinson, Mountain View, Calif), the total percentage of CD25+ and CD4+CD25high cells of the population was calculated.

Statistical analyses.

Statistical significance for differences in CD4+CD25+ Treg cells, CD4+CD25+ effector T cells, and Foxp3 levels between RA and control groups, age, and sex were determined using Mann-Whitney U-test via the program SPSS V.13.0. P values less than 0.05 were considered significant.

RESULTS.

CD4+CD25+ and CD4+CD25high regulatory T lymphocytes in peripheral blood of RA patients compared with healthy controls.

Given the immunosuppressive actions of regulatory T cells and their role in autoimmune disease, we explored the frequency of these cells in patients with rheumatoid arthritis. Conceivably, the impaired development and decrease in number or function of Treg cells could lead to autoimmunity, especially in the subpopulation of cells with the highest immunosuppressive properties, CD4+CD25high Treg cells.

Table 2. Peripheral blood frequency and phenotype of CD4+CD25+ Treg cells, CD4+CD25+ effector T cells, and Foxp3.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RA group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+/CD25+ ± SD</td>
<td>5.94 ± 3.07</td>
<td>4.78 ± 2.83</td>
<td>0.066</td>
</tr>
<tr>
<td>CD4+/CD25high ± SD</td>
<td>0.75 ± 0.97</td>
<td>0.81 ± 1.28</td>
<td>0.292</td>
</tr>
<tr>
<td>CD4+/CD25+ ± SD</td>
<td>51.10 ± 17.23</td>
<td>53.80 ± 8.48</td>
<td>0.823</td>
</tr>
<tr>
<td>CD4+/Foxp3+ ± SD</td>
<td>0.73 ± 0.40</td>
<td>0.78 ± 0.33</td>
<td>0.190</td>
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</table>

Regulatory T cells and effector T cells obtained from peripheral blood samples of 70 patients with RA and healthy controls were evaluated by flow cytometry. The results did not show statistically differences in the frequencies of these cells in RA patients compared to control group. P value was determined at 95% interval confidence. Values are expressed in means ± standard deviations.

Flow cytometry for regulatory T cells in RA patients and healthy controls is shown in figure 1. Comparing the RA and control groups in table 2, we found that the frequency of CD4+CD25+ and CD4+CD25high Treg cells was not significantly different between these groups (P = 0.066 & 0.292, respectively). Similarly, mean percentages of Treg cells did not differ between age and sex. Although findings have been conflicting, these results are consistent with earlier studies which found no significant variations in the numbers of Treg cells in peripheral blood from RA patients when compared to controls.
As shown in figure 2, there was a non-significant increase in the mean percentage of CD4⁺CD25⁺ T<sub>reg</sub> cells in the RA group compared with healthy controls (5.94% vs. 4.78%, respectively). Fig. 3 shows a non-significant decrease in the mean percentage of CD4⁺CD25<sub>high</sub> T<sub>reg</sub> cells in RA patients when compared with controls (0.75% vs. 0.81%, respectively). Data for CD4⁺CD25<sub>high</sub> T<sub>reg</sub> cells was also observed to be asymmetrically distributed. Outliers in these groups were included due to their large number and the detrimental effect of removing these data. These findings suggest that there are no significant differences in peripheral blood regulatory T cells between RA and healthy patients.

**CD4⁺CD25⁻ effector T lymphocytes in peripheral blood of RA patients compared with healthy controls.**

Effector T cells are crucial for the human immune response. Due to the increase in lymphocytes in inflammatory states, it was expected that the CD4⁺CD25⁻ effector T cell population would be increased in RA patients. However, as shown in table 2, our findings revealed a non-significant decrease in effector T cells in the RA group when compared with healthy controls ($P = 0.082$). Whereas a mean of 53.8% of lymphocytes in the control group were comprised of effector T cells, the mean of the same cell population comprised only 51.1% of lymphocytes in the RA group (Fig. 4). This observation suggests that levels of effector T cells in peripheral blood are comparable between RA patients and healthy controls.
Fig. 2. Peripheral blood CD4⁺CD25⁺ Treg cells in each group were analyzed by flow citometry from CD3⁺ cells. In the graph, dark line represents means and rectangles indicated standard error. There was no significant difference in RA group compared with healthy subjects. P= p value at interval confidence of 95%.

Fig. 3. CD4⁺CD25⁺H⁺ Treg cells obtained from peripheral blood of 70 RA patients and 32 healthy subjects were studied and frequencies of these Treg cells did not show statistically significant difference. P= p value at 95% IC.

Fig. 4. CD4⁺CD25⁻ effector T cells in peripheral blood from control group and RA patients group were compared. The result revealed a non-significant decrease in effector T cells in the RA group when compared with healthy controls.

**Foxp3 levels in peripheral blood of RA patients compared with healthy controls.**

Foxp3 is widely believed to be exclusively expressed in CD4⁺CD25⁺ high regulatory T cells and is subsequently the most commonly used marker for this subpopulation of lymphocytes. As table 2 indicates, there were no significant differences in peripheral blood levels of Foxp3 between the RA and control groups (P = 0.19). Mean Foxp3 expression in RA patients was 0.73 ± 0.40%, compared to 0.78 ± 0.33% in healthy controls. Because Foxp3 levels are expected to reflect the frequency of CD4⁺CD25⁺ high Treg cells, this finding is consistent with the non-significant differences in this cell population found between RA patients and healthy controls. These data demonstrate that Foxp3 displays a similar level of expression in the peripheral blood of RA patients and healthy controls.

**DISCUSSION.**

The non-significant variation of peripheral blood levels of CD4⁺CD25⁺/CD4⁺CD25⁺ high T reg cells between RA and healthy patients suggests that peripheral changes in regulatory T cell levels are not apparent in RA. Although conflicting results exist, these findings are consistent with other studies which demonstrated comparable frequencies of these cell populations in peripheral blood of RA patients and healthy controls (Liu et al. 2005, Möttönen et al. 2005, Van Amelsfort et al. 2004).

A number of possibilities can explain the comparable percentages of effector and regulatory T cells between RA and control groups found in the present study. Previous investigations examining Treg functionality have revealed similar immunosuppressive capabilities between RA and healthy controls (Kunitasu et al. 2000, Van Amelsfort et al. 2004). However, a recent study provided evidence that CD4⁺CD25⁺ Treg cells isolated from peripheral blood of RA patients were unable to suppress pro-inflammatory cytokine production from activated T cells and monocytes, and were also incapable of conveying suppressive function to effector CD4⁺CD25⁻ T cells (Ehrestein et al. 2004). The same study showed that anti-TNF-α therapy restored the suppressive capacity of regulatory T cells. These findings subsequently reveal a qualitative difference in effector and Treg cells in patients with RA, which would explain why no quantitative differences were found when compared with healthy counterparts. In our study, the patients received mainly DMARD and NSAID drugs and we

did not observed any differences in the numbers of regulatory T cells in both, RA group and healthy patients. These data seems to suggest that these drugs do not modify the frequencies of regulatory T cells and effector T cells, even diminished the inflammatory process. We thought there are qualitative defects in immune cells that could have a significant impact on the regulation of RA pathology. Another plausible explanation for the similar frequencies may arise from the patient population studied. To our knowledge, no prior studies have correlated ACR disease status classification with CD4+CD25\(^{+}\)/CD4+CD25\(^{\text{high}}\)/CD4+CD25\(^{\text{low}}\) T cell levels in peripheral blood. It is conceivable that more severe disease status would result in increased local recruitment of T\(_{\text{reg}}\) cells, leading to significantly altered levels of regulatory cells in both the peripheral blood and synovium as local T\(_{\text{reg}}\) cells are induced from peripheral effector T cells (Walker et al. 2003, Cao et al. 2004). In the present study, over 90% of RA patients were classified as having relatively mild disease (ACR class I & II). Thus, the non-significant differences in T\(_{\text{reg}}\) and effector cells between RA and control groups may be a reflection of the severity of disease studied, as the majority of RA patients studied had only mild-moderate disease. Extrapolating from the findings of our study, the lack of correlation between ACR status and T\(_{\text{reg}}\) cells parallels similar studies which failed to find a relationship between regulatory T cell levels and markers of RA disease such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) (Liu et al. 2005, Van Amelsfort et al. 2004). Although we would have liked to further investigate the relationship between ACR status and T\(_{\text{reg}}\) cell levels, the homogeneity in disease status of our patient population prevented such a study.

The simplest explanation for the similar frequencies of regulatory and effector T cells in RA and control groups is that peripheral differences in these cell populations do not exist. Despite conflicting findings, the preponderance of data on the matter supports this conclusion (Liu et al. 2005, Möttönen et al. 2005, Van Amelsfort et al. 2004). Conversely, numerous studies have demonstrated convincing localized changes in T\(_{\text{reg}}\) cells in the joint synovial fluid of individuals affected by RA, as demonstrated by increased levels of functional T\(_{\text{reg}}\) cells in synovial fluid when compared with peripheral blood (Cao et al. 2003, Möttönen et al. 2005, Van Amelsfort et al. 2004). Because lymphocytic infiltrates in the synovium of affected joints are characteristic of RA, it is rational to assume that effector and regulatory T cell concentrations will differ locally in the synovium more so than in peripheral blood. Furthermore, research has shown that human CD4+CD25\(^{+}\) T\(_{\text{reg}}\) cells are prone to apoptosis, which leads to the possibility that the accumulation and survival of this specialized cell population may differ peripherally and locally in the synovium, explaining the significantly different frequencies found in these compartments (Van Amelsfort 2004, Taams 2001).

The lack of peripheral changes in regulatory T cells in RA patients is consistent with our finding that CD4+Foxp3\(^{+}\) T cells displayed non-significant differences between RA and control groups. The similar percentages of CD4\(^{+}\) cells that expressed both CD25\(^{\text{high}}\) and Foxp3 support the widely accepted belief that Foxp3 is exclusively expressed by CD4+CD25\(^{\text{high}}\) T\(_{\text{reg}}\) cells. This theory is further supported by the finding that CD4+CD25\(^{\text{low}}\) effector T cells express low quantities of Foxp3 transcripts (Möttönen et al. 2005).

Due to the immunosuppressive functions exhibited by T\(_{\text{reg}}\) cells and the fact that these cells are the main cell population found in infiltrating synovial lymphocytes, they have been hypothesized to be crucial in the RA disease process. However, more specific characterization of the role of regulatory T cells in RA is challenging, as evidenced by the substantial discrepancies in research findings on the subject. Further studies are needed to determine their exact role in the pathogenesis of RA, for example whether T\(_{\text{reg}}\) cells play a role in the prevention and/or inhibition of RA. Future investigations correlating ACR disease status and regulatory T cell levels may also be helpful to better define the role of this cell population in RA. Finally, additional identification of the influences regulating function and synovial accumulation, including peripheral and local apoptosis in T\(_{\text{reg}}\) cells, may shed light on the therapeutic potential of this cell population for RA, as well as other autoimmune disorders.

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