Quantification of regulatory T cells and TGF-β expression in patients with Rheumatoid Arthritis

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Abstract

Introduction

Rheumatoid arthritis is a chronic and idiopathic autoimmune disease, manifested mainly through a chronic inflammation of the joints [1–4]. It is characterized by the destruction and progressive wear of the cartilage, and can lead to deformities and destruction of the joints, due to the erosion that takes place in the cartilage and bones [4–6]. The initial occurrence of the disease is the inflammatory process that occurs in the sinovial membrane, with a lymphocyte and macrophage infiltration [7].

A common characteristic of autoimmune diseases also implied in the development and progression of rheumatoid arthritis is the loss of self-tolerance [8, 9, 11]. The activation of T cells has been suggested as the cause of chronic inflammation for this pathology [10, 11].

The main components of the inflammatory process in rheumatoid arthritis are T lymphocytes and pro-inflammatory cytokines [1, 3]. Suppressor T cells were considered as regulatory T (Treg) cells in the late ‘90s, when several subpopulations of T cells were identified as having the ability to inhibit the proliferation of other cells [3, 12]. Treg cells (CD4+ CD25++) are naturally originated in the thymus, performing functions in the maintenance of immunological tolerance and in the prevention of autoimmunity [4, 13–15]. In humans these cells represent 1–2% of the T CD4+ cells in peripheral blood [14–16].

Despite the high expression of CD25 being a typical surface cells marker for Treg cells, it is not specific, as it can be equally expressed by activated effector T cells [4, 15, 16].

The Forkhead transcription factor boxp3 (Foxp3) is a specific marker for Treg cells CD4+CD25++ [17, 18]. This transcription factor is crucial to the development of Treg cells in the thymus but also for maintaining the suppressor function of mature Treg cells in the peripheral blood [4, 14, 15, 17].

Recently, the CD127 (interleukin-7 receptor – IL7R) has been used for a better and easier characterization of Treg cells (14). It may be considered as a biomarker, useful for identifying Treg cells, since a low expression on the cell surface allows an accurate assessment of the number of Treg cells [18].

The immunomodulatory cytokine TGF-β (transforming growth factor) is crucial for maintaining the Treg function [18, 19]. TGF-β is a mul-

Methods

The sample comprised 33 individuals with rheumatoid arthritis, 14 with high disease activity and 19 with low activity of rheumatoid arthritis and in control individuals.

Results

The results suggest that in patients with rheumatoid arthritis, there is a tendency to the decrease of the regulatory T cells frequency in peripheral blood, compared to controls. However, it was observed an increased number of mRNA copies of TGF-β also in these cells.

Conclusion: the numeric and functional variation of regulatory T cells in the different study groups suggests a contribution to the etiology and pathophysiology of rheumatoid arthritis.

Keywords: Rheumatoid arthritis; regulatory T cells; TGF-β; CD127, CD4, CD25

Introduction

Rheumatoid arthritis is a chronic and idiopathic autoimmune disease, manifested mainly through a chronic inflammation of the joints [1–4]. It is characterized by the destruction and progressive wear of the cartilage, and can lead to deformities and destruction of the joints, due to the erosion that takes place in the cartilage and bones [4–6]. The initial occurrence of the disease is the inflammatory process that occurs in the sinovial membrane, with a lymphocyte and macrophage infiltration [7].

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tifunctional cytokine which belongs to a superfamily comprised by more than 35 members [19]. It is secreted as an inactive complex and requires activation to be able to bind to its receptor [19].

TGF-β plays a central role in the maintenance of immune tolerance by inhibiting either lymphocyte or macrophage [20]. It has also been proposed in recent studies that this cytokine could not only play a role as a protecting agent of cartilage, but also mediate this degeneration during aging and the development of osteoarthritis [19].

In this context we proceed to quantify the CD4+CD25++CD127−/− Treg cells in the peripheral blood of patients with high and low disease activity as well as in control subjects, further evaluating the mRNA expression of TGF-β in these cells in the same patients.

**Material and methods**

**Study population**

The sample comprised 33 patients with diagnosed rheumatoid arthritis from the central region of Portugal; 14 were in the active phase of the disease and 19 under low disease activity. Disease activity was assessed according to the disease activity score (DAS), in which values above 3.2 show high activity and values below 3.2 represent low activity of the pathology. The sampling also includes a control group comprising 15 patients without clinical and family history of the disease or other autoimmune diseases.

From the 33 patients with the disease, 28 were female and of these, 12 were in the active phase of the disease and 16 under low disease activity. From the 5 males, 2 were in the active phase of the disease and 3 had low disease activity. The mean age of patients was 60 ± 10. The control group consisted of 11 women and 2 men with a mean age of 30 ± 12.

The patients included in this study were followed by the Rheumatology Service of the University Hospitals of Coimbra.

The study complies with the principles of the Declaration of Helsinki and all participants signed the informed consent.

**Biological samples**

The peripheral blood samples were obtained from the University Hospitals of Coimbra, and collected into tubes with tripotassium ethylene diamine tetraacetic acid (K3 EDTA) 1.2 mg/ml.

**Blood count**

Blood counts were performed on all 43 studied samples with the hematological counter Coulter Ac Tdiff™ (Fullerton, California, USA) using the impedance method.

**Direct marking of regulatory T cells and flow cytometry**

100 µl of blood were placed in a polystyrene tube and added 10 µl from each monoclonal antibody: anti-CD25 conjugated with FITC (fluorescein isothiocyanate, Pharmingen™ BD, San Diego, USA), anti-CD127 labeled with PE (phycoerythrin, BD Biosciences, San Jose, USA) and anti-CD4 conjugated with PerCP (piperidine chlorophyll, BD Biosciences, San Jose, USA), for each sample. The tubes were incubated for 10 minutes at room temperature in the dark.

Then, 2 ml of Facs Lysing solution (FACSTM Lysing solution: BD Biosciences, USA) were added, previously diluted 1/10 with distilled water and incubated for 10 minutes in the dark at room temperature. The diluted lysing solution was centrifuged at 1,500 rpm for 5 minutes and the supernatant was decanted. Finally, 1 ml of PBS buffer (Phos. Buffered Saline: pH 7.4, Gibco BRL-Life Technologies, Vienna, Austria) was added to the solution and then centrifuged for 5 minutes at 1,500 rpm. The supernatant was decanted and resuspended in 300 to 400 µl of PBS. The acquisition was made on the flow cytometer (FACS Calibur, BD, San Jose, USA) in a total of 300,000 cells using the Cell Quest software for Mac and the results were analyzed using the Infinicyt software (Cytognos, Spain) for Windows.

**Cell-Sorting**

1 ml of sample was lysed with NH4Cl for 20 minutes, centrifuged at 1,500 rpm for 5 minutes and then the supernatant was decanted. Thereafter, the direct surface marking of the cells was made according to the procedure already described above. The solution was incubated for 20 minutes in the dark at room temperature. Afterwards, the cells were washed with PBS (Phos. Buffered Saline: pH 7.4, Gibco BRL-Life Technologies, Vienna, Austria), the supernatant was decanted and the cells were resuspended. The sample was then processed on the flow cytometer BD FACS Aria™ Cell Sorter (BD, San Jose, USA) to undergo the cell separation.

The separated regulatory T cells were collected into eppendorfs with PBS buffer (Phos. Buffered Saline: pH 7.4, Gibco BRL, Life Technologies, Vienna, Austria).

**Gene expression analysis**

The study of the TGF-β mRNA expression was performed on isolated Treg cells. The extraction of total RNA from the sorting of Treg cells was performed using the RNeasy® Micro kit (Qiagen, Hilden, Germany). 1 µg of mRNA underwent reverse transcription through the Superscript® VI LOTM cDNA Synthesis kit (Invitrogen, California, USA) for real-time PCR, using oligo(dT) and some random hexamers according to the manufacturer’s instructions. Relative quantification of the
gene expression by RT-PCR was performed using the LightCycler 480 II thermal cycler (Roche, Rotkreuz, Switzerland).

For the RT-PCR reaction we used Quantitect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for TGF-β (QTO00000728) together with the gene expression kit QuantiTect® SYBR® Green PCR (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The reactions were conducted according to the following thermal profile: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. The results of the RT-PCR were analyzed using the LightCycler 480 software (Roche, Basel, Germany) and quantified using the qBasePlus software (Biogazelle, Zulte, Belgium).

**Statistical Analysis**

The statistical analysis was performed using the parametric Student’s T test for paired samples. Results were expressed as mean ± standard deviation (SD).

All statistical analyzes were performed using the Statistical Package for Social Sciences 15.0 (SPSS Inc, Chicago, USA) for Windows and differences were considered statistically significant when p value < 0.05.

**Results**

**Quantification of CD4⁺ CD25++ CD127+/– Treg cells**

As it can be seen in Table I (analysis of the absolute value calculated as the number of CD4⁺ CD25++ CD127+/– regulatory T cells/µl), the absolute value is greater for individuals with high activity of the disease in comparison to those with low activity. It can also be seen that statistical significance was found only for the relationship between the low activity group and the control group.

From the analysis of the percentage of regulatory T cells in the total cellularity of the peripheral blood, it can be seen that the frequency of these cells in patients with low activity is greater than the frequency of CD4⁺ CD25++ CD127+/– regulatory T cells in patients with high activity. In this analysis we can also observe that the results have shown statistical significance for the relationship between patients with low activity and the control group and between patients with high activity and the control group.

Regarding the percentage of regulatory T cells within the subpopulation of CD4⁺ T cells, there were no statistically significant results.

**TGF-β expression in CD4⁺ CD25++ CD127+/– Treg cells**

Analyzing the values of TGF-β mRNA in the same cells, obtained by RT-PCR, it was found that the number of copies in patients with high activity is higher than that of patients with low activity. It was also observed that the values were statistically significant when comparing the group of high activity with the control group, as well as the relationship between patients with low activity and the control group.

**Discussion**

Treg cells play a role in the maintenance of immunological tolerance and in the prevention of autoimmunity, since they eliminate effector T cells [14, 15].

From the results obtained in this study about the quantification of regulatory T cells, it can be observed (Tab. I) that the percentage of CD4 T cells and the total percentage of regulatory T cells is lower than in the control group and it was also observed a tendency for a decreasing proportion of these cells in patients with high activity.

Different publications about the relative frequencies of Treg cells in peripheral blood of patients with rheumatoid arthritis show that there is no concordance at this point [1, 9], since some papers refer that those cells appear to be normal in such individuals [9, 10]. Others refer that the percentage of those cells is increased [10] and some refer a decrease in peripheral blood, thus being concordant with the findings of this work [19].

An explanation for the observed results may be due to the fact that these Treg cells can also be encountered in the synovial fluid of patients with rheumatoid arthritis, and that in this location such cells are relatively increased compared to peripheral blood [9, 10, 21]. This suggests that there may be a recruitment of regulatory T cells from the periphery to the affected joints, thus justifying the decreased frequency of these cells in peripheral blood [9, 10, 22].

In relation to TGF-β, as it can be seen in Table I, there was an increase in the number of its copies in the group of patients with high and low activity.

### Table 1:

<table>
<thead>
<tr>
<th>TGF-β expression in CD4⁺ CD25++ CD127+/– Treg cells</th>
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<tr>
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| Table I – Quantification of Treg Cells in percentage and absolute value (number of cells/µl) and of TGF-β in both groups with illness, in comparison with the control group. Results are expressed as mean ± standard deviation. |
activity compared to the control group. It is known that this cytokine plays an essential role in inducing and maintaining the function of regulatory T cells, which could explain the increased TGF-β in patients in comparison to the control group, since these cells attempt to suppress autoreactive T cells [19, 20].

Conclusion

The obtained results suggest that in the patients with rheumatoid arthritis occurs a decrease of the Treg cell frequency, in comparison with the control group. However, the number of TGF-β mRNA copies suffered an increase in the patients compared to the control individuals.

Given this numeric and functional variation of Treg cells in the study groups, the results suggest that these cells play a role in the etiology and pathophysiology of this disease.

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References