ORIGINAL ARTICLE

Expression Patterns of HLA-DR$^+$ or HLA-DR$^-$ on CD4$^+$/CD25^{++}/CD127^{low} Regulatory T cells in Patients With Allergy

L Geraldes,[1,3] J Morgado,[2] A Almeida,[2] A Todo-Bom,[1,3] P Santos,[2] A Paiva,[2] C Cheira,[1] ML Pais[2]

[1]Immunoallergology Department, Coimbra University Hospitals, Coimbra, Portugal
[2]Histocompatibility Centre of the Central Region, Coimbra, Portugal
[3]Global Allergy and Asthma European Network (GA2LEN)

Abstract

Background: Allergic rhinoconjunctivitis induced by pollen is a highly prevalent chronic inflammatory disease in Europe. *Parietaria judaica* is a frequent trigger in the Mediterranean area. The function of regulatory T cells (Treg cells) in allergy has recently been investigated, but further data are necessary to better understand their role and to find new strategies to treat allergic diseases such as allergic rhinoconjunctivitis.
Objective: To characterize gene expression of HLA-DR$^+$ or HLA-DR$^-$ on peripheral CD4$^+$/CD25^{++}/CD127^{low} Treg cells in patients with allergy.
Methods: Peripheral Treg cells (CD4$^+$/CD25^{++}/CD127^{low}) were quantified using flow cytometry and sorted according to HLA-DR expression during the pollen season in patients with allergic rhinoconjunctivitis caused by *P judaica*. The results were compared with those of nonatopic controls. Expression of associated cytokines and their receptors was measured using quantitative reverse transcription-polymerase chain reaction after extraction of mRNA in sorted populations.
Results: During the pollen season, no significant differences were observed between allergic patients with rhinoconjunctivitis and healthy controls in terms of the absolute number or the percentage of Treg cells in peripheral blood. All patients had a higher number/percentage of HLA-DR$^-$ Treg cells than HLA-DR$^+$ Treg cells. In both groups we found high levels of *FOXP3* mRNA expression. Despite being lower in number, HLA-DR$^+$ Treg cells presented higher expression of *CD28, PRF1, GZMB*, and *FASL* than HLA-DR$^-$ Treg cells.
Conclusions: The most relevant results obtained suggest that HLA-DR$^+$ Treg cells tend to present higher gene expression of molecules associated with contact-dependent cell activation and cytotoxicity.

Key words: Gene expression. Regulatory T cell (Treg). Rhinoconjunctivitis. *Parietaria judaica* allergy.

Resumen

Antecedentes: La rinoconjuntivitis alérgica inducida por el polen es una enfermedad inflamatoria crónica muy frecuente en Europa. La especie *Parietaria judaica* es un desencadenante común en la región mediterránea. Recientemente se ha investigado la función de los linfocitos T reguladores (linfocitos Treg) en la alergia. Sin embargo, son necesarios más datos para comprender mejor dicha función y encontrar nuevas estrategias para tratar enfermedades alérgicas como la rinoconjuntivitis alérgica.
Objetivo: Caracterizar la expresión de HLA-DR$^+$ o HLA-DR$^-$ en linfocitos Treg periféricos CD4$^+$/CD25^{++}/CD127^{bajo} en pacientes alérgicos.
Métodos: Se cuantificaron linfocitos Treg periféricos (CD4$^+$/CD25^{++}/CD127^{bajo}) mediante citometría de flujo y se clasificaron según la expresión de HLA-DR durante la estación polínica en pacientes con rinoconjuntivitis alérgica causada por *P. judaica*. Los resultados se compararon con los de controles no atópicos. En ambas poblaciones se determinó la expresión de las citocinas asociadas y sus receptores mediante transcripción inversa y reacción en cadena de la polimerasa cuantitativa.
Resultados: Durante la estación polínica no se observaron diferencias significativas entre los pacientes alérgicos con rinoconjuntivitis y los controles sanos en términos de número absoluto o porcentaje de linfocitos Treg en sangre periférica. Todos los pacientes presentaron un número/porcentaje de linfocitos Treg HLA-DR$^-$ superior al de linfocitos Treg HLA-DR$^+$. En ambos grupos se registraron niveles de expresión elevados del ARNm de FOXP3. A pesar de ser inferiores en número, los linfocitos Treg HLA-DR$^+$ presentaron una mayor expresión de *CD28, PRF1, GZMB* y *FASL* que los linfocitos Treg *HLA-DR*$^-$.

Conclusiones: Los resultados más relevantes obtenidos parecen indicar que los linfocitos Treg HLA-DR$^+$ presentan una mayor expresión génica de moléculas asociada a la activación y la citotoxicidad de células dependientes del contacto.

Palabras clave: Expresión génica. Linfocito T regulador (Treg). Rinoconjuntivitis. Alergia a *Parietaria judaica*.

Introduction

Allergic diseases are chronic disorders characterized by hyperresponsiveness to allergens (eg, airborne particles) involving recruitment and activation of immune and inflammatory cells [1]. Atopy and allergic diseases can result from impaired inhibition of allergen-specific helper T cell (T_H)–type responses by regulatory T cells (Treg), a major subset of immune cells [2,3].

Modulation of inflammatory diseases depends on Treg cells, as these maintain immune self-tolerance in the periphery, thus limiting inflammatory responses [4,5]. These cells actively and dominantly suppress both activation and effective functioning of autoreactive T cells, which evade other tolerance mechanisms and regulate the pathogenicity of T_H1 and T_H2 cells [6]. At the cellular level, several types of CD4$^+$ Treg cells have been associated with regulatory function in allergy, in which peripheral T lymphocytes are characterized by higher expression of activation markers, such as the interleukin (IL) 2 receptor α chain (CD25) [5]. CD25 is not only a marker for CD4$^+$-activated T cells, but it is also present in Treg cells.

At gene level, *FOXP3*, a member of the forkhead box protein family, identifies the natural Treg population, and seems to play a crucial role in the differentiation of Treg cells from antigen-naïve T cells. Furthermore, *FOXP3* is believed to be a master regulator of the development and function of CD25$^+$ Treg cells [5].

Immune responses in both healthy and allergic individuals appear to result from the reciprocal modulation of allergen-specific Treg, T_H1, and T_H2 cells. Activated T_H2 cells regulate production of immunoglobulin (Ig) E and recruit inflammatory cells by releasing a wide range of cytokines such as IL-4, IL-5, and IL-13. Interestingly, the suppression of T_H2 cells from effector cells by CD4$^+$CD25^{++} Treg cells has been described in atopic individuals, although it is not effective during the pollen season [2,7,8].

Most patients with allergic rhinitis classify their disease as severe. Allergic rhinitis can lead to impairment of daily activities, quality of sleep, and productivity, with the consequent social and economic costs [9,10]. The disease is defined as a symptomatic disorder induced by an IgE-mediated inflammation of the nasal mucosa after exposure to an allergen [11]. Symptoms are triggered by the interaction of the allergen with IgE, which binds to mast cells in the nasal mucosa or to circulating basophils. Plant pollen is one of the most prevalent allergens [12], and in the Mediterranean area and the temperate climates of Central and Eastern Europe, the highly allergenic *Parietaria* pollen is a frequent cause of pollinosis [13-16].

Characterization of the cellular and genetic events involved in Treg cell function is important in order to understand and treat allergic diseases, such as allergic rhinoconjunctivitis. Treg cells expressing HLA-DR induce early contact-dependent suppression and cytotoxicity, and are involved in downregulation of different inflammatory responses [17].

We investigated the role of Treg cells (CD4$^+$/CD25^{++}/CD127^{low}) and gene expression profile after sorting HLA-DR$^+$ and HLA-DR$^-$ Treg cells from the blood of patients with pollen allergy during pollen season and demonstrated mRNA differences in a list of transcripts of interest. These Treg cells were collected from patients with allergic rhinoconjunctivitis induced by *Parietaria judaica* and compared with nonatopic controls. Shevach et al [18] identified natural Treg cells that were not activated by antigen, but by major histocompatibility complex (MHC) class II self-peptide and cytokines released by activated effector cells. In our study we analyzed the cytokines involved in the effector functions of T cells (eg, IL-13, IL-4, IL-2, interferon [INF] γ and IL-6) and membrane receptors (CCR7, CD28, and CXCR3) that facilitate cell adhesion and migration. Natural Treg cells can also differentiate into potent granzyme B–dependent cells, and into partially perforin-independent cytotoxic cells that can kill antigen-presenting B cells [18]. We also analyzed the expression of cytokines involved in apoptosis and cytotoxicity, namely, Fas ligand, granzyme B, and perforin-1. Finally, expression of IL-10, INF-γ and transforming growth factor (TGF) ß1 was measured, although the role of these cytokines in regulatory function is not dependent on cellular contact.

Methods

Participants

This study was approved by the Coimbra University Hospitals Ethics Board. Ten consecutive female patients with rhinoconjunctivitis caused by exposure to *P judaica* were enrolled after giving their oral informed consent (Group I). Mean (SD) age was 33 (13) years (range, 17-62). Diagnosis and classification of rhinitis were based on the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines [19].

No patient had persistent asthma according to the Global Initiative for Asthma (GINA) Guidelines [20]. Smokers and patients with recent (last 6 weeks) infectious, autoimmune, or neoplastic diseases were excluded. Patients treated with specific immunotherapy were also excluded.

Eight healthy nonallergic volunteers with a mean age of 29 (6) years (range, 22-40) were included as controls (Group II). The presence of rhinitis in group II was excluded using a detailed clinical questionnaire.

Skin Testing

All participants underwent skin prick tests (SPT) to 29 aeroallergens (ALK-ABELLO, Madrid, Spain) using disposable 1-mm–tip sterile lancets (Stallergènes, Antony, France), as follows: *Corylus avellana, Alnus cordata, Betula verrucosa, Platanus acerifolia, Cupressus arizonica, Graminea mixture, Olea europaea, Artemisia vulgaris, Ambrosia elatior, Alternaria tenuis, Cladosporium herbarum, Aspergillus fumigatus, Parietaria judaica, Canis familiaris, Felis catus, Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blatella germanica, Lepidoglyphus destructor, Tyrophagus putrescenciae, Quercus rober, Salix alba, Pinus radiata, Castanea sativa, Chenopodium album, Plantago lanceolata, Rumex azoricus, Tilia cordata*, and *Populus alba*. Histamine dihydrochloride was used as a positive control (10 mg/mL) and 0.9% saline solution as a negative control. The SPT results were recorded 15 minutes after the start of the test and were considered positive if the wheal and flare diameter of each allergen was at least 3 mm greater than that of the negative control.

All patients included had a positive SPT result to *P judaica* pollen and their clinical symptoms were typical of patients who are allergic to this pollen. The study was performed during the 2007 grass-pollen season (April and May).

Determination of Total and Specific IgE

Peripheral blood was withdrawn from the vein of the forearm of all individuals and placed into 2 tubes with sodium-heparin and into 1 Paxgene RNA tube (PreAnalytix, Hombrechtikon, Switzerland).

The differential blood cell count was performed using the Coulter Act Diff 2 hematological analyzer (Beckman Coulter, Fullerton, California, USA).

Quantitative determination of total serum IgE was performed using nephelometry. Total IgE concentration (IU/mL) was determined by comparing the results with the calibrators provided.

Serum specific IgE to *P judaica* was determined using the ImmunoCAP fluorescence enzyme immunoassay technique (Phadia, Uppsala, Sweden). To evaluate the test results, the response from the patient samples was converted to concentrations using a calibration curve. The range of measurements was 0.35 to 100 kU_A/L. The allergic patients selected had values higher than 3.51 kU_A/L (Class III) as part of their inclusion criteria.

Basophil Activation Test

The basophil activation test (BAT) is based on flow-cytometric quantification of the allergen-induced upregulation of the granule-associated marker CD63 or gp53 in peripheral basophils. Two tubes were prepared with 100 µL of blood plus 100 µL of stimulation buffer with or without 0.05 mg of *P judaica* allergen [20 mg/mL]. Cells were incubated for 40 minutes at 37ºC. The degranulation process was stopped by dipping the tubes in ice for 5 minutes. Peripheral blood was incubated with the fluorochrome-conjugated monoclonal antibodies—anti-CD63 fluorescein isothiocyanate (FITC), anti-CD123 phycoerythrin (PE), anti-HLA-DR mixture peridinin chlorophyll protein (PerCP) (H5C6/9F5/L243 [G46-6]; BD Biosciences, San Jose, California, USA) and kept in the dark and on ice for 15 minutes. Erythrocytes were lysed with 2 mL of FACS Lysing Solution (BD Biosciences) for 10 minutes. Cells were washed twice with phosphate-buffered saline (PBS 1x, Biochrom, AG, Berlin, Germany). The percentage of degranulated cells was measured using flow cytometry (FACSCalibur, BD Biosciences). The test was considered positive if the degranulation percentage was higher than 5% and the ratio of stimulated to nonstimulated cells was higher than 2.

Regulatory T cell Gene Expression

Isolation of regulatory T cells: Peripheral blood containing the anticoagulant sodium heparin was centrifuged at 1500 rpm for 5 minutes at room temperature. The buffy coat was recovered and the remaining erythrocytes were lysed with NH_4Cl-EDTA/$KHCO_2$.

The samples were incubated to identify Treg cells, as follows: anti-CD127 in PE (hIL-7R-M21; BD Biosciences, Pharmingen, San Diego, California, USA); anti-CD25 in FITC (M-A251; BD Bioscience Pharmingen); anti-CD4 in PerCP (JK3; BD Biosciences); and anti-HLA-DR in allophycocyanin (L243 [G46-6]; BD Biosciences) for 30 minutes at room temperature and washed twice with PBS 1x (Biochrom, AG) and centrifuged.

$CD4^+/CD25^{++}/CD127^{low}/HLA\text{-}DR^+$ and $CD4^+/CD25^{++}/CD127^{low}/HLA\text{-}DR^-$ cells were isolated using fluorescence-activated cell sorting (BD FACSAria, BD Biosciences) and separated according to HLA-DR expression. Cells were collected in individual polymerase chain reaction (PCR) tubes with 5 µL of PBS treated with 0.1% diethyl pyrocarbonate and cooled to –80ºC.

Treg cells were quantified using a double-platform approach (hematological analyzer and FACS).

Reverse transcription: Cells isolated by FACS were lysed, cooled to –80ºC, heated to 65ºC for 2 minutes, and cooled again to 4ºC before reverse transcription. Quantitative reverse transcription-PCR (qRT-PCR) was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The reactions were performed in a thermocycler (MyCycler, Biorad, Hercules, California, USA) using the following steps: 10 minutes at 25ºC, 50 minutes at 50ºC, 5 minutes at 85ºC, and a final hold at 4ºC. The cDNAs were stored at –80ºC.

Real-time PCR: PCR assays were performed in a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). Prepared cDNA was amplified using 1XQuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) on a 96-well PCR plate following the manufacturer's recommendations. qRT-PCR was performed using specific QuantiTect Primer Assays (Qiagen) for the following genes: *CCR7* (QT00045507), *CD28* (QT00001267), *CXCR3* (QT00213493), *FASL* (QT00001281), *FOXP3* (QT00048286), *GZMB* (QT01001875), *HLADRB1* (QT00090993), *IL10* (QT00041685), *IL13* (QT00000511), *IL2* (QT00015435), *IL4*

(QT00012565), *IL6* (QT00083720), *IFNG* (QT00000525), *PRF1* (QT00199955), *TGFB1* (QT00000728), *TNFA* (QT00029162), and *TNFRSF1A* (QT00216993). Quantification of gene expression was preceded by a normalization step using the geNorm Housekeeping Gene Selection kit for *Rattus norvegicus* (Primer Design, Southampton, United Kingdom) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes for this study [21]. Relative quantification was normalized with the housekeeping genes ACTB (QT01680476) and GAPDH (QT01192646) for both HLA-DR$^+$ and HLA DR$^-$ Treg cells.

Real-time PCR cycle conditions were as follows: an initial activation stage of 15 minutes at 95ºC followed by a second stage comprising 15 seconds at 94ºC, 30 seconds at 55ºC, and 30 seconds at 72ºC (with 50 repeats).

Calculation of results: mRNA expression was analyzed through the relative quantification of mRNA from HLA-DR$^+$ Treg cells and mRNA from HLA-DR$^-$ Treg cells using the comparative ΔΔCT method described by Livak et al [22].

Statistical Analysis

The statistical analysis was performed using SPSS 15.0. A Mann-Whitney test was applied to evaluate differences between groups. A *P* value of less than .05 was considered statistically significant.

Results

Age, sex, serum total IgE, serum specific IgE to *P judaica*, eosinophil count, and the BAT results are summarized in Table 1. All individuals enrolled in the study were female. Allergic patients reported the mean age for the onset of symptoms to be 11 (7) years.

Most patients with rhinoconjunctivitis had higher total serum IgE levels (8 out of 10) (*P*=.02) and peripheral eosinophil counts (*P*=.13) than nonallergic patients. Allergic patients had positive SPT results to *P judaica* and at least 1 of the other allergens tested.

The number and the percentage of total leukocytes, lymphocytes, CD4$^+$ lymphocytes, Treg cells (CD4$^+$CD25^{++}CD127^{low}), HLA-DR$^+$ Treg cells, and HLA-DR$^-$ Treg cells were calculated using the double-platform approach (hematological analyzer and FACS) are presented in Table 2.

No significant differences were observed between the allergic and the nonallergic group for number (*P*=.63) or percentage (*P*=.25) of Treg cells from peripheral blood (Table 2 and Figure 1).

The Treg cells identified by CD4$^+$CD25^{++}CD127^{low} were also analyzed according to HLA-DR expression, revealing 4 groups: HLA-DR$^+$ Treg cells from allergic patients; HLA-DR$^-$ Treg cells from allergic patients; HLA-DR$^+$ Treg cells from controls and HLA-DR$^-$ Treg cells from controls. All participants had a smaller number or percentage of HLA-DR$^+$ Treg cells. We could not find significant differences in the number or percentage of these cells between allergic and nonallergic individuals. Therefore, the difference observed in the percentage of HLA-DR$^+$ Treg cells between the 2 groups was too close to be statistically significant (*P*=.08) (Figure 2).

mRNA expression of HLA-DR$^+$ and HLA-DR$^-$ Treg cell subpopulations in allergic and nonallergic individuals is presented in Figure 3 through the relative quantification of $2^{-\Delta CT}$(log) mRNA between the differences in expression of the gene of interest and the housekeeping gene (*ACTB* and *GAPDH*).

No significant differences in gene expression were found between the 4 groups. Nevertheless, relative gene expression between allergic patients and controls tended to be different. The Treg cells of allergic patients showed higher expression of *FASL, GZMB, PRF1, CCR7, CXCR3, TGFB, TNFA*, and *IL13* and lower expression of *IL2* and *IFNG*. Expression of *FOXP3, CD28, IL10*, and *IL4* was very similar in patients and in controls. *FOXP3* and *CD28* showed high levels and *IL10* and *IL4* showed low levels in relation to endogenous gene expression.

Our results are presented in Figure 4 as a relative quantification (arbitrary units), namely, the average fold increase in gene expression in HLA-DR$^+$ Treg cells compared with the average fold increase in gene expression in HLA-DR$^-$ Treg cells in allergic and nonallergic individuals.

Table 1. Characteristics of the Study Groups[a]

	Patients (n=10)	Controls (n=8)	*P* Value
Sex (Female/Male)	10/0	8/0	
Age, y	33 (13)	29 (6)[b]	>.05
Serum total IgE, kU_A/L	317.8 (293.4)	30.7 (15.2)[c]	.02
Serum specific IgE to *Parietaria judaica*, kU_A/L	31.1 (23.2)	<0.35[c]	.02
Eosinophils, cells/μL	407.1 (337.4)	166.7 (81.7)[b]	>.05
BAT	Positive	Negative	

Abbreviations: BAT, basophil activation test; Ig, Immunoglobulin.
[a]Data are expressed as mean (SD).
[b]*P* value is nonsignificant.
[c]*P*<.05.

Table 2. Complete Blood Count Performed Using a Double-Platform Approach (Hematological Analyzer and FACS)[a]

	Patients	Controls	*P* Value
Leukocytes, cells/μL	7667 (2848)	7387 (1423)[b]	>.05
Lymphocytes			
Cells/μL	2296 (1728)	2634 (1110)[b]	>.05
% Leukocytes	29.6 (15.2)	34.8 (11.5)	
Lymphocytes T-CD4+			
Cells/μL	881 (627)	1268 (636)[b]	>.05
% Lymphocytes	38.6 (8.5	48.1 (8.5)	
Treg cells ($CD4^+CD25^{++}CD127^{low}$)			
Cells/μL	70 (54)	86 (56)[b]	>.05
% Lymphocytes	8.4 (2.6)	6.8 (3.99)	
Treg cells HLA-DR+ ($CD4^+CD25^{++}CD127^{low}DR^+$)			
Cells/μL	18 (14)	15 (11)[b]	>.05
% Treg cells	26.8 (5.0)	18.3 (9.5)	
Treg cells HLA-DR− ($CD4^+CD25^{++}CD127^{low}DR^-$)			
Cells/μL	51 (399)	70 (48)[b]	>.05
% Treg cells	72.5 (4.7)	80.6 (8.9)	

Abbreviation: Treg, regulatory T cells.
[a]Data are expressed as mean (SD).
[b]*P* value is nonsignificant.

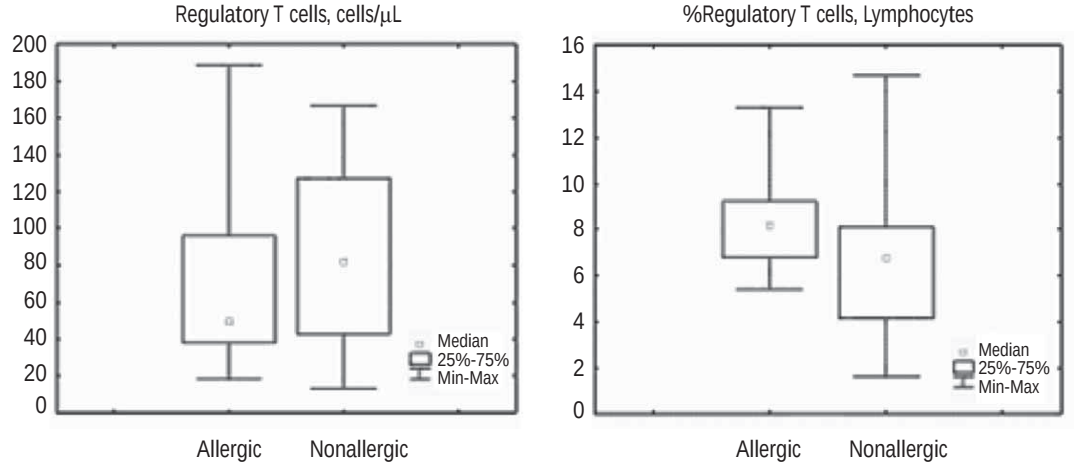

Figure 1. Distribution of regulatory T cells in allergic and nonallergic individuals.

The HLA-DR+ Treg cells from the control group presented higher expression values than HLA-DR− Treg cells in most of the variables studied. The exceptions were *CXCR3* and *PRF1*. In the allergic group, every mRNA expression value of HLA-DR+ Treg cells was higher than those of HLA-DR− Treg cells, despite HLA-DR+ Treg cells being lower in number. The difference in gene expression between HLA-DR+ and HLA-DR− Treg cells was not statistically significant.

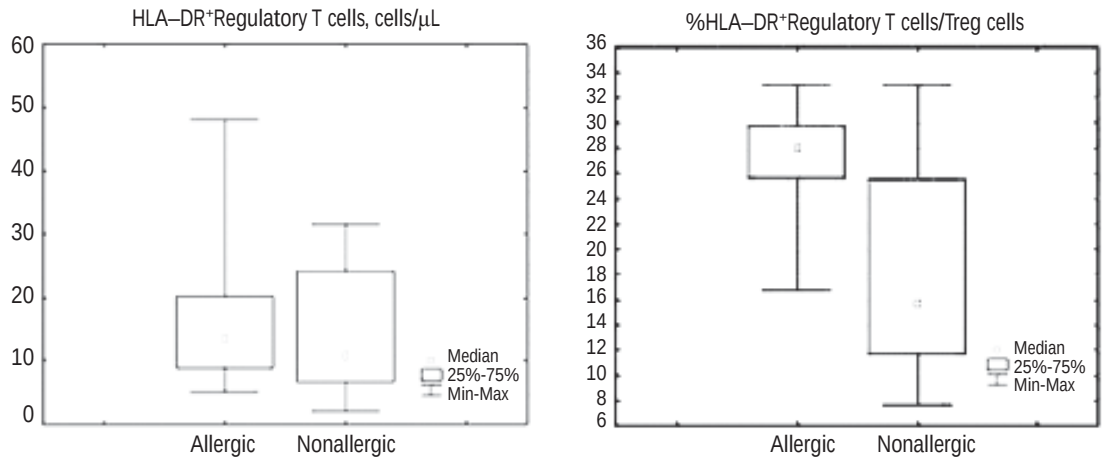

Figure 2. Distribution of HLA-DR+ regulatory T cells in allergic and nonallergic individuals.

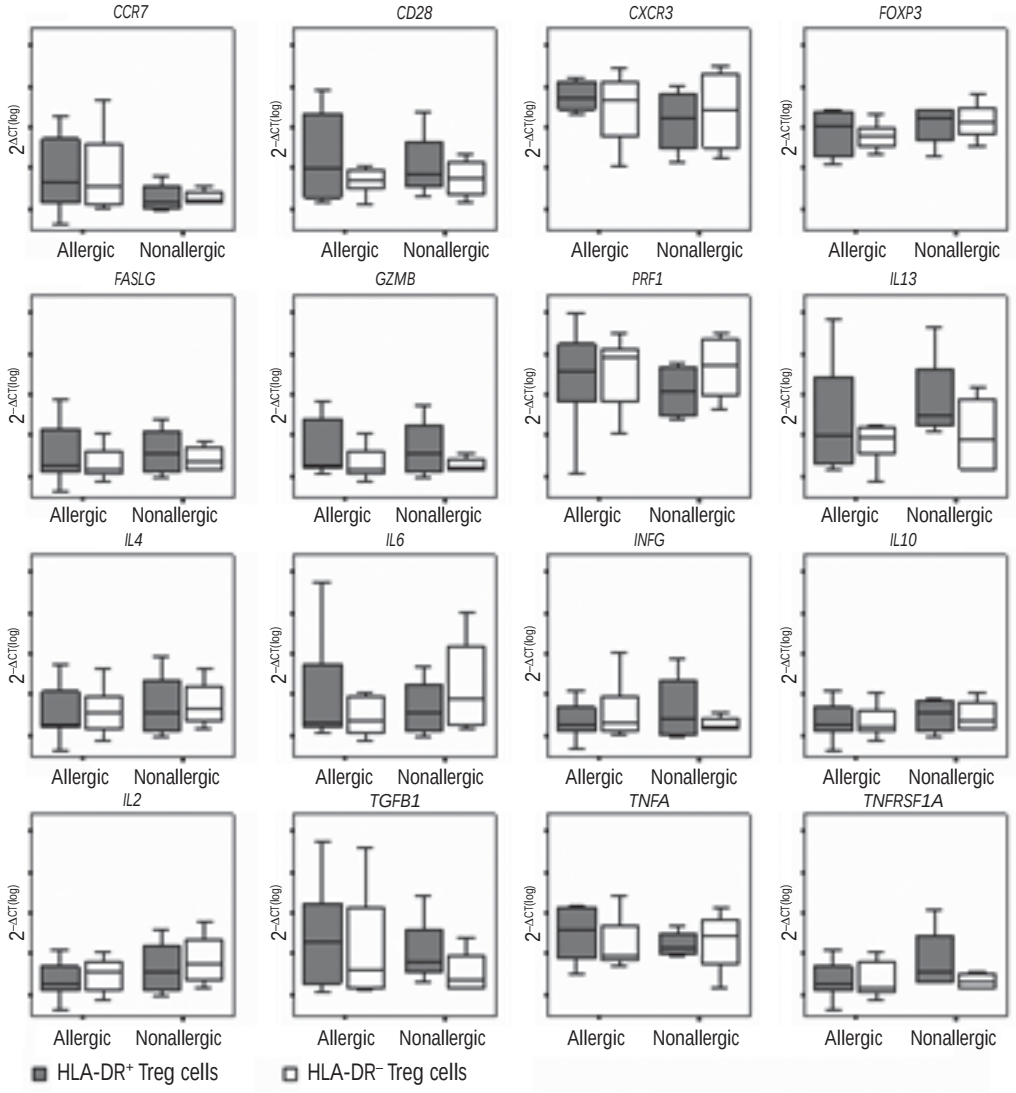

Figure 3. Gene expression profile from HLA-DR+ Treg cells and HLA-DR− Treg cells in allergic and nonallergic (controls) individuals.
The values above represent the relative quantification of $2^{-\Delta CT(log)}$ mRNA between the different expression of the gene of interest and the housekeeping genes (*ACTB* and *GAPDH*).

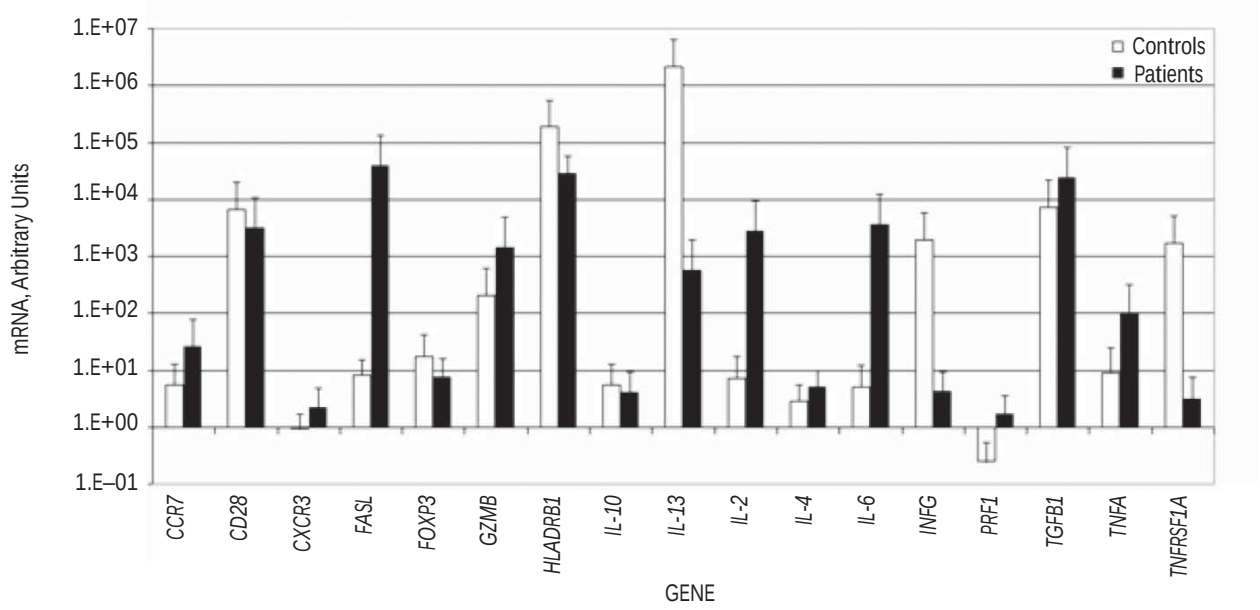

Figure 4. Gene expression profile from Treg cells in allergic and nonallergic (controls) individuals. The values above represent the relative quantification (arbitrary units): the average fold increase in gene expression from HLA-DR+ Treg cells in relation to the average fold increase in gene expression from HLA-DR− Treg cells in allergic and nonallergic individuals.

Discussion

This study was carried out to evaluate expression of HLA-DR in CD4+/CD25++/CD127low Treg cells in a chronic inflammatory disease such as allergic rhinoconjunctivitis.

Circulating human CD4 cells can express MHC-II, a marker of activated cells that reduces T-cell responsiveness to new antigens [23]. In circulating CD4+CD25++ T cells, MHC-II expression reveals functionally distinct Treg populations [17], which show suppression activity on contact with the membrane and downregulate different inflammatory responses. This cell population also participates in homeostatic maintenance of Treg in vivo by presenting self-antigens to other Treg cells. In contrast, the HLA-DR− Treg population first induces a T_H2 immune response through release of soluble mediators and later activates a *FOXP3*-associated contact-dependent suppression mechanism.

We analyzed the differences in mRNA expression between HLA-DR+ and HLA-DR− Treg subpopulations.

As expected, patients had high levels of total IgE. In addition, BAT confirmed in vitro that *P judaica* pollen was an allergen in the allergic individuals studied. *FOXP3* expression was high in all groups, and this result was consistent with our accurate Treg selection using FACS.

The percentage of Treg cells was within the normal range, from 5% to 10% of CD4+ T cells in peripheral blood [6]; no statistical differences were found between patients and controls. This may indicate that the number of Treg is not such an important factor in the disease process. Grindebacke et al [8] found that the number of CD25+ Treg cells in blood and their ability to suppress allergen-specific proliferative responses were similar in allergic patients and healthy controls outside the pollen season, although suppressive activity diminished during the pollen season. It seems unlikely that there was a complete dysfunction of the Treg repertoire targeting the antigen repertoire. Other authors, however, have found that allergic patients have fewer Treg cells and that therapeutic approaches such as specific immunotherapy can re-establish cells with suppressive potential [24].

All the participants had a smaller percentage of HLA-DR+ Treg cells—between 20% and 30% of the CD4+CD25++ regulatory population—in much the same proportion reported by others authors [17]. Data from the control group varied widely. There were no significant differences in the number or percentage of these cells between patients and controls, although a trend toward statistical significance was observed (P=.08) in the percentage of HLA-DR+/Treg cells in allergic patients in relation to controls. As HLA-DR+ cells are activated in order to exert contact-dependent suppression, their higher percentage in allergic patients could represent a controlled response to the allergic stimulus. In addition, the difference in gene expression between HLA-DR+ and HLA-DR− Treg cells was not statistically significant. This could be explained by the low number of study patients who also had highly variable results for relative gene expression. Nevertheless, relative gene expression between the 2 groups tended to be different.

In accordance with contact-dependent suppression activity through HLA-DR, both allergic and nonallergic individuals

had high levels and similar amounts of costimulatory *CD28* expression. After antigen exposure, interactions between CD28 and CD80 activate T lymphocytes and induce proliferation and differentiation of effector T cells [25]. When CD28 monoclonal antibody is added, Treg seem to expand without T-cell receptor enrolment or cross-linking.

Chemokine receptors play an essential role in leukocyte migration and chemotaxis. There is some controversy over whether these receptors can function as phenotypic markers in certain cell subsets: the chemokine receptor profile can be changed without a concomitant change in cytokine profile [26]. *CXCR3* has been reported to be a T_H1 marker and is abundant on memory T lymphocytes [27]. This homing receptor gene, which has also been associated with entry and permanence of T cells in lymph nodes, showed higher expression in all the individuals studied, although with a reduced ratio among HLA-DR$^+$ cells [28]. The lymphoid-homing chemokine receptor gene *CCR7* is not expressed as much as *CXCR3*, and higher levels of CCR7 were found in patients than in controls. There is evidence that *CCR7* mediates Treg migration to the paracortical areas of lymph nodes under steady-state conditions, although *CCR7*-independent migration through high endothelial venules also takes place in the medulla [29].

Analysis of mRNA by qRT-PCR demonstrated different cytokine gene expression profiles in HLA-DR$^+$ and HLA-DR$^-$ Treg cells (CD4$^+$/CD25^{++}/CD127^{low}). We observed low levels of mRNA expression of cytokines IL-2 and IL-6 in relation to endogenous gene expression, suggesting less important inflammatory activity in Treg cells. Besides being responsible for the differentiation of T cells into cytotoxic cells, and B cells into plasma cells, IL-6 contributes to the development of T_H17 cells and inhibits Treg cells [30]. In our study, mRNA expression of IL-6 was more evident in HLA-DR$^+$ Treg cells than in HLA-DR$^-$ Treg cells in the patients. The same results were observed for IL-2 mRNA expression.

We also found low levels of expression of IL-10 and IL-4 with similar patterns in both patients and controls. Curiously, IL-13 and TGFß mRNA expression seem to be more associated with HLA-DR$^+$ Treg cells.

The patients' Treg cells tended to have more transcripts involved in cytotoxic activity. Their HLA-DR$^+$ Treg cells overexpressed *PRF1* and *GZMB* mRNA. Cytolytic proteins stored in secretor granules (eg, perforin) from cytotoxic lymphocytes and NK cells, are crucial for immune surveillance and homeostasis. Perforin synergizes with pro-apoptotic serine proteases, such as granzymes, thus targeting the cell and inducing apoptosis. Granzyme B is the most powerful pro-apoptotic member of the granzyme family [31].

Moreover, apoptotic FasL (a TNF family member that is involved in the selection of thymocytes and cytotoxicity mediated by T cells and NK cells) also had higher mRNA levels in activated HLA-DR$^+$ Treg cells in patients. It seems that FasL may play a role as an accessory/costimulatory molecule sending a retrograde transduction signal into FasL-expressing cells [32]. In our study, both FasL mechanisms probably played a role in patients, whose high mRNA levels of *FASL* in HLA-DR$^+$ Treg cells associated with the high mRNA expression of *PRF1* and *GZMB* could promote apoptosis of autoreactive cells and selected cells in the allergic response.

The methods used to identify the different expression profiles could not fully reproduce all the mRNA expressed by the cells studied. This could be due to the short life span of mRNA and the fact that we only examined peripheral blood cells at a specific point in time. There are unlikely to be important immunological differences between groups. What is likely is that allergic and nonallergic individuals display different functions of their allergen-specific Treg cells during the spring allergy season. We think that our results have brought some new insights to the study of immune regulation of Treg cells by cell contact activation.

In summary, we found no evidence that a numerical difference in Treg cells in blood exists between adult patients with allergic rhinoconjunctivitis and healthy controls during the pollen season. The most relevant results obtained suggest that HLA-DR$^+$ Treg cells tend to present higher gene expression of molecules associated with contact-dependent cell activation and cytotoxicity.

Acknowledgments

The data presented in this study are to be submitted as an abstract to the XXVIII Congress of the European Academy of Allergology and Clinical Immunology.

References

1. Thompson PJ. Unique role of allergens and the epithelium in asthma. Clin Exp Allergy. 1998;28:110-6.
2. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber C, Blaser K, Akdis C. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med. 2004;199:1567-75.
3. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr V, Robinson D. Relation of CD4$^+$CD25$^+$ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet. 2004;363:608-15.
4. Trzonkowski P, Szmit E, Myśliwska J, Myśliwski A. CD4+CD25+ T regulatory cells inhibit cytotoxic activity of CTL and NK cells in humans—impact of immunosenescence. Clin Immunol. 2006;119:307-16.
5. Seroogy CM, Gern JE. The role of T regulatory cells in asthma. J Allergy Clin Immunol. 2005;116:996-9.
6. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol. 2005;116:949-59.
7. Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. Curr Opin Immunol. 2003;15:627-33.
8. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4$^+$CD25$^+$ regulatory T cells in birch allergics during birch pollen season. Clin Exp Allergy. 2004;34:1364-72.
9. Crystal-Peters J, Crown WH, Goetzel RZ, Schutt D. The cost of productivity losses associated with allergic rhinitis. Am J Manag Care. 2000;6:373-8.

10. Todo-Bom A, Loureiro C, Almeida MM, Nunes C, Delgado L, Castel-Branco G, Bousquet J. Epidemiology of rhinitis in Portugal: evaluation of the intermittent and persistent types. Allergy. 2007;62:1038-43.
11. Johansson SG, Bieber T, Dahl R, Friedmann P, Lanier B, Lockey R, Motala C, Martell JA, Platts-Mills T, Ring J, Thien F, Cauwenberge PV, Williams HC. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol. 2004;113:832-6.
12. Behrendt H, Becker WM. Localization, release and bioavailability of pollen allergens: the influence of environmental factors. Curr Opin Immunol. 2001;13:709-15.
13. D'Amato G, Ruffilli A, Sacerdoti G, Bonini S. Parietaria pollinosis: a review. Allergy. 1992;47:443-9.
14. Colombo P, Bonura A, Costa MA, Izzo V, Passantino R, Locorotondo G, Amoroso S, Geraci D. The allergens of Parietaria. Int Arch Allergy Immunol. 2003;130:173-9.
15. D'Amato G. Urban air pollution and plant-derived respiratory allergy. Clin Exp Allergy. 2000;30:628-36.
16. Cortes L, Carvalho AL, Todo-Bom A, Faro C, Pires E, Veríssimo P. Purification of a novel aminopeptidase from the pollen of Parietaria judaica that alters epithelial integrity and degrades neuropeptides. J Allergy Clin Immunol. 2006;118:878-84.
17. Baecher-Allan C, Wolf E, Hafler A. MHC class II expression identifies functionally distinct human regulatory T cells. J Immunol. 2006;176:4622-31.
18. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestlyle of naturally occurring CD4+CD25+Foxp3+ regulatory T cells. Immunol Rev. 2006;212:60-73.
19. Bousquet J, Khaltaev A, Cruz AA, Denburg J, Fokkens WJ, Togias A, Zuberbier T, Baena-Cagnani CE, Canonica GW, van Weel C, Aqache L, Aït-Khaled N, Bachert C, Blaiss MS, Bonini S, Boulet LP, Bousquet PJ, Camargos P, Carlsen KH, Chen Y, Custovic A, Dahl R, Demoly P, Douaqui H, Durham SR, van Wijk RG, Kalavci O, Kaliner MA, Kim YY, Kowalski ML, Kuna P, Le LT, Lemiere C, Li J, Lockey RF, Mavale-Manuel S, Meltzer EO, Mohammad Y, Mullol J, Naclerio R, O'Hehir RE, Ohta K, Ouedraogo S, Palkonen S, Papadopoulos N, Passalacqua G, Pawankar R, Popov TA, Rabe KF, Rosado-Pinto J, Scadding GK, Simons FE, Toskala E, Valovirta E, van Cauwenberge P, Wang DY, Wickman M, Yawn BP, Yorgancioglu A, Yusuf OM, Zar H, Annesi-Maesano I, Bateman ED, Ben Kheder A, Boakye DA, Bouchard J, Burney P, Busse WW, Chan-Yeung M, Chavannes NH, Chuchalin A, Dolen WK, Emuzyte R, Grouse L, Humbert M, Jackson C, Johnston SL, Keith PK, Kemp JP, Klossek JM, Larenas-Linnemann D, Lipworth B, Malo JL, Marshall GD, Naspitz C, Nekam K, Niggemann B, Nizankowska-Mogilnicka E, Okamoto Y, Orru MP, Potter P, Price D, Stoloff SW, Vandenplas O, Viegi G, Williams D. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA2LEN and AllerGen). Allergy. 2008;63:8-160.
20. Global Initiative for Asthma. Global strategy for Asthma Management and Prevention. Washington, DC: National Institutes of Health; National Heart, Lung and Blood Institute, 2002. Updated 2007. Available from: www.ginasthma.com.
21. Vandesompele Jo, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002:18;3(7):RESEARCH0034.1-11.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. Methods. 2001;25:402-8.
23. Bhandoola A, Tai X, Eckhaus M, Auchincloss H, Mason K, Rubin S A, Carbone KM, Grossman Z, Rosenberg AS, Singer A. Peripheral expression of self-MHC-II influences the reactivity and self-tolerance of mature CD4(+) T cells: evidence from a lymphopenic T cell model. Immunity. 2002;17:425-36.
24. Lin YL, Shieh CC, Wang JY. The functional insufficiency of human CD4+CD25high T-regulatory cells in allergic asthma is subjected to TNF-α modulation. Allergy. 2008:63:67-74.
25. Sansom DM, Walker LS. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T cell biology. Immunol Rev. 2006;212:131-48.
26. Holse M, Assing K, Poulsen LK. CCR3, CCR5, CCR8 and CXCR3 expression in memory T helper cells from allergic rhinitis patients, asymptomatically sensitized and healthy individuals. Clin Mol Allergy. 2006;4:1-6.
27. Okazaki H, Kakurai M, Hirata D, Sato H, Kamimura T, Onai N, Matsushima K, Nakagawa H, Kano S, Minota S. Characterization of chemokine receptor expression and cytokine production in circulating CD4+ T cells from patients with atopic dermatitis: up-regulation of C-C chemokine receptor 4 in atopic dermatitis. Clin Exp Allergy. 2002;32:1236-42.
28. Alam R, Gorska M. Lymphocytes. J Allergy Clin Immunol. 2003;111:S476-85.
29. Ueha S, Yoneyama H, Hontsu S, Kurachi M, Kitabatake M, Abe J, Yoshie O, Shibayama S, Sugiyama T, Matsushima K. CCR7 mediates the migration of Foxp3+ regulatory T cells to the paracortical areas of peripheral lymph nodes through high endothelial venules. J Leukoc Biol. 2007;82:1230-8.
30. Romagnani S. Regulation of T cell response. Clin Exp Allergy. 2006;36:1357-66.
31. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol. 2006;6:940-52.
32. Lettau M, Paulsen M, Kabelitz D, Janssen O. Storage, expression and function of Fas ligand, the key death factor of immune cells. Curr Med Chem. 2008;15:1684-96.

Manuscript received February 1, 2009; accepted for publication September 9, 2009.

Luísa Geraldes

Departamento de Ciências Pneumológicas
e Alergológicas dos Hospitais da Universidade
de Coimbra
Av. Bissaya Barreto e Praceta Prof. Mota Pinto
3000-075 Coimbra
Portugal