

Naturally Occurring Regulatory T Cells and Interleukins 10 and 12 in the Pathogenesis of Idiopathic Warm Autoimmune Hemolytic Anemia

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■ Abstract

Objectives: Naturally occurring regulatory T cells (nTregs) play an important role in immunologic tolerance and control immune-mediated pathology in murine models of autoimmune hemolytic anemia. Our aim was to measure nTregs and levels of interleukin (IL) 10 and IL-12 in peripheral blood mononuclear cell (PBMC) cultures from patients with idiopathic warm autoimmune hemolytic anemia (wAIHA) in an attempt to unravel some of the mysteries behind the pathogenesis of this autoimmune disorder.

Methods: Twenty-seven patients with idiopathic wAIHA and 15 age- and sex-matched controls underwent flow cytometric analysis of CD4⁺CD25^{high}FoxP3⁺T cells (nTregs) and analysis by enzyme-linked immunosorbent assay of IL-10 and IL-12 in the supernatants of basal and lipopolysaccharide (LPS)-stimulated PBMC cultures.

Results: The mean (SD) percentage of circulating CD4⁺ nTregs in peripheral blood was significantly lower in patients (4.63% [1.0%]) than in controls (9.76% [0.78%]) ($P < .001$). PBMCs from patients had significantly higher basal levels of IL-10 and IL-12, with a dramatic reduction in responsiveness to LPS *in vitro* compared to controls. There was a significantly negative correlation between the percentage of nTregs and reticulocyte count (RC), basal IL-10, and LPS-stimulated IL-10, and a significantly positive correlation with haptoglobin (Hp) ($P < .05$). Basal IL-10 and LPS-stimulated IL-10 were positively correlated with RC ($P < .001$ in both cases) and negatively correlated with Hp ($P < .01$ and $P < .05$, respectively).

Conclusion: Our study indicates that a reduced percentage of nTregs and IL-10/IL-12 imbalance may play an essential role in the onset and/or maintenance of this AIHA.

Key words: Autoimmune hemolytic anemia. Regulatory T cells. Interleukin10. Interleukin12.

■ Resumen

Objetivos: Los linfocitos T reguladores naturales (Treg) desempeñan un papel importante en la tolerancia inmunológica y controlan la patología inmunomediada en modelos murinos de anemia hemolítica autoinmunitaria. El objetivo de esta investigación fue determinar los Treg y los niveles de interleucina (IL) 10 e IL 12 en cultivos de células mononucleares de sangre periférica (CMSP) de pacientes con anemia hemolítica autoinmunitaria por anticuerpos calientes (AHAlc) idiopática, con el fin de resolver alguna de las incógnitas que rodean la patogenia de esta enfermedad autoinmunitaria.

Métodos: Se realizaron análisis mediante citometría de flujo de linfocitos T (Treg) CD4⁺ CD25^{elevado} FoxP3⁺ y enzimoimmunoanálisis de adsorción (ELISA) para la detección de IL-10 e IL-12 en los sobrenadantes de cultivos de CMSP al inicio y tras la estimulación con lipopolisacáridos (LPS) en 27 pacientes con AHAlc idiopática y 15 controles emparejados por edad y sexo.

Resultados: La media (DE) porcentual de Treg CD4⁺ circulantes en sangre periférica fue significativamente menor en los pacientes (4,63% [1,0%]) que en los controles (9,76% [0,78%]) ($p < 0,001$). Las CMSP de los pacientes presentaron niveles basales de IL 10 e IL 12 significativamente más elevados, con una disminución notable de la sensibilidad al LPS *in vitro* en comparación con los controles. Se observó una correlación significativamente negativa entre el porcentaje de Treg y el recuento de reticulocitos (RR), la IL-10 basal y la IL-10 estimulada por LPS, y una correlación significativamente positiva con la haptoglobina (Hp) ($p < 0,05$). La IL-10 basal y la IL 10 estimulada por LPS presentaron una correlación positiva con el RR ($p < 0,001$ en ambos casos) y una correlación negativa con la Hp ($p < 0,01$ y $p < 0,05$, respectivamente).

Conclusión: Este estudio indica que un pequeño desequilibrio entre los Treg y la IL 10/IL 12 puede desempeñar un papel importante en la aparición y/o el mantenimiento de la AHAlc.

Palabras clave: Anemia hemolítica autoinmunitaria. Linfocitos T reguladores. Interleucina 10. Interleucina 12.

Introduction

Warm autoimmune hemolytic anemia (wAIHA) accounts for about 50% to 70% of all cases of autoimmune hemolytic anemia and is characterized by the production of autoantibodies that target determinants on red blood cells (RBCs) [1]. These antibodies are typically of polyclonal origin, belong to the immunoglobulin (Ig) G isotype, and arise either idiopathically or in the context of lymphoproliferative disorders or autoimmune diseases. IgG-coated RBCs undergo accelerated Fc gamma receptor-mediated extravascular clearance [2]. Specific tolerance of self-antigens is achieved by complex dynamic interactions at the cellular and humoral levels of the immune system. In wAIHA, self-tolerance of Rh protein on the surface of RBCs is usually broken [3]. Regulatory T cells (Tregs) are a specialized subpopulation of T cells that act to suppress immune responses, thereby maintaining homeostasis and self-tolerance [4]. Naturally occurring Tregs (nTregs) originate in the thymus and account for 5% to 10 % of mature peripheral CD4⁺ T cells. It has been shown that Tregs are able to inhibit T-cell proliferation and cytokine production and play critical roles in preventing autoimmunity [5]. Impaired Treg development and/or function is a characteristic of a variety of autoimmune diseases; higher frequencies of Tregs, in contrast, can render the immune system hyporesponsive to pathogens [6]. In vitro studies have shown that CD4⁺CD25^{high} T cells appear to be a homogeneous population of suppressor cells that do not contain memory or activated T cells. The suppression effect is mediated by a cytokine-independent, cell contact-dependent mechanism that requires activation of these cells via a T-cell receptor [7]. The primary method to detect Tregs is flow cytometry with gating on CD4⁺CD25^{high} cells. Tregs have been further characterized by the expression of the transcription factor regulator forkhead box P3 (FoxP3), which, when overexpressed in activated T cells, downregulates cytokine production [8].

Interleukin (IL) 10 is known to promote antibody production by B cells, and it has been suggested that it might play a role in the development of autoantibody-mediated diseases such as systemic lupus erythematosus (SLE) [9,10] and AIHA [11]. On the other hand, it has been shown that, in human autoimmune diseases, IL-12 promotes cell-mediated immunity through its ability to induce interferon (IFN) γ , but suppresses humoral immune responses and autoantibody production [12]. Therefore, studies of peripheral blood lymphocyte-monocyte activation and interleukin production are valuable tools for investigating the relationship between the immunoregulatory cytokine network and AIHA.

In the present study we analyzed peripheral blood mononuclear cells (PBMCs) to measure the percentage of nTreg cells in CD4⁺ cells in the peripheral blood of patients with idiopathic wAIHA and healthy controls. We also measured levels of IL-10 and IL-12 in basal and lipopolysaccharide (LPS)-stimulated PBMC cultures in an attempt to unravel some of the mysteries behind the pathogenesis of this autoimmune disorder.

Participants and Methods

Patients and Controls

This study comprised 27 patients (9 men and 18 women) with idiopathic wAIHA. Their ages ranged from 28 to 63 years (mean [SD] age, 45.3 [11] years). Fifteen healthy individuals matched for age and sex were randomly selected from a group of blood donors as controls; they were aged between 23 and 54 years (mean [SD], 44.6 [9.2] years). Written informed consent was obtained from all patients and controls. Patients known or suspected to have SLE or other autoimmune diseases, lymphoproliferative or other neoplastic diseases, infections, or immune deficiency syndrome, were not included in the study. Patients were observed for 6 months to exclude primary malignancy. Blood samples were obtained at the time of acute hemolysis, which was defined by a sudden fall in hemoglobin (Hb), a haptoglobin (Hp) concentration of <50 mg/dL, lactate dehydrogenase (LDH) activity of >480 U/L, a serum bilirubin level of >1.0 mg/dL, and a high reticulocyte count (RC). A direct antiglobulin test (DAT) with polyspecific DiaMed Micro Typing ID gel system (DiaMed, Cressier sur Morat, Switzerland) was performed for all cases. All patients were DAT positive.

Procedures

Patients enrolled in the study underwent full history taking, clinical examination, liver and kidney function tests, complete blood count, flow cytometric analysis of CD4⁺CD25^{high}FoxP3⁺ T cells, and analysis of IL-10 and IL-12 in the supernatants of basal and LPS-stimulated PBMC cultures by enzyme-linked immunosorbent assay (ELISA).

Lymphocyte Separation

PBMCs were purified from heparinized blood samples by Ficoll-Hypaque gradients (Biochrom, Berlin, Germany). PBMCs separated for flow cytometry were washed twice with FACs buffer, with suspension of the cell pellet in FACs buffer at a concentration of 1.0×10^6 /mL. PBMCs for cultures were washed twice in Hank's balanced salt solution and resuspended at a concentration of 1.0×10^6 /mL in RPMI 1640 complete medium supplemented with 5% heat-inactivated fetal bovine serum.

Flow Cytometric Analysis of nTregs

Sample preparation and flow cytometric analysis. Cell-surface staining was performed using mouse anti-human monoclonal antibodies (mAbs), anti-CD25 fluorescein isothiocyanate, anti-CD4 peridinin chlorophyll protein conjugate, and anti-CD3 phycoerythrin conjugate (PE). Staining was performed by adding 20 μ L of each mAb to 100 μ L of separated PBMCs in the same tube, followed by incubation for 30 minutes in the dark at 4°C. The tubes were washed twice with FACs buffer. FoxP3 staining was performed according to the manufacturer's protocol (PE antihuman Foxp3 Staining Set; BD Biosciences, San Jose, California, USA). After surface staining of the cells, fixative and permeabilizing solutions were added, followed by intracellular staining using

20 μ L of FoxP3 mAbs. The cells were incubated for 30 minutes in the dark at 40°C and also washed twice with FACs buffer. Finally, 0.5 mL of phosphate buffered saline was added to the washed cells prior to measurement of CD4⁺CD25⁺FoxP3⁺ cells. We used appropriate isotype controls of anti-human mAbs to prevent nonspecific Fc receptor staining. Sample analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA). FACs-acquisition and analysis were performed with FACs Cell Quest software (BD Biosciences). Samples were first examined for the frequency of CD3⁺CD4⁺ T cells. The percentages of CD4⁺CD25⁺FoxP3⁺ T cells in the total CD4⁺ T-cell population were then determined and the intensity of CD25 surface expression was measured using mean fluorescence intensity (MFI) as previously described [13]. T cells with a CD25 expression level of ≥ 120 were considered to be CD25^{high} (mean [SD] MFI, 160 [25]).

Lymphocyte culture. Two-mL cell suspensions were plated into 5-mL plastic Petri dishes (Falcon; Becton-Dickinson) and cultured at 37°C in a 5% CO₂ humidified atmosphere under basal conditions and with the addition of 100 ng/mL of LPS (Sigma Aldrich, Saint Louis, Missouri, USA) to stimulate monocyte IL-10 and IL-12 production. After 48 hours, the supernatants were collected and frozen at -80°C until use.

Cytokine assay. Quantitative determination of IL-10 and IL-12 was performed using quantitative sandwich ELISA (Quantikine; R&D systems, Minneapolis, Minnesota, USA). The detection limits for IL-10 and IL-12 were 4.61 pg/mL and 2.0 pg/mL, respectively.

Statistical Analysis

Data were entered, checked, and analyzed using the SPSS software package (version 16) for Windows. Results were expressed as means (SD). The χ^2 test and the *t* test were used for statistical comparisons between parametric data from pairs of groups. The paired *t* test was used for statistical comparisons

Table 1. Comparison of Clinical and Laboratory Data for Patients With Warm Autoimmune Hemolytic Anemia and Healthy Controls^a

	Patients (n=27)	Controls (n=15)	<i>P</i>
Age, y	45.3 (11)	44.6 (9.2)	.76
Male to female ratio	1.0:1.5	1.0:1.5	.86
Hemoglobin, g/dL	6.8 (1.1)	13.5 (1.6)	.001 ^b
Reticulocyte count, %	21.5 (4.1)	0.3 (0.2)	.001 ^b
Bilirubin, mg/dL	2.4 (0.8)	0.2 (0.1)	.001 ^b
Lactate dehydrogenase, U/L	1153 (383)	326.0 (69)	.001 ^b
Haptoglobin, mg/dL	14.5 (10.8)	76.2 (10.8)	.001 ^b

^aData are expressed as mean (SD) unless otherwise indicated.

^bVery highly significant.

within the same group. Correlation analysis was performed using the Pearson correlation test. *P* values below .05 were considered significant.

Results

Twenty-seven patients with idiopathic wAIHA and 15 healthy age- and sex-matched controls participated in the study. Clinical and routine laboratory data for the 2 groups are summarized in Table 1. No statistically significant differences were found for age or sex, but there was a highly significant difference in Hb, RC, bilirubin, lactate dehydrogenase, and Hp between the 2 groups (*P*<.001).

PBMCs from patients and controls were first analyzed for the frequency of CD4⁺CD25⁺FoxP3⁺ T cells in the total CD3⁺CD4⁺ T-cell population (Figure 1). The percentage of nTregs (CD25^{high}) was then determined using MFI. The results showed a significantly lower percentage of nTreg cells in CD4⁺

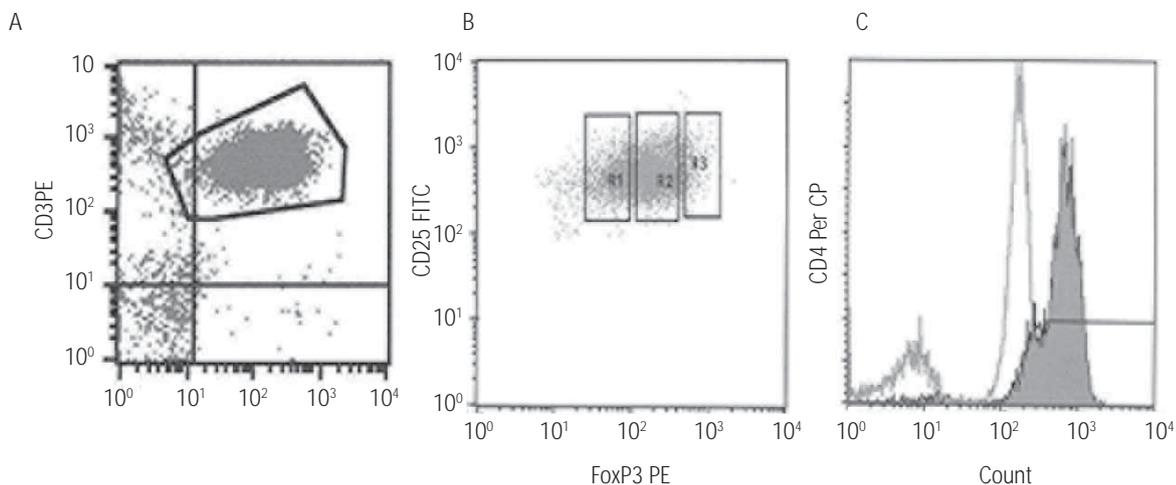


Figure 1. Flow cytometry analysis for the percentage of regulatory T cells (Tregs) in warm autoimmune hemolytic anemia. A, peripheral blood mononuclear cells were stained for CD3⁺CD4⁺ T cells and gated with exclusion of dead cells. B, CD25 gate set to determine CD25^{high} cells (5%), CD25^{int} and CD25^{low} cells in CD4⁺ T-cell populations. C, Forkhead box P3 (FoxP3) staining was measured in CD25^{high} populations using selected gates in which FoxP3 was brightly expressed in the CD25^{high} Treg population (shaded) while it was negative in CD25^{int} and CD25^{low} populations (line). FITC indicates fluorescein isothiocyanate; PerCP, peridinin-chlorophyll proteins.

Table 2. Comparison of Regulatory T cells, Interleukin (IL) 10 and IL-12 in Patients With Warm Autoimmune Hemolytic Anemia and Healthy Controls^a

Parameter	Patients (n=27)	Controls (n=15)	P
CD4 ⁺ CD25 ^{high} FoxP3, %	4.63 (1.0)	9.76 (0.78)	<.001
Basal IL-10, pg/mL	132 (19.1)	18.4 (1.9)	<.001
LPS-stimulated IL-10, pg/mL	834.9 (169)	728 (88)	<.05
Basal IL-12, pg/mL	1.2 (0.3)	0.46 (0.06)	<.01
LPS-stimulated IL-12, pg/mL	1.59 (1.62)	2.4 (0.3)	.26

^aData are expressed as mean (SD) unless otherwise indicated.
^bP<.05, significant; P<.01, highly significant; P<.001, very highly significant.

Table 3. Paired t Test Results for Basal Interleukin (IL) 12 and LPS-stimulated IL-12 Cultures in Patients With Warm Autoimmune Hemolytic Anemia and Healthy Controls^a

Parameter	Mean (SD)	t	P
Controls			
Basal IL-12	0.46 (0.06)	30.9	.001 ^a
LPS-stimulated IL-12	2.4 (0.3)		
Patients			
Basal IL-12	1.2 (0.3)	9.2	.17
LPS-stimulated IL-12	1.59 (1.62)		

Abbreviation: LPS, lipopolysaccharide.
^aVery highly significant.

cells in peripheral blood in patients than in controls (4.63% [1.0%] vs 9.76% [0.78%]; P<.001).

IL-10 and IL-12 are primarily secreted by monocytes, not T cells. We evaluated the levels of these cytokines in RPMI culture medium before and after LPS stimulation (LPS preferentially stimulates monocytes). Before stimulation, PBMCs from patients showed a constitutively increased level of IL-10 compared to controls (132 [19.1] vs 18.4 [1.9], P<.001). With stimulation, the levels increased 6-fold in patients (834.9 [169]) and 70-fold in controls (728 [88]), minimizing the difference between the 2 groups (P<.05) (Table 2). The levels after LPS stimulation were significantly higher than the basal levels in both the patient group (P<.01) and the control group (P<.001) (Table 3).

Table 4. Paired t Test Results for Basal Interleukin (IL) 10 and LPS-stimulated IL-10 Cultures in Patients With Warm Autoimmune Hemolytic Anemia and Healthy Controls^a

Parameter	Mean (SD)	t	P ^a
Controls			
Basal IL-10	18.4 (1.9)	29.4	<.001
LPS-stimulated IL-10	728 (88)		
Patients			
Basal IL-10	132 (19.1)	22.9	<.01
LPS-stimulated IL-10	834.9 (169.7)		

Abbreviation: LPS, lipopolysaccharide.
^aP<.01, highly significant; P<.001, very highly significant.

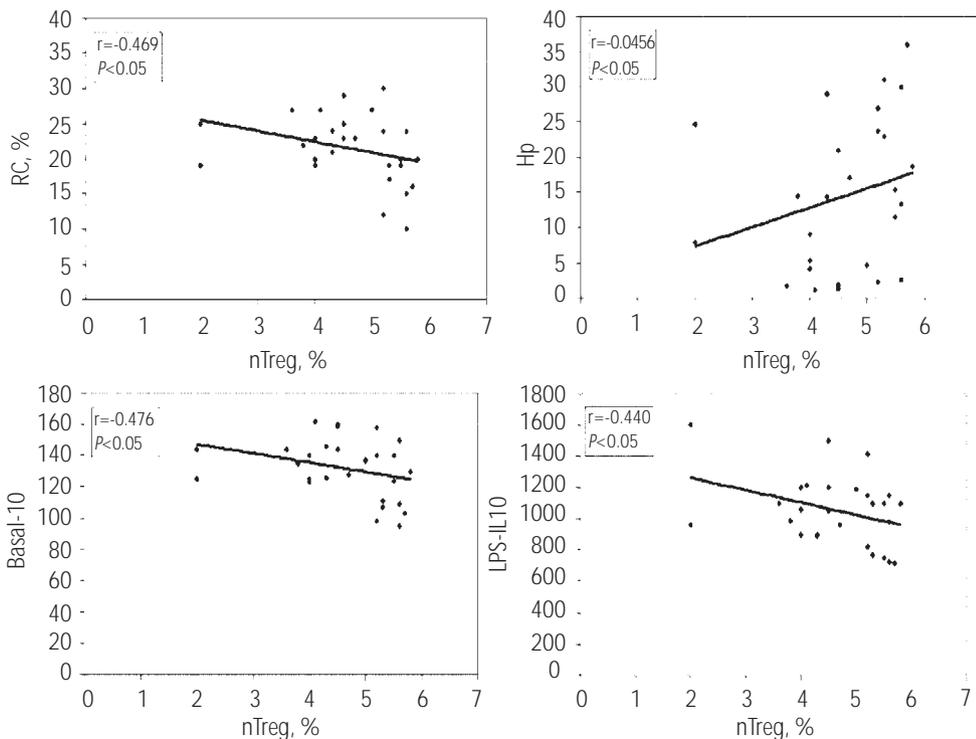


Figure 2. Correlations of the percentage of naturally occurring regulatory T cells (nTregs) in warm autoimmune hemolytic anemia. A, negative correlation between percentage of nTregs and reticulocyte count (RC). B, Positive correlation between percentage of nTregs and haptoglobin (Hp). C,D, Negative correlation between percentage of nTregs with interleukin (IL) 10 in basal and LPS-stimulated peripheral blood mononuclear cells. LPS indicates lipopolysaccharide.

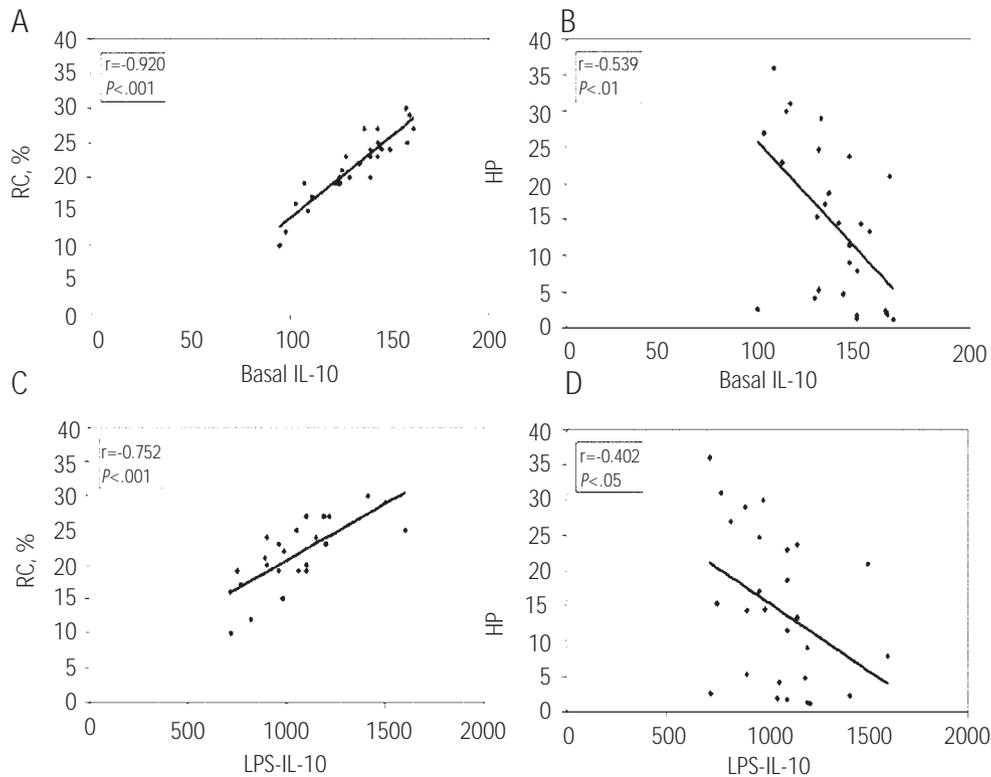


Figure 3. Correlations of interleukin (IL) 10 in basal and LPS-stimulated peripheral blood mononuclear cell (PBMC) cultures in warm autoimmune hemolytic anemia. A, Positive correlation between basal IL-10 and RC. B, Negative correlation between basal IL-10 and haptoglobin (Hp). C, Positive correlation between LPS-stimulated IL-10 and reticulocyte count (RC). D, Negative correlation between LPS-stimulated IL-10 and Hp. LPS indicates lipopolysaccharide.

In unstimulated culture media, IL-12 production was significantly greater in patients (1.2 [0.3]) than in controls (0.46 [0.06]) ($P<.01$) (Table 2). In LPS-stimulated culture media, in contrast, there was no significant difference between patients and controls (1.59 [0.62] vs 2.40 [0.3]) ($P=.26$). The difference in levels before and after LPS stimulation was significant in controls ($P<.001$) but not in patients ($P=.17$) (Table 4).

Correlations

In the group of patients, there was a significantly negative correlation between the percentage of nTregs and RC ($P<.05$) and a significantly positive correlation between the percentage of nTregs and Hp ($P<.05$). The correlation between IL-10 levels (both basal and LPS-stimulated) and the percentage of nTregs was significantly negative ($P<.05$). For RC, the correlations with basal and LPS-stimulated IL-10 were significantly positive ($P<.001$) while for Hp, they were significantly negative ($P<.01$ for basal IL-10 and $P<.05$ for LPS-stimulated IL-10) (Figures 2 and 3). Basal IL-10, LPS-stimulated IL-10, and the percentage of nTregs were not found to be significantly correlated with hemoglobin, serum bilirubin, or LDH levels. No significant correlations were observed between either basal or LPS-stimulated IL-10 and any of the other parameters studied. Finally, in the control group, no statistically significant correlations were observed.

Discussion

nTregs have a potent immunosuppressive function and contribute to immunologic self-tolerance by suppressing potentially autoreactive T cells [14]; although they are considered to play an important role in the pathogenesis of AIHA in animal models, their role in human AIHA remains unexplored.

Our findings showed that patients with wAIHA had a significantly decreased percentage of CD4⁺ nTreg cells in peripheral blood compared to healthy controls, suggesting that these cells have a specific role in the pathogenesis of the disease. It is important to recall that the Tregs in our patients were not diluted with activated CD25⁺ effector T cells. Although it is difficult to rule out this possibility entirely, the approaches taken and the results obtained make it quite unlikely. First, the brightest (CD25^{high}) cells were gated using MFI to exclude activated T cells, which usually have intermediate CD25 expression. Second, only Tregs expressing FoxP3 were analyzed and counted [15,16]. In this regard, Fontenot et al [17] reported that FoxP3 is a unique marker of nTregs, distinguishing them from activated CD4⁺CD25⁺ T cells and playing a pivotal role in their development and maturation. Torgerson [18] also showed that nTregs in the peripheral blood of healthy humans preferentially resided

within the CD4⁺CD25^{high} T-cell population. In our study, nTregs were defined as CD4⁺CD25^{high}Foxp3⁺ cells (flow cytometry enrichment of Foxp3⁺ cells at the transcriptional level has been confirmed by reverse transcription polymerase chain reaction [19]). Our results support findings of previous studies of animal and human autoimmune diseases. Mqadmi et al [20], for example, analyzed the role of nTregs in a murine AIHA model and showed that a depletion of Tregs resulted in an increase in the incidence of disease from 30% to 90% [20]. Additionally, several recent studies of human autoimmune disorders have reported decreased levels of circulating CD4⁺CD25⁺ Tregs in patients with acute and chronic idiopathic thrombocytopenic purpura [21], multiple sclerosis [22], SLE [23], autoimmune liver disease [18], and rheumatoid arthritis [24]. Other studies have identified functional abnormalities in Tregs with regard to their suppressive capacity [25,26]. Yan et al [26], for example, found that Tregs in patients with rheumatic arthritis showed decreased *in vitro* activity in terms of suppressing the production of IFN- γ and TNF- α by CD25⁻ T cells, which may contribute to ongoing inflammation.

Treg-mediated suppression is suspected to be either contact-dependent, driven by soluble factors, or fueled by IL-2 in a passive manner. Contact-dependent suppression involves interaction between cytotoxic T lymphocyte antigen-4 (CTLA-4) or transforming growth factor (TGF) β on Tregs and cognate receptors on the target cell, B7 (CD80/86) and TGF- β RII, respectively [27]. This direct physical interaction may result in the suppression or death of the target cell through granzyme B secretion [28]. Ward et al [29] reported that blockage of CTLA-4 expression by regulatory CD4⁺CD25⁺T cells but not by CD4⁺CD25⁻T cells leads to the development, acceleration, or exacerbation of autoimmune disease in mice. In the passive mechanism, nonregulatory T cells produce IL-2 upon activation, fueling the expansion of Tregs and their acquisition of suppressor function. As the IL-2 receptor alpha chain I (CD25) on the surface of Tregs binds its ligand (IL-2), the cytokine is also sequestered from the nonregulatory T-cell population, meaning that effector T cells are deprived of this essential growth factor [30]. Regardless of the mechanism, the degree of Treg-mediated suppression is dependent on the frequency of CD4⁺CD25⁺ T cells, access to sufficient concentration of antigen presented by antigen-presenting cells, and localization with effector T-cell targets. The cytokine environment required to support Treg growth, maintenance, and activation of suppressor function in the periphery at the time of antigen encounter is also critical [31].

We found a close association between the percentage of nTregs and parameters known to reflect the degree of hemolysis: RC and Hp. These findings raise the possibility that nTregs may regulate disease phenotype, particularly in relation to degree of hemolysis. In this respect, Lin et al [32] found that expression of circulating CD4⁺CD25⁺ Tregs derived from SLE patients inversely correlates with disease activity. Zhang et al [33] also found that the percentage of circulating Tregs in SLE decreased during active disease and that the extent of the decrease correlated with disease severity.

In the present study, IL-10 and IL-12 production was significantly higher in wAIHA patients than in controls prior to stimulation of the culture medium with LPS; this may be

related to the high basal activation state in the patient group. This significant difference was not maintained following LPS stimulation, indicating a state of cell exhaustion. Absence of IL-12 response to LPS stimulation could be also ascribed to the suppressive effect of IL-10 on IL-12 producer cells. Our results are in agreement with those of Toriani-Terenzi et al [34], who reported increased basal synthesis of IL-10 and levels of IL-12 in 55% of AIHA PBMC cultures compared to controls. The authors concluded that the production of autoantibodies in AIHA may be secondary to the imbalance between IL-10 and IL-12.

We also found that IL-10 levels were strongly correlated with RC and Hp. An explanation to this can be found in a study by Liorent et al [35], which reported that the continuous administration of anti IL-10 antibodies delays the onset of autoimmunity of SLE in experimental animals due to elevation of TNF- α ; IL-10 administration, in contrast, was found to accelerate disease progress. Since recombinant IL-10 strongly inhibits the *in vitro* production of IL-12 in PBMC cultures from patients with SLE and healthy controls [36], and since IL-12 inhibits *in vitro* antibody production by lupus PBMCs and downregulates IL-10 production, it has been suggested that IL-10 and IL-12 might form an immunoregulatory circuit that can regulate the development and maintenance of autoimmune diseases [37].

A significant inverse correlation was observed in this study between the percentage of CD4⁺ nTregs and IL-10 levels in both basal and LPS-stimulated PBMC cultures. This could be attributed to paucity in the suppressive potential of nTregs in terms of cytokine production owing to their reduced number. Ward et al [29] described autoantigen-specific, IL-10-secreting Treg clones recovered *ex vivo* from a patient with AIHA. They found that Treg levels decreased markedly with predominant secretion of IL-10 and little IFN- γ or IL-4 when the cells were specifically activated with peptide or Rh protein. Franzle et al [38] reported that Tregs are able to inhibit T-cell proliferation and cytokine production and play critical roles in preventing autoimmunity in different autoimmune diseases [38]. It has also been postulated that cytokine production is closely linked and inversely correlated to FoxP3 expression on nTregs [39].

To conclude, although there may be multiple factors that contribute to the induction of AIHA, our data indicate that a reduced percentage of nTreg cells and IL-10/IL-12 imbalance may play an essential role in the onset and/or maintenance of this autoimmune disease. Of the various kinds of Tregs, nTregs have been found to play an important role in autoimmune diseases. Further investigations into AIHA syndrome hold the promise of identifying new molecular defects that will further elucidate basic mechanisms of self-tolerance and provide new targets for therapy.

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