Influence of Functional Interleukin 10/Tumor Necrosis Factor-α Polymorphisms on Interferon-α, IL-10, and Regulatory T Cell Population in Patients with Systemic Lupus Erythematosus Receiving Antimalarial Treatment

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ABSTRACT. ***Objective.*** As interleukin 10 (IL-10)/tumor necrosis factor-α (TNF-α) polymorphisms have been shown to influence TNF-α inhibition and clinical response to antimalarial treatment in patients with systemic lupus erythematosus (SLE), we investigated involvement of these variants in antimalarial effects on cytokine serum levels and regulatory T cell population (Treg).

Methods. The alleles present at –308 TNF-α and –1082 IL-10 genes; serum concentrations of interferon-α (IFN-α), IL-10 and TNF-α; and size and function of CD4+CD25^{high} (Treg) population were determined in SLE patients and in healthy controls. These data were related to treatment and clinical manifestations.

Results. Patients were observed to have increased IFN-α serum levels that did not correlate with any treatment. Among patients receiving antimalarial drugs, high IL-10/low TNF-α producers presented higher levels of IFN-α and IL-10 than carriers of other genotypes. In contrast, patients with the converse, low IL-10/high TNF-α genotype who were receiving antimalarial treatment presented increased size and function of Treg population. The percentage of CD4+CD25^{high} cells was inversely correlated to TNF-α levels.

Conclusion. Our findings suggest that the beneficial effect of antimalarials in low IL-10/high TNF-α patients with SLE may be partially attributable to the increase in Treg activity, whereas patients with the converse genotype did not show this phenomenon, yet did have significantly upregulated levels of IFN-α and IL-10, 2 cytokines that have been associated with SLE activity. (First Release June 15 2008; J Rheumatol 2008;35:1559–66)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS ANTIMALARIALS CYTOKINES
SINGLE-NUCLEOTIDE POLYMORPHISMS REGULATORY T LYMPHOCYTES

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a disorder of immune regulation, resulting in chronic inflammation that affects many organs. Several cytokines, including interferon-α (IFN-α), tumor necrosis factor-α (TNF-α) and interleukin 10 (IL-10), have long been considered essential elements in the etiopathology of the disease. Since the earliest reported presence of IFN-α activity in the serum of patients with SLE[1], further findings have confirmed the central role played by this cytokine in the disease. Studies have demonstrated the induction of autoantibodies and autoimmune diseases during IFN-α therapy[2,3]. Recently, a high level of expression of IFN-α-inducible genes has been reported in patients with SLE[4-6]. Further, the existence of circulating inducers of IFN-α in SLE patients, acting specifically on plasmacytoid dendritic cells (pDC), the natural IFN-α-producing cells, supports a pathogenic role for IFN-α in SLE[7-9].

Although antimalarial drugs (hydroxychloroquine, chloroquine, and quinacrine) have been widely used as disease modifying antirheumatic agents in the treatment of several autoimmune diseases, their antiinflammatory mechanisms have not been fully defined. Antimalarial agents are thought to reduce autoantigen presentation by inhibition of endolysosomic acidic proteases. However, it has also been

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shown that chloroquine inhibits the production of proinflammatory cytokines after *in vitro* stimulation of human monocytes[10,11]. This interesting antiinflammatory effect has recently been documented in patients with SLE, in whom antimalarial treatment has been shown to downregulate serum levels of TNF-α, IL-6, and IL-18[12,13].

The production of IL-10 and TNF-α, 2 mutually-regulated cytokines that have complex and predominantly opposing roles in systemic inflammatory responses, has also been found to be elevated in SLE patients, suggesting that these proteins may be involved in the pathogenesis of the disease[14,15]. Genetic polymorphisms at the promoter of both genes have been linked to different cytokine production. Thus, homozygotes for the –1082G* allele at the IL-10 gene and the presence of the rare –308A* allele at the TNF-α gene have been associated with the highest cytokine production[16,17]. In addition, high TNF-α genotype has been shown to be linked to SLE susceptibility[18,19]. Moreover, we have reported that carriage of the low IL-10/high TNF-α genotype is associated with good clinical response to antimalarial treatment and with the greatest decrease in serum levels of TNF-α[12], suggesting that this therapy may only be completely effective for patients with a particular genotype. Although these results appear to indicate that clinical response to antimalarial drugs could be mediated by TNF-α inhibition, nothing is known about the possible modulation of other cytokines or variables affecting SLE pathology. Thus, an increase in the population of regulatory T cells should be a desirable effect for treatments used in autoimmune diseases[20]. Naturally-arising regulatory T lymphocytes (Treg) are a population of suppressor CD4+ cells that maintain peripheral immune tolerance by inhibiting the activation and expansion of self-reactive T cells. A constitutive high-level expression of the IL-2 receptor-α chain (CD25) and expression of the transcription factor Foxp3 define the subset of CD4+CD25^{high} T cells[21,22]. It has recently been reported that treatment with anti-TNF-α antibodies restored the suppressive function of Treg cells[23] in patients with rheumatoid arthritis (RA). However, the effect of antimalarial-mediated TNF-α inhibition has not been investigated.

We assessed the involvement of combined IL-10/TNF-α functional polymorphisms in the ability of antimalarial drugs to regulate the levels of IFN-α and IL-10 and to influence the size and function of the Treg population in patients with SLE.

MATERIALS AND METHODS

Patients. Approval for this study was obtained from the Regional Ethics Committee for Clinical Investigation. All patients included in the study (n = 208) were on the Asturian SLE Register[24,25], were all Caucasian in origin, and fulfilled the American College of Rheumatology (ACR) criteria for SLE[26]. Information on clinical manifestations and treatments during the disease course was obtained after a detailed review of clinical histories. At the time of sampling patients were asked precise questions regarding the treatment received over the previous 3 months. Sex and age-matched healthy controls (n = 215 for cytokine quantification and n = 45 for Treg analysis) were enlisted from the Asturian Blood Transfusion Center. Consent was obtained from all individuals prior to participation in the study.

Cytokine quantification. Serum samples for IFN-α determination were collected from 172 SLE patients and 109 controls; concentrations were determined by ELISA (PBL Biomedical, Piscataway, NJ, USA)[27] following the manufacturer's instructions. Serum levels of IL-10 were determined by a commercial ELISA (BioSource Europe, Nivelles, Belgium) in 157 SLE patients. Quantification of serum TNF-α in 171 SLE patients and 215 controls was carried out using an in-house ELISA[12]. The lower limit of detection was 3.12 pg/ml for IFN-α, 0.2 pg/ml for IL-10, and 7.5 pg/ml for TNF-α.

Promoter polymorphism genotyping. DNA was obtained from peripheral blood cells of 208 SLE patients by standard procedures. Single-nucleotide polymorphisms (SNP) at positions –1082 on the IL-10 gene and –308 on the TNF-α gene were determined after amplification and hybridization with fluorescent-labeled probes (LightCycler, Roche Diagnostics, Mannheim, Germany), as described[15,18]. The primers were 5'-ATC CAA GAC AAC ACT ACT AAG GC and 5'-ATG GGG TGG AAG AAG TTG AA for –1082 IL-10 and 5'-CCT GCA TCC TGT CTG GAA GTT A and 5'-CTG CAC CTT CTG TCT CGG TTT for –308 TNF-α. The hybridization probes (designed by TIB Molbiol, Berlin, Germany) were GGA TAG GAG GTC CCT TAC TTT CCT CTT ACC-F and LC Red 640-CCC TAC TTC CCC CTC CCA AA for –1082 IL-10 and AAC CCC GTC CCC ATG CCC C-F and LC Red 640-CCA AAC CTA TTG CCT CCA TTT CTT TTG GGG AC for –308 TNF-α.

Treg quantification. Whole-blood cells were stained with anti-CD4 peridinchlorophyl protein (PerCP) and anti-CD25 fluorescein isothiocyanate (FITC) or their respective isotype and fluorochrome-matched control antibodies (BD-Pharmingen, San Diego, CA, USA). The lymphocyte population was gated according to forward and side-scatter properties, and CD4+ cells were gated using anti-CD4 PerCP antibodies. Isotype and fluorochrome-matched controls were used to set up quadrants. CD4+CD25+ T cells were subdivided into CD25^{low} and CD25^{high} populations, as described[28]. The percentage of CD25^{high} cells in the total CD4+ lymphocyte population was determined in 93 SLE patients and 34 controls after acquisition of 10,000 CD4+ lymphocytes. Analyses were performed on a FACScan flow cytometer using CellQuest software (BD-Pharmingen).

mRNA isolation and quantification. Sample mRNA (poli-A+) was isolated from whole blood in 56 SLE patients using the mRNA isolation kit for blood/bone marrow (Boehringer Mannheim, Indianapolis, IN, USA). Reverse transcription was carried out by standard procedures. Real-time reverse transcription-polymerase chain reaction (RT-PCR; LightCycler) was used to quantify Foxp3 mRNA, monitoring the fluorescence emitted by SYBR-Green I dye using an external standard to generate a calibration curve (T cells stimulated for 48 h with platebound anti-CD3/anti-CD28 plus 500 U/ml IL-2). Since glucocorticoids and other treatments could modify the size of granulocyte or lymphocyte populations, quantification of the CD3 epsilon chain was used to normalize Foxp3 expression leading to the determination of Foxp3/CD3 mRNA relative units. The primers used were 5'-GAA ACA GCA CTA TCC CAG AGT TC-3' and 5'-ATG GCC CAG CGG ATG AG-3' for Foxp3 and 5'-CGT TCA GTT CCC TCC TTT TCT T-3' and 5'-GAT TAG GGG GTT GGT AGG GAG TG-3' for CD3.

Functional characteristics of Treg cells. For assessment of regulatory properties, 5×10^4 CD4+CD25– responder T cells were stimulated with allogenic monocyte-derived dendritic cells in 96-well U-bottom plates, and purified autologous CD4+CD25+ T cells (CD4+CD25+ regulatory T cell isolation kit; Milteny) were added at a 2:1 ratio. After 4 days of culture in RPMI-1640 supplemented with 10% fetal calf serum, 50 µl of supernatant were removed from each well and used for TNF-α quantification, adding 1 µCi/well of ^{3}[H]-thymidine for an additional 16 h. ^{3}H-thymidine incorporation was measured using a liquid scintillation counter. All cultures were performed in triplicate. Immature dendritic cells were generated from CD14+ monocytes isolated from peripheral blood mononuclear cells (PBMC) by magnetic bead-based purification (Miltenyi) and cultured 7

days in medium at 1×10^6 cells/ml supplemented with granulocyte macrophage-colony stimulating factor (70 ng/ml) and IL-4 (35 ng/ml). Dendritic cells were matured for 2 days with TNF-α (50 ng/ml).

Statistical analysis. As cytokine serum levels, the percentage of CD4+CD25^{high} T lymphocytes, and Foxp3/CD3 mRNA relative units were not distributed normally, nonparametric testing was used throughout (Mann-Whitney U-test or Kruskal-Wallis test). Correlations between cytokine concentrations, CD4+CD25^{high} cells, and clinical variables were performed using Spearman's rank correlation test. Cytokine values and percentage of CD4+CD25^{high} cells were described by median and interquartile range. SPSS 14.0 statistical software was used for all calculations.

RESULTS

Increased concentrations of IFN-α in serum of SLE patients did not correlate with treatment. Quantification of IFN-α levels in the serum of 172 SLE patients and 109 controls (Table 1) showed a significantly higher amount of this cytokine in the entire patient population compared to controls. However, no significant differences were detected among patients receiving different treatments. To assess the possible relationship between amounts of IFN-α and levels of IL-10 or TNF-α (2 cytokines usually related to SLE activity), we determined serum levels of TNF-α in 215 controls and 171 patients and IL-10 serum levels in 157 patients. The immunoassay used for IL-10 quantification was unable to detect this cytokine in most healthy controls. As previously reported, serum levels of TNF-α were elevated in the entire SLE patient group, although significantly higher levels, compared to controls, were found only in untreated patients and those not receiving antimalarial treatment[12]. Interestingly, a positive correlation between TNF-α and IFN-α was observed in all individuals (n = 273; r = 0.275, p = 0.000004, Spearman's rank correlation test) to a similar degree, both in healthy controls (r = 0.281, p = 0.003) and in SLE patients (r = 0.225, p = 0.004). IL-10 serum levels, on the other hand, did not seem to be significantly influenced by treatments, although a positive correlation with IFN-α was observed in patients receiving antimalarial drugs (n = 93; r = 0.237, p = 0.023).

IL-10/TNF-α genotype influences serum levels of IFN-α and IL-10 in SLE patients receiving antimalarial treatment. It has been shown that antimalarial treatment is particularly effective in SLE patients carrying the low IL-10/high TNF-α genotype. This has been attributed to the greater down-regulation of TNF-α levels in these patients[12], although no other cytokine has been tested. We therefore wished to assess the possible effect of the IL-10/TNF-α genotype on the levels of IFN-α and IL-10 in patients receiving antimalarial treatment. After determination of the alleles present at the –1082 IL-10 and –308 TNF-α genes, SLE patients were classified as genetically high (AA/AG) or low (GG) TNF-α producers and high (GG/AG) or low (AA) IL-10 producers, as well as in the 4 combined IL-10/TNF-α genotypes, as previously established[19,29]. No significant differences were detected in the levels of IFN-α between users and nonusers of antimalarial drugs, whereas an increase in IL-10 levels was observed in high IL-10/low TNF-α patients receiving this treatment (Table 2). Additionally, among patients receiving antimalarials, carriers of a high IL-10/low TNF-α genotype had the highest amounts of both IFN-α and IL-10, and this finding was statistically significant compared with the rest of the genotypes (Table 2). In contrast, however, no differences in cytokine levels were observed among patients not receiving the treatment. To exclude the possible influence of genotype on disease variables, we compared demographic and clinical characteristics of high IL-10/low TNF-α patients with carriers of other genotypes. Table 3 shows that patients did not differ significantly, sug-

Table 1. Serum levels of IFN-α, TNF-α, and IL-10 in healthy controls and SLE patients.

	IFN-α			TNF-α			IL-10		
	n	pg/ml	p	n	pg/ml	p	n	pg/ml	p
Controls	109	9.96 (16.50)		215	19.66 (53.93)			ND	
			< 0.0001			0.020			
SLE patients	172	18.64 (51.01)		171	33.57 (132.55)		157	0.50 (2.74)	
Patient treatment									
None/NSAID	21	15.58 (20.99)		21	60.78 (158.15)		21	0.20 (2.14)	
Antimalarials	36	18.33 (51.52)		36	16.64 (165.57)		34	0.59 (2.96)	
Antimalarials and CS	57	21.64 (87.07)		54	24.95 (88.49)		49	0.95 (2.69)	
			0.534			0.136			0.115
CS	25	27.26 (101.55)		26	60.01 (145.67)		23	0.20 (2.45)	
Immunosuppressive drugs* alone or with CS	22	16.04 (30.66)		22	105.34 (195.29)		19	0.50 (2.94)	
Antimalarials, CS, and immunosuppressive drugs*	11	15.50 (31.14)		12	16.89 (50.70)		11	2.74 (0.49)	

Values are median (interquartile range). Differences evaluated by Mann-Whitney or Kruskal-Wallis nonparametric testing. * Methotrexate, azathioprine, cyclophosphamide, cyclosporine A, or mycophenolate mofetil. NSAID: nonsteroidal antiinflammatory drugs; CS: corticosteroids.

Table 2. Serum levels of IFN-α and IL-10 in SLE patients with different IL-10/TNF-α genotypes, both users and nonusers of antimalarial drugs.

	IFN-α					IL-10				
	High IL-10 Low TNF-α		Other Genotype		p	High IL-10 Low TNF-α		Other Genotype		p
Antimalarial Treatment	n	pg/ml	n	pg/ml		n	pg/ml	n	pg/ml	
Users	16	57.64 (232.59)	88	15.15 (54.54)	0.010	13	3.04 (1.79)	81	0.50 (2.69)	0.035
Nonusers	8	28.59 (256.34)	60	17.39 (36.91)	0.641	6	0.20 (0.83)	57	0.23 (2.59)	0.234
p		0.298		0.950			0.046		0.486	

Values are median (interquartile range). Differences evaluated by Mann-Whitney U-test.

Table 3. Demographic characteristics and disease variables of SLE patient carriers of the high IL-10/low TNF-α genotype compared with other genotypes.

Combined Genotype	High IL-10 Low TNF-α, n = 27	Other Genotype, n = 181
Female/male	26/1	167/14
Age at diagnosis, yrs, mean ± SD	35.70 ± 12.05	33.52 ± 14.56
Disease duration, yrs, mean ± SD	14.88 ± 8.54	12.82 ± 7.72
Clinical features, n (%)		
Malar rash	15 (55.6)	100 (55.2)
Cutaneous lesions	21 (77.8)	123 (68.0)
Photosensitivity	18 (66.7)	93 (51.4)
Oral ulcers	9 (33.3)	63 (34.8)
Arthritis	20 (74.1)	130 (71.8)
Serositis	8 (29.6)	43 (23.8)
Renal disorder	7 (25.9)	53 (29.3)
Neurological disorder	2 (7.4)	13 (7.2)
Hematological disorder	10 (40.0)	110 (60.8)
Presence of anti-dsDNA antibodies	21 (77.8)	123 (68.0)

gesting that differences in cytokine levels are due to antimalarial treatment.

All these results indicate that in those receiving antimalarial treatment, the highest levels of IFN-α and IL-10 were exhibited by high IL-10/low TNF-α producer patients, the genotype contrary to the one that responds best to these agents.

IL-10/TNF-α genotype influences Treg population in SLE patients treated with antimalarials. Since the use of antimalarial agents in SLE patients reduces levels of TNF-α, a cytokine associated with inhibition of the Treg function[23], we investigated the possible effect of this treatment on the population of Treg cells. We thus determined the percentage of $CD25^{high}$ cells among CD4+ T lymphocytes in 34 healthy controls and 108 SLE patients, who were also analyzed for serum cytokine levels and IL-10/TNF-α polymorphisms. Additionally, Foxp3 mRNA expression was quantified in 56 SLE patients by real-time PCR. Interestingly, the percentage of $CD4+CD25^{high}$ cells in SLE patients correlated negatively with the amount of circulating TNF-α ($r = –0.247$, $p = 0.017$), although no significant relationships were detected with IFN-α or IL-10. Stratification of patients by treatment (Table 4) showed that patients receiving antimalarial therapy did not have a significantly higher percentage of $CD4+CD25^{high}$ cells than healthy controls or nontreated

Table 4. Relation between percentage of $CD4+CD25^{high}$ T cells and patient therapy.

	n	$CD4+CD25^{high}$, %	p
Healthy controls	34	5.43 (3.28)	
Patient treatment			
None/NSAID	20	5.58 (4.12)	0.979
Antimalarials alone	27	6.52 (3.96)	0.135
Glucocorticoids alone	15	8.25 (6.17)	0.027
Glucorticoids and antimalarials	30	7.77 (6.00)	0.035
Immunosuppressors* alone or with other treatment	16	6.09 (7.84)	0.240

Values are median percentage (interquartile range). Differences between each patient group and controls were evaluated by Mann-Whitney U-test. * Methotrexate, azathioprine, cyclophosphamide, cyclosporine A, or mycophenolate mofetil. NSAID: nonsteroidal antiinflammatory drugs.

patients, although an increased percentage was observed in glucocorticoid-treated patients, as reported[28]. Thus, quantification of the Treg marker Foxp3 did not show a significant increase in patients receiving antimalarial drugs, but confirmed corticosteroid-mediated upregulation of Treg (Figure 1). However, analysis of the IL-10/TNF-α genotype suggested a differential effect of antimalarial treatment. As shown in Table 5, patients receiving antimalarials who were low IL-10/high TNF-α producers had an increased percentage of CD4+CD25^{high} cells, significantly higher than those with another genotype. No differences were observed among nonusers of antimalarials.

Finally, we assessed the influence of genotype on Treg function in patients receiving antimalarial treatment. CD4+CD25+ cells isolated from healthy controls (n = 11) and SLE patients receiving antimalarial treatment who were carriers of low IL-10/high TNF-α (n = 5) or low IL-10/low TNF-α (n = 5) genotypes were co-cultured with stimulated autologous CD4+CD25– cells at a ratio of 1:2, and Treg function was assessed by determining the ability of CD25+ cells to inhibit both the proliferation (Figure 2A) and the production of TNF-α (Figure 2B). Results showed that Treg cells from low IL-10/high TNF-α patients treated with antimalarials had a greater ability to suppress proliferation than CD4+CD25+ cells isolated from healthy controls or from low IL-10/low TNF-α patients receiving the same treatment. Similarly, the percentage of inhibition of TNF-α production was diminished in low IL-10/low TNF-α patients, although not in low IL-10/high TNF-α patients, as compared to controls. Thus, all these results indicate that the IL-10/TNF-α genotype influences the size and activity of Treg cells in SLE patients receiving antimalarial treatment, suggesting

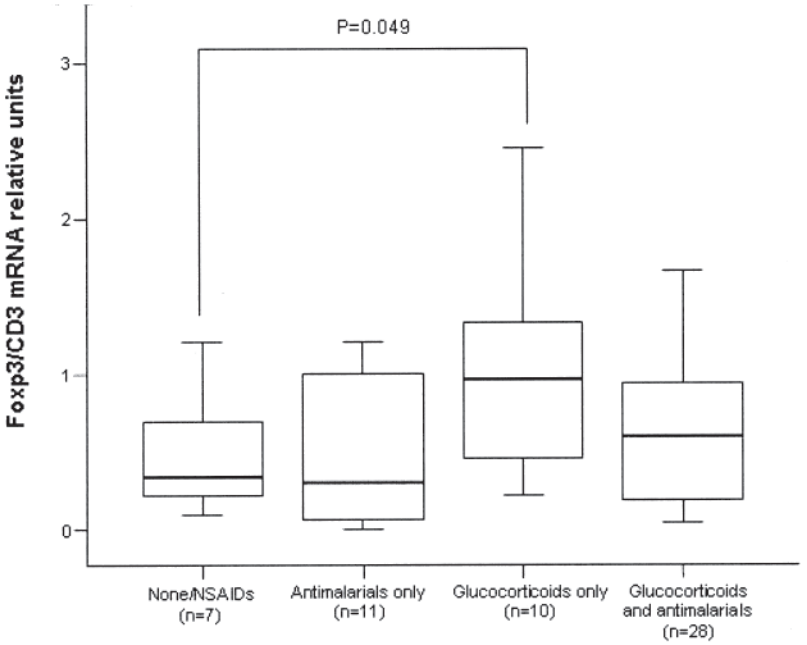

Figure 1. Foxp3 mRNA expression in SLE patients receiving different treatments. Whole-blood samples were collected from 56 unselected SLE patients and mRNA (poly-A+) was extracted. After cDNA synthesis, Foxp3 expression was quantified by RT-PCR and normalized to CD3 gene. Box plots represent Foxp3/CD3 relative units of SLE patients classified by treatment over the 3 months prior to sampling. Differences evaluated by Mann-Whitney U-test.

Table 5. Percentage of Treg cells in SLE patients with different IL-10/TNF-α genotypes, both users and nonusers of antimalarial treatment.

Group	Low IL-10/High TNF-α n	Low IL-10/High TNF-α CD4+CD25^{high}, %	Other Genotypes n	Other Genotypes CD4+CD25^{high}, %	p
SLE patients receiving antimalarial treatment (alone or in combination)					
Users	27	8.12 (6.37)	33	6.15 (4.63)	0.026
Nonusers	14	6.60 (3.82)	34	6.43 (5.57)	0.973
Healthy controls	8	6.65 (3.99)	26	5.41 (3.33)	0.236

Values are median percentage (interquartile range). Differences evaluated by the Mann-Whitney U-test.

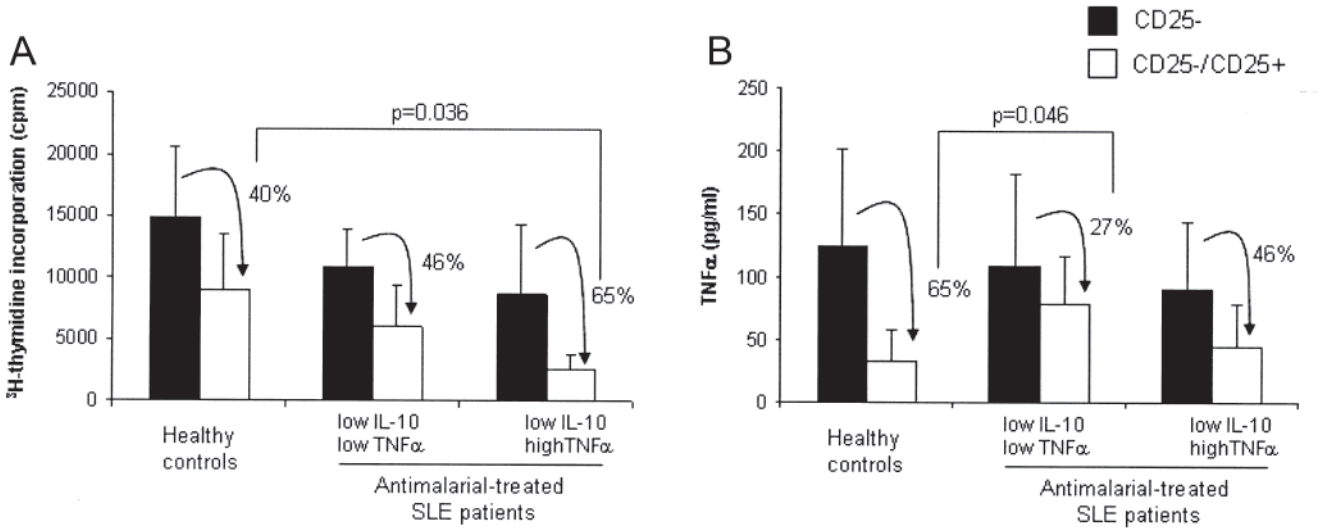

Figure 2. Treg function in SLE patients receiving antimalarials who were carriers of a different genotype. CD4+CD25– responder cells (5×10^4/well) were cultured with allogenic monocyte-derived dendritic cells either alone or with autologous CD4+CD25+ Treg cells at a 2:1 ratio. After 5 days, ^{3}H-thymidine incorporation was determined (A). Culture supernatants were analyzed to determine the amount of TNF-α (B). Bars represent mean ± SD. Mean values of percentage of inhibition and significant differences between healthy controls (n = 11) and patient groups (n = 5 in each group) evaluated by Mann-Whitney U-test are also shown.

that the clinical effect of these agents could be partly mediated by an increase in the Treg function.

DISCUSSION

Our study confirms the previously reported high expression of IFN-α in patients with SLE and shows for the first time the influence of the functional IL-10/TNF-α genotype on concentrations of this cytokine in patients receiving antimalarial treatment. Since the report that prolonged exposure to IFN-α therapy frequently resulted in development of autoimmunity[2,3], studies have supported the role of this cytokine in the etiopathogenesis of SLE[4,5,30], probably a consequence of the numerous immunostimulatory effects attributed to IFN-α. Indeed, the notable positive correlation with levels of TNF-α in our study is consistent with the elevated amounts of both cytokines reported in SLE patients with active disease[4-6,14,15], and supports the role of IFN-α in promoting inflammation. It has accordingly been demonstrated that IFN-α is able to induce phosphorylation of STAT4 in monocytes, macrophages, dendritic cells, and T lymphocytes, promoting cell-mediated immunity and Th1 differentiation[31,32].

Antimalarial drugs have been widely used as disease modifying antirheumatic agents in treatment of SLE and other autoimmune diseases. However, their antiinflammatory mechanisms are not fully understood. It has been shown that antimalarial therapy downregulates the levels of TNF-α in patients with SLE[12,13], suggesting that the beneficial effect of this treatment could be mediated by reduction of proinflammatory cytokines. Curiously, the low clinical effect of antimalarial treatment observed in SLE patients who smoke could be explained by the increase in TNF-α due to cigarette smoking[33]. *In vitro* studies have shown inhibition of the release of IL-1, IL-6, and TNF-α in monocytes activated with lipopolysaccharide (LPS) or CpG oligonucleotides[10,11,34], which could be mediated by interfering with LPS-induced TNF-α gene expression via a non-lysosomotropic mechanism[34] and/or by blocking the interaction between TLR9 and CpG in monocyte endosomes[35]. Indeed, it has been suggested that antimalarials, acting as TLR7/9 antagonists[36,37], may prevent apoptotic debris from stimulating and sustaining autoimmunity[38]. However, apart from inhibition of TNF-α, little is known about the immunomodulatory effects of antimalarial treatment *in vivo*.

The increased levels of IL-10 observed in a subset of our SLE patients receiving antimalarial treatment were in accord with *in vitro* studies showing that exposure of PBMC to chloroquine increased the number of IL-10-producing cells[39]. On the other hand, although this is the first report indicating that the use of antimalarial therapy could imply an increase in IFN-α levels, this result should not be surprising. *In vitro* experiments have shown that neutralization of endogenous TNF-α sustains IFN-α release by pDC[40]. Moreover, patients with systemic-onset juvenile idiopathic arthritis have displayed increased transcription of IFN-α-regulated genes after anti-TNF-α therapy[40], suggesting that the increased autoimmunity and autoantibody production observed in a fraction of patients undergoing therapy with TNF-α antagonists[41,42] could be mediated by an increase in IFN-α production.

We reported that patients with SLE and the low IL-10/high TNF-α genotype receiving antimalarial treatment had lower levels of TNF-α and improved clinical response compared to patients with another genotype[12]. Our present

results suggest that the diminished response observed in patients with the high IL-10/low TNF-α genotype could be partially mediated by the upregulation of IFN-α and IL-10 levels. In SLE patients, overproduction of IL-10 has usually correlated with disease activity and it has been assumed that elevated production of this cytokine might be capable of promoting generation of anti-dsDNA antibodies[15,43]. Similarly, expression of IFN-α-inducible genes was correlated with clinical disease activity and severity[6,44,45], and recent reports suggest that the continuing IFN-α production observed in SLE patients with active disease is related to the presence of DNA-anti-DNA immune complexes, endogenous IFN-α-inducers acting specifically on pDC, the main source of this cytokine[7-9,46]. We hypothesize that the high levels of IL-10 produced by high IL-10/low TNF-α patients receiving antimalarial treatment could lead to generation of antibodies against dsDNA, which would, in turn, form immune complexes able to stimulate pDC to secrete IFN-α. Since it has been shown that IFN-α increases IL-10 production[47], this cycle cannot be interrupted.

Another interesting finding was the increase in Treg size and function observed in SLE patients with the low IL-10/high TNF-α genotype receiving antimalarials. This valuable effect has not previously been reported for antimalarial drugs, although it has been reported, yet again, after treatment with TNF-α blockers. CD4+CD25^{high} Treg cells from patients with RA increased Foxp3 mRNA and protein expression and restored their suppressive activity after anti-TNF-α therapy[23,48,49]. All these data suggest an improvement of Treg function related to downregulation of TNF-α. Indeed, we found a significant negative correlation between TNF-α levels and the percentage of CD4+CD25^{high} cells, supporting this novel action of TNF-α in modulating autoimmunity by inhibiting Treg activity. Thus, the increase in Treg activity, probably mediated by downmodulation of TNF-α, seems to be involved in the clinical response to antimalarial drugs observed in patients with the low IL-10/high TNF-α genotype, i.e., patients with the lowest TNF-α levels after antimalarial treatment.

Our results support the influence of IL-10 and TNF-α genotypes on the immunomodulatory effects of antimalarial treatment in patients with SLE. The advantage of this therapy for genetically low IL-10/high TNF-α patients may be partially attributable to the increase in Treg activity, whereas patients with the converse, high IL-10/low TNF-α genotype not only did not present this phenomenon but, in addition, showed significantly upregulated levels of IFN-α and IL-10, 2 cytokines that have been associated with SLE activity. However, these trials are continuing and more data are being gathered to confirm our report.

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