



Effect of Interferon Alfa-2a on Peripheral Blood CD4+CD25+ T Regulatory Cells in Patients with Behçet Uveitis: Preliminary Study

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Abstract

Purpose: To evaluate the phenotypical and functional effect of interferon alfa-2a therapy on peripheral blood CD4+CD25+ T regulatory (Treg) cells in patients with Behçet uveitis.

Methods: Blood was taken from 5 patients with refractory Behçetpanuveitis and 5 age-matched healthy controls. Flow cytometric analysis of CD4+CD25+Treg cells was performed. CD4+CD25+Treg cells were separated by magnetic-assisted cell sorting and co-cultured. Cytokine levels in the supernatants were determined by ELISA.

Results: The percentage of CD4+CD25+Foxp3+Treg cells and the intensity of Foxp3 expression of CD4+CD25+Treg cells were slightly elevated in patients when compared to controls. Interferon alfa-2a led to a borderline significant decline of CD4+CD25+Foxp3+Treg cells and elevation of interleukin-10 ($p=0.06$).

Conclusions: Interferon alfa-2a therapy might lead to a decline in the dysfunctional CD4+CD25+Foxp3+Treg cell population. Interleukin-10 may play a major role in IFN α -2a mediated control of Behçet uveitis.

Keywords

Behçet uveitis, Interferon alfa-2a, CD4+CD25+ T regulatory cells

Introduction

Uveitis associated with Behcet Disease (BD) is characterized by bilateral intraocular inflammation mostly affecting the posterior segment of the eye as retinal vasculitis and has a relapsing remitting course [1]. Interferon alfa-2a is a cytokine proven to be effective in controlling refractory and sight-threatening uveitis in patients with BD [2-12]. However, the exact mechanism by which interferon alfa-2a controls the intraocular inflammation in patients with BD remains to be elucidated. CD4+CD25+ regulatory T (Treg) cells are characterized by their immunoregulatory ability to inhibit the

development of certain autoimmune diseases in animal models [13,14]. It has been reported that approximately 5% to 10% of the human CD4+ T-cell subpopulation from peripheral blood expresses CD25 and that of these cells only 1% to 2% express high levels of CD25. These CD4+CD25 high cells have regulatory properties and are designated CD4+CD25 high regulatory T (Treg) cells [15]. Forkhead box p3 (Foxp3) has been a reliable marker for CD4+CD25+Treg cells and is critical for maintaining immune tolerance and preventing autoimmune diseases [16,17]. Either reduced frequency or impaired function of CD4+CD25+Treg cells has been reported in patients with a number of autoimmune diseases, including multiple sclerosis, psoriasis, systemic lupus erythematosus, and rheumatoid arthritis [18-21]. The possible role of CD4+CD25+Treg cells in regulating active inflammation in BD has been emphasized in previous studies [22,23]. One study also pointed on the predictive value of decreased percentage of CD4+CD25+Treg cells as a marker of uveitis flare up in BD patients [24]. The effect of interferon alfa-2a on lymphocyte subpopulations and monocytes has also been studied in patients with BD [25,26]. Our study aimed to specifically focus on the effect of interferon alfa-2a therapy on CD4+CD25+Treg cells; with known immunoregulatory function(s), in patients with Behçet uveitis [27].

Material and Methods

Patients

This prospective study was conducted from January 1, 2009, to December 30, 2009. A total of five patients with Behcetpanuveitis refractory to conventional immunosuppressive therapy were included in the study. Diagnosis of BD was based on the criteria established by the International Study Group for Behcet's Disease [28]. In our uveitis routine biologics are used as second-line therapy and BD patients with uveitis involving the posterior segment are started on conventional immunosuppressive agent(s) upon diagnosis. Because Behçet uveitis patients are initially treated with conventional immunosuppressive agents blood samples were taken thrice: (1)

Citation: Koc A, Onal S, Tulunay A, Kazokoglu H, Demiralp EE, et al. (2015) Effect of Interferon Alfa-2a on Peripheral Blood CD4+CD25+ T Regulatory Cells in Patients with Behçet Uveitis: Preliminary Study. Int J Ophthalmol Clin Res 2:012

Received: January 08, 2015: **Accepted:** February 11, 2015: **Published:** February 13, 2015

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before initiation of any systemic therapy while the patients had active Behcet uveitis with posterior segment involvement (sample 1), (2) at the termination of conventional immunosuppressive agent due to unresponsiveness of uveitis/before the initiation of interferon alfa-2a (sample 2), and (3) while Behcet uveitis was inactive on interferon alfa-2a therapy (sample 3). All five patients had panuveitis that was refractory to azathioprine. Interferon alfa-2a (Roferon-A; Roche Pharmaceuticals, Whitehouse Station, New Jersey) was administered based on a low-dose and dose escalating treatment protocol described previously by us [10-12]. Based on this protocol interferon alfa-2a is given 3 million international-units (IU) subcutaneously (sc) daily for 14 days for induction. Maintenance is achieved with interferon alfa-2a given as 3 million IU 3x/week sc. The dose is increased sequentially to 4,5,6 and 9 million IU 3x/week, if uveitis relapses occur. Inactive anterior uveitis was defined as 0.5 cells or less [29]. Vitritis, evidenced by the presence of cells, not haze, was graded from 0 to 4 and considered inactive when there were 0.5 cells or less [30]. Inflammation of the posterior segment was documented by the presence of retinal vasculitis, retinitis, cystoid macular edema, and papillitis. Inactivity of uveitis while on maintenance therapy of IFN α -2a was defined as inactivity of anterior chamber and vitreous inflammation along with absence of posterior segment intraocular inflammatory signs [29,30]. In case the uveitis specialist decided that uveitis was inactive, a fluorescein angiography was also performed. Absence of any pathologic leakage on fluorescein angiography was also set as a criteria in order to collect the third blood sample from the patients. High-dose systemic corticosteroids were used to control uveitis flare up at presentation and during follow-up. Blood samples were always collected before the initiation of high-dose systemic corticosteroid therapy. Systemic corticosteroids were ideally discontinued or tapered to a maximum dose of 8mg/day methyl-prednisolone during maintenance therapy with conventional immunosuppressive and interferon alfa-2a therapy. Blood was also collected from 5 age and sex-matched healthy control subjects (sample 4).

The study was approved by the ethics committee of the School of Medicine, Marmara University (approval number: MR-YC-08-0105), and conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients and control subjects.

Flow cytometry

EDTA-preserved whole blood samples obtained from Behcet uveitis patients and control subjects were used to determine the frequencies of Foxp3+ expressions on CD4+CD25+Treg cells. Foxp3 Staining Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used for intracellular staining according to the manufacturer's instructions. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by erythrocyte lysing solution (155mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA) followed by washing the cells with phosphate buffered saline (PBS). Cells were then stained with Fluorescein Isothiocyanate (FITC) conjugated Anti-CD4 and Allophycocyanin (APC) conjugated anti-CD25 monoclonal antibodies and their isotypic controls. After

staining of the surface receptors, cells were fixed and permeabilized by using Foxp3 Buffer Set (BD Biosciences), then incubated with Phycoerythrin (PE) conjugated anti-Foxp3 monoclonal antibody for 30 minutes. Samples were acquired with FACSCanto flow cytometry using Diva software (BD Biosciences). Both percentage and Mean Fluorescence Intensity (MFI) of Foxp3 were quantified in the CD4+CD25+ gate.

Cell purification

CD4+CD25+ Treg and CD4+CD25-T cells were purified by magnetic separation with CD4+CD25+ Regulatory T Cell Isolation Kit (MACS, MiltenyiBiotec, Germany). Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation for cell purification. First, non-CD4 cells were labeled with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ / δ , and CD235a. Then labeled cells were incubated with microbeads conjugated to anti-biotin monoclonal antibodies. Indirectly labeled non-CD4+ T cells were magnetically depleted and CD4+ T cells were collected using a LS MACS Column with MidiMACS Separator (both MiltenyiBiotec). Further purified CD4+ T cells were labeled with CD25 microbeads for the isolation of CD4+CD25+ and CD4+CD25-T cells. Directly labeled CD25+ T cells were isolated with a MS MACS Column with MiniMACS Separator (both MiltenyiBiotec). CD25+ Treg cells were enriched with positive selection and CD25 depleted CD4+ T cells were purified with negative selection. Purification of enriched cell populations and purified cells were analyzed by FACS. The purity of the cell populations was >50% for CD4+CD25+T cells and >95% for CD4+CD25-T cells.

Proliferation assay

Freshly isolated CD4+CD25+Treg cells were suspended in complete RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin and streptomycin (all from Sigma-Aldrich Inc., St Louis, MO, USA). 1×10^6 cells/mL cells were cultured in 24 well plates in the presence of 10 μ g/mL phytohaemagglutinin (PHA, Sigma-Aldrich Inc.) at 37°C in 5% CO₂. After 3 days of culture, supernatants were collected and preserved at -80°C for ELISA testing.

ELISA

Cytokine secretions of CD4+CD25+ were determined by ELISA from culture supernatants. The levels of IFN- γ , TNF- α , IL-4, IL-17, IL-18 and IL-10 were evaluated using standard human ELISA kits (Biosource Europe S.A., Belgium) according to the manufacturer's instructions. The detection ranges were 15.6-1000 pg/mL for IFN- γ , TNF- α , IL-17, IL-18, and 7.8-500pg/mL for IL-4 and IL-10.

Statistical analysis

SPSS statistical software, version 16.0 (SPSS Inc, Chicago, Illinois), was used for the statistical analysis. The Friedman and Wilcoxon tests and the Mann-Whitney test used to test for differences in the paired and unpaired blood samples, respectively. No correction was made for multiple testing.

Table 1: Demographic and ocular clinical features and treatment properties at the time of blood sampling of patients with Behcet uveitis.

Patient No.	Age/ gender	Age at ocular involvement	Feature of uveitis	AZA and maintenance systemic corticosteroid dose / duration	Maintenance dose of IFN α -2a and systemic corticosteroid dose / duration
1.	21/M	19	Panuveitis OU, vitritis OU, retinal vasculitis OU, retinitis OU, macular edema OU	3mg/kg/day AZA, 8mg/kg/day ster, 3 mo.	4.5 million IU 3x/week, no ster, 6 mo.
2.	29/M	23	Panuveitis OU, vitritis OU, retinal vasculitis OU, retinitis OU, macular edema (OS)	2mg/kg/day AZA, 8mg/kg/day ster, 3 mo.	4.5 million IU 3x/week, 8 mg/kg/day ster, 5 mo.
3.	54/F	29	Panuveitis OU, vitritis OU, retinal vasculitis OU	2mg/kg/day AZA, no ster, 3 mo.	3 million IU 3x/week, no ster, 6 mo.
4.	29/M	21	Panuveitis OU, vitritis OU, retinal vasculitis OU, retinitis OU	2mg/kg/day AZA, 8mg/kg/day ster, 7 mo.	4.5 million IU 3x/week, 8 mg/kg/day ster, 4 mo.
5.	34/F	32	Panuveitis OU, vitritis OU, retinal vasculitis OU, macular edema OU	2mg/kg/day AZA, no ster, 4 mo.	4.5 million IU 3x/week, no ster, 6 mo.

F: Female, M: Male, AZA: Azathioprine, IFN α -2a: Interferon alfa-2a, OU: Bilateral, OD: Right Eye, OS: Left Eye, IU: International Units, ster: Systemic corticosteroid, methyl-prednisolone

Results

Demographical and clinical features and treatment properties at the time of blood sampling of patients with Behçet uveitis are shown in **Table 1**. Sex-matched controls composed of 3 male and 2 female subjects. Median age of Behçet uveitis patients and control subjects was 29 years (range: 21-54 years) and 28 years (range: 26-32 years), respectively and there was no significant difference ($p=0.54$). Median duration of Behçet uveitis was 6 years (range: 2-25 years). All patients included in the study had panuveitis with vitritis and retinal vasculitis. Patients were started on azathioprine 2mg/kg/day upon diagnosis. At the time of the second blood sampling median duration of azathioprine therapy was 3 months (range: 3-7 months). Maintenance dose of interferon alfa-2a was 4.5 million IU 3x/week sc in 3 patients and 3 million IU 3x/week in 1 patient. The median duration of interferon alfa-2a therapy was 6 months (range 4-6 months) at the time of the third blood sampling.

Table 2 shows comparison of CD4+CD25+Treg cell percentage, the intensity of Foxp3 expression of CD4+CD25+Treg cells indicated by MFI and level of cytokines in the supernatants between samples 1 and 4. Although not significant statistically there was a slightly elevated percentage of CD4+CD25+Treg cells and Foxp3 MFI in patients with active Behçet uveitis when compared with healthy controls (median \pm SEM; $5.30 \pm 4.09\%$ vs. $5.10 \pm 1.21\%$ and 582.4 ± 151.59 vs. 454.4 ± 73.47 , respectively). This suggested that BD patients with active panuveitis had a slightly increased circulating Treg cells.

Table 3 summarizes comparison of CD4+CD25+Treg cell percentage, Foxp3 expression of CD4+CD25+Treg cells (MFI) and cytokine levels in the supernatants between samples 1, 2 and 3. As shown in **Table 2**, IFN α -2a therapy did not cause a significant change in IFN- γ , TNF- α , IL-4, IL-17 and IL-18 levels. However, IFN α -2a led to a borderline significant decline of CD4+CD25+Treg cells [median

\pm SEM; sample (1): $5.30 \pm 4.06\%$ vs. sample (3): $0.80 \pm 0.87\%$, $p=0.06$] and an elevation of IL-10 [median \pm SEM; sample (1): $0.12 \pm 0.45\text{pg}/\text{ml}$ vs. sample (3): $2.39 \pm 0.51\text{pg}/\text{ml}$, $p=0.06$].

Discussion

In this in-vitro study, we showed that interferon alfa-2a decreased Foxp3+CD4+CD25+ Treg cells and increased the level of IL-10 in the culture supernatant of refractory Behçet uveitis patients. The Foxp3+CD4+CD25+ Treg cells are a unique lineage of T cells, capable of suppressing effector cell responses. T regulatory cells have been extensively studied in many autoimmune and inflammatory diseases [31]. Although there is a controversy among the studies regarding their frequency in peripheral blood of BD patients, Hamzaoui et al. [22] reported that CD4+ CD25+ Treg cells are increased in the peripheral blood of active BD patients [22]. Similar to their results we found a slightly elevated percentage of CD4+CD25+Foxp3+ Treg cells in patients with active Behçet uveitis.

Behçet disease is a systemic inflammatory disorder in which the presence of prolonged inflammation causes clinical manifestations including ocular involvement. It has been shown that the frequency of Treg cells is higher in the synovial fluid compared to peripheral blood of patients with rheumatic diseases [33-35]. According to these studies, Treg cells accumulate at sites of inflammation where they perform their suppressive functions on reactive T cells. However, for the efficient suppression of reactive cells that reside at inflammation sites, functional trafficking of Treg cells is required. In various autoimmune and inflammatory diseases, homing receptors on Treg cells have been shown to be defective and suggested to be involved in the pathogenesis of diseases [36,37]. Our finding of elevated percentage of CD4+CD25+Foxp3+ Treg cells at the peripheral blood of patients with active Behçet uveitis may indicate the defective migration of these cells to the inflamed ocular tissue. Further studies

Table 2: Comparison of CD4+CD25+Treg cells, Foxp3 mean fluorescence index, and cytokine levels from CD4+CD25+Treg cell supernatants offive Behçet uveitis patients at baseline-during acute panuveitis attack (sample 1) and five healthy control subjects (sample 4).

Blood samples	Median \pm SD Mean \pm SEM Range	Percentage of CD4+CD25+Treg cells	Foxp3 MFI	IFN- γ (pg/ml)	TNF- α (pg/ml)	IL-4 (pg/ml)	IL-17 (pg/ml)	IL-18 (pg/ml)	IL-10 (pg/ml)
Sample 1	5.30 \pm 4.06 9.86 \pm 9.10 1.50-22.40	543 \pm 67.79 582.4 \pm 151.59 398-792	1.34 \pm 8.48 9.82 \pm 18.96 0-43.7	1.34 \pm 0.67 1.75 \pm 1.50 0.52-4.20	20.5 \pm 5.55 23.02 \pm 12.41 14.0-44.20	7.67 \pm 3.42 8.22 \pm 7.66 0-17.2	1.62 \pm 0.38 1.45 \pm 0.85 0.16-2.47	0.12 \pm 0.45 0.81 \pm 1.01 0-2.11	
Sample 4	5.10 \pm 1.21 4.10 \pm 2.70 0.70-7.20	473 \pm 32.58 454.4 \pm 73.47 345-544	7.50 \pm 1.62 8.86 \pm 3.64 5.89-15.2	6.30 \pm 8.92 13.47 \pm 19.96 1.99-48.90	23.40 \pm 2.90 26.50 \pm 6.50 21.40-36.60	1.54 \pm 3.47 4.93 \pm 8.37 0-19.7	1.62 \pm 1.51 3.25 \pm 3.38 0.16-7.43	1.98 \pm 0.44 2.11 \pm 1.0 0.89-3.63	
p*	0.54	0.22	0.15	0.05	0.30	0.59	0.83	0.11	

MFI: Mean Fluorescence Index, IFN: Interferon, TNF: Tumor Necrosis Factor, *: Mann-Whitney U

Table 3: Comparison of CD4+CD25+Treg cells, Foxp3 mean fluorescence index, and cytokine levels from CD4+CD25+Treg cell supernatants between baseline-during acute panuveitis attack (sample 1), at termination of azathioprine therapy (sample 2), and while panuveitis was inactive on maintenance therapy of interferon alfa-2a (sample 3) of five patients with Behçet uveitis.

Blood samples	Median \pm SD Mean \pm SEM Range	Percentage of CD4+CD25+Treg cells	Foxp3 MFI	IFN- γ (pg/ml)	TNF- α (pg/ml)	IL-4 (pg/ml)	IL-17 (pg/ml)	IL-18 (pg/ml)	IL-10 (pg/ml)
Sample 1	5.30 \pm 4.06 9.86 \pm 9.10 1.50-22.40	543 \pm 67.79 582.4 \pm 151.59 398-792	1.34 \pm 8.48 9.82 \pm 18.96 0-43.7	1.34 \pm 0.67 1.75 \pm 1.50 0.52-4.20	20.5 \pm 5.55 23.02 \pm 12.41 14.0-44.2	7.67 \pm 3.42 8.22 \pm 7.66 0-17.2	1.62 \pm 0.38 1.45 \pm 0.85 0.16-2.47	0.12 \pm 0.45 0.81 \pm 1.01 0-2.11	
Sample 2	1.80 \pm 2.89 4.62 \pm 6.46 0.30-15.90	340 \pm 75.86 415 \pm 169.63 248-680	2.17 \pm 0.70 2.33 \pm 1.58 0.12-4.45	2.22 \pm 3.95 6.03 \pm 8.85 0.82-21.80	15.7 \pm 1.24 16.14 \pm 2.78 12.4-19.4	3.44 \pm 2.05 4.52 \pm 4.59 0-12.3	3.68 \pm 0.96 3.58 \pm 2.16 0.16-6.15	1.43 \pm 0.42 1.42 \pm 0.94 0-2.52	
Sample 3	0.80 \pm 0.87 1.62 \pm 1.94 0.60-5.10	439 \pm 28.41 444.6 \pm 63.53 347-514	2.59 \pm 0.77 2.30 \pm 1.72 0.12-4.74	2.56 \pm 2.26 5.68 \pm 5.06 2.10-13.90	17.7 \pm 3.75 19.79 \pm 8.38 9.99-29.4	7.67 \pm 2.89 9.72 \pm 6.47 3.44-19.70	1.17 \pm 0.46 1.12 \pm 1.04 0-2.26	2.39 \pm 0.51 2.69 \pm 1.15 1.43-3.91	
p*	0.09	0.09	0.94	0.36	0.95	0.36	0.36	0.09	
Paired groups comparison p[†]									
1 vs. 3	0.06	0.12	>0.99	0.18	0.81	0.62	0.62	0.06	
2 vs. 3	0.18	0.81	>0.99	0.81	0.81	0.37	0.12	0.31	
1 vs. 2	0.31	0.06	>0.99	0.12	0.31	0.43	0.18	0.43	

MFI: Mean Fluorescence Index, IFN: Interferon, TNF: Tumor Necrosis Factor, *: Friedman; [†]: Wilcoxon

are needed to clarify the involvement of homing receptors in the pathogenesis of BD.

Accordingly, Nanke et al. [24] showed that peripheral blood Treg cells of patients with BD were significantly decreased before and elevated during a uveitis flare-up [24]. In this study, we demonstrated that interferon alfa-2a therapy led to a borderline significant decline of CD4+CD25+Foxp3+Treg cells, indicating that interferon alfa-2a contributes to the reversal of inflammatory process.

Two important classes of Treg cells within the CD4+ subset are CD4+CD25+Foxp3+Treg cells and T regulatory type 1 (Tr1) cells [36-38]. These two regulatory subsets differ in a number of biological features, including their cytokine profile, cellular markers, transcription factors, and mechanism of immune suppression. The Tr1 subset are CD4+ T lymphocytes defined by their production of IL-10 and suppression of helper T cells. T regulatory 1 cells are inducible cells, arise from naive precursors, and can be differentiated both *ex-vivo* and *in-vivo*. T regulatory 1 cell differentiation is characterized by a massive secretion of IL-10 and bystander CD4+T cell suppression [38]. The increased level of IL-10 after interferon alfa-2a therapy can be accepted as clear evidence that this treatment affects the altered Treg cells, either enabling them to regain their suppressive functions or by inducing the Tr1 cell subset. Furthermore, the protective role of IL-10 in patients with Behcet uveitis has been shown recently [39]. Interleukin-10 significantly reduced the production of nitric oxide in peripheral mononuclear cell culture supernatants of patients with Behcet uveitis.

However, there are some limitations of our study. First, our patient had a severe form of Behcet uveitis that was refractory to conventional immunosuppressive therapy. Therefore we had to switch to interferon alfa-2a therapy immediately after azathioprine had failed to control uveitis flare-ups. Despite the fact that IL-10 level did not seem to be affected by azathioprine therapy, the effect(s) of azathioprine on the results can not to be ruled out. However, the current practice of Behcet uveitis management dictates to start interferon alfa-2a therapy in those who fail conventional treatment and interferon alfa-2a is used as second-line agent. Second, our study group was rather small to draw any strong conclusions.

In summary, our results suggest that IFN α -2a therapy might cause Treg cells to regain their suppressive function and thereof beneficial in suppressing the intraocular inflammation as well as in preventing uveitis flare-ups to occur in patients with refractory Behcet uveitis. Further studies are warranted to understand the exact mechanisms of interferon alfa-2a in controlling Behcet uveitis.

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