Mod Rheumatol (2008) 18:354–358
DOI 10.1007/s10165-008-0064-x

ORIGINAL ARTICLE

Decreased percentages of regulatory T cells in peripheral blood of patients with Behcet's disease before ocular attack: a possible predictive marker of ocular attack

Yuki Nanke · Shigeru Kotake · Mari Goto · Hiroshi Ujihara · Masao Matsubara · Naoyuki Kamatani

Received: 29 November 2007 / Accepted: 15 February 2008 / Published online: 22 April 2008

Abstract The current study investigates the role of regulatory T (Treg) cells in the pathogenesis of ocular attack in patients with Behcet's disease (BD). Nineteen BD patients with ocular complications (BDo), including 11 BD patients with ocular attack (BDa) and eight BD patients with inactive ocular complications (BDi), were studied. Four BD patients without ocular complications (BDwo) were also evaluated as controls. All patients were prospectively followed by our outpatient clinic between autumn 2004 and spring 2005. CD4+ CD25+^{bright} T cells (Treg cells) from peripheral blood were measured by flow cytometry. The percentages of Treg cells in CD4+ T cells from BDo were significantly decreased before ocular attack compared with those after ocular attack. Moreover, surprisingly, these levels before attack were significantly lower than normal level, whereas the percentages of Treg cells in both BDi and BDwo patients were normal. Treg cells were significantly decreased in BDa before active ocular attack. These findings suggest that Treg cells play an important role in ocular attack in BD patients. In addition, decreased percentages of Treg cells may be a predictive marker of ocular attack in BD patients allowing treatment of BD patients before an ocular attack.

Keywords Ocular attack · Behcet's disease · Regulatory T

Y. Nanke (✉) · S. Kotake · N. Kamatani
Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan
e-mail: ynn@ior.twmu.ac.jp

M. Goto · H. Ujihara · M. Matsubara
Department of Ophthalmology, Medical Center East, Tokyo Women's Medical University, Tokyo, Japan

Abbreviations
BD Behcet's disease
Treg regulatory T

Introduction

Behcet's disease (BD) is polysymptomatic with recurrent oral and genital ulceration, uveitis with a chronic course and an unknown cause [1]. Pathology of the lesions consists of widespread vasculitis. Eyes, skin, joints [2], oral cavity, blood vessels, and central nervous system are usually involved. Although the pathogenesis of the disease remains unclear, it has been proposed that its causes might involve (a) genetic predisposition, (b) abnormalities of the immune system such as immunoactive cells, (c) T cells infiltrating into the affected lesions with activation of circulating T and B cells, followed by chemotaxis of neutrophils or vice versa or (d) endothelial damage [1]. Recently, we reported that both total and mature adrenomedullin levels are elevated in plasma from BD patients, which may be related to endothelial damage [3].

CD4+ CD25+^{bright} T cells are a population of regulatory T cells (Treg) cells responsible for active suppression of autoimmunity [4]. Treg cells constitute 5–15% of peripheral CD4+ T cells in humans, suppressing T cell responses against autoantigens. Treg cells play an important role in pathogenesis in autoimmune disorders, such as diabetes mellitus [5], arthritis [6,7] and lupus [8]. These autoimmune disorders can be prevented by infusion of Treg cells [4]. Treg cells also inhibit previously activated CD4+ CD25(−) autoreactive T cell clones [4]. These findings indicate that these cells may contribute to regulation of the immune response in humans. The role of Treg

Table 1 Clinical features of Behcet's disease (BD) patients with ocular attack

Case	Sex/age	Type	Duration (y)	OU	GU	Skin	Therapy	HLA
1	M/58	C	19	+	+	–	C,P,Cs	ND
2	M/38	IC	7	+	–	–	P,Cs	B51
3	F/50	C	28	+	+	Acne,EN	–	B51,A26
4	M/47	C	13	+	+	EN,T	PSL,Cs	–
5	M/47	IC	18	+	–	Acne	PSL,Cs,C	B51
6	M/40	C	5	+	–	EN	PSL,M	B51
7	M/55	IC	13	+	+	T	PSL,C	B51,A26
8	M/45	IC	12	+	–	EN,Acne	PSL,M,Cs	A26
9	M/63	C	1	+	+	EN,Acne	C	–
10	M/37	C	1	+	–	EN	C	B51
11	M/52	C	15	+	+	Acne,T	PSL	ND

OU oral ulcer, *GU* genital ulcer, *C* complete type according to the Japanese Behcet's Disease Research Committee Criteria, *EN* erythema nodosum, *T* thrombosis, *PSL* predonisolone, *C* colchicines, *Cs* cyclosporin, *M* mizoribine, *ND* not done

Table 2 Clinical features of Behcet's disease (BD) patients without ocular attack

Case	Sex/age	Type	Duration (y)	OU	GU	Skin	Therapy	HLA
12	M/69	C	32	+	+	Acne	–	ND
13	F/56	C	9	+	–	EN	C	ND
14	M/60	C	14	+	+	Acne	C	ND
15	M/48	C	19	+	+	Acne	–	ND
16	F/67	C	18	+	+	Acne	C	ND
17	F/54	C	13	+	+	–	C,M	A26
18	F/71	C	20	+	+	EN	C	A26
19	M/38	C	8	+	+	Acne	C,PSL	B51,A26

OU oral ulcer, *GU* genital ulcer, *C* complete type according to the Japanese Behcet's Disease Research Committee Criteria, *EN* erythema nodosum, *T* thrombosis, *PSL* prednisolone, *C* colchicines, *Cs* syslosporin, *M* mizoribine, *ND* not done

cells has been investigated in only a few autoimmune diseases, including SLE [8], Sjögren's syndrome [9], rheumatoid arthritis [7], and type-1 diabetes [5].

Ocular involvements are common in BD. In addition, uveitis in BD patients sometimes causes blindness. We hypothesized that Treg cells are involved in the pathogenesis of BD. In the current study, we investigated the role of Treg cells in BD patients with ocular complications (BDo), including in both BD patients with ocular attack (BDa) and BD patients with inactive ocular complications (BDi), compared with BD patients without ocular complications (BDwo) as well as those with other collagen diseases.

Patients and methods

Patients

All BD patients who were regularly followed by our outpatient clinic between autumn 2004 and spring 2005 were entered into this study. We chose the cold seasons because uveitis attack frequently occurs in low temperature. All patients visit our outpatient clinic once a month and blood samples were obtained each time. Nineteen BDo included 11 BDa (ten men and one woman aged 37–63 years, Table 1), case 9, 10, and 11 had a weak attack and eight BDi in the inactive phase (four men and four women, aged 38–71, Table 2). Another four BDwo (one men, three women) aged 21–58, six sarcoidosis patients (three men and three women) aged 38–78, and five systemic sclerosis patients (five women) aged 47–68 were also included as controls. All sarcoidosis patients had inactive uveitis. Four normal volunteers (two men and two women, aged 38–47) were also included. All patients were Japanese and diagnosed as having BD based on both the Behcet's Disease Research Committee Criteria of Japan and International Study Group for Behcet's Disease Criteria [10, 11]. All systemic neural, enteric, or vascular symptoms were inactive. Uveitis was diagnosed by two ophthalmologists according to the International Uveitis Study Group guidelines. If only a few cells infiltrate, ophthalmologists diagnosed the uveitis as "weak attack" uveitis. We obtained informed consent from all patients. This study was approved by the ethics committee at our institution.

Treg analysis

Dual color flow cytometry was performed to quantify all populations of Treg cells. Cells were stained with anti-CD4-FITC (Beckman Coulter Inc., FL, USA) and anti-CD25-PE (Beckman Dickinson Immunocytometry System, CA, USA). Flow cytometry was performed using a Beckman Dickinson FACSCAN instrument (Becton Dickinson, Franklin Lakes, NJ, USA). The proportion of total CD4+ CD25+ T cells was determined by level of isotype control fluorescence. CD4+ CD25+^{bright} fraction was determined by more than 10^2 of fluorescence intensity, with the same settings used in all patients and controls (Fig. 1). Lymphocytes were gated according to forward

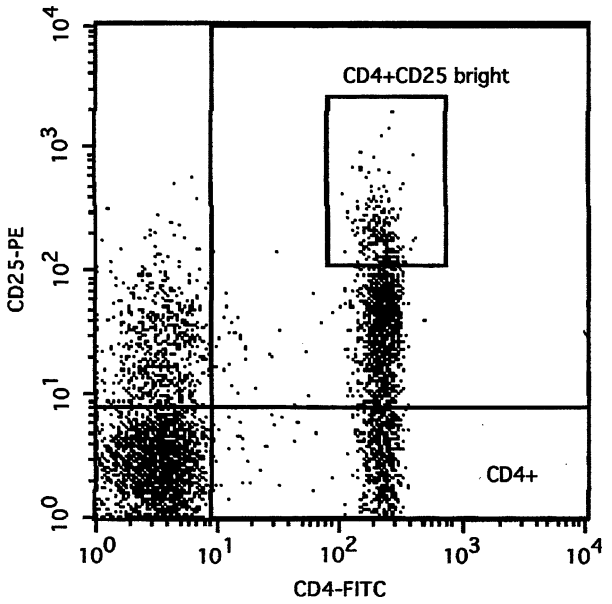

Fig. 1 Lymphocytes were gated according to forward and side scatter (FSC/SSC) from peripheral blood mononuclear cells, then screened by flow cytometry for the presence of CD4+ CD25+ T and CD4+ CD25+^{bright} T cells. Representative FACS staining from one normal control subject is shown

and size scatter and 10,000 cells were analyzed with CellQuest software (Becton Dickson). Results were compared using arbitrary units (AU) of mean fluorescence intensity (MFI) as a relative measure of CD25 expression.

Statistical analysis

Significance was analyzed using Mann–Whitney test and Wilcoxon's rank sum test. The results were expressed as mean ± SD and considered significant when the P value was <0.05.

Results

Table 1 shows the clinical features of BDo. Cases 9, 10, and 11 demonstrated weak inflammation. Table 2 describes the clinical features of BDwo. All patients fulfilled both the International Study Group Criteria [10] and Behcet's Disease Research Committee Criteria for Japan [11].

We quantified the percentages of Treg cells present in peripheral blood. The percentage of Treg cells in normal controls was more than 1.51%. The percentages of Treg cells among CD4+ T cells from the peripheral blood of BDo were significantly decreased before ocular attack compared with those after ocular attack ($P = 0.0117$) (Fig. 2). Case 12 developed a left ocular attack 1 month after the study finished. The percentage of Treg cells in pre-and post-ocular attack of cases 9, 10, and 11 were as follows: 3.0–4.4, 4.5–5.1, and 3.1–2.7%, respectively (Fig. 2). However, the levels of C-reactive protein (CRP), white blood cells (WBC) and immunoglobulin D (IgD) did not reflect ocular activity (data not shown). Treg cells in BDi showed normal percentages during the follow-up period. We showed the first and the last data during the follow-up period (Fig. 2). The percentages of Treg cells among CD4+ T cells in BDwo were 2.45 ± 0.81%. Those patients with sarcoidosis and patients with systemic scleroderma were 2.67 ± 1.19 and 3.10 ± 0.78%, respectively. The percentage of Treg cells among CD4+ T cells in normal volunteers was 1.51 ± 0.29% (Fig. 2). Thus, in BDwo, sarcoidosis patients, systemic sclerosis patients and normal volunteers, the percentages of Treg cells among CD4+ T cells remained within normal ranges.

Discussion

We demonstrated for the first time in a prospective study, the percentages of Treg cells in CD4+ T cells in BD patients with or without ocular complications. Our study further demonstrated that the percentages of Treg cells among CD4+ T cells from BDo were significantly decreased before ocular attack compared with those after ocular attack. In addition, surprisingly, those before ocular attack were significantly lower than the normal level. However, percentages of Treg cells in all BDi except case 12, discussed below, remained within the normal level during the follow-up period. In addition, the percentages of Treg cells among CD4+ T cells in the group of BDwo, sarcoidosis, and systemic sclerosis were all within normal ranges. Thus, these findings suggest that we can predict ocular attack by a low level of Treg cells.

Percentages of Treg cells of BDi remained within the normal range except in case 12; the percentage of Treg cell in case 12 was decreased to less than 1.1% as shown in Fig. 2. Surprisingly, the patient notably demonstrated ocular attack 1 month after the current follow-up period finished. Thus, the decreased percentage of Treg cells was again associated with the development of ocular attack in this patient. The finding in this case supported our conclusion that ocular attack can be predicted by a low level of Treg cells, although another study is needed for validation.

Jiao Z et al. [12] reported that Treg cells were increased significantly in synovial fluid compared to paired peripheral blood from rheumatoid arthritis patients. In the current study, we demonstrated that the percentages of Treg cells in CD4+ T cells from BDo were significantly decreased before ocular attack compared with those after ocular attack. Thus, the decreased percentage of Treg cells in peripheral blood in BDa may reflect trafficking to the eyes.

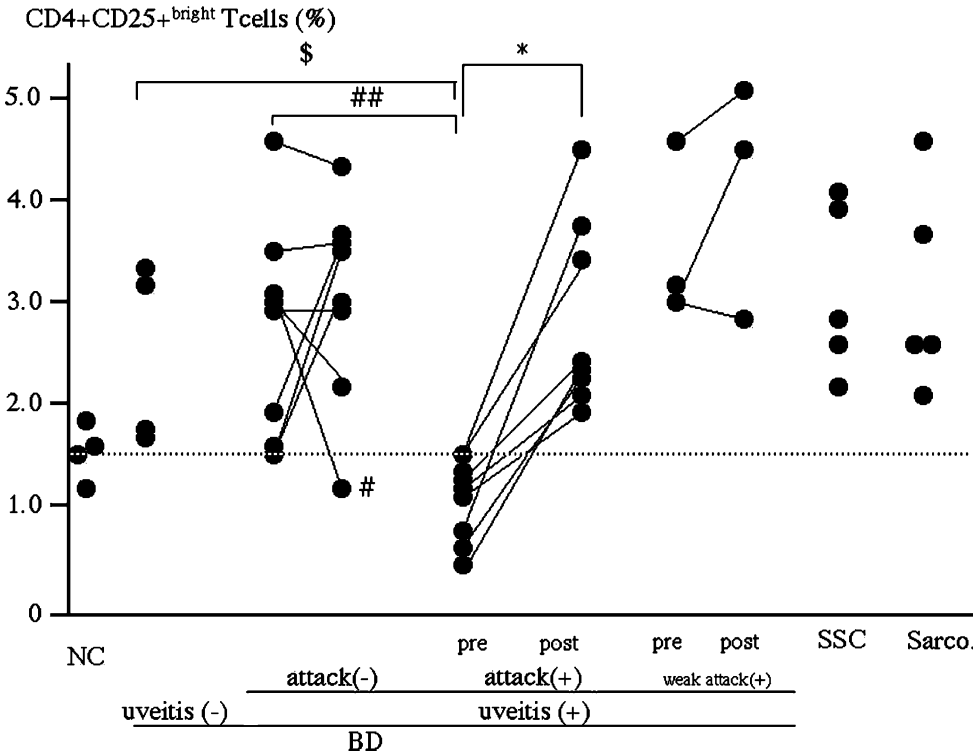

Fig. 2 The percentage of CD4+ CD25+^{bright} T cells from the peripheral blood of BD patients with ocular attack ($n = 8$) and BD patients with weak ocular attack ($n = 3$), BD patients with inactive ocular complications and BD patients without ocular complications. *Horizontal line* shows the average value of four normal controls. *SSC* Sytemic scrlosis; Sarco; Sarcoisdosis, *NC* normal control $*P = 0.0117$, $\$P = 0.0065$, $\#\#P = 0.0016$ # case 12 developed ocular attack 1 month later after the current study finished

Since this study was a prospective study, we did not measure the percentages of Treg cells before the study period. However, we followed the patients measuring the percentages of Treg cells in seven out of eight BDa for 6 months after the study period (data not shown). The percentages of Treg cells of those patients remained within the normal range. Thus, again we speculated that the decreased percentages of Treg cells were associated with the development of ocular attack.

The decreased percentage of Treg cells may also reflect not only ocular activity but also other disease activity in BD patients. In fact, during the current follow-up period, one patient with a decreased percentage of Treg cells developed oral ulcers and arthritis of the right ankle, but not an ocular attack. However, a further study is needed to clarify the role of Treg cells in other lesions since the current study was a prospective study investigating on attacks of uveitis only.

The balance between activated responder T cells and Treg cells may influence the extent of immunoregulation during an ocular attack in BD patients. Grajewski et al. [13] reported that experimental autoimmune uveitis (EAU) that is induced in mice with retinal antigen interphotorecepter retinoid-binding protein (IRBP) is controlled by injection of Treg cells. The number of Treg cells is increased by immunosuppressive treatments that down-regulate inflammation [14]. TNF-α has a direct effect on Treg cell viability, such as the induction of apoptosis, which would explain the increased number of Treg cells after TNF-α neutralizations [15]. In fact, anti-TNF-α therapy was shown to modulate the proportion and function of blood Treg cells in patients with rheumatoid arthritis [14]. Thus, anti-TNF-α therapy may be useful for treatment in BD patients with ocular complications, showing a decreased percentage of Treg cells.

In BD, ocular lesions occur in the uvea and retina causing various symptoms, including blurred vision, eye pain, photophobia, lacrimation, floaters, and periglobal hyperemia. Recurrent and explosive attacks decrease visual acuity; uveitis in BD patients sometimes causes blindness. Thus, not only ophthalmologic evaluations but also a predictive marker of ocular activity in peripheral blood may facilitate treatment before ocular attack in BD patients, improving the prognosis in ocular complications.

In this study, we did not use FoxP3 as a marker to detect human Treg cells. It has been reported that in humans CD4+ CD25 ^{bright} exhibit all properties of Treg cells [16]. In addition, in contrast to mice, expression of FoxP3 is not entirely restricted Tregs in humans, because activated human CD4+ T cells also express FoxP3 [17]. Thus, we measured high expression of CD25 on helper T cells as demonstrated in this study.

More recently, Hamzaoui et al. [18] reported that BD in active phase had significantly higher Treg cells, as compared with BD in the remission stage and healthy controls. In addition, Treg cells in active BD were impaired in the proliferative response, suppressing the proliferation of their CD4+ CD25(−) counterparts [17]. However, they did not measure the levels of Tregs in peripheral blood of

patients with ocular attack in the pre-attack phase. Thus, we for the first time reported decreased levels of Tregs in the pre-attack phase.

In summary, the percentages of Treg cells among CD4+ T cells from BDa were significantly decreased before ocular attack compared with those after ocular attack. In addition, more importantly, these levels before ocular attack were significantly lower than the normal level. These findings suggest that the Treg cells play a pivotal role in ocular attack in BD patients. In addition, percentages of Treg cells in peripheral blood may be a predictive marker of ocular activity in BD patients, allowing prophylactic treatments of BD patients before ocular attack.

References

1. Sakane T. Behcet's disease. N Eng J Med. 1999;341:1284–91.
2. Nanke Y, Kotake S, Tateishi M, Yamanaka H, Kamatani N. Synovial histology in three Behcet's disease patients with orthopedic surgery. Clin Exp Rheumatol. 2002;20:35–44.
3. Nanke Y, Kotake S, Ogasawara K et al. Raised plasma adrenomedullin level in Behcet's disease patients. Mod Rheumatol. 2003;13:139–42.
4. Sakaguchi S, Sakaguchi N, Shimizu J. Immunological tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev. 2001;182:18–32.
5. Kukreja A, Cost G, Marker J et al. Multiple immuno-regulatory defects in type-1 diabetes J. Clin Investig. 2002;109:131–40.
6. Amelsfort J, Jacvobs K, Bijlsma J, Lafeber F, Taams L. CD4+ CD25+ regulatory T cells in rheumatoid arthritis. Difference in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. 2004;50:2775–85.
7. Cao D. Isolation and functional characterization of regulatory CD25brightCD4 T cells from the target organ of patients with rheumatoid arthritis. Eur J Immunol. 2003;33:215–23.
8. Crispin JC, Martinez A, Varela JA. Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun. 2003;21:273–6.
9. Gottenberg JE, Lavie F, Abbed K et al. CD4+ CD25+ high regulatory T cells are not impaired in patients with primary Sjögren's syndrome. J Autoimmun. 2005;24:235–42.
10. International Study Group for Behcet's Disease: criteria for diagnosis of Behcet's disease. Lancet. 1999;335:1078–80.
11. Behcet's Disease Research Committee of Japan. Behcet's disease: a guide to diagnosis of Behcet's disease. Jpn J Ophthalmol. 1974;18:291–4.
12. Jiao Z, Wang W, Jia R et al. Accumulation of FoxP3-expressing CD4(+)CD25(+) T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis. Scand J Rheumatol. 2007;36:428–33.
13. Grajewski R, Silver P, Agarwal RK et al. Endogenous IRBP can be dispensable for generation of natural CD4+ CD25+ regulatory T cells that protect from IRBP-induced retinal autoimmunity. JEM. 2006;203:851–6.
14. Ehrenstein MR, Evans JG, Singh A et al. Compromised function of regulatory T cells in Rheumatoid arthritis and reversal by anti-TNFα therapy. J Exp Med. 2004;200:277–85.
15. Sarin A, Conan-Cibotti M, Henkart P. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. J Immunol. 1995;155:3716–8.
16. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Fox P3. Science. 2003;299:1057–61.
17. Allan SE, Crome SQ, Crellin NK et al. Activation-induced FoxP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol. 2007;19:345–54.
18. Hamzaoui K, Hamzaoui A, Houman H. CD4+ CD25+regulatory T cells in patients with Behcet's disease. Clin Exp Rheumatol. 2006;24:S71–8.