
Imbalance of Th17 to Th1 cells in Behçet's disease

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ABSTRACT

Objectives. Behçet's disease (BD) is a helper T cell-mediated autoimmune disease characterised by recurrent orogenital ulcers, uveitis and skin lesions. The helper T cells are divided into Th1, Th2 and Th17 cells according to the pattern of cytokine secretion. Th1 and Th17 cells can contribute to the development of the disease with their respective proinflammatory cytokines, IFN- γ and IL-17. In this study, we investigated the relative role of Th17, Th1 and Th2 cells in BD.

Methods. Peripheral blood mononuclear cells were isolated from 30 patients with BD, for whom the detailed clinical manifestations and medication history were investigated and recorded. Surface markers and intracellular levels of IL-17, IFN- γ and IL-4 in isolated CD4⁺ T cells were measured using flow-cytometry from these patients and from two control groups, 34 rheumatoid arthritis patients and 24 healthy blood donors to analyse the relationship of Th1, Th17 and Th2 cells in BD.

Results. The ratio of Th17/Th1 cells was significantly increased in BD compared to healthy controls (0.16 ± 0.09 vs. 0.10 ± 0.04 , $p=0.012$), while there was no difference in the ratios of Th1/Th2 or Th17/Th2 cells. Th17/Th1 ratio was elevated in BD patients with uveitis or folliculitis compared to those without it (0.21 ± 0.10 vs. 0.13 ± 0.06 , $p=0.045$ for uveitis; 0.18 ± 0.10 vs. 0.12 ± 0.05 , $p=0.036$ for folliculitis).

Conclusion. Our results confirm that TH17 cells are instrumental in Behçet's uveitis and folliculitis. Furthermore, our findings suggest that the role of Th17 cells should be interpreted in the context of their ratio to Th1 cells.

Introduction

Behçet's disease (BD) is an autoimmune inflammatory disease characterised by recurrent aphthous stomatitis, uveitis, genital ulcers, skin lesions and vasculitis (1-3). Activation of T cells

and neutrophils plays an essential role in the pathogenesis of the disease (4-6). Since IFN- γ and IL-12 from Th1 cells can mediate the inflammatory response between T cells and neutrophils, Th1 response has been considered to be an important step in the development of BD (4, 6-11).

IL-17, which is predominantly produced by Th17 cells, is a strong proinflammatory cytokine that mobilises and activates inflammatory neutrophils and macrophages (12-16). The proinflammatory nature of this cytokine suggests that it is a critical mediator of BD, as it is in other autoimmune diseases. In fact, the levels of IL-17 and its related cytokine IL-23 are increased both in the serum of BD patients and in the aqueous humor of those with active uveitis (11, 17). Furthermore, the proportion of peripheral blood Th17 cells is also elevated in BD patients with active uveitis (17).

Each subset of helper T cells (Th1, Th2 and Th17) differentiates and functions under the influence of the other subsets. Th1-derived cytokines prevent development of Th2 cells and affect the expansion and function of Th17 cells (18). Therefore, we decided to investigate the relative role of each subset of helper T cells in BD with intracellular flow cytometry, focusing on Th17 cells.

Patients and methods

Patients

A total of 30 patients with BD (19 women, 11 men; age: 43.7 ± 10.8 years) were enrolled from Seoul National University Hospital between May 2006 and June 2008. The patients were diagnosed according to the criteria of the International Study Group for Behçet's Disease (19). The presence of BD-related manifestations was ascertained at the blood sampling; oral ulcers, genital ulcers, uveitis, erythema nodosum, folliculitis, arthritis /arthralgia, colitis and deep vein thrombosis. Patients with active disease were defined as those with at

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least one active manifestation when samples were obtained. Medication use was also noted during enrollment, including colchicines, nonsteroidal anti-inflammatory drugs, prednisolone, sulfasalazine, azathioprine and cyclosporine.

As a disease control, 33 patients with rheumatoid arthritis (RA) (27 women, 6 men; 55.5±12.1 years) were enrolled from the same hospital during the same period as the BD patients. RA was diagnosed according to the classification criteria by American College of Rheumatology (20). As a healthy control, 24 normal blood donors were enrolled for the study (19 women, 5 men; 45.2±21.6 years).

The Institutional Review Board of Seoul National University Hospital approved the study and informed written consent was obtained from all of the subjects.

Methods

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples with Ficoll-Paque™ Plus (GE healthcare). CD4⁺ T-cell subsets were purified from PBMC by positive selection using anti-CD4 MACS Microbeads (Miltenyi Biotec). The purity of the CD4⁺ T-cell populations was >97%.

Surface staining of PBMC was performed with the following antibodies: PerCP- anti-human CD3 (Beckton-Dickinson), phycoerythrin (PE)-anti-human CD4 (Beckton-Dickinson), fluorescein isothiocyanate (FITC)-anti-human CD8 (Beckton-Dickinson), FITC-anti-human CD45RA and Allophycocyanin (APC)- anti-human CD45RO monoclonal antibodies.

Isolated CD4⁺ T cells (4x10⁵ cells) were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich), ionomycin (250 ng/ml; Sigma-Aldrich) with 2 µL of monensin (Golgistop®;

BD Biosciences) in RPMI 1640 with 10% fetal bovine serum in a 96-well culture plate at 37°C for 5 hours and frozen at -70°C until flow cytometry was performed.

For intracellular cytokine analysis, we thawed the activated CD4⁺ T cells, blocked them with mouse IgG1, permeabilised with BD Cytotfix/Cytoperm™ solution (BD Pharmingen), and stained with the following monoclonal antibodies: FITC anti-IFN-γ (BD Biosciences), PE anti-IL-17 (eBioscience) and PE anti-IL-4 antibodies (BD Pharmingen). Th1 cells were defined as those which have intracellular IFN-γ, Th2 cells as those with IL-4 and Th17 cells as those with IL-17. All flow cytometry was performed with a FACS Calibur (Becton Dickinson) to study the surface markers in PBMC and intracellular levels of these cytokines in CD4⁺ T cells. The WinMDI (version 2.9) was used to analyse the flow cytometry data.

Statistical analysis

Analysis of variance (ANOVA) and post-hoc Tukey's test or Student *t*-test was used to compare the mean levels of cytokine-producing T cells. Categorical variables were analysed using Fisher's exact test. All statistical tests were two-tailed, and *p*-values less than 0.05 were considered significant. SPSS version 12 for Windows was used for all statistical tests.

Results

Clinical characteristics and subset distribution of T cells in BD patients compared with RA and HC

Mean age of the enrolled patients was 43.7±10.8 years and the mean duration of the disease was 13.1± 2.0 years. All the patients showed oral ulcers, while genital ulcers were found in 21(70.0%),

erythema nodosum in 23 (76.7%), pseudofolliculitis in 18 (60.0%), uveitis in 11 (36.7%), colitis in 3 (10.0%), deep vein thrombosis in 2 (6.7%), arthritis in 20 (66.7%) and positive pathergy test in 9 patients (30.0%). The number of patients with active disease was 25 (83.3%). The most commonly used medication was colchicines (43.3%) followed by prednisolone (26.7%), azathioprine (16.7%) and cyclosporine (6.7%). The proportions of CD4⁺ and CD8⁺ T cells were not different among the three groups as the ratio of naïve (CD3⁺CD4⁺CD45RA⁺) versus memory T cells (CD3⁺CD4⁺CD45RO⁺).

Distribution of Th1, Th2 and Th17 cells in BD, RA and healthy controls

There was no difference in the proportions of Th1, Th2 and Th17 cells between BD, RA and healthy controls (HC) (Table I, Fig. 1). However, the ratio of Th17/Th1 cells were significantly different among the 3 groups (0.16±0.09 in BD, 0.10±0.04 in HC and 0.13±0.06 in RA; *p*=0.012 by ANOVA; Table I). Post-hoc analysis showed a significantly increased ratio of Th17/Th1 cells in BD when compared to healthy controls. The ratios of Th1/Th2 and Th17/Th2 did not show any difference among the 3 groups (*p*=0.39 and *p*=0.79, respectively by ANOVA; Table I).

Clinical characteristics and the ratio of Th17/Th1 cells

We then investigated the relationship between helper T-cell subsets and the clinical manifestations of BD (Table II). The ratio of Th17/Th1 was significantly higher in the patients with uveitis than those without it (0.21±0.10 vs. 0.13±0.06, *p*=0.045, Table II). Those with uveitis tend to have more Th17 cells than those without it (3.08±2.44%

Table I. Th17, Th1 and Th2 cells in patients with Behçet's disease, rheumatoid arthritis and healthy controls.

	IL-17 (%)	IFN-γ (%)	IL-4 (%)	IL-17 ⁺ /IFN-γ ⁺	IL-17 ⁺ /IL-4 ⁺	IFN-γ ⁺ /IL-4 ⁺
Behçet's disease*	2.21 ± 1.90	14.46 ± 10.09	2.70 ± 1.29	0.16 ± 0.085	0.64 ± 0.65	4.443 ± 2.9494
Healthy controls*	1.68 ± 0.91	17.22 ± 9.17	3.19 ± 1.35	0.10 ± 0.043	0.54 ± 0.26	6.06 ± 3.10
Rheumatoid arthritis*	1.31 ± 0.07	16.04 ± 10.48	3.20 ± 1.85	0.13±0.063	0.58 ± 0.38	5.43 ± 4.59
<i>p</i> -value [†]	0.23	0.60	0.49	0.012	0.79	0.39

*Mean±SD, [†]*p*-value by ANOVA

Table II. The proportion of Th17 cells and the ratio Th17/Th1 cells in relation to the clinical manifestations of Behçet's disease.

Clinical manifestations (Number)*		Th17 (%)	<i>p</i> -value	Th17/Th1	<i>p</i> -value*
Genital ulcers	Presence (21)	1.90 ± 1.62	0.17	0.15 ± 0.09	0.24
	Absence (9)	2.96 ± 2.40		0.19 ± 0.07	
Erythema nodosum	Presence (23)	2.13 ± 1.65	0.66	0.17 ± 0.09	0.42
	Absence (7)	2.50 ± 2.73		0.14 ± 0.08	
Folliculitis	Presence (18)	2.56 ± 2.21	0.24	0.18 ± 0.10	0.036
	Absence (12)	1.70 ± 1.23		0.12 ± 0.05	
Uveitis	Presence (11)	3.08 ± 2.44	0.057	0.21 ± 0.10	0.045
	Absence (19)	1.72 ± 1.35		0.13 ± 0.06	
Colitis	Presence (3)	2.50 ± 2.03	0.79	0.22 ± 0.14	0.23
	Absence (27)	2.19 ± 1.92		0.15 ± 0.08	
Arthritis	Presence (20)	1.81 ± 1.37	0.19	0.14 ± 0.08	0.14
	Absence (10)	3.03 ± 2.57		0.19 ± 0.09	
Deep vein thrombosis	Presence (2)	3.35 ± 3.32	0.39	0.24 ± 0.19	0.60
	Absence (28)	2.13 ± 1.84		0.15 ± 0.08	
Pathergy	Presence (9)	2.96 ± 2.57	0.45	0.17 ± 0.11	0.79
	Absence (16)	2.34 ± 1.45		0.16 ± 0.08	
Activity	Active (15)	2.04 ± 1.57	0.50	0.16 ± 0.086	0.71
	Inactive (5)	3.12 ± 3.20		0.17 ± 0.089	

**p*-value by Student's *t*-test.

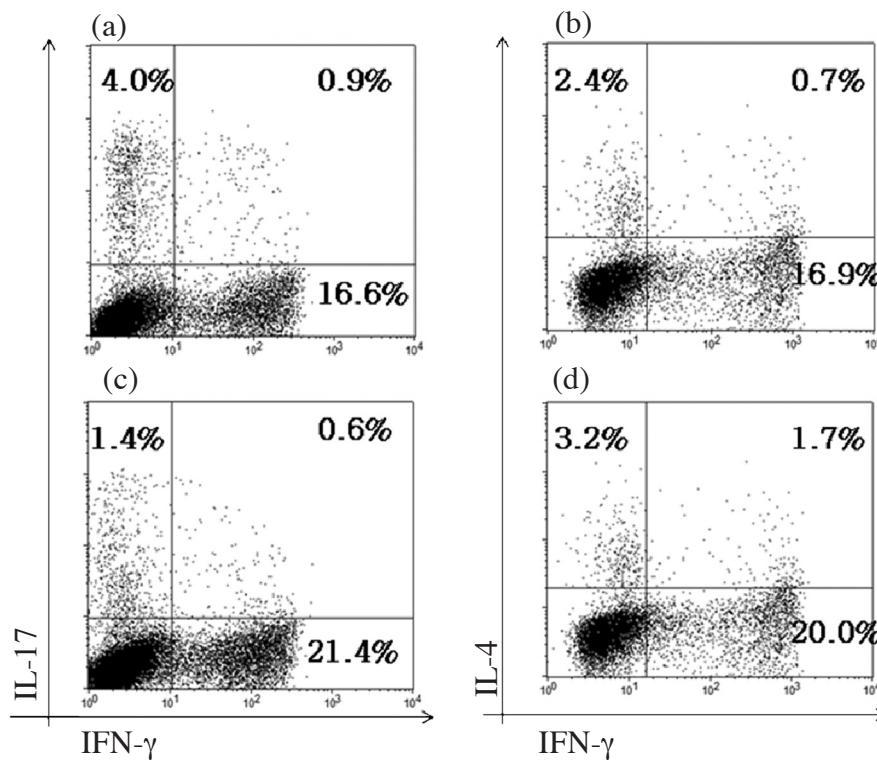


Fig. 1. Representative figures of intracellular cytokine flowcytometry. After isolating CD4⁺ T cells from peripheral blood mononuclear cells using magnetic beads, we stimulate the cells with phorbol 12-myristate 13-acetate and ionomycin for 6 hours and measured intracellular cytokines with flowcytometry. (a) interferon- γ (IFN- γ) and IL-17 in Behçet's disease (BD) (b) IFN- γ and IL-4 in BD (c) IFN- γ and IL-17 in healthy controls (HC) (d) IFN- γ and IL-4 in HC.

vs. 1.72±1.35%, *p*=0.057 by Student's *t*-test; Table II). Patients with folliculitis also showed a higher ratio of Th17/Th1 cells than controls (0.18±0.10 vs.

0.12±0.05, *p*=0.036; Table II). Disease activity or pattern of medication use was not associated with the proportion of Th17 cells or the Th17/Th1 ratio. The

ratios of Th17/Th1 cells were not different according to the use of medications except azathioprine. The patients who took azathioprine showed significantly higher ratio of Th17/Th1 cells than those who did not (0.23±0.10 vs. 0.14±0.18, *p*<0.05 by Student's *t*-test). All patients who took azathioprine had uveitis. There was no difference of Th17/Th1 ratios between uveitis patients who took azathioprine and those who did not (0.23±0.10 vs. 0.19±0.11, *p*=0.48)

Discussion

In this study, we found that the ratio of Th17/Th1 cells was significantly higher in patients with BD than in healthy controls. The ratio was even more prominent in BD patients with uveitis or folliculitis.

In Behçet's disease, CD4⁺ T cells and neutrophils play important roles in the pathogenesis of the disease (4-7). IFN- γ and IL-17, which are secreted from Th1 and Th17 cells, respectively, are two representative pro-inflammatory cytokines which can affect the activity of these cells. Accordingly, Th1 cells have been reported to be involved in the pathogenesis of BD, and Th17 was also recently suggested to be a pathogenic factor (8-11, 17, 21-24).

IFN- γ , a representative Th1-derived cytokine, is pro-inflammatory. However, it can also regulate the other pro-inflammatory Th17 cells in certain clinical settings (18, 25,26). Inadequate control of the pro-inflammatory Th17 cells by IFN- γ may lead to an excessive inflammatory state in the susceptible tissues such as eye and skin. The successful treatment of posterior uveitis with IFN- γ supports our hypothesis (27-29). Balancing of Th17/Th1 cells could be a new target for the development of novel drugs for BD, especially for uveitis, which is incapacitating.

BD is a heterogeneous disease affecting various organs and tissues (30, 31). It can be clinically clustered into several diseases. The results of our study suggest that uveitis and folliculitis in BD may be immunologically different from other subsets of the disease, as they exhibit elevated ratios of Th17/Th1. Large-scale studies which include

enough patients in the different subsets could suggest new classification of the disease based on Th17/Th1 cell ratios. Alternatively, direct phenotyping of T cells in the affected lesions could reveal the direct role of Th17/Th1 ratios in this disease.

In this study, azathioprine was associated with an increased ratio of Th17/Th1. However, all patients taking azathioprine had uveitis. No difference of Th17/Th1 ratio was found between uveitis patients who took azathioprine and those who did not, which suggests that the increased ratio of Th17/Th1 comes from the uveitis itself, not from the azathioprine.

While the balance of Th17/Th1 was skewed in BD compared with healthy controls, this was not true when compared with RA. This result came from the elevated, although not statistically significant, level of Th17 in RA compared with healthy control group. This reflects the possible role of Th17 in the pathogenesis of RA (32). Furthermore, our study results suggest that the balance of Th17/Th1 may play a more important role in BD than RA.

In addition to the classical role of Th1 cells in Behçet's disease which was proven in functional and pathologic investigations, our results suggest that the balance of Th17 and Th1 cells may also play a crucial role in the pathogenesis of BD, especially in the subsets of uveitis and folliculitis.

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