Skewed TGFβ/Smad signalling pathway of T cells in patients with Behçet's disease

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ABSTRACT

Objective. *Behçet's disease (BD) is a multi-systemic inflammatory disease, characterised by recurrent oral aphthosis, genital ulcers, skin lesions and uveitis. We have reported excessive Th1 cell activity in patients with BD. More recently, Th17 cells were suggested to associate with several autoimmune diseases. This study was designed to investigate the role of Th17 related cytokines and signalling molecules in patients with BD.*

Methods. *We examined mRNA expressions of Th1 and Th17 related cytokines and related signalling molecules in PBMC of 12 patients with BD and 14 normal controls (NC) using quantitative RT-PCR. We studied expressions of the Th17 related cytokines in other four BD patients' skin lesions by immunofluorescence.*

Results. *Major Th17 related cytokines were not detected in unstimulated PBMC in patients with BD. After stimulation, mRNA expressions of TGFβ receptor type 1, IL-12 receptor β2 and suppressor of cytokine signalling protein (SOCS) 1 on PBMC were significantly enhanced in patients with BD, as compared with NC ($p<0.05$). mRNA expression of RORC, a key transcription factor for Th17 cell differentiation, was comparable between BD and NC.*

$CD4^+$ T cells infiltrating into BD skin lesion expressed TGFβ1 much more than those infiltrating into non-Behçet's disease erythema nodosum.

Conclusion. *These findings suggest that TGFβ/Smad signalling pathway of T cells is overactive in patients with BD.*

Introduction

Behçet's disease (BD) is a systemic inflammatory condition that is presumably associated with autoimmunity (1). We previously reported that IFNγ-producing cells were detected in erythema nodosum (EN) of patients with BD (2). Heavy infiltration of $CD4^+$ and $CD8^+$ T cells and enhanced mRNA expressions of proinflammatory and Th1 cytokines/chemokines at intestinal lesions of BD were reported (3). There were several reports that described dominance of the Th1 cytokines by immunohistochemistry (4-6).

Some researchers have investigated intracellular cytokine production and have found predominant expression of IFNγ in BD T cells (7, 8). Th1 dominance was observed in BD uveitis (9) and stomatitis as well (10).

Recently, the Th1/Th2 paradigm was challenged by the discovery of several subsets of helper T cells.

Th17 cells produce a number of proinflammatory cytokines, such as IL-17, IL-17F, IL-21, and IL-22. TGFβ and IL-6 are essential for Th17 cell development in mice, while, adding to them, IL-23 is important in human Th17 pathophysiology.

TGFβ activates TGFβ receptor/Smad signalling pathway and induces expression of retinoic acid-related orphan receptor-C (RORC), which is a master transcription factor of Th17 cells (11).

There have been several reports mentioning abnormal differentiation of $CD4^+$ T cells to Th17 cells in BD (12, 13). Recently, several researchers described that TGFβ was overexpressed in rheumatoid arthritis (RA) (14), whereas it was decreased in systemic lupus erythematosus (SLE) (15).

It was reported that IL-17 concentrations were elevated and Th17 cell frequencies were increased in patients with BD (12, 13, 16-18).

The role of TGFβ/Smad signalling pathway remains largely unclear in BD. Therefore, we here studied the condition of TGFβ/Smad signalling pathway in patients with BD.

Table I. Clinical characteristics of 12 Behcet's disease patients.

Patients	Age/Sex	Condition	Medications
1	32/M	OA, A, S, GIS	colchicine
2	49/F	OA, S, GIS	steroid, cyclosporine, colchicine
3	35/M	OA, S	steroid
4	44/F	OA, GU, S	steroid, colchicine
5	51/F	OA, S, GIS	steroid, cyclosporine, colchicine
6	46/F	OA, GU, S	steroid, colchicine
7	37/M	OA, S	steroid
8	47/M	OA, S	colchicine
9	52/M	OA, S	colchicine
10	25/F	OA, S	colchicine
11	64/F	OA, U, CNS	steroid, colchicine
12	30/F	OA, S	colchicine

OA: oral aphthosis; GU: genital ulcers; A: arthritis; S: skin involvement; U: uveitis; GIS: gastrointestinal system lesions; CNS: central nervous system involvement. None of the patients have been treated with intermediate-high dose corticosteroid therapy (more than 5 mg prednisone/day) or colchicine therapy (more than 0.5 mg/day).

Patients and methods

Patients

PBMC were collected from 12 patients (5 females and 7 males) with BD. Their mean age (±SD) was 42.7±11.12 years (range 25-64 years). The patients fulfilled the diagnostic criteria proposed by the International Study Group of BD (19). Table I summarises the clinical characteristics of the patients. Age and sex matched 14 normal controls (NC) blood donors served as control subjects. None of the patients had been treated with intermediate-high dose corticosteroid therapy (more than 5 mg prednisone/day) or colchicine therapy (more than 0.5 mg/day).

Then we studied specimens of EN with other four BD patients (BD-EN) by skin biopsy (two females and two males), compared with 3 specimens of primary EN without any other systemic immune diseases (primary EN) and 2 normal specimens with non-specific mild inflammation.

This study was conducted with the approval of the institutional review boards and was registered with the University hospital Medical Information Network-Clinical Trials Registry (UMIN000003806). Informed consent was obtained from all the individuals prior to enrolment in the study.

Cell culture and quantitative RT-PCR

PBMC were stimulated for 24 hours with phytohemagglutinin (PHA-M, Sigma-Ardrich, St. Louis, MO).

Total RNA was isolated from PBMC with an RNeasy kit (Qiagen, Venlo, Netherlands). Complementary DNA was synthesised with TaqMan Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA), using random hexamers as primers in accordance with the manufacturer's instruction.

Eukaryotic 18S ribosomal RNA was used as an endogenous control. We studied 25 combinations of TaqMan primers and probes from Applied Biosystems as follows: IL-10, IL-17, IL-17F, IL-21, IL-22, IL-23, TGFβ1, IFNγ, IL-12 receptor β1, 2, IL-23 receptor, TGFβ receptor type1, 2, 3, Smad2, 3, 7, IL-6ST, STAT 1, 3, SOCS 1, 3, Foxp3, ROR-C and Aryl hydrocarbon receptor (AhR).

Expression level of the gene in patients with BD was calculated with the 2^{ddCt} method, and was compared with that in NC.

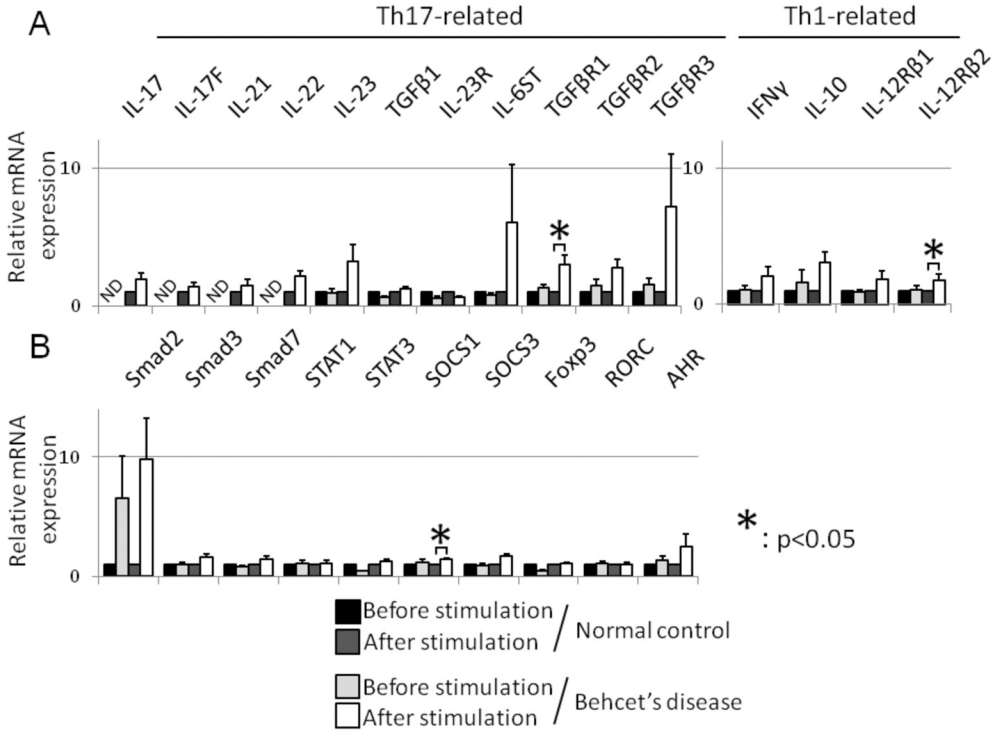

Fig. 1. Relative mRNA expressions of Th17-related cytokines/receptors (**A**) and signalling molecules (**B**) before and after lectin stimulation.

The level of each expression was calculated by the 2^{ddCt} method. Expression level in patients with BD was compared with that in normal controls (NC).

(**A**) We detected mRNA expressions of IL-17, IL-17F, IL-21, and IL-22 on PBMC in neither BD patients nor NC before stimulation. After stimulation, mRNA expressions of TGFβ receptor type 1 and IL-12 receptor β2 in patients with BD were significantly increased as compared with NC.

(**B**) mRNA expression of SOCS1 were significantly enhanced in patients with BD as compared with NC after PHA stimulation. Smad2 mRNA was clearly increased without significance because of its patient to patient variation.

Mean ± SEM of 12 BD patients and 14 NC was shown. An asterisk indicates that the *p*-value was less than 0.05.

Immunofluorescence of skin specimens

We deparaffinised skin tissues and retrieved the relevant antigen with Histo VT One (Nacalai tesque, Kyoto, Japan). The primary antibodies included anti-CD4 (DAKO, Glostrup, Denmark), anti-CD8 (DAKO), anti-TGFβ1 (LifeSpan BioScience, Seattle, WA), anti-TGFβ receptor type 1 (LifeSpan BioScience), anti-IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IFNγ (Bioworld Technology, Minneapolis, MN) and anti- phosphorylated Smad2 (LifeSpan BioScience). We conducted double staining, using a confocal laser scanning microscope.

Statistical analysis

Quantitative data were expressed as the mean ± standard error of the mean (SEM). Wilcoxon Rank Sum tests were performed with JMP statistical software 7.0 (SAS Institute Inc., Cary, NC). A *p*-value of less than 0.05 was considered significant.

Results

mRNA expressions of PBMC before and after lectin stimulation

– Th17 cell related cytokines

We detected mRNA expressions of IL-17, IL-17F, IL-21, and IL-22 in neither BD patients nor NC before stimulation. After stimulation, mRNA expressions of TGFβ receptor type 1 and IL-12 receptor β2 were significantly upregulated in BD (Fig. 1A).

There were no significant differences in other mRNA expressions of Th17 related cytokines between BD and NC. mRNAs of IFNγ, IL-10, IL-23, IL-6ST, TGFβ receptor type 2 and 3 were clearly increased without significance because of their patient to patient variation (Fig. 1A).

– Th17 cell associated signalling molecules

After stimulation, SOCS1 mRNA expression was significantly enhanced in BD compared to NC (Fig. 1B). mRNAs of Smad2 was clearly increased without significance because of their patient to patient variation. There were no significant differences in other mRNA expressions of Th17 associated signalling molecules between BD and NC. Neither RORC nor Foxp3 mRNA expressions showed significant elevation even after stimulation.

These results suggested that mRNA expressions of TGFβ/Smad signalling pathway was enhanced in BD PBMC, irrespective of RORC mRNA expression.

Confocal microscopic analysis of skin lesions in BD

We investigated specimens of EN with 4 BD patients (BD-EN), 3 specimens of primary EN without any other systemic immune diseases (primary EN) and 2 normal specimens with non-specific mild inflammation.

Histological examination revealed that T cells infiltrated into perivascular sites of superficial and deep dermal layers in both BD-EN and primary EN (data not shown). Then we performed double staining with anti-CD4 antibody and antibodies against TGFβ1, TGFβ receptor type 1, IL-17, IFNγ and phosphorylated Smad2. These proteins were mainly expressed on $CD4^+$ and $CD8^+$ T cells and other mononuclear cells in BD-EN and primary EN skin specimens. We demonstrated representative results of a BD-EN (Fig. 2A-E) and a primary EN (Fig. 2F-I).

BD-EN showed infiltrating $CD4^+$ T cells with strong staining intensity of TGFβ (Fig. 2A), while a primary EN showed infiltrating $CD4^+$ T cells with

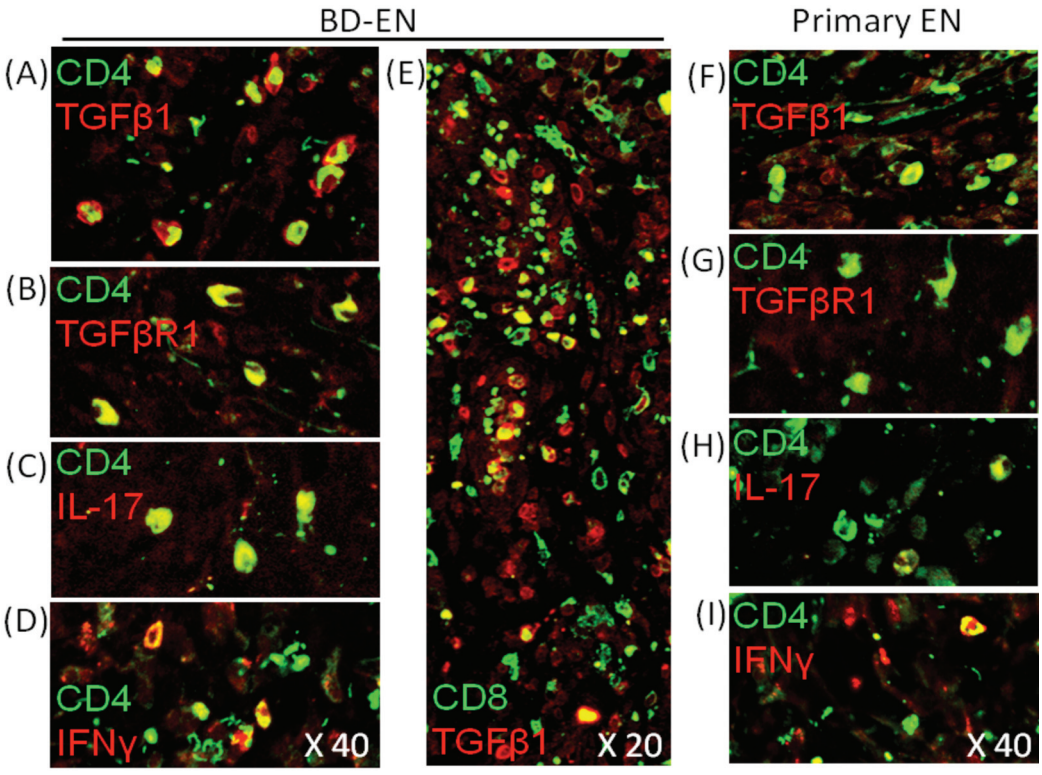

Fig. 2. Confocal analysis of TGFβ expression on skin lesions in patients with BD.
As representative staining results, a skin lesion of a BD patient (BD-EN: **A-E**) and a primary erythema nodosum (Primary EN: **F-I**) were shown.
(**A-D**, **F-I**) High power field views (x40).
(**A, F**) Two-colour staining with CD4 (green) and TGFβ1 (red).
(**B, G**) Two-colour staining with CD4 (green) and TGFβ receptor type 1 (red).
(**C, H**) Two-colour staining with CD4 (green) and IL-17 (red).
(**D, I**) Two-colour staining with CD4 (green) and IFNγ (red).
(**E**) A low power field view of BD-EN (x20). Two-colour staining with CD8 (green) and TGFβ1 (red). This staining shows TGFβ1 positive CD8+ T cells (yellow), TGFβ1 negative CD8+ T cells (green) and CD8- TGFβ1 positive cells (red).

Table II. Immunofluorescence of skin infiltrating CD4+ T cells.

	BD-EN				Primary EN			Normal control	
	pt.1	pt.2	pt.3	pt.4	pt.1	pt.2	pt.3	n.1	n.2
TGFβ1	2+	1+	1+	0	1+	0	0	0	0
TGFβR1	1+	1+	0	0	1+	0	0	0	0
IL-17	2+	1+	1+	0	1+	0	0	0	0
IFNγ	2+	1+	1+	0	2+	1+	0	0	0
pSmad2	1+	1+	1+	1+	1+	1+	1+	1+	1+

'2+' indicates more than 3 infiltrating CD4+ T cells, '1+' indicates 1-2 infiltrating CD4+ T cells in each high power field. Less than above was scored zero. TGFβR1: TGFβ receptor type 1, pSmad2: phosphorylated Smad2.
Erythema nodosum of 4 BD patients (BD-EN), primary erythema nodosum lesions without any other systemic autoimmune diseases from 3 patients (primary EN) and 2 normal controls with non-specific inflammation were examined for cytokine production by immunofluorescence staining.

weak staining intensity of TGFβ (Fig. 2F).

Numbers of tissue infiltrating CD4+ T cells with respective cytokine production are summarised in Table II. Differences in staining intensity of TGFβ were remarkable between BD-EN and primary EN. Figure 2E showed TGFβ1 positive CD8+ T cells (yellow) and TGFβ1 negative CD8+ T cells (green). TGFβ1 positive CD8- cells were also prevalent.

We observed phosphorylated Smad2 protein on epidermal cells, vascular cells and almost all of infiltrating T cells in BD-EN, primary EN and normal specimens (Table II), suggesting that phosphorylation of Smad2 was hardly affected in BD.

Discussion

We demonstrated here that TGFβ/Smad signalling pathway was skewed in patients with BD.

In mouse models, Th17 cells were generated from naïve CD4+ T cells in the presence of IL-6 and TGFβ. In human physiology, cytokine requirement for Th17 cells differentiation remains controversial. Several investigations suggested that a combination of IL-1β and IL-23 induced Th17 differentiation in the absence of TGFβ in humans (20-22).

Against these findings, several reports supported that TGFβ played a role in human Th17 cell differentiation (23-25). It was reported that Th1 and Th2 cells were more susceptible to inhibitory effects of TGFβ and, as a result, TGFβ caused Th17 cell expansion in human peripheral blood (11). Recent studies suggested that TGFβ was dispensable for Th17 differentiation even in mouse models (26, 27).

Collectively, the relationship between Th17 cell differentiation and TGFβ was not fully understood even in physiological conditions.

In patients with BD, Th17 cells were increased and signalling molecules of Th17 cell differentiation were upregulated (12, 13, 16-18). The role of TGFβ in skewed Th17 cell differentiation in patients with BD remains largely unclear. This is an initial report elucidating a skewed TGFβ/Smad signalling pathway in patients with BD.

TGFβ/Smad signalling pathway regulates several biological functions such as morphogenesis, embryonic development, adult stem cell differentiation, immune responses and wound healing. It is thus reasonable that alteration of TGFβ/Smad signalling pathway causes a wide range of human diseases (28, 29).

We found several articles which discussed the involvement of TGFβ/Smad signalling pathway in human diseases (14, 15, 30-33). In patients with BD, serum TGFβ concentrations were not elevated even in active disease phases, whereas synovial fluid TGFβ level was clearly increased (34, 35).

We have reported that naïve CD4+ T cells excessively responded to both Th1 (IL-12 and anti-IL-4 antibody) and Th17 cytokines (IL-1β, IL-6, IL-23, anti-IFNγ antibody and anti IL-4 antibody) in patients with BD (36). In this study, we found a skewed TGFβ/Smad signalling pathway of T cells in patients with BD. The results suggested the existence of an unknown mechanism in T cells to enhance TGFβ/Smad signalling pathway in patients with BD. Further investigations focusing on subsequent T cell signal transduction systems are needed in order to understand the pathogenic role of TGFβ/Smad signalling pathway in patients with BD.

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