Preferential activation of circulating CD8+ and γδ T cells in patients with active Behçet's disease and HLA-B51

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ABSTRACT

Objective. *To evaluate the activation status of circulating CD4+, CD8+, and γδ T cells in patients with active and inactive Behçet's disease (BD).*

Methods. *We studied 11 subjects with active BD, 28 with inactive BD, and 13 healthy controls. The expression of CD4, CD8, pan-γδ, Vδ1, and Vδ2 along with the early activation marker CD69 was analyzed by 3-color flow cytometry.*

Results. *Proportions of activated CD8+ and γδ T cells were significantly greater in patients with active BD than in those with inactive BD or healthy control subjects, but the proportion of activated CD4+ T cells did not differ among these 3 groups. In addition, significantly greater proportions of the Vδ1+ and Vδ2+ γδ T-cell subsets were activated in patients with active BD than in those with inactive BD or healthy controls; in active BD, the balance of activation between these subsets favored the Vδ1+ T cells. No significant differences in these proportions were found between subjects with inactive BD and healthy controls. These findings were observed exclusively in patients with HLA-B51. A comparison of samples from 5 patients taken during active BD and after resolution of BD-related symptoms showed the proportions of activated CD8+ and γδ T cells dropped when the patients' BD became inactive.*

Conclusion. *CD8+ and γδ T cells, rather than CD4+ T cells, were activated in vivo in patients with active BD and HLA-B51, but not in those with inactive BD, suggesting that these potentially cytotoxic T cells play a critical role in BD flares.*

Introduction

Behçet's disease (BD) is an inflammatory disorder characterized by uveitis, oral aphthous ulcers, genital ulcers, and skin lesions. The etiology and immunopathogenesis of this disease remain unclear, although one hypothesis suggests that it is induced by a dysregulation of immune responses to microorganisms in genetically predisposed individuals (1). Previous studies on the pathogenesis of BD have mainly focused on CD4+ T cells, especially T helper (Th) cytokine balance, and have shown excessive Th1-polarized T-cell functions in BD, based on an increased interferon-γ-producing T cells in circulation and affected tissues of BD patients (2, 3). However, CD8+ T cells and γδ T cells also accumulate in the affected lesions (4, 5). To investigate which T-cell subset contributes to the pathogenic inflammatory response of BD, we evaluated the activation status of circulating CD4+, CD8+, and γδ T cells in BD patients in relation to disease status.

Materials and methods

Patients and controls

We studied 39 patients with BD (17 males, 45.8±13.5 years) who fulfilled the criteria proposed by an International Study Group (6). Thirteen healthy individuals (7 males, 30.2±7.7 years) served as control subjects. Clinical characteristics during the entire disease course and drugs used at blood examination in BD patients are summarized in Table I. The BD of the patients was classified as active in 11 and inactive in 28 at the time of blood sampling. Active disease was defined as the presence of characteristic BD symptoms, including severe oral/genital ulcers and ocular involvement that required introduction or increase of systemic corticosteroids, intraocular administration of corticosteroids, and/or cyclosporine (7). Some patients with active disease were re-evaluated after their symptoms improved. All blood samples were obtained after the patients and control subjects had given their written informed consent, as approved by the Keio University Institutional Review Board.

Table I. Clinical features of 39 BD patients analyzed in this study.

Male	17	(44%)
HLA-B51	20	(51%)
Oral ulcer	39	(100%)
Ocular lesion	26	(67%)
Skin lesion	36	(92%)
Genital ulcer	24	(62%)
Intestinal involvement	6	(15%)
Vascular involvement	4	(10%)
Neurological involvement	7	(18%)
Current use of corticosteroids	15	(38%)
Current use of colchicine	10	(26%)
Current use of cyclosporine	4	(10%)

HLA-B51 genotyping

HLA-B51 was detected by polymerase-chain reaction using sequence-specific primers and sequence-based typing (8).

Flow cytometric analysis

Unfixed peripheral blood mononuclear cells were stained with fluorescence-conjugated monoclonal antibodies to CD4, CD8α, CD69, NKG2D (Beckman Coulter, Fullerton, CA, USA), pan-γδ (BD Biosciences, San Diego, CA, USA), Vδ1, and Vδ2 (Endogen, Woburn, MA, USA). Cells incubated with fluorescence-labeled isotype-matched antibodies to irrelevant antigens were the negative controls. The cells were analyzed by 3-color flow cytometry using a FACS®Calibur flow cytometer (BD Biosciences) with CellQuest software. Viable lymphocytes were selected by exclusion of apoptotic cells stained with propidium iodide (Sigma, St. Louis, MO, USA). Lymphocytes were then identified by gating on forward and side scatter, and the proportion of $CD69^+$ activated T cells was calculated for $CD4^+$, $CD8^+$, pan-$\gamma\delta^+$, $V\delta1^+$, or $V\delta2^+$ T-cell subsets. In some samples, the relative expression level of NKG2D, a potential T cell-activating molecule, on cells gated for $CD8^+$ or γδ T cells was evaluated using the mean fluorescence intensity (MFI) as an index. The NKG2D expression levels in individual samples were adjusted by the result of stored peripheral blood mononuclear cells derived from a single healthy donor, which were included in each experiment. Consistent settings for detector sensitivity, compensation, and scatter gating were used in the analyses of all the samples.

Statistical analysis

All results were expressed as the mean ± standard deviation. Statistical comparisons between 2 groups were performed using the Mann-Whitney U-test. Data obtained at serial time points were compared by the Wilcoxon test. All statistical procedures were performed using StatView software version 5.0 (SAS Institute, Cary, NC, USA).

Results

Activation status of circulating $CD4^+$, $CD8^+$, and γδ T cells

To evaluate the activation status of $CD4^+$, $CD8^+$, and γδ T cells, the proportions of $CD69^+$ activated T cells in individual T-cell subsets in 11 patients with active BD, 28 with inactive BD, and 13 healthy controls were measured using flow cytometry (Fig. 1A, left panel). As shown in Figure 1B, the proportions of activated $CD8^+$ and γδ T cells were significantly greater in patients with active BD than in those with inactive BD or healthy controls, but these proportions did not differ between patients with inactive BD and healthy controls. In contrast, there was no statistically significant difference in the proportion of activated $CD4^+$ T cells among the groups. These findings suggest that $CD8^+$ and γδ T cells were activated *in vivo* in patients with active BD, but $CD4^+$ T cells were not.

NKG2D expression on $CD8^+$ and γδ T cells

Patients with active BD showed a relatively large population of circulating $CD8^+$ and γδ T cells with the activated phenotype, suggesting that these cells are activated through a receptor expressed on both $CD8^+$ and γδ T cells, rather than through the engagement of T-cell receptors with particular antigens. One such potential molecule is a natural killer cell-activating receptor, NKG2D, which is expressed on the majority of $CD8^+$ and γδ T cells, but on only a minor subset of $CD4^+$ T cells (9). However, we found that NKG2D was expressed on >80% of the $CD8^+$ and γδ T cells in 17 patients with BD and 9 healthy controls. We further examined the expression level of NKG2D on $CD8^+$ and γδ T cells, as reflected by the MFI (Figure 1A, right). The NKG2D expression in 6 patients with active BD, 11 with inactive BD, and 9 healthy controls were almost equivalent for the $CD8^+$ T cells (23±11, 25±11, and 25±17, respectively) and γδ T cells (27±12, 30±20, and 30±20, respectively), indicating that enhanced NKG2D expression is not responsible for the specific increase of T-cell activation in active BD.

Activation status of circulating $V\delta1^+$ and $V\delta2^+$ γδ T cells

Since the majority of γδ T cells in the human peripheral blood are classified as $V\delta1^+$ and $V\delta2^+$ T cells (10), we examined which γδ T cell subsets were preferentially activated in patients with active BD. As shown in Figure 1C, the proportions of $V\delta1^+$ and $V\delta2^+$ γδ T-cell subsets among the $CD69^+$ activated T cells were significantly higher in patients with active BD than in those with inactive BD or healthy controls, and no significant difference in these proportions was found between the latter two groups. Interestingly, the ratio of activated $V\delta1^+$ T cells to activated $V\delta2^+$ T cells was significantly higher in patients with active BD than in patients with the inactive disease or healthy controls.

Associations of T cell activation status with clinical parameters

To further evaluate potential associations between activation status of $CD8^+$ and γδ T cells and clinical parameters, BD patients were divided into two groups based on the presence or absence of individual clinical features listed in Table I, and T cell activation status was

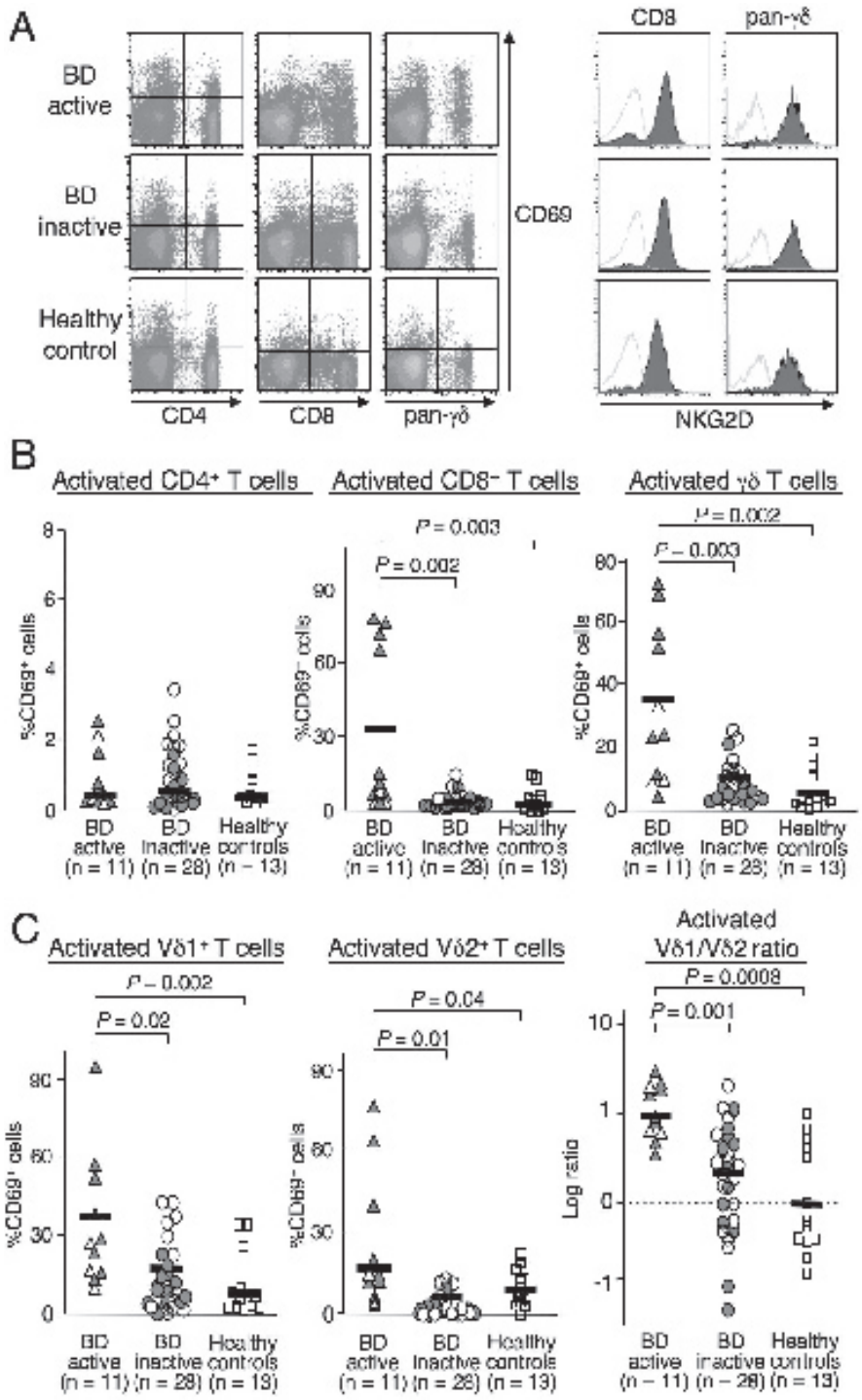

Fig. 1. Detection of CD69+ activated T cells in CD4+, CD8+, and γδ T-cell subsets in patients with active BD, patients with inactive BD, and healthy controls. A. Representative flow cytometric findings for CD69+ activated CD4+, CD8+, and γδ T cells (*left*) and expression of NKG2D on CD8+ and γδ T cells (*right*) from a patient with active BD, a patient with inactive BD, and a healthy control. Activated T cells were identified as cells double-stained for CD69 together with CD4+, CD8+, or pan-γδ. The expression of NKG2D was evaluated for T cells gated on CD8+ or γδ. Gray solid lines showed the cells stained with isotype-matched control antibody, and black solid lines with filling showed anti-NKG2D antibody-treated cells. B. Proportions of activated CD4+, CD8+, and γδ T cells in peripheral blood from 11 patients with active BD, 28 with inactive BD, and 13 healthy controls. C. Proportions of activated Vδ1+ and Vδ2+ γδ T cells and the ratio of activated Vδ1+ T cells to activated Vδ2+ T cells in peripheral blood from 11 patients with active BD, 28 with inactive BD, and 13 healthy controls. HLA-B51-positive patients are shown as closed triangles or circles, and HLA-B51-negative patients are shown as open triangles or circles. The bar in each data set denotes the mean. Statistical comparisons between 2 groups were performed using the Mann-Whitney *U*-test.

compared between patients with active disease and those with inactive disease. As a result, the sole parameter that correlated with activation status of CD8+ and γδ T cells was the presence or absence of HLA-B51. Specifically, increased proportions of activated CD8+ and γδ T cells and increased activated Vδ1+/Vδ2+ ratio were observed exclusively in HLA-B51-positive patients, although the number of active BD patients lacking HLA-B51 was small (Table II).

Serial analysis of T cell activation status

Five patients, including 3 positive for HLA-B51, who had active BD at the time of blood sampling were evaluated again after their symptoms resolved. All 5 patients had ocular attacks of uveitis and required systemic or intraocular administration of corticosteroids. Second evaluation was performed when ocular involvement was quiescent, and intervals between two evaluations ranged from 2 to 26 months. As shown in Figure 2, the proportions of activated γδ and Vδ1+ T cells decreased with the BD-related symptoms, and these changes were statistically significant. In addition, activated CD8+ T cells were markedly decreased in all except one patient who lacked HLA-B51 and had recurrent oral and genital ulcers without requiring systemic corticosteroids.

Discussion

In this study, we demonstrated that circulating CD8+ and γδ T cells were activated *in vivo* in patients with active BD, especially in those with HLA-B51. The activated phenotype of these potentially cytotoxic T cells was associated with the active disease status rather than with BD itself, because (i) there was no difference in these proportions between patients with inactive BD and healthy controls; and (ii) the increased proportions of activated CD8+ and γδ T cells returned to the levels seen in healthy controls after the BD-related symptoms resolved. Thus, CD8+ and γδ T cells with activated phenotype may play an important role in the pathogenic processes of BD, potentially by exerting their capacity to directly damage cells

Table II. Proportions of CD69$^+$ activated T cells in CD4$^+$, CD8$^+$, and γδ T-cell subsets in patients with active and inactive BD according to the presence or absence of HLA-B51.

Activated lymphocyte subsets	HLA-B51-positive			HLA-B51-negative		
	Active (n=8)	Inactive (n=12)	*p*	Active (n=3)	Inactive (n=16)	*p*
Activated CD4$^+$ T cells (%)	0.7 ± 0.9	0.6 ± 0.9	NS	0.9 ± 1.2	0.4 ± 0.4	NS
Activated CD8$^+$ T cells (%)	33 ± 40	2 ± 2	0.0005	5 ± 3	3 ± 4	NS
Activated γδ T cells (%)	30 ± 28	6 ± 5	0.002	14 ± 13	9 ± 8	NS
Activated Vδ1$^+$ γδ T cells (%)	40 ± 28	17 ± 24	0.002	17 ± 8	16 ± 16	NS
Activated Vδ2$^+$ γδ T cell (%)	27 ± 29	4 ± 3	0.0003	5 ± 4	5 ± 3	NS
Activated Vδ1/Vδ2 ratio	1.0 ± 0.6	- 0.1 ± 0.1	0.002	0.8 ± 0.4	0.3 ± 0.5	NS

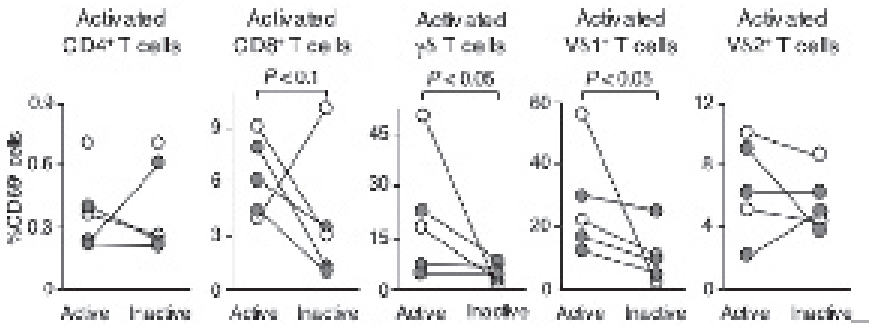

Fig. 2. Comparative analysis of the proportions of CD69$^+$ activated CD4$^+$, CD8$^+$, γδ, Vδ1$^+$, and Vδ2$^+$ T cells in peripheral blood taken from 5 patients during active BD and after their BD-related symptoms had resolved. HLA-B51-positive patients are shown as closed circles, and HLA-B51-negative patients are shown as open circles. Statistical significance was examined by the Wilcoxon test.

at the site of inflammation, such as the skin and mucous membrane.

In contrast, there was no apparent increase in the proportion of activated CD4$^+$ T cells in BD patients, regardless of their disease status. This finding was rather unexpected, but does not necessarily indicate that there is no role for CD4$^+$ T cells in BD pathogenesis. The expression of activation markers on T cells is principally induced upon recognition of antigenic peptides presented by antigen-presenting cells via T-cell receptors. Considering the huge diversity of T-cell receptors, antigen-specific activation of a restricted T-cell repertoire would not alter the proportion of circulating CD69$^+$ T cells. Since circulating CD4$^+$ T cells from patients with active BD tend to generate a Th1 response to appropriate mitogenic or antigenic stimulation (2, 3, 11), the activation of a limited repertoire of CD4$^+$ T cells should be sufficient to promote a Th1-dominant cytokine response.

Regarding γδ T-cell subsets activated *in vivo* in BD patients, activation of both Vδ1$^+$ and Vδ2$^+$ subsets were detected in patients with active disease status. Although accumulation of γδ T cells was previously reported in circulation, skin, oral mucous membrane, and cerebrospinal fluid in patients with BD, but mechanisms for dysregulated γδ T cells still remain unclear (4, 12-16). Previous researches focused on the role of Vδ2$^+$ T cells in the pathogenesis of BD (12-15), and found an increased proportion of activated Vδ2$^+$Vδ9$^+$ T cells in patients with active BD. Since Vδ2$^+$ T cells are activated by recognition of bacterial components (17), activation of Vδ2$^+$ T cells in patients with active BD may explain clinical observations that BD flare is often triggered by infection. On the other hand, we reported here that activation of Vδ1$^+$ T cells was more prominent, compared with Vδ2$^+$ T cell activation, in HLA-B51-positive patients with active BD. This finding supports accumulation of cytotoxic Vδ1$^+$ T cells at inflammatory sites of BD patients (16). In this regard, a subset of Vδ1$^+$ T cells are shown to recognize the MHC class I chain-related complex A (MICA) molecule (18), which is selectively expressed by epithelial and endothelial cells in a stress-inducible manner (19). Since MICA molecule is a ligand for the Vδ1$^+$ γδ T-cell receptor, but not for the Vδ2$^+$ γδ T-cell receptor (18), preferential activation of Vδ1$^+$ T cells observed in patients with active BD could be explained, in part, by increased interactions between the Vδ1$^+$ γδ T-cell receptor and MICA.

Activation of circulating CD8$^+$ and γδ T cells was prominent in BD patients possessing HLA-B51. In this regard, we have recently reported that HLA-B51-positive patients with active BD have HLA-B51-restricted CD8$^+$ cytotoxic T cells that are autoreactive to MICA (7). In patients with HLA-B51, after non-specific minor injuries and microbial infection, MICA-reactive cytotoxic T cells might be activated upon recognition of the MICA-derived peptide presented on the epithelium and endothelium and subsequently lead to excessive and prolonged inflammatory responses at the site of stress by enhancing the MICA-mediated cytotoxicity induced by NKG2D expressed on both CD8$^+$ and γδ T cells.

In summary, CD8$^+$ and γδ T cells are activated *in vivo* in HLA-B51-positive BD patients with clinically active disease, suggesting a direct involvement of CD8$^+$ and γδ T cells in the disease flare. Further studies are necessary to clarify the mechanisms of activating CD8$^+$ and γδ T cells and the role of these potentially cytotoxic T cells in the development of the inflammatory response in HLA-B51-positive patients with BD.

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