T and NK cell subset changes with microbial extracts and human HSP60-derived peptides in Behçet's disease

A. Kibaroglu[1], E. Eksioglu-Demiralp[1], T. Akoglu[1], H. Direskeneli[2]

Departments of [1]Hematology-Immunology and [2]Rheumatology, Marmara University Medical School, Istanbul, Turkey.

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Please address correspondence to: H. Direskeneli, MD, Department of Rheumatology, Marmara University Medical School Hospital, Tophanelioglu Cad. 13/15, 81190 Altunizade, Istanbul, Turkey. E-mail: direskeneli@superonline.com

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ABSTRACT

Objective. *Microorganisms such as streptococcus and autoantigens such as 60 kD heat-shock protein (HSP60) are implicated in the etiopathogenesis of Behcet's disease (BD).*

Methods. *Peripheral blood mononuclear cells from patients with BD (n = 16) and healthy controls (HC) (n = 11) were cultured for 5 days with extracts of* S. sanguis-*KTH-1 (SS),* E. coli *(EC) and a mixed peptide combination from human HSP60 (aa 136-50, 179-97, 224-58 and 336-51) reported to be associated with BD. T and NK cell subset changes were determined by flow cytometry.*

Results. *In unstimulated 5-day cultures* $\gamma\delta^+$ *(both* $CD4^+\gamma\delta^+$ *and* $CD8^+\gamma\delta^+$*),* $CD8^+\alpha\beta^+$*,* $CD4^+CD56^+$ *and* $CD8^+CD11b^+$ *cells were increased in BD compared to HC. In antigen-stimulated cultures of BD patients* $CD3^+$ *and* $\alpha\beta^+$ *T cells responded to HSP60 peptides whereas EC stimulated only* $CD16/CD56^+$ *NK cells. In the control group, similar to BD,* $\alpha\beta^+$ *and* $CD4^+$ *T cells responded to HSP60 peptides, however SS and EC mainly activated cytotoxic T cell subsets (*$CD8^+CD11b$ *and* $CD4^+CD56^+$ *Tcells).*

Conclusion. *Significant increases in unstimulated T cell subsets suggest the presence of an* in vivo *T cell activation in BD. In both patients and controls similar patterns of responses were observed against different microorganisms, however the role of human HSP-60 peptides as immunodominant, cross-reactive antigens could not be demonstrated.*

Introduction

Various microorganisms such as Streptococcus and Herpes Simplex virus are implicated in the etiopathogenesis of Behcet's disease (BD), a systemic vasculitis of unknown etiology (1, 2). Clinical observations such the presence of oral apthae as the first manifestation in 70% of the patients, increased oral manifestations after dental manipulations, hypersensitivity to streptococcal skin tests, dominance of atypical streptococcal species in BD patients' oral flora, and the recent reports of benefical anti-bacterial therapy put forward the role of streptococcus in BD (3-8).

In elucidating the immune mechanisms of streptococcal pathology, KTH-1 (a crude extract of *Streptococcus sanguis* SSH-83, SS) has been found to stimulate IL-6 and IFN-γ secretion by the peripheral blood T cells of BD patients (9). Oral bacterial flora is also shown to activate $\gamma\delta^+$ T cells in BD (10). KTH-1 upregulates $\gamma\delta^+$ T cells in short-term T cell cultures and KTH-1 specific $\gamma\delta^+$ T-cell lines secrete pro-inflammatory mediators IL-6, IL-8 and TNF-α (11,12). The main antigenic stimuli of *S. sanguis* is suggested to be a 95 kD BES-1 gene product consisting of 849 amino acid residues (13).

However, the specificity of the anti-streptococcal response has not been studied in detail in comparison to other microorganisms such as gram negatives in BD (2). Although not significantly different compared to controls, *Escherichia coli* (EC) also caused a higher incidence of skin reactions in BD patients (4). In addition to streptococcal antigens, *E. coli* and *Staphylococcus aureus* also activate BD lymphocytes to release increased amounts of IFN-γ and IL-6 (9). Behcet's T lymphocytes secrete IFN-γ in response to very low (1-10 pg/ml) doses of Staphylococcal superantigens SEB and SEC1 compared to controls and Hirohata *et al.* proposed a T cell hyperreactivity in BD, unrelated to a species-specific response (14).

Heat-shock proteins (HSP) are highly conserved proteins among microorganisms and mammals and HSP60/65 has been suggested as an immunodominant antigen in BD, associated with the "cross-reactivity" of microbial HSP60 with its human homologue (3, 15). Anti-mycobacterial HSP65 antibodies

are shown to be cross-reactive with oral mucosal homogenates and oral streptococcus (16). Four epitopes of mycobacterial HSP65 (amino acid sequences 111-25, 154-72, 219-33 and 311-26) and their human counterparts with 50-80% homology, were recognized to be immuno-dominant antigens for T and B cell responses in BD in studies from UK, Japan and Turkey (15, 17-19).

With this background, in this study we aimed to investigate the effects of microbial (*E. coli* and *S. sanguis*) and auto immune stimulation with human HSP-60 peptides on peripheral blood lymphocyte subsets in BD patients to test whether anti-microbial responses against streptococcus are specific and associated with human HSP60 responses.

Materials and methods

Patients and controls

Sixteen patients with BD (6 males, 10 females; mean age: 35, range 24-51), diagnosed according to the International Study Group Criteria for BD (20) and followed in the multidisciplinary Behçet's Disease Out-patient Clinic at Marmara University Hospital in Istanbul were studied. At the time of the study, all patients had clinically active disease with at least two clinical manifestations. All patients had a history of oral ulcers, 13 (81%) had folliculitis, 12 (75%) had genital ulcers, 10 (63%) had erythema nodosum, 5 (31%) had venous thrombosis and 4 each (25%) had arthritis or ocular involvement. The pathergy test was positive in 9 (56%) of the patients. Nine patients were HLA-B51-positive. Eleven patients were treated with colchicine (1-2 mg/day), whereas no patients were using immunosuppressive treatment at the time of sampling. Eleven healthy volunteers (6 males, 5 females; mean age: 33, range 21-46) with no signs or symptoms of BD or any other disease, were studied as normal controls. The study was approved by the Institutional Review Board and informed consent was obtained from all patients.

Preparation of antigenic stimulants

Streptococcus sanguis SSH-83 (KTH-1) were kindly provided by F. Kaneko, Japan. Bacteria were washed 3 times by centrifugation, re-suspended in PBS, and incubated at 42°C for 30 minutes to stimulate HSP expression. After killing at 85°C for 20 minutes, whole bacterial cells were subjected to sonification for obtaining their soluble extracts. Extracts were then centrifuged and washed twice with PBS. Protein contents of the extracts were estimated acccording to Lowry method. In each experiment, 10 mg/ml of soluble bacterial extracts were used.

Human HSP60 derived peptides 136-150, 179-197, 224-258 and 336-351, previously demonstrated to be immunodominant in BD (17,19), were synthesized (Albachem, Ireland). As T-cell responses to single peptides were low in frequency and magnitude (19), we mixed the four peptides together in equal concentrations of 50 mgr each in serum-free RPMI-1640 medium.

Cell seperation and culture

Blood was taken from the patients and controls into heparinized tubes and peripheral blood mononuclear cells (PBMNCs) were separated by standard Hypaque/Ficoll density gradient centrifugation. Cell counts were determined by using automatic blood count analyser (Coulter, USA). Serum samples were also isolated using heparin-free tubes and inactivated at 56°C for 30 minutes. All cultures were carried out in RPMI-1640 medium supplemented with 1% Penicillin-Streptomycin, 1% Sodium pyruvate, 0.3 mg/ml L-Glutamine and 10% freshly prepared autologous serum. 3-4 x 10^6 T-cells and monocytes were cultured in flat-bottom 6-well plates (Greiner, Germany) in a total volume of 3 ml in the presence or absence of antigenic stimulators. The cells were incubated for 5 days until the immunophenotypical analysis at 37°C in a humified atmosphere of 5% CO_2.

Immunophenotyping

After 5-day incubation period, non-adherent cells were harvested and washed twice with PBS. Cells were resuspended in PBS and incubated with saturating amount of appropriate antibodies for 30 min at room temperature. Flow cytometric analysis were performed using a FACSort instrument (Becton Dickinson, Mountainview, CA). Following MoAbs were used for double stainings; anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD16, anti-CD56, anti-αβ TCR and anti-γδTCR. Background fluorescence was eliminated by running isotype matched PE- and FITC-conjugated mouse IgGs as negative controls (Pharmingen, San Diego, CA). Data were acquired and analyzed using the Cell Quest software. The lymphocye population was identified on a scatter plot, based on forward angle light scatter and 90° light scatter. To eliminate cell debris and electronic noise contaminating the lymphocyte gated population, a leucocyte common antigen, anti-CD45, was used. An anti-CD14 was used to examine monocyte contamination which was consistently less than 1.0%. For dual color fluorescence a cytogram was generated and 20000 cells were analyzed.

Statistical analysis

Kruskal-Wallis and Mann Whitney-U tests are used for statistical analysis. $P < 0.05$ was considered as significant.

Results

In unstimulated cultures, $\gamma\delta^+$ T cells, (both $CD4^+\gamma\delta^+$ and $CD8^+\gamma\delta^+$ subsets), $CD8^+\alpha\beta^+$, $CD4^+CD56^+$ and $CD8^+CD11b^+$ T lymphocyte subsets were found to be upregulated significantly in BD compared to HC after 5-day incubations (Table I). Only $\alpha\beta/CD4^+$ T cells were significantly higher in HC compared to BD ($p = 0.02$). Although not reaching statistical significance, $CD16^+/CD56^+$ NK subset were also elevated in HC ($p = 0.08$).

Although mild changes were also observed in $\alpha\beta^+$, $\gamma\delta^+$ and $CD8^+CD11b^+$ T cell subsets, only $CD16^+CD56^+$ NK cells increased significantly with *E. coli* stimulation in BD (Table II). A similar trend was also observed with *S. sanguis*, without reaching statistical significance. On the other hand, *E. coli* and *S. sanguis* both increased $CD56^+$ (especially of $CD4^+CD56^+$T cells) and $CD8^+CD11b^+$ cytotoxic T cell subsets in HC (Table III). $\gamma\delta^+CD8^+$ T cells are also increased significantly with *S. sanguis* in HC, with a similar but not significant trend with *E. coli*.

Table I. T and NK cell subsets in BD patients and controls after unstimulated (medium only) 5-day cultures (mean ± SD).

Cell subsets	Behçet's disease (n = 16)	Healthy controls (n = 11)	p
CD3	79.1 ± 9.2	71.0 ± 12.1	
CD4	50.7 ± 10.9	51.8 ± 2.8	
CD8	35.0 ± 5.7	30.1 ± 6.5	
CD11b	11.4 ± 8.6	4.7 ± 3.3	0.01
CD16	11.7 ± 7.0	14.3 ± 7.4	
CD56	12.6 ± 3.7	12.9 ± 5.5	
αβ TCR	73.5 ± 11.5	70.5 ± 4.6	
γδTCR	5.9 ± 3.9	2.8 ± 1.4	0.01
αβ / CD4	39.8 ± 11.9	48.1 ± 5.8	0.02
γδ/ CD4	1.9 ± 0.7	0.7 ± 0.6	0.04
αβ / CD8	27.8 ± 6.9	22.5 ± 4.0	0.03
γδ/ CD8	5.6 ± 4.4	1.4 ± 1.1	0.002
CD16 / CD56	6.2 ± 2.6	8.5 ± 3.6	
CD4 / CD16	0.9 ± 0.8	0.9 ± 0.3	
CD4 / CD56	2.7 ± 1.7	1.3 ± 0.4	0.03
CD8 / CD11b	7.1 ± 5.1	2.1 ± 1.5	0.008

Table II. T and NK cell subsets after *in vitro* stimulation in patients with Behçet's disease (mean ± SD).

	Medium only	*E. coli*	*S. sanguis*	HSPPeptide Mix
CD3	79.1 ± 9.2	81.4 ± 1.3	80.9 ± 10.0	86.9 ± 9.00*
CD4	50.7 ± 10.9	50.6 ± 9.8	51.0 ± 8.2	55.6 ± 11.6
CD8	35.0 ± 5.7	34.5 ± 7.6	36.7 ± 8.6	33.3 ± 7.7
CD11b	11.4 ± 8.6	12.3 ± 7.1	11.6 ± 7.2	9.2 ± 6.9
CD16	11.7 ± 7.0	13.2 ± 6.5	13.9 ± 6.5	10.4 ± 7.7
CD56	12.6 ± 3.7	14.1 ± 3.6	14.1 ± 4.8	11.7 ± 5.5
αβ TCR	73.5 ± 11.5	75.0 ± 12.3	75.3 ± 12.6	80.8 ± 13.1*
γδTCR	5.9 ± 4.0	7.9 ± 3.7	7.5 ± 4.6	7.7 ± 5.1
αβ / CD4	39.8 ± 12.0	44.7 ± 11.4	43.0 ± 12.9	48.9 ± 13.5**
γδ/ CD4	1.9 ± 0.7	2.2 ± 2.5	1.3 ± 1.4	1.8 ± 3.1
αβ / CD8	27.8 ± 6.9	27.2 ± 7.5	29.9 ± 9.1	28.6 ± 8.3
γδ/ CD8	5.6 ± 4.4	7.5 ± 5.5	7.9 ± 5.4	7.6 ± 9.9
CD16 / CD56	6.2 ± 2.6	8.0 ± 3.7 *	7.9 ± 4.0	5.5 ± 3.8
CD4 / CD16	0.9 ± 0.8	0.8 ± 0.4	0.8 ± 0.6	0.9 ± 0.4
CD4 / CD56	2.73 ± 1.73	2.6 ± 1.7	3.1 ± 2.7	2.9 ± 1.9
CD8 / CD11b	7.1 ± 5.1	10.0 ± 7.0	8.7 ± 5.5	6.5 ± 4.3

*p < 0.05; **p = 0.06 – 0.07.

With HSP60 peptide stimulation, only $\alpha\beta^+$ T cell subset (mainly of $CD4^+\alpha\beta^+$ T cells) increased significantly in BD. In HC, in addition to $\alpha\beta^+$, $CD4^+CD56^+$ T cells are also increased compared to unstimulated cultures. Although mild elevations were observed, changes in $\gamma\delta^+$ T cell subsets did not reach significance in both groups. There was also no correlation between the individual HLA-B51 expressions, colchicine treatment and cell surface antigen expressions after stimulation (data not shown).

Discussion

The innate immune system detects infection using a variety of pattern-recognition receptors that recognize pathogen-associated molecular patterns on microorganisms and trigger various effector responses. Interaction with and control of the adaptive immune system is also a crucial role of the innate immunity. Viral or streptococcal infections have been suggested to be associated with BD and a dysfunction of the innate immune system might predispose to persistant immune activation after infectious stimuli. Abnormal adaptive immune responses, such as tissue lymphocyte/phagocyte infiltrations and a Th1-type, pro-inflammatory cytokine profile with enhanced IL-12 and IFNγ expressions are then induced (2,15). In this context, lymphocyte and NK-mediated cytotoxic responses are possibly also augmented in BD.

In our study, BD patients had higher levels of both $CD4^+$ and $CD8^+\gamma\delta^+$ T cells in addition to cytotoxic-supressor T cell subsets $CD8^+\alpha\beta^+$, $CD4^+CD56^+$ and $CD8^+CD11b^+$ T cells compared to healthy controls in unstimulated 5-day cultures. These results are in agreement with our previous report of increased $CD4^+CD56^+$ T cells in BD in unstimulated, directly analysed PBMCs (21).

Two different cell populations, $CD8^+$ T cells and NK cells, mediate the cytotoxic function of the immune system. Recent studies have shown that a subset of $CD4^+$ T cells can also have cytotoxic activity (22). This subset expressing CD56 antigen is generally found in close proximity to inflammatory sites and only a small proportion has been detected in the peripheral blood (23, 24). Therefore, $CD4^+CD56^+$ phenotype is regarded to be associated with organ and dermal associated cytotoxic T cells (25).

When we compared responses against two different microorganisms *S. sanguis* and *E. coli*, although similar trends were observed in most subsets, the only subset with a significant response was $CD16^+CD56^+$ NK cells with *E. coli*. The role of NK cells is controversial in BD, with normal, decreased or elevated reports in the peripheral blood (21, 26, 27). NK cell activity measured by K562 cytotoxicity was observed to be low, explained by immature NK cells in active patients (27). However, increased levels of NK cells after bacterial stimulation is not previously reported in BD. The similarity of responses with two different microorganisms suggest that this is possibly a general anti-

Table III. Tand NK cell subsets after *in vitro* stimulation in healthy controls (mean ± SD).

	Medium only	*E. coli*	*S. sanguis*	HSPPeptide Mix
CD3	71.0 ± 12.1	74.2 ± 10.9	76.7 ± 12.5	79.5 ± 9.7*
CD4	51.8 ± 2.8	51.6 ± 4.1	54.9 ± 5.0	56.5 ± 5.7*
CD8	30.1 ± 6.5	31.9 ± 6.8	31.8 ± 6.9	29.2 ± 6.9
CD11b	4.7 ± 3.3	7.0 ± 2.4**	6.8 ± 2.8**	5.3 ± 2.1
CD16	14.3 ± 7.4	16.0 ± 8.9	15.6 ± 7.9	12.2 ± 6.6
CD56	12.9 ± 5.5	18.2 ± 6.2*	18.9 ± 4.3*	13.6 ± 4.4
αβ TCR	70.5 ± 4.6	72.0 ± 7.4	75.5 ± 7.7	77.9 ± 5.5*
γδTCR	2.8 ± 1.4	4.0 ± 2.3	3.7 ± 2.3	3.91 ± 3.1
αβ / CD4	48.1 ± 5.8	47.9 ± 5.0	51.6 ± 4.4	47.5 ± 15.5
γδ / CD4	0.7 ± 0.6	1.1 ± 1.0	1.4 ± 1.5	1.0 ± 0.6
αβ / CD8	22.5 ± 3.9	22.9 ± 4.3	24.2 ± 5.4	24.7 ± 5.1
γδ / CD8	1.3 ± 1.1	2.6 ± 2.0	2.5 ± 2.2**	2.0 ± 2.2
CD16 / CD56	8.5 ± 3.6	11.2 ± 5.9	10.8 ± 5.6	6.9 ± 3.6
CD4 / CD16	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	1.9 ± 1.4
CD4 / CD56	1.3 ± 0.4	2.3 ± 1.7*	2.5 ± 1.1*	2.5 ± 2.4**
CD8 / CD11b	2.1 ± 1.5	3.5 ± 1.3*	3.4 ± 1.6*	2.7 ± 0.9

*p < 0.05, **p = 0.06 – 0.07.

bacterial response which might be of innate nature.

On the other hand, responses in HC showed a similar pattern for both *S. sanguis* and *E. coli* with increases of mainly cytotoxic subsets $CD8^+CD11b^+$, $CD4^+CD56^+$ and $CD8^+\gamma\delta^+$ T lymphocytes. As an interesting observation, the same subsets are elevated in BD patients'peripheral blood without "stimulation", suggesting an *in vivo* activation of lymphocytes which may limit their proliferation capacity further in *in vitro* conditions.

Elevations of $\gamma\delta^+$ T cells in unstimulated samples in our study are also similar to most, but not all studies done in BD in directly analysed PBMCs (10, 28, 29). However, unlike previous reports, we could not observe increased $\gamma\delta^+$T cell subsets after *S. sanguis* stimulation (10-12). Similar to a previous study from Japan, we observed mainly TCR-$\alpha\beta^+$ responses against HSP60 peptides in our study, with responses of $CD4^{+-}$ $CD56^+$ cytotoxic T cells also in HC. This is in contrast to Hasan *et al*. whom reported significant $\gamma\delta^+$ T cell responses to HSP60 derived peptides in BD patients from UK (17). This discrepancy might be explained with the background level of $\gamma\delta^+$ T cells which is observed to be different according to ethnical origin. A higher percentage of $\gamma\delta^+$ T cells were reported from the individuals of Mediterranean origin compared to Scandinavians (30). However, we also have not observed a very high level of $\gamma\delta^+$ T cells in our healthy controls and elevated levels of $\gamma\delta^+$ T cells mainly in unstimulated samples in our BD patients suggest that antigens other than HSPs may upregulate this subset in our population. In this respect, non-peptide prenyl pyrophosphates are recently described as the dominant antigens for $\gamma\delta^+$ T cell lines derived from intra-ocular fluid of uveitis patients with BD (31).

Possibly the most important part of our data is the similarity of immune responses against two different (gram positive and negative) microorganisms, revealing the problem of the specificity of anti-streptococcal immunity in BD. As Hirohata *et al.* first proposed (14), antigens possibly common to a variety of different microorganisms might be immuno-dominant in BD. In this respect, highly conserved heat shock proteins are logical candidates. However, the differences between anti-HSP responses and bacterial extracts suggest that antigens other than HSP60 are possibly dominant for immune recognition of *S. sanguis* and *E. coli*. In this respect, further analysis of other bacterial antigens such as BES-1 gene product in different ethnic populations is required (13).

There are some points in our study which are to be taken into consideration in the interpretation of our data. We have not performed a direct, freshly isolated unstimulated analysis of PBMNCs and our 5-day unstimulated cultures might have down- or upregulating effects on certain cell surface expressions. This makes comparing our data difficult with most studies having analysis of unstimulated samples done immediately.

In conclusion, our results suggest that responses to whole bacteria extracts do not correlate with anti-HSP60 peptide responses which are mainly recognized by $\alpha\beta^+$ T cells in BD. We also found no evidence of a species-specific response against *S. sanguis* in our population. Further studies on anti-bacterial immune responses in BD should also include control microorganisms such as *E. coli* to clarify the role of infectious agents in the pathogenesis.

References

1. SAKANE T, TAKENO M, SUZUKI N, INABA G: Behçet's disease. *N Engl J Med* 1999; 341: 1284-91.
2. DIRESKENELI H: Review: Behçet's disease: Infectious etiology, new auto-antigens and HLA-B51.*Ann Rheum Dis* 2001;82:996-1002.
3. LEHNER T: The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. *Int Rev Immunol* 1997; 14: 21-32.
4. THE BEHCET'S DISEASE RESEARCH COMMITTEE OF JAPAN: Skin hypersensitivity to streptococcal antigens and the induction of systemic symptoms by the antigens in Behçet's disease – A multicenter study. *J Rheumatol* 1989; 16: 506-11.
5. CALGUNERI M, KIRAZ S, ERTENLI I, BENEKLI M, KARAASLAN Y, CELIK I: The effect of prophylactic penicillin treatment on the course of arthritis episodes in patients with Behçet's disease. A randomized clinical trial. *Arthritis Rheum* 1996; 39: 2062-65.
6. KANEKO F, OYAMA N, NISHIBU A: Streptococcal infection in the pathogenesis of Behçet's disease and clinical effects of minocycline on the disease symptoms. *Yonsei Med J* 1997; 38: 444-54.
7. ISOGAI E, OHNO S, KOTAKE S *et al*.: Chemiluminescense of neutrophils from patients with Behcet's disease and its correlation with an increased proportion of uncommon serotypes of *Streptococcus sanguis* in the oral flora. *Arch Oral Biol* 1990; 35: 43-8.
8. MIZUSHIMA Y, MATSUDA T, HOSHL K, OHNO S: Induction of Behçet's disease symptoms after dental treatment and streptococcal

antigen skin test. *J Rheumatol* 1988; 15: 1029-30.

9. HIROHATA S, OKA H, MIZUSHIMA Y: Streptococcal-related antigens stimulate production of IL-6 and interferon-gamma by T cells from patients with Behçet's disease. *Cell Immunol* 1992; 140: 410-19.
10. BANK I, DUVDEVANI M, LIVNEH A: Expansion of γδT-cells in Behçet's disease: Role of disease activity and microbial flora in oral ulcers. *J Lab Clin Med* 2003; 141: 33-41.
11. NISHIDA T, HIRAYAMA K, NAKAMURA S, OHNO S: Proliferative response of CD8+ gamma delta+ T cells to *Streptococcus sanguis* in patients with Behçet's disease. *Ocul Immunol Inflamm* 1998; 3: 139-44.
12. MOCHIZUKI N, SUZUKI N, TAKENO M *et al.*: Fine antigen specificity of human gamma delta T cell lines (V gamma 9+) established by repetitive stimulation with a serotype (KTH-1) of a gram-positive bacterium, *Streptococcus sanguis. Eur J Immunol* 1994; 24: 1536-43.
13. YOSHIKAWA K, KOTAKE S, KUBOTA T, KIMURA K, ISOGAI E, FUJII N: Cloning and sequencing of BES-1 gene encoding the immunogenesis antigen of *Streptococcus sanguis* KTH-1 isolated from the patients with Behçet's disease. *Zent Bl Bakteriol* 1998; 287: 449-60.
14. HIROHATA S, HASHIMOTO T: Abnormal T cell responses to bacterial superantigens in Behçet's disease (BD). *Clin Exp Immunol* 1998; 112: 17-24.
15. DIRESKENELI H, SARUHAN-DIRESKENELI G: The role of heat-shock proteins in Behcet's disease. *Clin Exp Rheumatol* 2003; 21: S44-S48.
16. LEHNER T, LAVERY E, SMITH R, van der ZEE R, MIZUSHIMA Y, SHINNICK T: Association between the 65-kilodalton heat shock protein, *Streptococcus sanguis*, and the corresponding antibodies in Behçet's syndrome. *Infect Immun* 1991; 59: 1434-41.
17. HASAN A, FORTUNE F, WILSON A *et al.*: Role of gamma delta T cells in pathogenesis and diagnosis of Behçet's disease. *Lancet* 1996; 347: 789-94.
18. KANEKO S, SUZUKI N, YAMASHITA N *et al.*: Characterization of T cells specific for an epitope of human 60 kD heat shock protein (hsp) in patients with Behçet's disease in Japan. *Clin Exp Immunol* 1997; 108: 204-11.
19. DIRESKENELI H, EKSIOGLU-DEMIRALP E, YAVUZ S *et al.*: T cell responses to 60/65 kD HSPderived peptides in Turkish Behçet's disease patients. *J Rheumatol* 2000; 27: 708-13.
20. INTERNATIONAL STUDY GROUP FOR BEHCET'S DISEASE: Criteria for diagnosis of Behçet's disease. *Lancet* 1990; i: 1078-80.
21. EKSIOGLU-DEMIRALP-E, DIRESKENELI H, ERGUN T, FRESKO I, AKOGLU T: Increased CD4+CD16+ and CD4+CD56+ T cell subsets in Behçet's disease. *Rheumatol Int* 1999; 19: 23-6.
22. LEVITZ MS, MATHEWS HL, MURPHY JW: Direct anti-microbial activity of T cells. *Immunol Today* 1995; 16: 387-91.
23. VERGELLI M, LE H, VAN NOORT JM, DHIBJALBUT S, MCFARLAND H, MARTIN R: A novel population of CD4+CD56+ myelin-reactive T cells lyses target cells expressing CD56/neural cell adhesion molecule. *J Immunol* 1996; 157: 679-88.
24. BARNABU U, FRANCO A, PAROLI M, BENVENUTO R, FERRARINI M: Selective expansion of cytotoxic T lymphocytes with a CD4+CD56+ surface phenotype and a T helper type 1 profile of cytokine secretion in the liver of patients chronically infected with hepatitis B virus. *J Immunol* 1994; 152: 3074-87.
25. DUMMER R, POTOCZNA N, HAFFNER AC, ZIMMERMANN DR, GILARDI S, BURG G: A primary cutaneous non-T, non-B CD4+ CD56+ lymphoma. *Arch Dermatol* 1996; 132: 550-3.
26. HAMZAOUI K, AYED K, HAMZA M, TOURAINE JL: Natural killer cells in Behcet's disease. *Clin Exp Immunol* 1988; 71: 126-31.
27. KANEKO F, TAKAHASHI Y, MURAMATSU R, ADACHI K, MIURA Y, NAKANE A, MINAGAWAT: Natural killer cell numbers and function in peripheral lymphoid cells in Behcet's disease. *Br J Dermatol* 1985; 113: 313-8.
28. DIRESKENELI H, EKSIOGLU-DEMIRALP E, KIBAROGLU A, YAVUZ S, ERGUN T, AKOGLU T: Oligoclonal T cell expansions in patients with Behcet's disease. *Clin Exp Immunol* 1999; 117: 166-70.
29. FREYSDOTTIR J, LAU SH, FORTUNE F: Gamma delta+ T cells in Behçet's disease (BD) and recurrent aphthous stomatitis (RAS). *Clin Exp Immunol* 1999; 118: 451-7.
30. ESIN G, SHIGEMATSU M, NAGAI S, EKLUND A, WIGZELL H, GRUNEWALD J: Different percentages of peripheral blood γδ+ T cells from different areas of the world. *Scand J Immunol* 1996; 43: 593-6.
31. VERJANS GMGM, VAN HAGEN PM, VAN DER KOOI A, OSTERHAUS ADME, BAARSMA GS: Vγ9δ2 T cells recovered from eyes of patients with Behçet's disease recognize non-peptide prenyl pyrophosphate antigens. *J Neuroimmunol* 2002; 130: 46-54.