Behçet’s disease is a multisystem disorder that is characterized by oral and genital ulcers, and mucocutaneous, ocular, joint, vascular and central nervous system involvement. It is particularly frequent in countries along the Silk Route, from the Mediterranean area to Japan, and is strongly associated with HLA-B51 [1]. Various micro-organisms such as streptococci and herpes simplex virus have been implicated in the pathogenesis of Behçet’s disease. There is also evidence of immunological dysregulation, including neutrophil hyperfunction, autoimmune manifestations, and several phenotypic and functional lymphocyte abnormalities, possibly resulting from complex interactions of genetic and environmental factors.
Histological findings in Behçet’s disease suggest a mixed or mainly mononuclear cell infiltration with a predominance of T cells in the inflammatory infiltrates of oral ulcers, erythema nodosum-like lesions and pathergy reactions [7,8].

In the present study we analyzed γδ T lymphocytes with phenotype Vγ9/Vδ2 in Italian patients with active and inactive Behçet’s disease. Among γδ T cells, Vγ9/Vδ2 T cells represent the majority of peripheral blood T cells in healthy individuals [18]. The response of Vγ9/Vδ2 cells to phoshoantigens was investigated. Because of their relatively low number, circulating Vγ9/Vδ2 T cells must be specifically activated by nonpeptidic phosphorylated antigens (so-called phoshoantigens) [19]. Subsequent to this stimulation by nonpeptidic ligands, Vγ9/Vδ2 T cells proliferate, release type 1 cytokines and acquire cytotoxic activity against tumour cells [20] or virus infected cells [21].

It has been shown that tumour necrosis factor (TNF)-α and IL-12 induce activation and proliferation of γδ T cells in vitro [22]. Plasma levels of TNF-α and IL-12 have been also found to be increased in Behçet’s disease [23]. In this regard, we examined the expression of TNF-α and IL-12 receptors on Vγ9/Vδ2 T cells before and after inducing their expansion.

Materials and methods

Patients

Twenty-five patients with Behçet’s disease (12 males and 13 females, mean age 42 ± 24 years), classified according to the International Study Group for Behçet’s disease [24], were studied. The activity of Behçet’s disease was assessed by the 1994 criteria for disease activity of Behçet’s disease, proposed by the Behçet’s Disease Research Committee of Japan [25]. At time of sampling, disease was active in 15 patients and inactive in 10. All patients were using colchicine, an immunosuppressant agent such as ciclosporin (n=8), azathioprine (n=2) and low dose corticosteroids (n=16). Forty-five healthy volunteers (age range 21–47 years, mean 38 years) were enrolled as controls. Human studies committee approval and individual informed consent from each patient were obtained.

Monoclonal antibodies and flow cytometry

mAbs specific for human surface antigens anti-CD3 phycoerythrin-labelled (PE) and anti-T-cell receptor (TCR) Vδ2 fluorescein isothiocyanate (FITC; Pharmingen, San Diego, CA, USA) were used as follows. Peripheral blood mononuclear cells (PBMCs; 10⁶ in 100 μl phosphate buffered saline [PBS] with 1% heat-inactivated foetal calf serum and 0.02% Na-azide) were incubated at 4°C for 30 min with anti-CD3-PE conjugated mAb and anti-TCR Vδ2 FITC conjugated mAb simultaneously. After washing, the cells were suspended in PBS with 1% foetal calf serum and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) by using forward scatter/side scatter gating to select the lymphocyte population for analysis.

Cell separation and expansion in vitro of Vγ9/Vδ2 T lymphocytes

PBMCs were obtained from each individual by separating heparinized venous blood on Ficoll (Euroclone, Wetherby, Yorkshire, UK). The cells were washed in RPMI-1640 medium (Euroclone), and cultured in 24-well plates (Costar, Cambridge, MA, USA) at a concentration of 5 x 10⁶ cells/ml in RPMI 1640 supplemented with 10% foetal calf serum (Euroclone), hepes 20 mmol/l (Euroclone) and penicillin/streptomycin 100 U/ml (Sigma, St Louis, USA), at 37°C and at 0.5% CO₂. For the expansion of Vγ9/Vδ2 T cells, PBMCs were cultured for 10 days in medium alone or in the presence of the following phoshoantigens: xylene 1-P (Sigma; 0.5 mmol/l final concentration); ribose 1-P (Sigma; 0.5 mmol/l final concentration); dimethylallyl pyrophosphate (DMAPP; Sigma; 0.5 mmol/l final concentration); isopenetyl pyrophosphate (Sigma; 0.5 mmol/l final concentration); or Mycobacterium tuberculosis derived TUBAg (1 nmol/l final concentration; generously provided by Dr JJ Fournie, CHU Purpan, Toulouse, France). After 72 hours, cultures were supplemented with a 0.5 ml medium containing 20 U/ml recombinant human interleukin (IL-2; Genzyme, Cambridge, MA, USA). Every 72 hours, 0.5 medium was replaced with a 0.5 ml fresh medium containing 20 U/ml IL-2. After 10 days, cells were washed three times in medium, and expansion of Vγ9/Vδ2...
T cells was assessed using FACSScan, as described above. The absolute number of $\gamma\delta$ T cells in each culture was calculated according to the following formula: 

$$%V_{\gamma\delta} \times \text{total cell count} / 100.$$ 

The $V_{\gamma\delta}$ expansion factor (EF) was then calculated by dividing the absolute number of $V_{\gamma\delta}$ T cells in specifically stimulated cultures by the absolute number of $V_{\gamma\delta}$ T cells cultured in the absence of any antigen [26].

**Expression of tumour necrosis factor receptor II and interleukin-12 receptor $\beta_1$ by $V_{\gamma\delta}$ T lymphocytes**

We studied the expression of TNF receptor II and IL-12 receptor $\beta_1$ on $V_{\gamma\delta}$ T cells from peripheral blood of patients with Behçet’s disease and from normal individuals, using anti-TNF receptor II PE or anti-IL-12 receptor $\beta_1$ PE mAbs (R&D systems, Minneapolis, MN, USA) and anti-V$\delta_2$ TCR FITC simultaneously. We also evaluated the expression of these receptors after stimulation of $V_{\gamma\delta}$ cells with phosphoantigens with and without the addition of exogenous human TNF-α (10 ng/ml = 100 U/ml Genzyme) for 10 days. Briefly, cell cultures were centrifuged at 500 g for 5 min and washed three times in an isotonic PBS buffer supplemented with 0.5% bovine serum albumin, to remove any residual growth factor that might have been present in the culture medium. Cells were then resuspended in the same buffer to a final concentration of $2 \times 10^6$ cells /ml, and 100 µl of cells were transferred to a 5 ml tube for staining with anti-TNF receptor II and anti-IL-12 receptor $\beta_1$ (10 µl/10^6 cells) and anti-V$\delta_2$ (1 µl/10^6 cells). After incubation for 30 min at 4°C and two washings, the cells were resuspended in 500 µl PBS buffer for flow cytometric analysis. As a control, cells were treated in a separated tube with phycoerythrin-labelled mouse IgG antibody (Sigma).

**Statistical analysis**

Student’s t-test was used to compare responses in different groups. $P<0.05$ was chosen for rejection of the null hypothesis.

**Results**

**Expression of $V_{\gamma\delta}$ T-cell receptor on lymphocytes in peripheral blood**

The percentage of $\delta\gamma$ T cells with phenotype $V_{\gamma\delta}$ was similar in both patients and normal individuals (2.38 ± 2.3% and 3.05 ± 1.34%, respectively). There was no statistical difference in the percentage of $V_{\gamma\delta}$ T cells between patients with active (2.63 ± 1.73%) and those with inactive (2.02 ± 1.26%) disease. The number of circulating $V_{\gamma\delta}$ T cells also was not substantially modified by different therapies.

**Expansion in vitro of $V_{\gamma\delta}$ T cells**

The expansion of $V_{\gamma\delta}$ T lymphocytes was evaluated in vitro by incubating the cells with five different phospho-

[Figure 1](#)

**Expansion of $V_{\gamma\delta}$ T lymphocytes from patients with active or inactive Behçet’s disease and healthy control individuals in response to various phosphoantigens.** The $V_{\gamma\delta}$ expansion factor (EF) was then calculated by dividing the absolute number of $V_{\gamma\delta}$ T cells in specifically stimulated cultures by the absolute number of $V_{\gamma\delta}$ T cells cultured in the absence of any antigen. DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; RIB, ribose 1-P; TUBAg, *Mycobacterium tuberculosis* related phosphorylated components; XYL, xylose 1-P.
occurrence of surface TNF-α and IL-12 receptors on resting Vγ9/Vδ2 T cells from all studied groups. This finding is reinforced by the knowledge that these receptors are not constitutively expressed on γδ T cells. TNF receptor II and IL-12 receptor β1 were detected on Vγ9/Vδ2 T lymphocytes after the addition of DMAPP or DMAPP plus TNF-α. TNF receptor II and IL-12 receptor β1 expression was increased after 10 days in all studied groups. In particular, the proportion of cells coexpressing Vγ9/Vδ2 and TNF receptor II or IL-12 receptor β1 was higher among patients with active disease (n=4; 17.8±1.1% and 49.2±5.5%, respectively) than in patients with inactive disease (n=4; 1.4±0.9% and 25.2±2.2%, respectively) or control individuals (n=4; 0.5±0.4% and 1.6±2.2%, respectively). When Vγ9/Vδ2 cells from patients with active Behçet’s disease were cultured in the presence of TNF-α there was a further increase in the cells coexpressing Vγ9/Vδ2 and TNF receptor II (24±5.6% in active Behçet’s disease; 0.65±0.2% in inactive Behçet’s disease; 1.26±1.02% in control individuals). Fig. 4 shows a typical cytofluorimetric analysis of TNF receptor II and IL-12 receptor β1 positive Vγ9/Vδ2 T cells.

Figure 2

Cytofluorimetric analysis of Vγ9/Vδ2 T lymphocytes from a patient with active Behçet’s disease (BD) and a healthy control individual in vitro, cultured with dimethylallyl pyrophosphate (DMAPP) or medium alone. The horizontal axis represents log10 fluorescence intensity of Vγ9/Vδ2 stained cells. Each analysis was repeated at least three times and was performed each time with cells from different donors. FITC, fluorescein isothiocyanate.

Discussion

The immunopathogenesis of Behçet’s disease is believed to be T-cell mediated. Oligoclonal expansion in CD4+ and CD8+ T-cell subsets were observed in clinically active Behçet’s disease [27]. However, γδ T lymphocytes appear to play an important role in the development of disease [9,15–17]. γδ T lymphocytes play a major role in mucosal immunity and in the first line of host defence [10,11]. The preferential localization of γδ T cells in epithelial layers was also considered evidence for their surveillance function at these important sites of microbial entry [28]. In addition, they may regulate the function of αβ T cells through the production of cytokines [14]. Associations with disease have been also reported for rheumatoid arthritis [29], autoimmune thyroid conditions [30], autoimmune liver disease [31] and multiple sclerosis [32]. Increased levels of γδ T cells have been demonstrated in Behçet’s disease [9,15–17], and a role in the pathogenesis of the disease has been also suggested.

In the present study we analyzed the in vitro expansion capacity, and TNF receptor II and IL-12 receptor β1
expression of Vγ9/Vδ2 T cells, which represent the majority of γδ T lymphocytes in the peripheral blood [18], after exposure to phosphoantigens. In fact, phosphoantigens are known to activate specifically Vγ9/Vδ2 T cells in a major histocompatibility complex unrestricted, but TCR-dependent manner [19].

A low number of circulating Vγ9/Vδ2 cells was found both in patients with active and in those with inactive Behçet’s disease, and this was comparable with the number in normal control individuals. Different results have previously been reported, but this discrepancy is probably due to inclusion of different populations of patients and/or stages of disease progression in those studies [33]. Indeed, Vγ9/Vδ2 cells from patients with active Behçet’s disease, but not from inactive patients or control individuals, responded to DMAPP in vitro with expansion and upregulation of TNF receptor II and IL-12 receptor β1 expression. This phenomenon might be explained by the fact that Vγ9/Vδ2 cells from active patients are pre-activated in vivo. In vivo activation of Vγ9/Vδ2 lymphocytes may be the result of the presence of cytokines (i.e. TNF-α and IL-12) [22]. Moreover, increased serum levels of proinflammatory cytokines, namely IL-1β, IL-6, TNF-α [34,35] and IL-12 [23], have been found in active Behçet’s disease. Alternatively, Vγ9/Vδ2 T cells in active disease might be less susceptible to apoptosis and account for the increased expansion. Indeed, our recent results in unfractionated T lymphocytes from patients with active Behçet’s disease, which show inhibition of spontaneous and CD95-induced apoptosis after exposure to IL-12 (unpublished data), might be in agreement with this hypothesis.

In the present study we found that peripheral Vγ9/Vδ2 lymphocytes from active patients do not express TNF and/or IL-12 receptors. However, enhanced expression of other activation receptors (IL-2 receptor β, HLA-DR, CD29 and CD69 antigens) have been reported in unstimulated γδ T lymphocytes from patients with active Behçet’s disease [15,32], this discrepancy probably being due to the fact that our cytfluorimetric analysis is not sensitive enough to measure membrane antigens in a relatively low number of cells.

After phosphoantigen stimulation a remarkable upregulation of TNF receptor II and IL-12 receptor β1 expression was observed, the expression being maximal in the presence of TNF-α.

Cell TNF receptor II and IL-12 receptor β1 expression was not investigated in Vγ9/Vδ2 T cells from Behçet’s disease patients. Increased serum levels of soluble TNF receptor II has been observed, however, during the active stage of disease [36], and a central role of IL-12 in the pathogenesis of Behçet’s disease has been postulated [24]. A possible role played by TNF receptor II could be to increase the local concentration of TNF-α, which would in turn promote TNF-receptor I engagement, with both TNF receptors II and I being directly involved in cytotoxic activity [37]. TNF-α, which has been reported also to be produced by γδ T cells [33], hence might stimulate the TNF receptor bearing γδ T cells in an autocrine or paracrine manner or both, to express CD25, proliferate and upregulate IL-12 receptor expression. It is also possible that the ability of IL-12 and TNF-α to upregulate mutual receptors may lead to a reciprocal amplification circuit in γδ T cells [22].
All together, these data clearly indicate that Vγ9/Vδ2 T lymphocytes from patients with Behçet’s disease are activated. Vγ9/Vδ2 T cells may play a key role in the pathogenesis and progression of Behçet’s disease. They may be responsible for the development of inflammatory processes through cytokine production and subsequent induction of adhesion molecules, which permit accumulation of reactive T lymphocytes at the sites of inflammation. In this regard, involvement of γδ T cells in the local injury process has been also demonstrated by their presence in the infiltrate of mucosal ulcerations [15]. Further definition and identification of effector functions of the Vγ9/Vδ2 cells are required to prove their role in the maintenance of disease. In addition, inhibition of γδ activation, and therefore of proinflammatory cytokine production, may provide an interesting therapeutic strategy for novel treatments for Behçet’s disease.

Competing interests
None declared.

Acknowledgements
This work was supported by a grant from Ministero della Istruzione, della Università e della Ricerca (MIUR) of Italy. Dr A Accardo-Palumbo is a PhD student and recipient of a fellowship from MIUR. This work was presented at the Annual European Congress of Rheumatology (Prague, 13–16 June 2001) and was published in abstract form in the *Annals of the Rheumatic Diseases* (volume 60, supplement 1, page 193). GT and AA-P have contributed equally to this work. The authors wish to thank Dr JJ Fournie (CHU Purpan, Toulouse, France) for providing TUBAg.

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