

Research article

# V $\gamma$ 9/V $\delta$ 2 T lymphocytes in Italian patients with Behçet's disease – evidence for expansion, and tumour necrosis factor receptor II and interleukin-12 receptor $\beta_1$ expression in active disease

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## Abstract

Behçet's disease is a multisystem disease in which there is evidence of immunological dysregulation. It has been proposed that  $\gamma/\delta$  T cells are involved in its pathogenesis. The aim of the present study was to assess the capacity of  $\gamma/\delta$  T cells with phenotype V $\gamma$ 9/V $\delta$ 2, from a group of Italian patients with Behçet's disease, to proliferate in the presence of various phosphoantigens and to express tumour necrosis factor (TNF) and IL-12 receptors. Twenty-five patients and 45 healthy individuals were studied. V $\gamma$ 9/V $\delta$ 2 T cells were analyzed by fluorescence activated cell sorting, utilizing specific monoclonal antibodies. For the expansion of V $\gamma$ 9/V $\delta$ 2 T cells, lymphocytes were cultured in the presence of various phosphoantigens. The expression of TNF receptor II and IL-12 receptor  $\beta_1$  was evaluated with the simultaneous use of anti-TNF receptor II phycoerythrin-

labelled (PE) or anti-IL-12 receptor  $\beta_1$  PE and anti-V $\delta$ 2 T-cell receptor fluorescein isothiocyanate. There was a certain hierarchy in the response of V $\gamma$ 9/V $\delta$ 2 T cells toward the different phosphoantigens, with the highest expansion factor obtained with dimethylallyl pyrophosphate and the lowest with xylose 1P. The expansion factor was fivefold greater in patients with active disease than in those with inactive disease or in control individuals. TNF receptor II and IL-12 receptor  $\beta_1$  expressions were increased in both patients and control individuals. The proportion of V $\gamma$ 9/V $\delta$ 2 T cells bearing these receptors was raised in active disease when V $\gamma$ 9/V $\delta$ 2 T cells were cultured in the presence of dimethylallyl pyrophosphate. These results indicate that V $\gamma$ 9/V $\delta$ 2 T cell activation is correlated with disease progression and probably involved in the pathogenesis.

**Keywords:** Behçet's disease, interleukin 12,  $\gamma/\delta$  T lymphocyte, tumour necrosis factor

## Introduction

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

DMAPP = dimethylallyl pyrophosphate; EF = expansion factor; FACS = fluorescence activated cell sorting; FITC = fluorescein isothiocyanate; IL = interleukin; mAb = monoclonal antibody; PBMC = peripheral blood mononuclear cell; PBS = phosphate buffered saline; PE = phycoerythrin-labelled; TCR = T-cell receptor; TNF = tumour necrosis factor.

[2–6]. 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].

Increases in  $\gamma/\delta$  T cells in peripheral blood and cerebrospinal fluid, and heightened  $\gamma/\delta$  T cell responses to heat shock protein derived peptides suggest a role for this T-cell subset in the aetiopathogenesis of Behçet's disease [9].  $\gamma/\delta$  T cells play a prominent role in immune regulation; they are the first line of host defence and control epithelial cell growth, thus participating in the maintenance of epithelial integrity [10,11]. In particular, it has been postulated that they recognize structures presented by micro-organism as well as by stressed, abnormal cells, preventing the entrance of pathogens into the subepithelial layer by a cytotoxic mechanism against infected and stressed epithelial cells [12]. Some populations of these cells are known to be involved in the initiation of acute inflammatory responses and in the persistence of chronic inflammation in several skin diseases [13]. Finally,  $\gamma/\delta$  T cells have been reported to produce several cytokines, with the cytokine profile dependent on the nature of the immune response. They also produce a panel of chemokines that may attract inflammatory cells within damaged epithelium [14]. On the basis of these observations, it has been hypothesized that  $\gamma/\delta$  T cells may trigger the development of Behçet's disease [9,15–17].

In the present study we analyzed  $\gamma/\delta$  T lymphocytes with phenotype V $\gamma$ 9/V $\delta$ 2 in Italian patients with active and inactive Behçet's disease. Among  $\gamma/\delta$  T cells, V $\gamma$ 9/V $\delta$ 2 T cells represent the majority of peripheral blood T cells in healthy individuals [18]. The response of V $\gamma$ 9/V $\delta$ 2 cells to phosphoantigens was investigated. Because of their relatively low number, circulating V $\gamma$ 9/V $\delta$ 2 T cells must be specifically activated by nonpeptidic phosphorylated antigens (so-called phosphoantigens) [19]. Subsequent to this stimulation by nonpeptidic ligands, V $\gamma$ 9/V $\delta$ 2 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)- $\alpha$  and IL-12 induce activation and proliferation of  $\gamma/\delta$  T cells *in vitro* [22]. Plasma levels of TNF- $\alpha$  and IL-12 have been also found to be increased in Behçet's disease [23]. In this regard, we examined the expression of TNF- $\alpha$  and IL-12 receptors on V $\gamma$ 9/V $\delta$ 2 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  $42 \pm 24$  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) V $\delta$ 2 fluorescein isothiocyanate (FITC; PharMingen, San Diego, CA, USA) were used as follows. Peripheral blood mononuclear cells (PBMCs;  $10^6$  in 100  $\mu$ l 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 V $\delta$ 2 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 V $\gamma$ 9/V $\delta$ 2

#### 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  $5 \times 10^5$  cells/ml in RPMI 1640 supplemented with 10% foetal calf serum (Euroclone), hepes 20 mmol/l (Euroclone), 2 mmol/l L-glutamine (Euroclone) and penicillin/streptomycin 100 U/ml (Sigma, St Louis, USA), at 37°C and at 0.5% CO<sub>2</sub>. For the expansion of V $\gamma$ 9/V $\delta$ 2 T cells, PBMCs were cultured for 10 days in medium alone or in the presence of the follow phosphoantigens: xylose 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); isopentenyl pyrophosphate (Sigma; 0.5 mmol/l final concentration); or *Mycobacterium tuberculosis* derived TUBAg (1 nmol/l final concentration; generously provided by Dr JJ Fourniè, 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 V $\gamma$ 9/V $\delta$ 2

T cells was assessed using FACScan, as described above. The absolute number of V $\gamma$ 9/V $\delta$ 2 T cells in each culture was calculated according to the following formula: %V $\gamma$ 9/V $\delta$ 2 positive cells before culture  $\times$  total cell count/100. The V $\gamma$ 9/V $\delta$ 2 expansion factor (EF) was then calculated by dividing the absolute number of V $\gamma$ 9/V $\delta$ 2 T cells in specifically stimulated cultures by the absolute number of V $\gamma$ 9/V $\delta$ 2 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 $\gamma$ 9/V $\delta$ 2 T lymphocytes

We studied the expression of TNF receptor II and IL-12 receptor  $\beta_1$  on V $\gamma$ 9/V $\delta$ 2 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 $\gamma$ 9/V $\delta$ 2 cells with phosphoantigens with and without the addition of exogenous human TNF- $\alpha$  (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  $\mu$ l of cells were transferred to a 5 ml tube for staining with anti-TNF receptor II and anti-IL-12 receptor (10  $\mu$ l/10<sup>5</sup> cells) and anti-V $\delta$ 2 (1  $\mu$ l/10<sup>6</sup> cells). After incubation for 30 min at 4°C and two washings, the cells were resuspended in 500  $\mu$ l 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

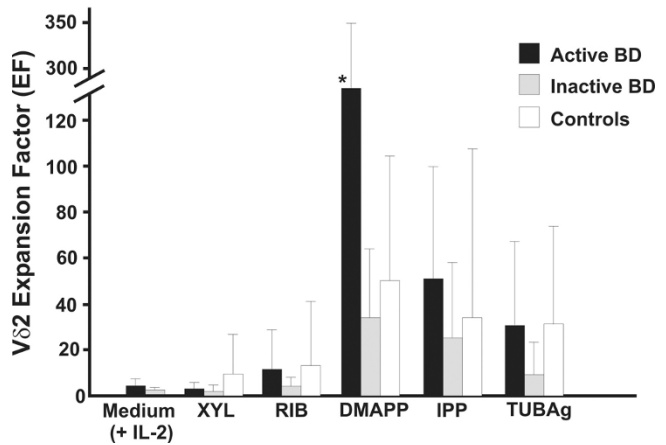
### Expression of V $\gamma$ 9/V $\delta$ 2 T-cell receptor on lymphocytes in peripheral blood

The percentage of  $\delta\gamma$  T cells with phenotype V $\gamma$ 9/V $\delta$ 2 was similar in both patients and normal individuals ( $2.38 \pm 1.56\%$  and  $3.05 \pm 1.34\%$ , respectively). There was no statistical difference in the percentage of V $\gamma$ 9/V $\delta$ 2 T cells between patients with active ( $2.63 \pm 1.73\%$ ) and those with inactive ( $2.02 \pm 1.26\%$ ) disease. The number of circulating V $\gamma$ 9/V $\delta$ 2 T cells also was not substantially modified by different therapies.

### Expansion *in vitro* of V $\gamma$ 9/V $\delta$ 2 T cells

The expansion of V $\gamma$ 9/V $\delta$ 2 T lymphocytes was evaluated *in vitro* by incubating the cells with five different phospho-

Figure 1

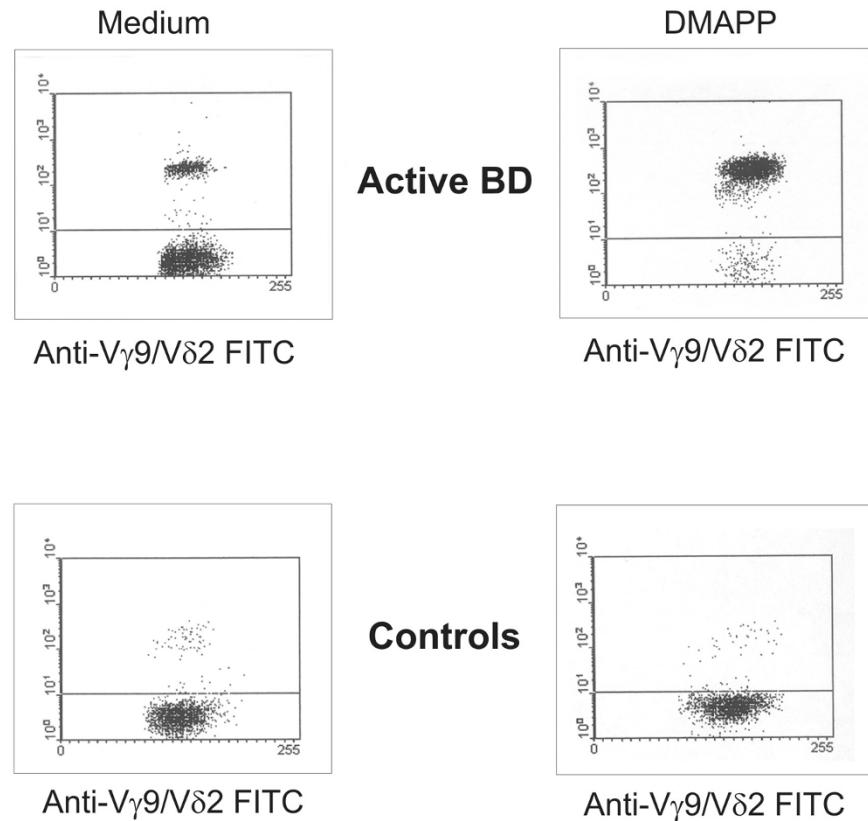


Expansion of V $\gamma$ 9/V $\delta$ 2 T lymphocytes from patients with active or inactive Behçet's disease and healthy control individuals in response to various phosphoantigens. The V $\gamma$ 9/V $\delta$ 2 expansion factor (EF) was then calculated by dividing the absolute number of V $\gamma$ 9/V $\delta$ 2 T cells in specifically stimulated cultures by the absolute number of V $\gamma$ 9/V $\delta$ 2 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.

antigens for 10 days or in medium (containing IL-2) alone. At this time the percentage of expansion was assessed by fluorescence activated cell sorting (FACS) analysis using the anti-TCR V $\delta$ 2 mAb. The results were expressed as EF (see Materials and methods). There was a certain hierarchy in the response of V $\gamma$ 9/V $\delta$ 2 cells toward different phosphoantigens, with the highest EF obtained with DMAPP and the lowest with xylose 1P (Fig. 1). A significant difference was found in the response to DMAPP in the tested groups. Specifically, the EF of V $\gamma$ 9/V $\delta$ 2 cells of patients with Behçet's disease was fourfold higher than that in healthy control individuals ( $113.4 \pm 153$  and  $28.5 \pm 22.5$ , respectively). In addition, the EF of V $\gamma$ 9/V $\delta$ 2 T cells from patients with active Behçet's disease was fivefold higher than that of cells from patients with inactive disease ( $170 \pm 180$  and  $34.1 \pm 30.6$ , respectively). Fig. 2 shows a typical cytofluorometric analysis of expansion of V $\gamma$ 9/V $\delta$ 2 cells from one patient with Behçet's disease and one healthy control individual on stimulation with DMAPP.

### Expression of tumour necrosis factor receptor II and interleukin-12 receptor $\beta_1$ on V $\gamma$ 9/V $\delta$ 2 T cells

We investigated the expression of TNF receptor II and IL-12 receptor  $\beta_1$ , as cell activation markers, in the V $\gamma$ 9/V $\delta$ 2 T cell population from Behçet's disease patients (*n* = 8) and from normal control individuals (*n* = 4; Fig. 3). The expression of these receptors was analyzed in V $\gamma$ 9/V $\delta$ 2 T cells of peripheral blood and in cells cultured in the presence of DMAPP with or without the addition of exogenous TNF- $\alpha$ . No difference was observed in the

**Figure 2**

Cytofluorimetric analysis of V $\gamma$ 9/V $\delta$ 2 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 log<sub>10</sub> fluorescence intensity of V $\gamma$ 9/V $\delta$ 2 stained cells. Each analysis was repeated at least three times and was performed each time with cells from different donors. FITC, fluorescein isothiocyanate.

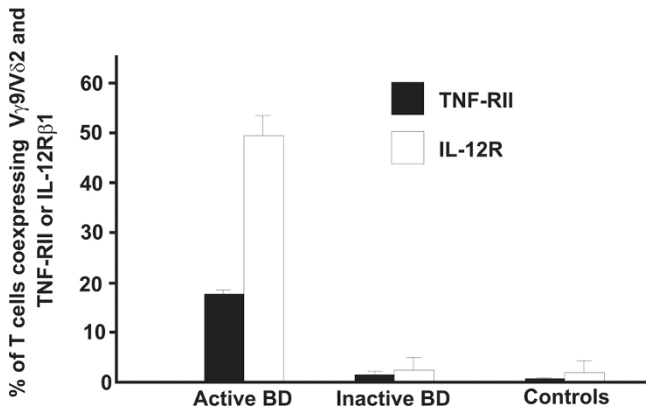
occurrence of surface TNF- $\alpha$  and IL-12 receptors on resting V $\gamma$ 9/V $\delta$ 2 T cells from all studied groups. This finding is reinforced by the knowledge that these receptors are not constitutively expressed on  $\gamma$ / $\delta$  T cells. TNF receptor II and IL-12 receptor  $\beta_1$  were detected on V $\gamma$ 9/V $\delta$ 2 T lymphocytes after the addition of DMAPP or DMAPP plus TNF- $\alpha$ . TNF receptor II and IL-12 receptor  $\beta_1$  expression was increased after 10 days in all studied groups. In particular, the proportion of cells coexpressing V $\gamma$ 9/V $\delta$ 2 and TNF receptor II or IL-12 receptor  $\beta_1$  was higher among patients with active disease ( $n=4$ ;  $17.8 \pm 1.1\%$  and  $49.2 \pm 5.5\%$ , respectively) than in patients with inactive disease ( $n=4$ ;  $1.4 \pm 0.9\%$  and  $25.2 \pm 2.2\%$ , respectively) or control individuals ( $n=4$ ;  $0.5 \pm 0.4\%$  and  $1.6 \pm 2.2\%$ , respectively). When V $\gamma$ 9/V $\delta$ 2 cells from patients with active Behçet's disease were cultured in the presence of TNF- $\alpha$  there was a further increase in the cells coexpressing V $\gamma$ 9/V $\delta$ 2 and TNF receptor II ( $24 \pm 5.6\%$  in active Behçet's disease;  $0.65 \pm 0.2\%$  in inactive Behçet's disease;  $1.26 \pm 1.02\%$  in control individuals). Fig.4 shows a typical cytofluorimetric analysis of TNF receptor II and IL-12 receptor  $\beta_1$  positive V $\gamma$ 9/V $\delta$ 2 T cells.

## Discussion

The immunopathogenesis of Behçet's disease is believed to be T-cell mediated. Oligoclonal expansion in CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets were observed in clinically active Behçet's disease [27]. However,  $\gamma$ / $\delta$  T lymphocytes appear to play an important role in the development of disease [9,15–17].  $\gamma$ / $\delta$  T lymphocytes play a major role in mucosal immunity and in the first line of host defence [10,11]. The preferential localization of  $\gamma$ / $\delta$  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  $\alpha$  $\beta$  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  $\gamma$ / $\delta$  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  $\beta_1$

**Figure 3**

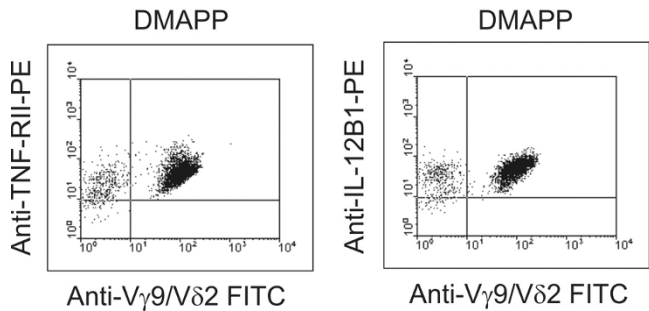


Expression of tumour necrosis factor receptor II (TNF-RII) and IL-12 receptor  $\beta_1$  (IL-12R $\beta_1$ ) on V $\gamma$ 9/V $\delta$ 2 T lymphocytes from patients with active or inactive Behçet's disease (BD) and healthy control individuals. Results are expressed as percentage of V $\gamma$ 9/V $\delta$ 2 T cells.

expression of V $\gamma$ 9/V $\delta$ 2 T cells, which represent the majority of  $\gamma/\delta$  T lymphocytes in the peripheral blood [18], after exposure to phosphoantigens. In fact, phosphoantigens are known to activate specifically V $\gamma$ 9/V $\delta$ 2 T cells in a major histocompatibility complex unrestricted, but TCR-dependent manner [19].

A low number of circulating V $\gamma$ 9/V $\delta$ 2 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, V $\gamma$ 9/V $\delta$ 2 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  $\beta_1$  expression. This phenomenon might be explained by the fact that V $\gamma$ 9/V $\delta$ 2 cells from active patients are pre-activated *in vivo*. *In vivo* activation of V $\gamma$ 9/V $\delta$ 2 lymphocytes may be the result of the presence of cytokines (i.e. TNF- $\alpha$  and IL-12) [22]. Moreover, increased serum levels of proinflammatory cytokines, namely IL-1 $\beta$ , IL-6, TNF- $\alpha$  [34,35] and IL-12 [23], have been found in active Behçet's disease. Alternatively, V $\gamma$ 9/V $\delta$ 2 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.

**Figure 4**



Tumour necrosis factor receptor II (TNF-RII) and IL-12 receptor  $\beta_1$  (IL-12R $\beta_1$ ) expression of V $\gamma$ 9/V $\delta$ 2 T lymphocytes from a patient with active Behçet's disease (BD). The ordinate indicates the expression of phycoerythrin-labelled (PE) conjugated TNF-RII or IL-12R $\beta_1$ , and the abscissa indicates the expression of fluorescein isothiocyanate (FITC)-conjugated anti-V $\gamma$ 9/V $\delta$ 2. Each analysis was repeated at least three times and was performed each time with cells from different patients. DMAPP, dimethylallyl pyrophosphate.

In the present study we found that peripheral V $\gamma$ 9/V $\delta$ 2 lymphocytes from active patients do not express TNF and/or IL-12 receptors. However, enhanced expression of other activation receptors (IL-2 receptor  $\beta$ , HLA-DR, CD29 and CD69 antigens) have been reported in unstimulated  $\gamma/\delta$  T lymphocytes from patients with active Behçet's disease [15,32], this discrepancy probably being due to the fact that our cytofluorimetric 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  $\beta_1$  expression was observed, the expression being maximal in the presence of TNF- $\alpha$ .

Cell TNF receptor II and IL-12 receptor  $\beta_1$  expression was not investigated in V $\gamma$ 9/V $\delta$ 2 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- $\alpha$ , which would in turn promote TNF-receptor I engagement, with both TNF receptors II and I being directly involved in cytotoxic activity [37]. TNF- $\alpha$ , which has been reported also to be produced by  $\gamma/\delta$  T cells [33], hence might stimulate the TNF receptor bearing  $\gamma/\delta$  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- $\alpha$  to upregulate mutual receptors may lead to a reciprocal amplification circuit in  $\gamma/\delta$  T cells [22].

All together, these data clearly indicate that V $\gamma$ 9/V $\delta$ 2 T lymphocytes from patients with Behçet's disease are activated. V $\gamma$ 9/V $\delta$ 2 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  $\gamma/\delta$  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 V $\gamma$ 9/V $\delta$ 2 cells are required to prove their role in the maintenance of disease. In addition, inhibition of  $\gamma/\delta$  activation, and therefore of proinflammatory cytokine production, may provide an interesting therapeutic strategy for novel treatments for Behçet's disease.

## Competing interests

None declared.

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