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γδ T cells in Behçet's disease (BD) and recurrent aphthous stomatitis (RAS)

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SUMMARY

The immunopathogenesis of BD is believed to be T cell-mediated. The objective of this study was to characterize the activation stage and cytokine profile of peripheral blood lymphocytes (PBL), with particular emphasis on γδ T cells. Venous blood was collected from 20 patients with BD, and for comparison, from 11 patients with RAS and from 15 healthy controls. Both the expression of activation markers (CD25, CD29, CD40 ligand, CD69 and HLA-DR) on freshly isolated PBL and T cell subsets, and the expression of intracellular cytokines (IL-4, IL-10, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α)) on mitogen-stimulated PBL and T cell subsets were analysed by double immunofluorescent staining and flow cytometry. Significantly decreased proportion of αβ T cells and increased proportion of γδ T cells, $CD56^+$ cells and $CD8^+$ γδ T cells were found in BD patients compared with healthy controls. This was also seen to a lesser extent in patients with RAS. Furthermore, in BD a significantly increased proportion of the γδ T cell population expressed CD69 and high levels of CD29 and were induced to produce IFN-γ and TNF-α compared with healthy controls. In contrast, an increased percentage of γδ T cells from RAS patients was induced to produce IFN-γ, but not TNF-α. These results indicate that in BD, activated γδ T cells, capable of producing IFN-γ and TNF-α, are present in peripheral blood, suggesting that γδ T cells are dynamic and may be regulating immunopathogenic events.

Keywords γδ T cells Behçet's disease recurrent aphthous stomatitis cytokines activation

INTRODUCTION

BD is a multisystemic disease with recurrent ulceration affecting both the oral and genital mucosa. Furthermore, patients with BD suffer from lesions affecting the eye, skin, joints, gastrointestinal tract, central nervous, vascular and respiratory systems. However, the aetiopathogenesis of BD remains unknown. Unlike BD, RAS is the most common oral mucosal disease, affecting about 15–20% of the population [1,2], with the oral mucosa being the prime and only site of lesions.

A major part of the immunopathogenesis in BD and RAS is believed to be a T cell-mediated immune response. We and others have shown a depressed number of $CD4^+$ T cells and elevated numbers of $CD8^+$ T cells in peripheral blood in both patient groups [3–5]. The proportion of γδ T cells has also been found to be increased in peripheral blood from patients with BD compared with controls [3], whereas the proportion of γδ T cells in peripheral blood from patients with RAS has been reported to be unchanged or raised [6,7]. γδ T cells are uncommon in a healthy oral mucosa ([8], Freysdottir *et al*., manuscript in preparation). However, γδ T cells are observed within the oral lesions of patients with BD and RAS (Freysdottir *et al*., manuscript in preparation). Immune regulation has been suggested as a role for γδ T cells in the immune system [9], with the γδ T cells acting as a first line of defence. The γδ T cells have also been associated with controlling epithelial growth, thus participating in maintaining epithelial integrity [10–12]. Furthermore, it is postulated that they recognize structures presented by microorganisms as well as by stressed cells but not normal cells [13,14], thus enabling them to prevent entrance of pathogens into the subepithelial layer by cytotoxicity against infected and stressed epithelial cells. γδ T cells have also been reported to produce several cytokines, with the cytokine profile dependent on the nature of antigen, enabling the γδ T cells to influence the nature of the immune response [15]. They also produce a panel of chemokines which may attract inflammatory cells within damaged epithelium [16].

In order to investigate the role of γδ T cells in the immunopathogenesis of BD, we analysed the cytokine production and activation profile of T cell subsets in peripheral blood from patients with BD. For comparison we also analysed peripheral blood from patients with RAS and from healthy individuals. An increased

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percentage of γδ T cells in peripheral blood from patients with BD compared with both RAS and healthy controls was found. An increased proportion of the γδ T cells from BD patients expressed CD25 (the α-chain of the IL-2 receptor), CD69 (an early activation marker) and high levels of CD29 (a β_1-integrin), indicating that the cells were at an activated stage. Furthermore, a raised percentage of γδ T cells from BD patients produced the inflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α).

PATIENTS AND METHODS

Patients and controls

Twenty patients with BD and 11 patients with RAS were recruited from out-patient clinics at Guy's Hospital, London. All the BD patients (nine females and 11 males; mean age 40 years; range 22–62 years) fulfilled the International Criteria [17] and had clinically active disease, i.e. with active oral ulceration plus evidence of clinical signs of having more than one other organ system involvement, at the time of analysis (Fig. 1). All the RAS patients (eight females and three males; mean age 35 years; range 23–44 years) had more than one ulcer present for not more than 24 h at the time of analysis. For comparison, 15 healthy individuals (10 females and five males; mean age 40 years; range 26–45 years) were included as controls. Care was taken to match the ethnic origin of the BD patients, since it has been reported that the frequency of γδ T cells in peripheral blood differs in individuals from different areas of the world [18]. Patients with RAS and controls were excluded from the study if they had evidence of anaemia or systemic disease. Patients were venesected at their initial clinic visit before high doses of either topical or systemic immunosuppressives were prescribed. None of the patients with RAS were taking any medication, whereas four patients with ocular BD were already taking either prednisolone 5 mg ($n=2$), azathioprine 100 mg ($n=1$) or cyclosporin 100 mg ($n=1$). Ethical approval for the blood samples was obtained from Guy's Hospital Ethics Committee.

Antibodies

Mouse MoAbs against human CD29 (clone K20), CD69 (clone TP1.55.3), CD40 ligand (CD40L; CD154, clone TRAP1) and γδ TCR (clone IMMU510) were all from Coulter Electronics (Luton, UK). FITC-labelled and unlabelled MoAbs against human αβ TCR (clone WT31), FITC-labelled MoAbs against human γδ TCR (clone 11F2), PE-labelled MoAbs against human CD4 (clone SK3) and CD8 (clone SK1) were all purchased from Becton Dickinson (Oxford, UK). For intracellular cytokine measurements, PE-labelled MoAbs against human IL-4 (clone 8D4-8), IL-10 (clone JES3-9D7), TNF-α (clone Mab11), IFN-γ (clone 4S.B3) and control mouse IgG1 were obtained from Cambridge BioScience (Cambridge, UK). MoAbs against human CD25 (clone M-A251) and FITC-labelled MoAbs against human CD56 (clone C5.9) were purchased from Serotec (Oxford, UK). PE- or FITC-labelled goat anti-mouse immunoglobulin, the $F(ab)_2$ fragments, were purchased from Dako (High Wycombe, UK). MoAbs against human HLA-DR (clone DA6.231) were tissue culture supernatant produced in our own laboratory. FITC- or PE-labelled control mouse IgG1 were obtained from Sigma (Poole, UK).

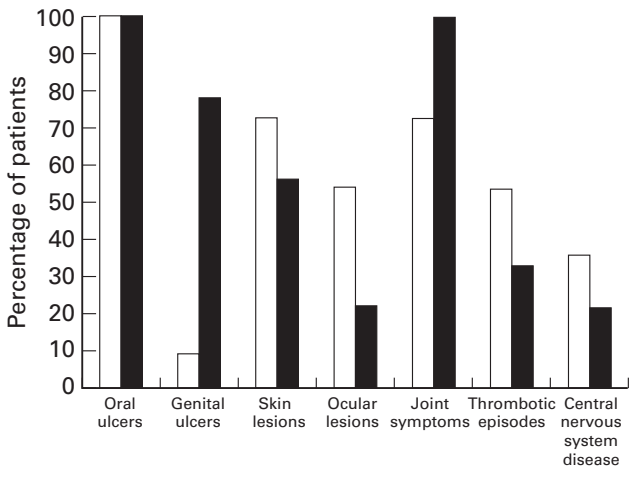

Fig. 1. Clinical pattern of disease activity of the BD patients. □, Male; ■, female.

Cell separation and culture

Peripheral blood mononuclear cells (PBMC) were obtained from each individual by separating heparinized venous blood on Histopaque (Sigma). The cells were washed in RPMI medium (Gibco, Paisley, UK) and either stained immediately or cultured before further analysis. The cells were cultured in 24-well plates at a concentration of 1×10^6 cells/ml in RPMI medium with 10% fetal calf serum (FCS; Gibco) for 4 h at 37°C, 5% CO_2 and 100% humidity.

Initially, in order to determine optimal culture conditions for induction of cytokine expression, PBMC were cultured for 2, 4, 6, 9 and 12 h in medium alone, in the presence of phytohaemagglutinin (PHA; Sigma) at concentrations of 2·5, 4·5 or 7·5 μg/ml, or in the presence of phorbol mysitrate acetate (PMA; Sigma) at concentrations of 1, 5 or 50 ng/ml in combination with 1 μmol/ml of ionomycin (Sigma). In order to prevent secretion of the cytokines, brefeldin A (Sigma) at 10 μg/ml was added during the last 4 h of the incubation. Maximum production of IFN-γ and TNF-α was observed after stimulation with PMA in combination with ionomycin, when used at either 5 or 50 ng/ml; whereas PHA was more effective in inducing production of IL-4 and IL-10, with maximum production observed at 7·5 μg/ml. All the cytokines were detectable after 2 h and maximum production was observed between 4 h and 9 h. Therefore, in this study the PBMC were cultured for 4 h in the absence or presence of 7·5 μg/ml of PHA for detection of IL-4 and IL-10, and in the absence or presence of 10 ng/ml of PMA in combination with 1 μmol/ml of ionomycin for detection of IFN-γ and TNF-α. Brefeldin A at 10 μg/ml was present from the beginning.

Double immunofluorescence staining for activation markers or co-receptors and T cell receptors

In order to analyse expression of activation markers on peripheral blood lymphocytes (PBL) and T cell subsets, 5×10^5 freshly isolated PBMC in 100 μl of staining buffer (PBS with 1% FCS and 0·02% NaN_3) were incubated with 5 μl of MoAbs against CD25 or CD40L, or 10 μl of MoAbs against CD29 or CD69, or 100 μl of MoAbs against MHC class II for 45 min on ice. Then the cells were washed twice with 1 ml of staining buffer at 180 ***g*** for 5 min at 4°C and subsequently incubated with 100 μl of PE-labelled goat anti-mouse immunoglobulin, diluted 1:100, for 30 min on ice, followed by two washes as before. Unbound goat antibody sites

were blocked by incubating the cells with 100 μl of PBS containing 5% normal human serum (NHS) and 5% normal mouse serum (NMS) for 10 min on ice before the cells were incubated with 10 μl of FITC-labelled MoAbs against αβ or γδ TCR or FITC-labelled control mouse IgG1. The cells were washed as before and fixed in 400 μl of 1% paraformaldehyde in staining buffer. For co-receptor measurements, 5×10^5 cells were incubated for 45 min on ice with 10 μl of PE-labelled MoAbs against CD4 or CD8 or PE-labelled control mouse IgG1 and 10 μl of FITC-labelled antibodies against CD56, αβ TCR, or γδ TCR or FITC-labelled control mouse IgG1.

Double immunofluorescence staining for intracellular cytokines and TCR

Stimulated and unstimulated cells were stained in parallel by incubating 5×10^5 cells in 100 μl of staining buffer for 45 min on ice with 10 μl of MoAbs against αβ TCR, diluted 1:5, or γδ TCR, diluted 1:10, followed by 100 μl of FITC-labelled goat anti-mouse immunoglobulin, diluted 1:100, for 30 min on ice. Staining buffer was used for washing and dilutions. Before the second staining, the cells were fixed in 500 μl of 4% paraformaldehyde in PBS for 10 min on ice and then unbound goat antibody sites were blocked by incubating the cells with 100 μl of PBS containing 5% NHS and 5% NMS for 10 min on ice. In order to permeabilize the cells, 0·1% saponin was included in the staining buffer used for subsequent incubations and washing. The cells were incubated with 10 μl of PE-labelled antibodies against cytokines (IL-4, IL-10, TNF-α or IFN-γ) or PE-labelled control mouse IgG1, diluted 1:16, for 45 min on ice, washed and then fixed in 400 μl of 1% paraformaldehyde in staining buffer.

Flow cytometric analysis

Fluorescent labelled cells were analysed by a flow cytometer (Epics XL; Coulter). Before each set of data analysis the machine was standardized for data reproducibility using fluorescent labelled beads (Coulter). Ten thousand cells were collected. Plots showing size and granularity were used to select the lymphocyte population (PBL) for further analysis. Cells stained with isotype-matched antibody or second antibody alone were used as controls with positive cells set at 0·5%. For intracellular cytokine analysis, unstimulated cells were used as negative controls.

Statistical analysis

Results were expressed as mean ± s.e.m. for each study group. Significance was evaluated using Student's *t*-test for non-paired samples with unequal variance, with $P < 0{\cdot}05$ being regarded as significant.

RESULTS

Expression of αβ TCR, γδ TCR, CD56, CD4 and CD8 on lymphocytes and T cell subsets in peripheral blood from patients with BD or RAS and healthy controls

The expression of αβ TCR, γδ TCR, CD56, CD4 and CD8 on lymphocytes and the expression of CD4 and CD8 on T cell subsets was analysed in peripheral blood from patients with BD or RAS and healthy controls. A significant decrease in the proportion of lymphocytes expressing αβ TCR and a significant increase in the proportion of lymphocytes expressing γδ TCR or CD56 was found in BD patients (63·2 ± 2·7%, 3·7 ± 0·5%, and 17·0 ± 2·5%, for αβ TCR, γδ TCR and CD56, respectively) compared with healthy controls (70·0 ± 1·7%, $P < 0{\cdot}05$, 1·8 ± 0·2%, $P < 0{\cdot}005$, and 8·6 ± 0·6%, $P < 0{\cdot}005$, for αβ TCR, γδ TCR and CD56, respectively), as shown in Fig. 2. Furthermore, BD patients had a significantly decreased proportion of αβ T cells and a significantly increased percentage of γδ T cells compared with patients with RAS (71·9 ± 2·1%, $P < 0{\cdot}05$, and 2·3 ± 0·4%, $P < 0{\cdot}05$, for αβ TCR and γδ TCR, respectively). The proportion of lymphocytes expressing CD56 was also significantly raised in RAS patients (13·3 ± 1·3%) compared with controls (8·6 ± 0·6%, $P < 0{\cdot}01$).

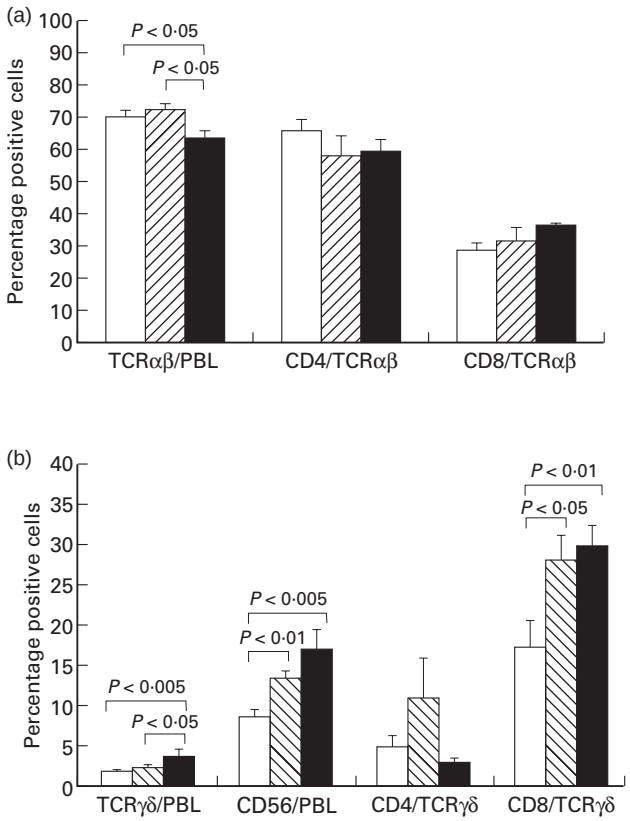

Fig. 2. The expression of αβ T cell receptor (TCR) on peripheral blood lymphocytes (PBL) and expression of CD4 or CD8 on αβ T cells (a) and the expression of γδ TCR or CD56 on PBL and expression of CD4 or CD8 on γδ T cells (b), analysed by double immunofluorescent staining and flow cytometry. Results are expressed as percentage positive cells, shown as mean ± s.e.m. for 20 BD patients (■), 11 RAS patients (hatched bars) and 20 healthy controls (□). Statistically significant differences between BD patients and controls, BD patients and RAS patients, and RAS patients and controls are indicated.

The proportion of $CD8^+$ lymphocytes was raised in both patient groups (31·7 ± 4·1% and 30·5 ± 2·1%, for RAS and BD, respectively) compared with controls (25·5 ± 1·9%), although the difference did not reach significance. However, there was no difference in the percentage of $CD4^+$ lymphocytes between either patient group and the controls (data not shown).

When the T cell subsets were analysed separately, a significantly raised proportion of γδ T cells from both BD patients (29·6 ± 2·6%) and RAS patients (27·9 ± 3·1%) co-expressed CD8 compared with γδ T cells from healthy controls (17·3 ± 3·3%, $P < 0{\cdot}01$ for BD and $P < 0{\cdot}05$ for RAS) (Fig. 2b). This was not observed for αβ T cells (Fig. 2a).

Expression of activation markers on lymphocytes and T cell subsets in peripheral blood from patients with BD or RAS and healthy controls

The expression of CD40L, CD69, CD29, CD25 and HLA-DR on lymphocytes and T cell subsets was analysed in peripheral blood from patients with BD or RAS and healthy controls.

A significantly increased percentage of $CD69^+$ lymphocytes was observed in the BD patients ($14{\cdot}6 \pm 1{\cdot}1\%$) compared with the controls ($10{\cdot}2 \pm 1{\cdot}3\%$, $P < 0{\cdot}05$) (Fig. 3a). This was reflected in a significantly raised proportion of $\alpha\beta$ and $\gamma\delta$ T cells expressing CD69 in BD patients ($5{\cdot}5 \pm 1{\cdot}3\%$ and $14{\cdot}2 \pm 2{\cdot}1\%$, for $\alpha\beta$ and $\gamma\delta$ T cells, respectively) compared with controls ($2{\cdot}6 \pm 0{\cdot}5\%$, $P < 0{\cdot}05$, and $8{\cdot}0 \pm 1{\cdot}9\%$, $P < 0{\cdot}05$, for $\alpha\beta$ and $\gamma\delta$ T cells, respectively) (Fig. 3a). Furthermore, BD patients had a significantly increased proportion of lymphocytes expressing the CD40L ($2{\cdot}4 \pm 0{\cdot}7\%$) compared with healthy controls ($1{\cdot}3 \pm 0{\cdot}2\%$, $P < 0{\cdot}05$) (Fig. 3a). Because a very low percentage of lymphocytes expressed CD40L, reliable results could not be obtained when the $\gamma\delta$ T cell subset was analysed for co-expression of the CD40L.

CD29 was expressed on all lymphocytes. Histogram analysis showed a biphasic population of cells with 'low' and 'high' expression of CD29. Significantly raised proportions of $\gamma\delta$ T cells from BD patients ($66{\cdot}5 \pm 3{\cdot}9\%$) or RAS patients ($60{\cdot}5 \pm 5{\cdot}0\%$) expressed high levels of CD29 ($CD29^{hi}$) when compared with healthy controls ($47{\cdot}8 \pm 3{\cdot}7\%$, $P < 0{\cdot}005$ for BD and $P < 0{\cdot}05$ for RAS) (Fig. 3b).

There was no significant difference in the proportion of lymphocytes or T cell subsets expressing CD25 or HLA-DR for either patient group compared with healthy controls (data not shown). However, an increase was observed for the proportion of $\gamma\delta$ T cells expressing CD25 in patients with BD ($7{\cdot}4 \pm 2{\cdot}9\%$) or RAS ($5{\cdot}6 \pm 3{\cdot}0\%$) compared with healthy controls ($4{\cdot}2 \pm 1{\cdot}3\%$) (Fig. 3b). This was not observed for the $\alpha\beta$ T cells (Fig. 3b).

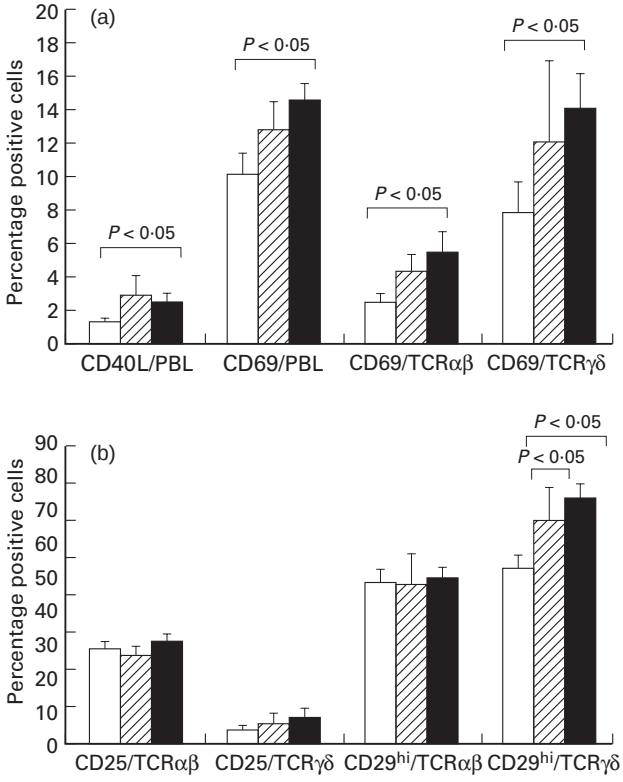

Fig. 3. The expression of CD40 ligand (CD40L) or CD69 on peripheral blood lymphocytes (PBL) and expression of CD69 on $\alpha\beta$ T cells or $\gamma\delta$ T cells (a) and the expression of CD25 and $CD29^{hi}$ on $\alpha\beta$ T cells and $\gamma\delta$ T cells (b), analysed by double immunofluorescent staining and flow cytometry. Results are expressed as percentage positive cells, shown as the mean ± s.e.m. for 20 BD patients (■), 11 RAS patients (hatched bars) and 20 healthy controls (□). Statistically significant differences between BD patients and controls, BD patients and RAS patients, and RAS patients and controls are indicated.

Expression of intracellular cytokines on lymphocytes and T cell subsets in peripheral blood from patients with BD or RAS and healthy controls

The expression of intracellular IL-4, IL-10, TNF-α and IFN-γ in lymphocytes and T cell subsets was analysed in peripheral blood from patients with BD or RAS and healthy controls.

Before stimulation, lymphocytes did not express any of the cytokines analysed (data not shown). However, after mitogen stimulation of lymphocytes for 4 h, there was a remarkable up-regulation of TNF-α and IFN-γ, and a low but consistent IL-4 and IL-10 expression. Maximal expression of IL-4 and IL-10 was observed when the PBMC were stimulated with PHA, whereas TNF-α and IFN-γ expression was preferably induced by stimulation with PMA and ionomycin (see Patients and Methods).

As seen in Fig. 4a, a significantly raised proportion of lymphocytes from BD patients could be induced to express TNF-α after mitogen stimulation for 4 h ($25{\cdot}3 \pm 2{\cdot}3\%$) compared with controls ($18{\cdot}9 \pm 2{\cdot}2\%$, $P < 0{\cdot}05$). This was reflected in a significantly increased frequency of $\gamma\delta$ T cells expressing TNF-α in PBL from BD patients ($48{\cdot}2 \pm 6{\cdot}1\%$) compared with controls ($28{\cdot}3 \pm 6{\cdot}3\%$, $P < 0{\cdot}05$). Interestingly, this was not seen for RAS patients (Fig. 4a).

An increased proportion of lymphocytes expressing IFN-γ was observed for the BD patients ($14{\cdot}6 \pm 2{\cdot}9\%$) compared with the controls ($9{\cdot}2 \pm 1{\cdot}5\%$), although the difference did not reach significance (Fig. 4b). This increase was reflected in a significantly increased proportion of $\gamma\delta$ T cells expressing IFN-γ from BD patients ($43{\cdot}3 \pm 6{\cdot}6\%$) compared with controls ($23{\cdot}5 \pm 6{\cdot}1\%$, $P < 0{\cdot}05$). In contrast to results obtained for TNF-α, RAS patients had an increased frequency of lymphocytes, as well as $\gamma\delta$ T cells, that expressed IFN-γ compared with healthy controls, although the difference did not reach significance (Fig. 4b).

Because of the low frequency of lymphocytes expressing IL-4 or IL-10 (<4%) it was not possible to obtain reliable results for IL-4 and IL-10 expression on $\gamma\delta$ T cells. However, results obtained did not indicate a difference between either patient group and controls (data not shown).

DISCUSSION

Healthy oral mucosa does not contain many $\gamma\delta$ T cells ([8], Freysdottir *et al*., manuscript in preparation). However, we have observed an increase in the number of $\gamma\delta$ T cells within the oral ulcers, extending deep within the oral mucosa (Freysdottir *et al*., manuscript in preparation). It is still not known whether these cells accumulate at the site of oral ulceration or whether they actively participate in the pathogenesis of mucosal ulceration. If non-specific ulceration was the only reason for accumulation of $\gamma\delta$ T cells, it could be postulated that there should be no difference between $\gamma\delta$ T cells in peripheral blood from patients with RAS and

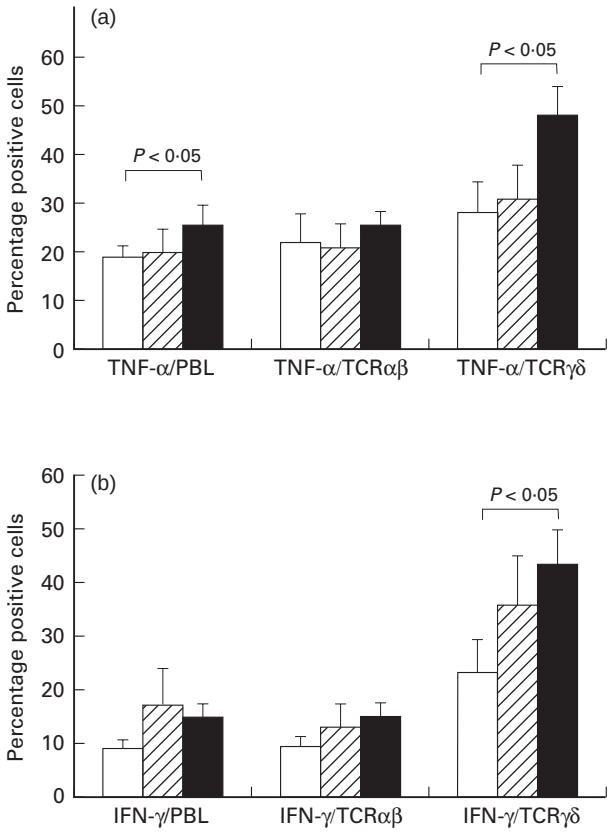

Fig. 4. The expression of tumour necrosis factor-alpha (TNF-α) on peripheral blood lymphocytes (PBL), $\alpha\beta$ T cells or $\gamma\delta$ T cells (a) and the expression of IFN-γ on PBL, $\alpha\beta$ T cells or $\gamma\delta$ T cells (b), analysed by double immunofluorescent staining and flow cytometry. Results are expressed as percentage positive cells, shown as the mean ± s.e.m. for 20 BD patients (■), 11 RAS patients (hatched bars) and 20 healthy controls (□). Statistically significant differences between BD patients and controls, BD patients and RAS patients, and RAS patients and controls are indicated.

patients with BD. We therefore analysed the activation and cytokine profiles of $\gamma\delta$ T cells in peripheral blood from patients with either BD or RAS and compared the results with those found in a healthy control population.

The proportion of $\gamma\delta$ T cells in peripheral blood from patients with BD was significantly higher than in RAS patients ($P<0{\cdot}05$) and in healthy controls ($P<0{\cdot}05$), as has been shown by us previously [3]. There was also an increase in the proportion of $\gamma\delta$ T cells in peripheral blood from patients with RAS. Although this increase did not reach significance, it supports the findings of Pedersen & Ryder [7], who reported a significant increase in the proportion of $\gamma\delta$ T cells in blood from RAS patients.

Both patient groups had a raised percentage of $CD8^+$ cells amongst the cells expressing the $\gamma\delta$ TCR. This suggests a role for $CD8^+$ cells in both these diseases, with the $\gamma\delta$ T cells being more prominent in BD. Furthermore, there was a significant increase in the proportion of $CD56^+$ cells in peripheral blood from BD and RAS patients compared with controls. CD56 is one of the markers of natural killer (NK) cells. Although CD56 has also been found on $\gamma\delta$ T cells [19], the large increase observed in the proportion of $CD56^+$ lymphocytes far exceeded $\gamma\delta$ T cells, indicating a genuine increase in the proportion of NK cells. The role of cytotoxicity in both RAS and BD has been proposed by the finding of peripheral blood leucocytes from the patients displaying a greater degree of cytotoxicity towards target epithelial cells than leucocytes from healthy controls [20,21]. This mechanism is further supported by the findings in this study of an increased percentage of lymphocytes expressing CD56, and $CD8^+$ $\gamma\delta$ T cells.

A classical way of analysing T cell activation is to measure the up-regulation of CD25, the α-chain of the IL-2 receptor. In BD patients it was clearly the $\gamma\delta$ T cell population that was in an activated stage, whereas the $\alpha\beta$ T cell subset showed no increase in CD25 expression compared with controls. This observation indicates that $\gamma\delta$ T cells in peripheral blood from BD patients had already been activated. Whether the activation occurs as a result of the disease or whether the activated $\gamma\delta$ T cells play a direct role in the pathogenesis needs to be elucidated.

It was of interest to investigate expression of other activation and adhesion molecules on the T cells that could interact with the various cells and extracellular matrix (ECM) proteins in the oral mucosa. Therefore, we analysed the expression of CD40L, CD29 and CD69.

An increased percentage of lymphocytes expressing CD40L was observed in patients with BD compared with controls. CD40L is primarily expressed on activated T cells, including $\gamma\delta$ T cells [22]. Its ligand, CD40, is expressed on B cells, endothelium, epithelium and some dendritic cells [23–25]. Interaction between CD40 and its ligand plays an important costimulatory role for B cells [23]. Whether interaction between CD40L on T cells and CD40 on the oral epithelium plays a role in maintaining healthy mucosa needs to be elucidated. The low frequency of $CD40L^+$ lymphocytes in peripheral blood of BD patients suggest that CD40L does not play a significant role in ulcer formation.

This study found a raised proportion of $\gamma\delta$ T cells expressing high levels of CD29 in both BD and RAS patients compared with controls. CD29, in combination with one of the α-integrin chains, comprises the very late activation (VLA) molecules. The VLA molecules interact with many of the ECM molecules, such as collagen, vimentin, laminin, fibronectin [26]. The major ligand for VLA-4 is the vascular cell adhesion molecule-1 (VCAM-1) molecule, which is expressed on activated endothelium [27]. Infiltrating T cells in oral lesional biopsies from BD patients are found surrounding blood vessels (Freysdottir *et al*., manuscript in preparation). Expression of raised levels of CD29 enables the $\gamma\delta$ T cells to interact with the endothelial cells, as well as with the ECM proteins that are present in the oral mucosa.

A raised percentage of $\gamma\delta$ T cells expressing CD69 was observed in BD patients compared with controls. CD69, an early activation marker on activated T and B cells, is involved in lymphocyte proliferation, and functions as a signal-transmitting receptor in T and B lymphocytes, NK cells and platelets [28]. The ligand for CD69 is still unknown, which makes it very interesting to analyse the distribution of the $CD69^+$ T cells at the lesional sites. This might cast light on to what extent $CD69^+$ T cells play a role in BD.

Unbalanced expression of Th1 or Th2 cytokines has been associated with inflammation in different animal disease models and may play a role in human inflammatory diseases. Previous studies analysing cytokines in BD and RAS have not been directed against individual cells, but have analysed cytokines in serum or in supernatants from bulk populations of PBMC [29–36]. These bulk populations consist of T cells and other lymphoid and non-lymphoid cells and secrete various cytokines, such as IL-1β,

IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), with the specific origin of the cytokines being unknown. The development of a method of detecting intracellular cytokines by flow cytometry [37] meant that it was possible to study the contribution of individual cells, in a heterogeneous cell population, to specific cytokine production.

Both patient groups had an increased proportion of $\gamma\delta$ T cells secreting IFN-γ when compared with healthy controls. Sugi-Ikai *et al*. [38] demonstrated an increased frequency of IFN-γ and IL-2 cytokine-producing $CD4^+$ and $CD8^+$ cells in peripheral blood from BD patients with active disease. Taken together, these two sets of data show that Th1 cytokine-producing cells, of both the $\alpha\beta$ and the $\gamma\delta$ TCR type, are present in BD and RAS patients. These Th1 cytokine-producing cells may play a specific role in the ulceration of both BD and RAS; or they may be up-regulated as part of the general inflammatory response.

TNF-α is another cytokine involved in inflammation which plays a major role in induction of adhesion molecules and inflammatory cytokine production. It is mainly produced by activated monocytes and macrophages. Our results show however that in BD a substantial proportion of $\gamma\delta$ T cells is able to participate in TNF-α production. Previous studies have also shown that the production of TNF-α is increased in patients with BD [30,31]. However, none have demonstrated which cell types participated in the TNF-α production.

If one assumes that mucosal ulceration causes an increase in TNF-α-producing $\gamma\delta$ T cells, no difference should be observed between patients with RAS or with BD. In this study however the BD patients had significantly raised percentages of TNF-α-producing $\gamma\delta$ T cells, indicating that this high percentage may be disease-specific. BD has been strongly associated with the HLA-B51 antigen, although it has been suggested that the important gene for BD is not the HLA-B51 gene but a gene located near the HLA-B gene [39,40]. One such gene is the gene encoding for TNF-α. Recent studies have shown that the levels of gene expression of TNF-α are related to the clinical activity of BD [41]. Whether there is a link between raised TNF-α production and any polymorphism within the TNF-α gene in BD needs to be investigated. Interestingly, thalidomide, which is known to suppress TNF-α production [42], causes healing of oral ulceration in BD ([43], personal observations).

The significant findings in this study were the increased percentage of $\gamma\delta$ T cells in peripheral blood of patients with BD, and that a greater proportion of these cells was in an activated stage and could be induced to secrete the inflammatory cytokines IFN-γ and TNF-α. These findings suggest that $\gamma\delta$ T cells may play an important role in the immunopathogenesis of BD. In order to confirm this, the presence of $\gamma\delta$ T cells in oral lesions from BD patients is being analysed with emphasis on their cytokine production and activation stage. The observation of an increased proportion of $\gamma\delta$ T cells which secrete TNF-α provides an interesting therapeutic strategy for the development of novel treatments for BD.

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