

EXTENDED REPORT

Systemic CD4⁺ T cell phenotype and activation status in intermediate uveitis

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Aim: To investigate peripheral blood lymphocyte phenotype in patients with intermediate uveitis using CD69, chemokine receptor, and cytokine expression.**Methods:** Peripheral blood lymphocytes of 18 patients with idiopathic intermediate uveitis and 6 patients with presumed sarcoid intermediate uveitis were evaluated for CD4⁺ T cell expression of CD69, CCR4, CCR5, CXCR3 and the intracellular cytokines IFN γ , TNF α , and interleukin (IL)-10 by flow cytometry, and for IL-2, IL-4, IL-5, IL-10, IFN γ , and TNF α production following unstimulated and activated culture using cytokine bead array and compared with healthy control subjects.**Results:** The expression of CD69 and TNF α by peripheral blood CD4⁺ lymphocytes of patients with idiopathic intermediate uveitis and presumed sarcoid intermediate uveitis was significantly higher than control subjects ($p=0.002$ and $p<0.05$, respectively). The ratios of the concentrations of IL-2:IL-5 and IFN γ :IL-5 in supernatants of activated peripheral blood lymphocyte cultures were significantly higher in patients with presumed sarcoid intermediate uveitis than control subjects.**Conclusions:** This study implicates TNF α in the pathogenesis of intermediate uveitis, highlighting the potential role of anti-TNF treatments for this disease. Studies of Th1:Th2 cytokine ratios suggested polarisation of the immune response towards Th1 in presumed sarcoid intermediate uveitis despite clinically quiescent systemic disease.

Intermediate uveitis is a chronic relapsing condition characterised primarily by inflammation of the pars plana, peripheral retina, and vitreous base. Typical clinical features include a mild anterior chamber reaction, cells and debris in the vitreous, peripheral retinal vasculitis, and cystoid macular oedema with minimal or no chorioretinal inflammation. Our understanding of the pathophysiology of intermediate uveitis and other posterior segment intraocular inflammatory (PSII) disorders is largely based on studies of the animal model, experimental autoimmune uveoretinitis (EAU), which suggest that intermediate uveitis and other forms of PSII are autoimmune diseases mediated by T helper (Th) type1 CD4⁺ T cells.¹ While an abundance of evidence supports the role of Th1 CD4⁺ T cells in EAU, such a clear Th1 bias has yet to be demonstrated in clinical studies. Increased levels of the Th1 cytokines interferon gamma (IFN γ) and interleukin (IL)-2 have been found in the ocular fluids and serum of patients with a wide range of uveitis diagnoses but definitive evidence of Th1 predominance, with the exception of Behçet's disease, is lacking.^{2,3} Tumour necrosis factor alpha (TNF α), a proinflammatory cytokine produced mainly by macrophages and T cells, is critical to the pathogenesis of a number of autoimmune diseases, including rheumatoid arthritis and multiple sclerosis, and has been shown to play a role in the development of inflammation in uveitis in both laboratory and clinical studies.^{4–9} Identification of the key mediators of uveitis might allow a more specific and targeted approach to treatment with biologic agents such as monoclonal antibodies and immunoadhesins.

Few studies have investigated T cell phenotype and cytokine production in either ocular fluid or serum specifically in intermediate uveitis. The presence of a preponderance of CD4⁺ T cells in the vitreous of patients with intermediate uveitis and in the area of snowbanking in pars planitis has been shown, substantiating its role as the main effector cell in intermediate uveitis.^{10–11} The finding of high levels of IL-12 in the vitreous of patients with active intermediate uveitis

may also suggest a role for Th1 cells in its pathogenesis, as IL-12 is a proinflammatory cytokine that is known to promote Th1 differentiation.¹²

Th1 and Th2 subsets have been traditionally identified on the basis of cytokine secretion; Th1 cells produce IL-2, and IFN γ , whereas Th2 cells secrete IL-4, IL-5, and IL-10. Preferential expression of certain chemokine receptors by Th1 and Th2 subsets has recently been demonstrated and presents an alternative way of identifying CD4⁺ T cell subsets.¹³ In this study we investigated peripheral blood CD4⁺ T cell phenotype in intermediate uveitis using cytokine and chemokine receptor (Th1 associated CCR5 and CXCR3, and Th2 associated CCR4) expression. We also measured CD4⁺ T cell expression of CD69, an early T cell activation marker, to evaluate the possible role of CD69 as a surrogate marker of systemic immune activation.

MATERIALS AND METHODS

Twenty four patients, comprising 18 patients with idiopathic intermediate uveitis and six patients with presumed sarcoid intermediate uveitis, were enrolled for this study. Mean age was 41 years (range 17–63) and 13 patients were female. The mean (SD) duration of uveitis was 4 (2.8) years. The diagnosis of sarcoidosis was presumed on the basis of radiological evidence of bilateral hilar lymphadenopathy but was not histologically proved and non-ocular clinical features of sarcoidosis were absent in all cases. At the time of blood sampling, 14 patients were on immunosuppressive therapy, including three patients on oral prednisolone alone and 11 patients on oral prednisolone and second line therapy with

Abbreviations: EAU, experimental autoimmune uveoretinitis; IFN γ , interferon gamma; IL, interleukin; PBS, phosphate buffered saline; PSII, posterior segment intraocular inflammation; Th, T helper; TNF α , tumour necrosis factor alpha

cyclosporin A, tacrolimus, azathioprine, or mycophenolate mofetil. Four patients were on topical steroids alone and six patients were not receiving any treatment. Clinical evaluation and appropriate investigations for other uveitis associated systemic diseases were negative in all cases. Ophthalmic examination was performed according to the international uveitis scoring system to evaluate uveitis activity and this was graded as inactive when vitreous and anterior chamber activity was scored as 1 or less and other signs of posterior segment inflammation were absent, or active.¹⁴ Clinical activity was assessed by the same observer (CCM) for all patients prior to and on the same day as peripheral blood lymphocyte analysis. The uveitis was active in five of 18 patients with idiopathic intermediate uveitis and two of six patients with presumed sarcoid intermediate uveitis. The remainder of the patients had had inactive intermediate uveitis for at least one month before lymphocyte analysis. Twenty three age and sex matched healthy control subjects (12 men and 11 women, mean age 38 years, range 20–54) with no history of ocular or systemic inflammation underwent peripheral blood lymphocyte analysis in parallel. Ethics Committee approval was received and informed consent was obtained from all participants.

PERIPHERAL BLOOD LYMPHOCYTE ANALYSIS

Isolation of lymphocytes

PrepaCyte and VitaLyse (BioErgonomics Inc, MN, USA) were used for the isolation and preparation of peripheral blood lymphocytes from 10 ml of heparinised venous blood obtained from each patient and control subject. The isolated lymphocytes were washed twice with phosphate buffered saline (PBS), centrifuged, resuspended in either PMA (phorbol 12-myristate 13-acetate) with ionomycin (activated culture) or basal medium (unstimulated culture) containing RPMI (ActiCyte PMA and basal media, BioErgonomics Inc) at a volume of 1 ml of culture medium to 1×10^6 lymphocytes and incubated for 4 hours at 37°C in 5% CO₂.

Flow cytometric analysis of CD4⁺ T cell CD69 and chemokine receptor expression

Four colour immunofluorescence using saturated concentrations of the following directly conjugated monoclonal antibodies (mAb) was undertaken: CD3FITC, CD4PerCP, CD69APC, and either CCR4PE, CCR5PE, or CXCR3PE. Isotype matched control antibodies were used for negative controls. All mAb were acquired from Becton Dickinson (San Jose, CA, USA) apart from CD69APC, which was obtained from Caltag Medsystems (Towcester, UK). 2.5×10^5 cells were incubated with the mAb for each of CD69, CCR4, CCR5, and CXCR3 for 30 minutes at 4°C in the dark and then washed twice in FACS buffer (PBS with 1% bovine specific albumin and 0.1% sodium azide) before immediate FACS acquisition. CD4⁺ T cell expression of chemokine receptors and CD69 was calculated by gating on the CD3⁺CD4⁺ cell population and 10 000 cells were analysed in each experiment using the CellQuest program (Becton Dickinson), after setting the quadrants using isotype controls.

Flow cytometric analysis of CD4⁺ T cell intracellular cytokine expression

Brefeldin A (Golgiplug, Becton Dickinson) and monensin (Golgistop, Becton Dickinson) were added at recommended concentrations immediately before cell culture in PMA/basal medium prior to intracellular staining of 2.5×10^5 lymphocytes for TNF α /IFN γ and IL-10, respectively. After surface staining for CD3 and CD4, cells were washed twice with FACS buffer, fixed with cellfix (Becton Dickinson) for 20 minutes at 4°C, then washed a further 2 times before being kept overnight in FACS buffer at 4°C. The following

morning the cells were permeabilised using 0.1% saponin and intracellular cytokine staining using saturated concentrations of IL-10PE, IFN γ PE, and TNF α APC (all from Becton Dickinson) was performed.

Flow cytometric cytokine bead array determination of peripheral blood lymphocyte cytokine production

After peripheral blood lymphocyte isolation, 5×10^5 lymphocytes were incubated in 0.5 ml of unstimulated or activated culture medium for 4 hours at 37°C without golgi inhibitor. The samples were then centrifuged and the supernatants removed and stored frozen until the day of BD cytokine bead array analysis (Becton Dickinson), which was performed as described elsewhere.¹⁵ Simultaneous quantification of IL-2, IL-4, IL-5, IL-10, IFN γ , and TNF α in the activated and the unstimulated lymphocyte culture supernatants was performed.

Evaluation of the Th1:Th2 balance using cytokine ratios

The Th1:Th2 balance was evaluated by calculating the ratio of the intracellular expression of IFN γ to IL-10 by unstimulated and activated peripheral blood CD4⁺ T cells and the ratio of the concentrations of IFN γ and IL-2 to IL-4, IL-5, and IL-10 in the supernatants of unstimulated and activated peripheral blood lymphocyte cultures.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test to detect significant differences between the CD4⁺ T cell expression of CD69, chemokine receptors, and cytokines of the groups described. Prism version 3.02 (GraphPad, San Diego, CA, USA) was used for all statistical calculations and significance was attributed when $p < 0.05$.

RESULTS

Expression of CD69, chemokine receptors, and intracellular cytokines by peripheral blood CD4⁺ T cells

Table 1 shows the median and interquartile range values for the flow cytometric measurement of CD4⁺ T cell expression of CD69, chemokine receptors, and intracellular cytokines of patients with idiopathic and presumed sarcoid intermediate

Table 1 Median (interquartile range) percentage expression of CD69, chemokine receptors, and intracellular cytokines by peripheral blood CD4⁺ T cells of patients with intermediate uveitis and normal controls (** $p = 0.002$, * $p < 0.05$ v controls)

	Idiopathic intermediate uveitis	Presumed sarcoid intermediate uveitis	Normal controls
CCR4	31.3 (26.0–37.1)	25.6 (20.8–27.8)	31.5 (27.2–33.0)
CCR5	5.5 (3.3–9.2)	6.0 (3.3–8.1)	6.0 (2.5–9.6)
CXCR3	21.0 (12.0–35.0)	13.7 (8.9–23.0)	21.1 (10.2–36.5)
CD69	52.5 (46.7–58.2)**	53.8 (52.2–56.9)*	38.4 (29.8–47.8)
IL-10 unstimulated	0.2 (0.2–0.6)	0.4 (0.3–0.5)	0.3 (1.2–0.7)
IL-10 activated	0.7 (0.3–2.9)	1.2 (1.0–1.6)	0.8 (0.6–1.6)
TNF α unstimulated	4.3 (2.9–6.1)	4.3 (4.1–6.8)	4.4 (1.2–9.1)
TNF α activated	70.7 (61.4–76.1)*	71.1 (68.9–74.3)*	56.5 (52.8–70.2)
IFN γ unstimulated	0.4 (0.2–0.6)	0.6 (0.5–0.6)	0.3 (0.2–0.4)
IFN γ activated	6.5 (4.9–10.4)	8.4 (8.3–14.5)	7.3 (5.5–12.1)

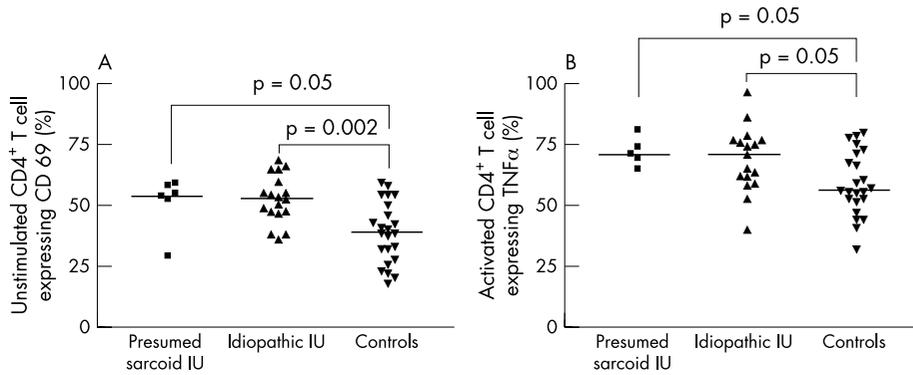


Figure 1 CD69 (panel A) and intracellular TNF α (panel B) expression by peripheral blood CD4⁺ T cells of patients with idiopathic intermediate uveitis (IU) and presumed sarcoid intermediate uveitis compared with healthy controls. The horizontal lines represent the median for each data set.

uveitis. CD4⁺ T cell expression of the T cell activation marker CD69 and the proinflammatory cytokine TNF α were significantly higher for both intermediate uveitis groups than for healthy controls, as shown in figure 1. Differences in the other variables measured between the intermediate uveitis groups and normal controls did not reach statistical significance. Furthermore, no difference was found in the CD4⁺ T cell expression of any of the variables measured when patients with active intermediate uveitis were compared with patients with inactive intermediate uveitis for the group as a whole ($n = 7$ v $n = 17$) or for idiopathic intermediate uveitis ($n = 5$ v $n = 13$).

Concentration of cytokines in the supernatants of peripheral blood lymphocyte cultures

Using the cytokine bead array, no significant difference was found in the concentration (pg/ml) of the cytokines TNF α , IFN γ , IL-2, IL-10, IL-4, and IL-5 in the supernatants of unstimulated or activated peripheral blood lymphocyte cultures of healthy controls compared with idiopathic intermediate uveitis and presumed sarcoid intermediate uveitis or on comparing active and inactive intermediate uveitis.

Peripheral blood lymphocyte Th1:Th2 cytokine ratios

The IL-2:IL-5 and IFN γ :IL-5 ratios, calculated from the cytokine concentrations in the supernatants of activated peripheral blood lymphocyte cultures, were significantly higher in patients with presumed sarcoid intermediate uveitis than in healthy controls (fig 2, $p < 0.05$ for both). The IFN γ :IL-10 ratio was also higher but this failed to reach statistical significance ($p = 0.08$). No difference was found in the ratio of the CD4⁺ T cell intracellular expression of IFN γ to IL-10 for patients with presumed sarcoid intermediate uveitis compared with normal controls. Finally, no difference was

found for any of the cytokine ratios for idiopathic intermediate uveitis compared with healthy controls, or for active compared with inactive intermediate uveitis, using both the CD4⁺ T cell intracellular expression of cytokines and the cytokine concentrations in supernatants of lymphocyte cultures.

DISCUSSION

We have shown in this study that the expression of the activation marker CD69 and the proinflammatory cytokine TNF α by circulating CD4⁺ T cells was increased in idiopathic and presumed sarcoid intermediate uveitis compared with healthy control subjects, but was not associated with disease activity. In addition, we found that the ratios of the concentrations of IL-2:IL-5 and IFN γ :IL-5 were increased in supernatants of activated peripheral blood lymphocyte cultures of patients with presumed sarcoid intermediate uveitis compared with healthy control subjects. This suggests polarisation of the immune response towards Th1 in this subgroup of patients.

Current treatments for uveitis aim to minimise intraocular inflammation and the frequency of sight threatening relapses without excessive immunosuppression and the associated risk of drug toxicity. In intermediate uveitis the course of the disease is often unpredictable, with some patients maintaining vision despite prolonged low grade intraocular inflammation, while others develop sight threatening disease that requires aggressive immunosuppression. The only method currently available for monitoring uveitis activity is clinical assessment. Deciding on when to reduce treatment once remission is induced can be difficult, and the immunosuppression requirement of individual patients is often determined by the occurrence of relapse on reducing treatment. Serological measurements of inflammatory activity using acute phase reactants such as C reactive protein, which can

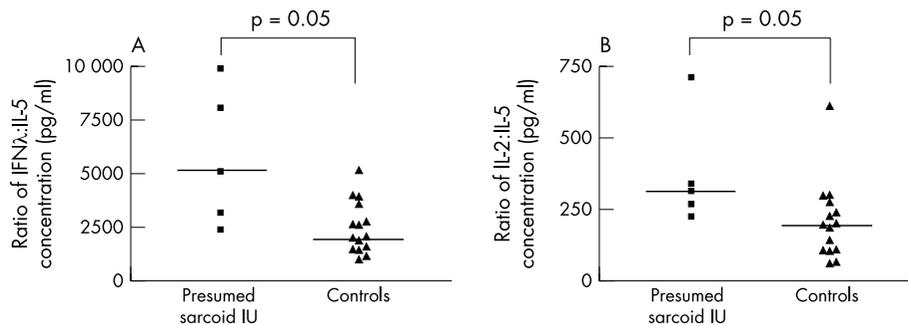


Figure 2 Ratio of the concentration of IFN γ :IL-5 (A) and the concentration of IL-2:IL-5 (B) in the supernatants of activated peripheral blood lymphocyte cultures of patients with presumed sarcoid intermediate uveitis and healthy controls. IFN γ concentrations greater than 5000 pg/ml were calculated from the mean fluorescent intensity by extrapolating the standard curve above 5000 pg/ml. The horizontal lines represent the median for each data set.

be sensitive indicators of systemic inflammation, are generally within normal limits in uveitis.¹⁶ Studies of circulating T cells and their inflammatory mediators have attempted to identify surrogate markers of disease activity, severity, and prognosis in uveitis. In a previous study our group showed that peripheral blood CD4⁺ T cell expression of CD69, an early activation antigen that can be expressed by T and B cells and a variety of other leukocytes,¹⁷ decreased in patients with posterior uveitis following mycophenolate mofetil treatment in parallel with control of clinical activity, suggesting that its measurement may help guide immunosuppressive therapy and even help predict relapses of intraocular inflammation.¹⁸ This contrasts with the results of the present study, which suggest that while idiopathic intermediate uveitis is accompanied by systemic immune activation, CD4⁺ T cell CD69 expression bears no correlation with uveitis activity when only a single rather than a series of measurements is evaluated. The high level of CD69 expression by circulating CD4⁺ T cells of both patients and healthy controls suggests that CD69 is a non-specific activation marker that may become upregulated by a variety of immunological insults. Thus, while CD69 may be a useful surrogate marker of ocular inflammation when measured longitudinally over a course of treatment, when it may also provide a measure of the response to treatment, a single measurement as in the present study is not useful in assessing disease activity. Klok and colleagues demonstrated increased levels of IL-8 and the intercellular adhesion molecule 1 (ICAM-1) in the serum of patients with active intermediate uveitis, and suggested that they may be used as both markers of ocular disease activity and predictors of the later development of an associated systemic disease.^{19, 20} However, these and other reports of the association of surrogate markers of immune activation with disease activity in uveitis, including sIL-2R levels and HLA-DR and CD25 expression by circulating CD4⁺ T cells,²¹⁻²³ will need to be confirmed and evaluated in longitudinal studies before they can become useful for predicting outcome of disease and monitoring of immunosuppressive therapy in clinical practice.

The enhanced expression of TNF α by peripheral blood CD4⁺ T cells in idiopathic intermediate uveitis in the present study suggests a role for this proinflammatory cytokine in the pathogenesis of this disease, and also implicates TNF α as a potential therapeutic target in intermediate uveitis. Recent reports have demonstrated that TNF α inhibition with the monoclonal antibody infliximab is effective at inducing remission in ocular Behçet's disease and juvenile uveitis, and anti-TNF α therapies are now in widespread use in severe cases of other autoimmune diseases in which TNF α has been shown to play a central role, including rheumatoid arthritis and Crohn's disease.²⁴⁻²⁷ To date, however, no reports have been published on the use of anti-TNF α therapies in intermediate uveitis.

The Th1:Th2 balance is considered to be important in the induction and regulation of autoimmune disease.²⁸ In EAU, Th1 type cytokines predominate during the active phase whereas IL-10 increases during recovery, indicating a shift towards Th2 as remission is achieved.²⁹ In the present study, we found no difference in the peripheral blood CD4⁺ T cell expression of the Th1 associated chemokine receptors CXCR3 and CCR5 or the Th2 associated chemokine receptor CCR4 between patients with intermediate uveitis and healthy controls, or on comparing patients with active and inactive disease. Although intracellular CD4⁺ T cell expression of TNF α was increased in idiopathic intermediate uveitis suggesting a proinflammatory CD4⁺ T cell phenotype, studies of Th1:Th2 cytokine ratios failed to identify a predominant CD4⁺ T cell subset in this type of intermediate uveitis. However, in presumed sarcoid intermediate uveitis we found

high IL-2:IL-5 and IFN γ :IL-5 ratios in the peripheral blood lymphocyte culture supernatants consistent with a Th1 phenotype. In all of these cases the diagnosis of sarcoidosis was presumed on the basis of radiological evidence of bilateral hilar lymphadenopathy associated with uveitis, with or without a raised serum angiotensin converting enzyme, and was not proved histologically. The T cell cytokine profile has been studied in bronchoalveolar lavage fluid and blood of patients with pulmonary sarcoidosis and a prominent shift towards a Th1 phenotype has been reported.^{30, 31} To our knowledge, this is the first report of a Th1 polarised immune response in sarcoid uveitis where systemic features of sarcoidosis were clinically quiescent.

In conclusion, this study implicates TNF α in the pathogenesis of intermediate uveitis, suggesting a potential role for anti-TNF α agents in the management of this disease. Although a clear Th1/Th2 bias in idiopathic intermediate uveitis was not found, the demonstration of high IL-2:IL-5 and IFN γ :IL-5 ratios in the peripheral blood lymphocyte culture supernatants of patients with presumed sarcoid intermediate uveitis suggests polarisation of the systemic immune response towards Th1 in ocular sarcoidosis. Finally, high CD69 expression by peripheral blood CD4⁺ T cells suggests that idiopathic intermediate uveitis is accompanied by systemic immune activation but its lack of correlation with clinical activity based on a single measurement suggests that its usefulness as a surrogate marker of inflammation is more suited to longitudinal studies.

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