CD69 Expression on Peripheral CD4⁺ T Cells Parallels Disease Activity and Is Reduced by Mycophenolate Mofetil Therapy in Uveitis

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**Purpose.** To assess the effects of mycophenolate mofetil (MMF) therapy on T helper cell activation status, using CD69 expression and cytokine profile with flow cytometry in relation to clinical activity in uveitis.

**Methods.** Patients with posterior or intermediate uveitis treated with MMF (n = 10), patients with active uveitis not treated with MMF and receiving no or minimal therapy (n = 10), and healthy volunteers (n = 21) had peripheral blood lymphocyte immunofluorescence analysis for T helper cell (CD4, CD3) markers, activation status (CD69), and intracellular cytokine (interleukin [IL]-2, interferon [IFN]-γ, and IL-4) levels. Patients were compared before and during MMF therapy in relation to T helper cell activation and clinical activity.

**Results.** Patients with active uveitis not treated with MMF and receiving no or minimal therapy had increased frequency of CD69-positive CD4 T cells (10.5% ± 4.6%, P = 0.0007) compared with healthy volunteers (3.3% ± 2.7%). Of all patients receiving MMF therapy, only patients with moderate to severe uveitis activity in the pre-MMF treatment group (n = 5; 15.5% ± 5.0%, P = 0.004) had increased frequency of CD69-positive CD4 T cells compared with healthy volunteers. During MMF therapy, a significant reduction in frequency of CD69-positive CD4 T cells occurred in patients with prior moderate to severe uveitis activity (to 8.9% ± 3.8%, P = 0.04). Levels of CD69-positive CD4 T cells in patients who had had inactive or mildly active disease (n = 5) before and during MMF therapy were comparable with levels in healthy volunteers. No significant changes in cytokine levels were found between the patient and control groups. A significant association between changes in frequency of CD69-positive CD4 T cells and changes in visual acuity (P = 0.008) and changes in vitreal haze (binocular indirect ophthalmoscopy score; P = 0.01) was observed in MMF-treated patients with prior moderate to severe uveitis activity.

**Conclusions.** Reduction in uveitis activity during MMF therapy correlates with reduction in frequency of peripheral blood CD69-positive CD4 cells. The frequency of CD69-positive CD4 T cells is a measure of activity in posterior uveitis and may guide adequate immunosuppression. (Invest Ophthalmol Vis Sci. 2001;42:1285-1292)

Mycophenolate mofetil (MMF) is a novel immunosuppressant with proven efficacy in preventing rejection in solid organ transplantation and autoimmune disease.¹ Posterior segment intraocular inflammation (PSII), a CD4 T-cell-mediated disease,² responds to MMF therapy, even when cyclosporine (CsA) or tacrolimus therapy has failed.³ MMF inhibits the de novo synthesis of purines and has a selective antiproliferative effect on lymphocytes.¹ The immunomodulatory action of MMF in vivo remains unclear. MMF does not interfere with either early T-cell activation, or cytokine production by CD4 T cells in vivo.⁴ However, mycophenolic acid (MPA), the active component of MMF, inhibits the production of a wide variety of cytokines after superantigen-stimulated peripheral blood lymphocytes (PBLs) in vitro.¹ ⁴

Evidence of systemic T-cell activation in active PSII is supported by observation of increased peripheral blood cell-associated CD25 expression and soluble interleukin (IL)-2 receptor (sIL-2R) levels.⁵ Such surrogate markers of immune activation are present in PSII without evidence of concomitant systemic inflammatory disease⁶ and serum IL-2R levels are reduced by effective CsA-mediated immunosuppression.⁵ Furthermore, unconfirmed reports show that IL-2R expression correlates with clinical disease activity.⁶ Confirmation of surrogate marker involvement in the effects of immunomodulation that allow us to either ensure adequate immunosuppression or predict clinical relapses is lacking in the management of PSII.

Animal models of PSII, particularly experimental autoimmune uveoretinitis (EAU), are CD4 T-cell (T helper) mediated, with a T helper (Th) type 1 cytokine profile with IL-2 and interferon (IFN)-γ secretion, rather than a Th2 profile, characterized by IL-4, IL-5, IL-6, and IL-10 secretion.⁷ ⁸ Such clear Th1-mediated effects have not been recorded in clinical studies of PSII. For example, enzyme-linked immunosorbent assay (ELISA) estimation of cytokine production in PSII shows no clear Th1/Th2 bias.⁹ Recently, however, a predominant Th1 cytokine secretion in active Behçet’s disease, detected at the single-cell level with flow cytometry, has been documented.¹⁰ Detecting changes in immune status in the peripheral blood in PSII is problematic because, first, sensitivity of assays may preclude detection of, for example, activation of antigen-specific T-cell clones and, second, the effector cells have largely homed to the target organ, where major effects may be seen.¹¹ However, increasingly sensitive single-cell assays permit recognition of surrogate markers of T-cell activation, such as CD69 upregulation.⁹ Although resting PBLs express CD69 at very low levels, with typical levels of 2.7% to 8.0%,¹⁰ CD69 is quickly induced after stimulation of the T-cell receptor (TCR)/CD3 complex, with detectable surface expression within 2 to 3 hours of antigen stimulation.⁹ Whereas levels of antigen-specific CD4 T cell responders in peripheral blood are low, higher CD69 expression on CD4 T cells may be found as a result of bystander activation, as may be the case in peripheral blood in PSII. The purposes of this study were to assess in peripheral blood by flow cytometry whether MMF immunomodulation affects CD69 expression, a marker of T-cell activation, on CD4

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T cells and T-cell cytokine profile in PSII and to determine whether such changes parallel clinical disease activity during MMF therapy.

**Methods**

**Patients**

Patients were recruited with informed consent from the uveitis clinics of Grampian University Hospitals National Health Service Trust who were participating in a small open-label study of MMF as rescue therapy in noninfectious PSII. Local ethics committee approval was obtained, and the study adhered to principles of the Declaration of Helsinki.

**MMF Group**

All MMF-treated patients ($n = 10$; mean age, $38.5 \pm 13.3$ years) had either current or previous CsA resistance, toxicity, or intolerance (Table 1). Of these, eight patients had PSII without systemic disease, and two had PSII with previous systemic manifestations (one sarcoidosis, one Behçet’s disease). Previous systemic therapy included combined corticosteroid (CST) and CsA therapy (six patients) or combined CST, CsA, and azathioprine therapy (four patients; Table 1).

The MMF treatment group was subdivided into two subgroups, a group with moderate to severe activity ($n = 5$) and a group with inactive to mild activity ($n = 5$)—that is, those with either no disease activity or mild activity before beginning MMF therapy. Patients with either no disease activity or mild activity had had intolerable or toxic adverse effects during CsA therapy and required alternative systemic immunosuppression to maintain inflammatory control. Azathioprine was withdrawn, and patients were changed to and maintained with MMF therapy at 2 g daily. Patients were treated with MMF alone (one patient) or in combination with CST (two patients), CsA (four patients), and combined CST and CsA therapy (three patients; Table 1). From baseline before beginning MMF, a reduction was observed in CST dosage during MMF therapy. Five patients were either not treated with CsA, or CsA dosage was reduced throughout MMF therapy (Table 1).

**Negative Control Subjects**

Negative control subjects were normal healthy clinic staff or university students ($n = 21$; mean age, $35.9 \pm 11.6$ years) with no previous history of ocular or systemic inflammation.

**Positive Control Patients**

Positive controls were patients not treated with MMF with clinically active noninfectious PSII ($n = 10$; mean age, $34.8 \pm 12.4$ years) receiving either no systemic therapy ($n = 5$) or suboptimal short-term low dose (<10 mg daily) CST monotherapy ($n = 5$). All patients with active PSII not receiving MMF therapy had moderate to severe uveitis activity deemed to require induction doses of systemic therapy or additional immunosuppression. Of these, five patients had panuveitis; four, intermediate uveitis; and one, posterior uveitis alone. Nine patients had isolated PSII, and one had PSII with systemic disease (Behçet’s disease).

**Clinical Activity Assessment**

Clinical disease activity was assessed by visual acuity, binocular indirect ophthalmoscopy (BIO) score (a measure of the clarity of fundal details affected by vitreal haze), and fundoscopy. Best corrected Snellen visual acuity was converted to log minimum angle of resolution (logMAR) for statistical comparison. Patients’ uveitis activity was deemed to be either moderate to severe or inactive to mild from the worse affected eye by observers (ADD, JVF) independently of estimation of PBL activation and cytokine profile (DJK). Severe uveitis activity was defined as active inflammation producing visual acuity of 6/60 or worse or BIO score greater than or equal to 4, moderate activity as visual acuity worse than 6/12 but better than 6/60 or BIO score of 2 or 3, and mild activity as low-grade uveitis with visual acuity better than or equal to 6/12 or BIO score less than 2.

**Blood Sampling and Time Course**

Blood sampling was performed from each negative control (normal subject), positive control (active PSII not treated with MMF and receiving no or minimal therapy) and pre-MMF treatment groups (pre-MMF moderate to severe and pre-MMF inactive to mild), with subsequent sampling at each clinic visit during MMF therapy (MMF previously moderate to severe and MMF previously inactive to mild), producing six groups for comparison.

**PBL Stimulation and Immunofluorescence**

**Monoclonal Antibodies, Reagents, and T-Cell Activation**

Five milliliters of heparinized whole venous blood was drawn from each subject and separated into 500-μl aliquots. Unstimulated PBL status was compared with 4 hours’ stimulation with phytohemagglutinin (PHA; 50 μg/ml, Sigma, St. Louis, MO) at 37°C. In parallel, for intracellular cytokine staining, the Golgi apparatus was stabilized by brefeldin A (BFA; 10 μg/ml, Sigma), which was included for the final 3 hours of incubation only, to allow antigen processing. To 50-μl aliquots of preincubated whole blood, 10 μl of directly conjugated monoclonal antibodies (mAbs) were added, using isotype-matched control mAbs, CD3 peridinin chlorophyll protein (PerCP), CD4 fluorescein isothiocyanate (FITC), CD69 phycoerythrin (PE), anti-IL-2 PE (Becton Dickinson, San Jose, CA), anti-IFN-γ FITC, and anti-IL-4 FITC (Pharmingen, San Diego, CA).

**Flow Cytometric Assessment of CD69 Expression.** For PBL activation, triple immunofluorescence staining was performed for both unstimulated and PHA-stimulated PBLs with CD3 (T lymphocytes)/CD4/Th/CD69 (activation marker), with isotype-matched control mAbs. Incubation with mAbs was performed for 30 minutes in the dark and room temperature (RT). Red blood cell lysis was then performed adding 2 ml of lysis buffer (Becton Dickinson) and incubating for 10 minutes at RT in the dark. Samples were then centrifuged at 322g for 5 minutes at 4°C. The cell pellets were washed in phosphate-buffered saline (PBS) 1% bovine specific albumin (BSA) 0.1% sodium azide and fixed in 400 μl FITC carboxymethylcellulose (CMC), which was included for the final 3 hours of incubation only, to allow antigen processing. To 50-μl aliquots of preincubated whole blood, 10 μl of directly conjugated monoclonal antibodies (mAbs) were added, using isotype-matched control mAbs, CD3 peridinin chlorophyll protein (PerCP), CD4 fluorescein isothiocyanate (FITC), CD69 phycoerythrin (PE), anti-IL-2 PE (Becton Dickinson, San Jose, CA), anti-IFN-γ FITC, and anti-IL-4 FITC (Pharmingen, San Diego, CA).

**Flow Cytometric Assessment of Intracellular Cytokine Production.** For parallel intracellular cytokine staining, surface staining was initially performed for both unstimulated and PHA-stimulated PBLs with CD3 PerCP and isotype-matched control mAbs. Samples were incubated, and red blood cell lysis performed. Cells were then permeabilized with 500 μl flow cytometry permeabilizing solution (FACS solution; Becton Dickinson) for 10 minutes at RT in the dark and washed. Intracellular staining was then performed with mAbs to IL-2, IFN-γ, and IL-4, by incubating 10 μl mAb with the cell pellet for 30 minutes at RT in the dark. Cells were then washed and fixed as previously described. Samples were acquired using a flow cytometer (FACSCalibur; Becton Dickinson).

**Background Fluorescence and Compensation.** Scatter characteristics, background fluorescence, and fluorescent compensation were established using isotype-matched and fluorochrome-matched control samples. Initially, background fluorescence and channel compensation were set using negative and positive mAb control samples. For negative mAb control samples, liberal gates were constructed using forward and side-scatter properties, after further backgating to exclude debris and dead cells. This reduced nonspecific staining that gives rise to the shoulder of fluorescence that occurs between negative and positive in control samples. This allowed a positive threshold to be clearly established—particularly important because CD69 expression was a graded increase in fluorescence from the negative value. For positive mAb control samples, PHA-stimulated CD69, and cytokine positivity, a large separation between peaks was obtained. Between 10,000 and 40,000 events were acquired for each sample, with data analysis by computer (CellQuest software; Becton Dickinson). The frequency of CD69 or cytokine-positive CD4 cells was...
### TABLE 1. Immunosuppressive Therapy before and during MMF Therapy

<table>
<thead>
<tr>
<th>Patient/Diagnosis*</th>
<th>Uveitis Activity†</th>
<th>Uveitis Duration‡ (y)</th>
<th>Cause of Visual Loss</th>
<th>MMF Indication</th>
<th>Previous Systemic Therapy</th>
<th>Final Systemic Therapy</th>
<th>Prednisolone§ Before</th>
<th>Prednisolone§ Final</th>
<th>Cyclosporine§ Before</th>
<th>Cyclosporine§ Final</th>
<th>Azathioprine§ Before</th>
<th>Azathioprine§ Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/Panuveitis</td>
<td>Moderate/severe</td>
<td>3.6</td>
<td>Vitritis, CME, cataract</td>
<td>CsA intolerance</td>
<td>CST, CsA</td>
<td>MMF, CST</td>
<td>20</td>
<td>10</td>
<td>4.4</td>
<td>4.4</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>2/Posterior</td>
<td>Moderate/severe</td>
<td>4.7</td>
<td>Macular edema</td>
<td>CsA toxicity (renal, HBP) and failure</td>
<td>CST, CsA, Aza</td>
<td>MMF, CsA</td>
<td>3.8</td>
<td>2.9</td>
<td>1.7</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/Intermediate</td>
<td>Moderate/severe</td>
<td>1.1</td>
<td>Macular edema, ERM</td>
<td>CsA intolerance</td>
<td>CST, CsA</td>
<td>MMF, CsA</td>
<td>15</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/Intermediate</td>
<td>Moderate/severe</td>
<td>1.5</td>
<td>Vitritis, CME</td>
<td>CsA toxicity (renal) and failure</td>
<td>CST, CsA</td>
<td>MMF, CST, CsA</td>
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<tr>
<td>5/Panuveitis</td>
<td>Moderate/severe</td>
<td>18.5</td>
<td>Macular ischemia and edema, vitritis</td>
<td>CsA toxicity (renal, HBP) and failure</td>
<td>CST, CsA, Aza</td>
<td>MMF, CST</td>
<td>10</td>
<td>7</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/Intermediate</td>
<td>Inactive/mild</td>
<td>4.7</td>
<td>Vitritis, macular edema</td>
<td>CsA toxicity (HBP) and failure</td>
<td>CST, CsA</td>
<td>MMF, CsA</td>
<td>5</td>
<td>0</td>
<td>1.4</td>
<td>1.4</td>
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<td></td>
</tr>
<tr>
<td>7/Panuveitis</td>
<td>Inactive/mild</td>
<td>5.5</td>
<td>Macular edema, vitritis</td>
<td>CsA toxicity (renal, HBP) and failure</td>
<td>CST, CsA</td>
<td>MMF, CsA</td>
<td>10</td>
<td>7</td>
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<tr>
<td>8/Panuveitis</td>
<td>Inactive/mild</td>
<td>3.2</td>
<td>Macular ischemia and edema, vitritis</td>
<td>CsA intolerance</td>
<td>CST, CsA, Aza</td>
<td>MMF, CsA, CsA</td>
<td>30</td>
<td>15</td>
<td>2.9</td>
<td>4</td>
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<td>9/Panuveitis</td>
<td>Inactive/mild</td>
<td>8.9</td>
<td>Cataract, vitritis, ERM</td>
<td>CsA toxicity (renal) and failure</td>
<td>CST, CsA, Aza</td>
<td>MMF, CsA, CsA</td>
<td>5</td>
<td>0</td>
<td>2.3</td>
<td>1.2</td>
<td></td>
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<tr>
<td>10/Intermediate</td>
<td>Inactive/mild</td>
<td>1.0</td>
<td>CME, cataract</td>
<td>CsA intolerance and failure</td>
<td>CST, CsA</td>
<td>MMF, CST, CsA</td>
<td>15</td>
<td>7</td>
<td>4.7</td>
<td>4.7</td>
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<td></td>
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</table>

CME, cystoid macular edema; ERM, epiretinal membrane; HBP, high blood pressure; Aza, azathioprine.

* Anatomic diagnosis as panuveitis or intermediate or posterior uveitis.

† Moderate/severe uveitis activity or inactive/mild activity as defined in the Methods section, patients 8 and 9 were included in the inactive/mild activity group but had visual acuity of 6/36. One of these also had a BIO score of 3, not due to moderate/severe uveitis activity but mainly due to cataract and macular ischemia.

‡ Duration of uveitis prior to starting MMF therapy.

§ Dose just before MMF therapy and final dose during MMF therapy at last clinic visit; CsA (patients 1, 5, and 7) and CST (patients 2, 3, and 7) were withdrawn before initiation of MMF therapy. Doses are expressed in milligrams per day for prednisolone and in milligrams per kilograms body weight per day for CsA and azathioprine.
determined after further gating on a live CD3⁺ population. To assess intertest variability, the average difference in frequency of CD69-positive CD4 T cells when the same subjects were evaluated on different days was 0.4% (range, 0.1%–0.7%) for normal subjects and 0.5% (range, 0.2%–0.8%) for patients with PSII with nonfluctuating clinical activity.

Statistics
The mean and SD were compared for most groups, except when the median value for each patient from data recorded at each visit during MMF therapy was used for comparison with single baseline values in the two pretreatment groups and two control groups.

Data from patient and control groups were tested for normality using the Kolmogorov–Smirnov test using statistical software (Prism 2.0; GraphPad, San Diego, CA). If normally distributed, groups were compared using an unpaired t-test with unequal variance. Otherwise, statistical comparison was performed using nonparametric tests (Mann–Whitney test and Pearson correlation coefficient) with significance attributed when two-tailed P ≤ 0.05.

RESULTS
CD69 Expression in Active PSII and during MMF Therapy
Increased frequency of CD69-positive CD4 T cells (10.5% ± 4.6%, P = 0.0007) was observed without mitogen stimulation in patients with active PSII not treated with MMF and receiving minimal or no treatment compared with normal subjects (3.4% ± 2.7%; Fig. 1). Of the MMF-treated patient groups (Fig. 2), only those in the pre-MMF treatment subgroup with moderate to severe activity showed a statistically significant increase in frequency of CD69-positive CD4 T cells (15.5% ± 5.0%, P = 0.004) compared with normal subjects. A significant reduction in frequency of CD69 cells (8.9% ± 3.8%, P = 0.04) was observed during MMF therapy, compared with pretreatment levels in patients who had moderate to severe activity at the beginning of MMF therapy. However, a significant increase in frequency of CD69 cells occurred during MMF therapy in patients who had inactive disease or mild activity before beginning MMF therapy (from 2.1% ± 1.4% to 6.1% ± 3.0%, P = 0.04; Fig. 2), but measurements were still within the 95% range (mean ± 2SD) of measurements from the normal group (Fig. 1).

The number of patients with CD69 positivity above a cutoff level of 2 SD from the mean of normal subjects was seven (70%) of the patients with active PSII not treated with MMF. All five patients in the pre-MMF treatment group with moderate to severe activity had positivity above this threshold level. Positivity in two patients remained above this level despite MMF therapy. None of the five patients in the pre-MMF treatment group with prior inactive disease or mild activity had positivity above the cutoff level, and positivity in four of these patients remained below the cutoff level during MMF therapy.
**DISCUSSION**

This study demonstrates that changes in frequency of CD69-positive CD4 T cells, an activation marker that represents a surrogate marker of immune activation in PSII, are parallel to changes in clinical activity in response to MMF therapy. Reduction in uveitis activity achieved by instituting MMF therapy in patients with previous CsA resistance or intolerance was accompanied by a reduction in frequency of CD69-positive peripheral blood CD4 T cells, with a corollary increase in frequency of CD69-positive cells during a period of disease reactivation. The severity of clinical uveitis is graded using a standardized uveitis scoring system, and changes in visual acuity and BIO score are the most important components. Visual acuity is the best single determinant of clinical uveitis activity, and changes in visual acuity had the strongest association with changes in CD69 positivity in patients with prior moderate to severe uveitis activity. In addition, BIO score has been found to significantly improve after MMF therapy, and reduction in BIO score was also significantly associated with reduction in CD69 positivity in this study.

Currently, the only method of monitoring disease activity in PSII is by clinical assessment. This method may not reflect the true status of underlying lymphocyte activation, as demonstrated in the present study in which media opacities or macular scarring or ischemia may produce a poor clinical assessment not due to active inflammation. Nonspecific measurements of inflammatory activity, such as erythrocyte sedimentation rate or acute phase reactants (for example, C-reactive protein), are generally normal in isolated ocular inflammation. More specific assays of immune activation, such as measurement of antineutrophil cytoplasmic antibody (ANCA) titers in ANCA-positive uveitides, CD25-positive CD4 T-cells CD25 or sIL-2R levels, serum IL-8 levels, and Th1 cytokine levels, have all been used to assess inflammatory activity in uveitis, but results of these studies are largely unconfirmed, and many markers have not been monitored longitudinally. Our study shows that longitudinal changes in frequency of CD69-positive CD4 T cells correlate and aid in the assessment of uveitis activity and may predict incipient disease reactivation and guide the need for adjusting immunotherapy with MMF.

A cutoff level for CD69 positivity implies a threshold degree of bystander activation and is arguably a more meaningful way of determining correlation with clinical activity. The assumption is that CD69 expression with T cell activation does not
have a continuous linear relationship with disease severity. Our results in part support this notion, in that all the patients with MMF with prior moderate to severe activity had CD69 positivity above this threshold level, whereas none of those with prior inactive or mild disease activity had positivity above this level. Moreover, only mild clinical improvement was observed in the two patients (patients 1 and 3, Table 1 and Fig. 2A) in the MMF treatment group with prior moderate to severe activity who had CD69 expression persistently increased above threshold despite MMF therapy. Although there was a significant increase observed in the frequency of CD69-positive cells during MMF therapy in the five patients with prior inactive or mild disease activity, in four of these patients CD69 positivity remained below the threshold level during therapy. This further supports the theory that a threshold level is a more clinically relevant means of assessing CD69 expression with T cell activation.

MMF acts to inhibit T- and B-cell proliferation and limits humoral responses. It may also act by inhibiting glycosylation of adhesion molecules reducing lymphocyte adhesion at sites of inflammation. Increased lymphocyte CD69 expression occurs very quickly after antigen stimulation, and persistent increased expression can occur with continuing antigen challenge. CD69 expression on lymphoid cells requires prolonged elevation of cytosolic Ca²⁺ concentration and resultant protein kinase C (PKC) activation. Although there are no previous reports of a direct effect of MMF on CD69 expression, reduction in CD69-positive PBLs after MMF therapy may result from reduced cyclic guanyl nucleotide levels, thereby reducing PKC activity. Reduction in CD69-positive PBLs may be an indirect bystander effect of MMF therapy, from reduced lymphocyte proliferation, that inhibits lymphocyte clonal expansion. Another indirect effect of introducing MMF therapy, reduction in the CST dosage required to control disease, may also account for the reduction in the frequency of CD69-positive cells.

Our data do not support a distinct Th1 or Th2 cytokine role in PSII, which is supported by previous work on ELISA cytokine analysis of peripheral blood and ocular fluid samples in patients with uveitis. However, unstimulated and anti-CD3 mAb-stimulated CD4-positive PBLs have been shown by flow cytometry to have a Th1 cytokine profile in patients with active Behçet’s disease treated with immunosuppressive drugs. Effective immunsuppression with CsA or tacrolimus reduced the frequency of IL-2- and IFN-γ-secreting CD4 T cells. Our patient group had PSII treated with combination immunotherapy, mostly with CsA, and this may explain why a distinct Th1 cytokine profile was not seen in the pre-MMF treatment group with active uveitis compared with those patients with inactive disease or normal subjects. Ideally, only patients with active untreated PSII should have been studied on MMF monotherapy to answer these questions, but in this study, MMF was reserved for patients with PSII that was intolerant or resistant to conventional immunotherapy.

**FIGURE 4.** Changes in CD4-positive T-cell CD69 expression (A, D) compared with changes in clinical uveitis activity, assessed by visual acuity (logMAR, B, E) and BIO score (C, F) during MMF therapy. Patients with prior moderate to severe uveitis activity (●, A, B, C) and prior inactive to mild uveitis activity (○, D, E, F) were monitored over time from the beginning of MMF therapy. Levels shown are mean ± SEM.
Although PHA stimulation produced appreciable cytokine staining, the fact that there were no significant differences in cytokine staining between patient and control groups is probably because of the natural heterogeneity in cytokine expression and the small sample size. MMF is reported to have no effect on the early production of IL-2, IFN-\(\gamma\), or IL-4, and this may also explain the absence of inhibition of PHA-induced cytokine secretion. For future work, it would be informative to sort CD69-positive cells from CD69-negative cells and repeat the cytokine analysis comparing the two subsets, specifically examining the effects of MMF therapy on cytokine secretion by CD69-positive cells.

Despite reports of systemic markers of immune activation in PSII, negative reports have suggested that such assays—for example, serum IL-2R levels—are not an accurate or appropriate measurement of ocular inflammatory activity. These discrepancies may be because such assays are insufficiently sensitive. One theory is that the increase in frequency of CD69-positive cells and subsequent decrease after MMF therapy may be due to systemic disease rather than ocular activity.

However, the present experimental evidence in predominantly isolated ocular disease strongly supports systemic activation of CD4 T cells and evidence of circulation of cells between the eye, regional lymph nodes, and spleen concomitant with breakdown of the blood–ocular barrier. Thus, we have been able to detect a clinically relevant surrogate systemic marker of immune activation in PSII. Although such studies of peripheral immune activation may not offer advances in our direct understanding of the immunopathogenesis of PSII, measurement of frequency of CD69-positive peripheral blood CD4 T cells may guide adequate immunosuppression and preempt clinical relapses, as we have shown in this small cohort of patients treated with MMF.

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References