Multiplex Bead Immunoassay Analysis of Aqueous Humor Reveals Distinct Cytokine Profiles In Uveitis

S. John Curnow,[1,2] *Francesco Falciani,*[3] *Omar M. Durrani,*[2] *C. M. Gemmy Cheung,*[2] *Emma J. Ross,*[1] *Kaska Wloka,*[1,2] *Saaeha Rauz,*[2] *Graham R. Wallace,*[1,2] *Mike Salmon,*[1] *and Philip I. Murray*[2]

Purpose. To extensively characterize the complex network of cytokines present in uveitis aqueous humor (AqH), and the relationships between cytokines and the cellular infiltrate.

Methods. AqH from noninflammatory control subjects and patients with idiopathic, Fuchs' heterochromic cyclitis (FHC), and herpes-viral or Behçet's uveitis were analyzed for IL-1β, -2, -4, -5, -7, -8, -10, -12, -13, -15, TNFα, IFNγ, CCL2 (MCP-1), CCL5 (RANTES), CCL11 (Eotaxin), TGFβ2, and CXCL12 (SDF-1), using multiplex bead immunoassays. The cellular infiltrate was also determined for each sample.

Results. Idiopathic uveitis AqH, compared with noninflammatory controls, was characterized by high levels of IL-6, IL-8, CCL2 and IFNγ, the levels of which correlated with each other. For IL-6 and IL-8 these levels were proportional to the number of neutrophils present. By contrast, the levels of both TGFβ2 and CXCL12 decreased in idiopathic uveitis AqH with increasing inflammation. Cluster analysis showed a degree of segregation between noninflammatory and idiopathic uveitis AqH. Further examination using random forest analysis yielded a complete distinction between these two groups. The minimum cytokines required for this classification were IL-6, IL-8, CCL2, IL-13, TNFα, and IL-2.

Conclusions. Application of multiplex bead immunoassays has allowed us to identify distinct patterns of cytokines that relate to both clinical disease and the cellular infiltrates present. Bioinformatics analysis allowed identification of cytokines that differentiate idiopathic uveitis from noninflammatory control AqH and are likely to be important for the pathogenesis of uveitis. (*Invest Ophthalmol Vis Sci.* 2005;46:4251–4259) DOI: 10.1167/iovs.05-0444

From the [1]Institute of Biomedical Research, MRC Centre for Immune Regulation, Division of Immunity and Infection, Medical School, The University of Birmingham, Birmingham, United Kingdom; [2]Academic Unit of Ophthalmology, Division of Immunity and Infection, University of Birmingham, Birmingham and Midland Eye Centre, City Hospital NHS Trust, Birmingham, United Kingdom; and [3]School of Biosciences, The University of Birmingham, Birmingham, United Kingdom.

Supported by grants from the Birmingham Eye Foundation, Arthritis Research Campaign (SO652) and Medical Research Council UK.

Submitted for publication April 8, 2005; revised June 29, 2005; accepted September 7, 2005.

Disclosure: **S.J. Curnow**, None; **F. Falciani**, None; **O.M. Durrani**, None; **C.M.G. Cheung**, None; **E.J. Ross**, None; **K. Wloka**, None; **S. Rauz**, None; **G.R. Wallace**, None; **M. Salmon**, None; **P.I. Murray**, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: S. John Curnow, Institute of Biomedical Research, Division of Immunity and Infection, Medical School, The University of Birmingham, Birmingham B15 2TT, UK; s.j.curnow@bham.ac.uk.

Uveitis is a group of diseases characterized by significant sight-threatening intraocular inflammation.[1,2] Many cases resolve rapidly, but a significant number of patients will develop persistent disease, with inflammatory damage to ocular structures resulting in severe visual impairment. A number of cytokines and chemokines known to play key roles in the control of inflammatory responses are elevated in uveitis aqueous humor (AqH).[3–12] Although some of these molecules can be increased in the serum of patients with uveitis,[13] their measurement in serum does not adequately describe the inflammatory processes occurring within the eye. In human uveitis, elevations in AqH IL-6, IL-8 (CXCL8), IL-10, IL-12, CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES), and IFNγ have all been reported.[6,8,11,12,14–19] These studies correlate well with experimental uveitis where, during the course of experimental autoimmune uveoretinitis and endotoxin-induced uveitis, levels of IL-6, TNFα, IL-1β, CCL2, CCL3, CCL5, and IFNγ increase with disease.[20–24]

Despite these studies, examination of the complex patterns of these molecules in human uveitis has been limited by the quantity of AqH available from each patient. For most studies, comparisons were made using either a few samples for each cytokine or by using a different cohort of patients for each molecule of interest. This has resulted in inconsistent reports in the literature for a number of cytokines, including IL-12 and IL-10,[8,10–12,25] and these studies have been unable to analyze the complex relationships between the cytokines and clinical disease. Recently, multiplex-bead-based immunoassays have been established that allow the identification of many molecules in a single small sample volume.[26,27] They have already been successfully used to measure cytokines in serum, cerebrospinal fluid, tears, and culture supernatants,[28–32] and this technique is ideally suited to the analysis of AqH. By applying conventional statistical tests to the multiplex data, it may be possible to identify cytokines and chemokines that are differentially expressed between two groups of patients (e.g., uveitis vs. noninflammatory controls). However, this approach examines only one molecule at a time and does not take into consideration that a combination of cytokines may be required to adequately describe the differences between two groups of patients. Methods to identify multimarker predictors of disease status have been developed and applied in a large number of clinical studies. In this study we have used a recently developed method, random forest analysis.[33] This method is based on the principal of decision trees and incorporates efficient methods to establish the importance of each variable in the classification and to perform an unbiased estimate of the classification error. Converting the structure of the trees into a two-dimensional model allows groups of patients to be visually represented in a graphical manner.

The aim of our study was to undertake multiplex immunoassay analysis of uveitis AqH for cytokines and chemokines and to apply both traditional and cluster analysis to the data. The ability to measure numerous molecules in a single sample and

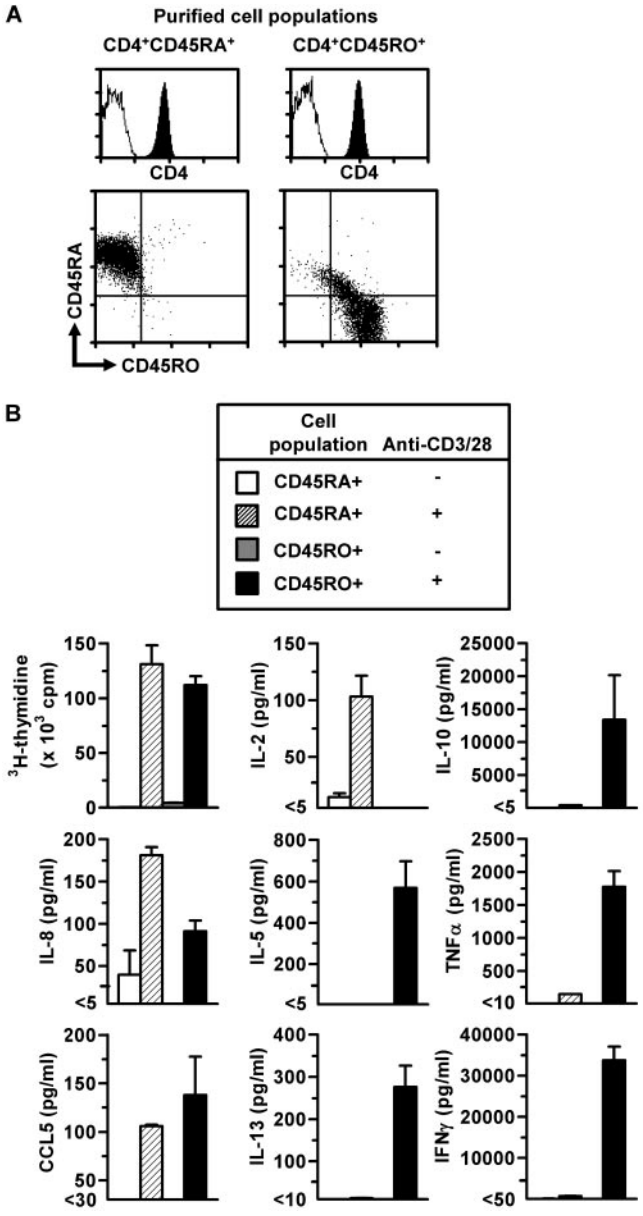

FIGURE 1. Distinct cytokine profiles can be detected from naïve and primed $CD4^+$ lymphocytes by multiplex bead immunoassay. $CD4^+CD45RA^+$ (>98%) and $CD4^+CD45RO^+$ (>95%) lymphocytes were purified from peripheral blood (**A**) and cytokine production or [^{3}H]thymidine incorporation was measured at 48 h (**B**). Results are expressed as the mean ± SD of triplicate wells and are representative of 3 separate experiments.

to perform complex analysis may allow a greater insight into the intraocular environment during active uveitis and underlying pathogenetic mechanisms.

METHODS

Patients, Diagnosis, and Aqueous Humor Samples

AqH samples (approximately 100 μL) were collected from 37 patients with active uveitis by using an insulin syringe. Sample collection followed the tenets of the Declaration of Helsinki, was approved by the West Birmingham Local Research Ethics Committee, and written informed consent was obtained from each patient. AqH samples were from patients with idiopathic uveitis (23/37), herpes-viral uveitis (5/37; 3 varicella-zoster virus [VZV], 2 herpes-simplex virus [HSV]—all confirmed by PCR on intraocular fluid), Behçet's disease (4/37—all fulfilled the International Study Group [ISG] criteria), and FHC (5/37). The uveitis was classified as idiopathic if investigations failed to reveal an associated or underlying cause. The uveitis was anterior in 24 and pan uveitis in the remainder. The majority of patients were on no treatment at the time of sampling (23/37) with the remainder on topical (12/37) or systemic (2/37) corticosteroids. Uveitis patients had a mean age of 40.2 years (range, 18–66 years). Noninflammatory control group specimens were collected from individuals undergoing routine cataract surgery (mean age, 73.3 years; range 59–94 years, $n = 12$).

AqH was centrifuged at 300*g* for 5 minutes, the cell-free supernatant (subsequently referred to as AqH) was removed and frozen in aliquots at −70°C. Cell pellets were resuspended, counted, and cytocentrifuged (Cytospin; Shandon Inc., Pittsburgh, PA). Slides were stained with Diff-Quik (Dade Behring, Marburg, Germany) and viewed by light microscopy for determination of the number and the percentage of lymphocytes, macrophages, and neutrophils.

Purification of $CD4^+CD45RA^+$ and $CD45RO^+$ Peripheral Blood T Cells

Naïve ($CD45RA^+$) and primed ($CD45RO^+$) $CD4^+$ T cells were isolated from the peripheral blood of healthy volunteers. Peripheral blood mononuclear cells isolated by density gradient centrifugation were depleted of adherent cells by two rounds of incubation on plastic at 37°C for 30 minutes each. Nonadherent cells were incubated for 30 minutes at 4°C with a combination of antibodies specific for CD8, CD11b, CD14, CD16, CD19, TCRγδ, glycophorin A, and either CD45RA or CD45RO. After washing, the cells were incubated for 30 minutes at 4°C, with continuous rotation, with sheep anti-mouse IgG Dynabeads (Dynal Biotech, Wirral, UK). The bead depletion was repeated to produce $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ populations of >95% purity (typically >98%).

Cytokine Measurement in AqH

AqH samples (50-μL volumes) were analyzed using multiplex bead analysis that uses microspheres as the solid support for immunoassays and allows the analysis of all molecules from each sample.[27] IL-1β, -2, -4, -5, -8, -10, -12, -13, TNFα, IFNγ, CCL2, CCL5, and CCL11 were measured according to the manufacturer's instructions (Upstate Biotechnology UK, Buckingham, UK). IL-7, IL-15, TGFβ2, and CXCL12 were measured using a multiplex bead immunoassay developed in the laboratory. Paired anticytokine antibodies, already tested for use in ELISA (R&D Systems, Abingdon, UK), were selected, because they have already been extensively tested for cross-reactivity to other cytokines. Carboxylated xMAP fluorescent microbeads (Luminex Corporation, Austin, TX) were centrifuged, the pellet sonicated, and 100-μL beads (1.25×10^6) were activated with 5 mg/mL N-hydroxysulfosuccinimide sodium salt and 5 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 0.1M NaH_2PO_4, pH 6.2, for 20 minutes at room temperature. Beads were centrifuged and washed twice with PBS. Monoclonal capture antibody (50 μg) was added in a volume of 250 μL for 2 hours at room temperature. Antibody-coupled beads were washed 3 times with PBS 0.05% Tween-20 and were resuspended in PBS, 1% BSA, 0.05% NaN_3. Successful coupling of the capture antibody to the beads was assessed by staining with FITC goat anti-mouse IgG (Southern Biotech, Birmingham, AL), followed by flow cytometric analysis. Each antibody was coupled to a different fluorescent bead. Cross-reactivities in the multiplex assay were rigorously tested and were always <0.01%.

For cytokine measurements, AqH samples, diluted with an equal volume of PBS, 1% BSA, 0.05% Tween 20, were incubated with monoclonal antibody-coated capture beads (2×10^3 for each cytokine) for 2 hours at 20°C. Washed beads were further incubated with biotin-labeled polyclonal anti-human cytokine antibody (2 μg/mL) for 2

TABLE 1. Detection of AqH Cytokines by Multiplex Bead Immunoassay

	Controls (n = 12)		Idiopathic (n = 23)		FHC (n = 5)		Herpes-viral (n = 5)		Behçet's (n = 4)	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
IL-1β	16*	<5–81	31	<5–96	7	<5–17	41	<5–147	14	<5–67
IL-2	19	10–31	<10	<10–21	<10	<10	<10	<10–15	<10	<10–64
IL-4	12	<10–22	<10	<10–19	<10	<10	<10	<10–21	<10	<10
IL-5	<5	<5	<5	<5–168	<5	<5	<5	<5	<5	<5
IL-6	79	<10–1128	60104	<10–800000	140	<10–2537	2651	1812–24998	11689	2259–14879
IL-7	<50	<50–182	78	<50–494	140	<50–370	59	<50–118	132	<50–265
IL-8	<5	<5–22	105	<5–1728	27	11–83	188	43–3700	417	51–2312
IL-10	<10	<10	<10)	<10–151	<10	<10–19	230	86–423	<10	<10
IL-12	46	<10–74	<10	<10–33	<10	<10–27	<10	<10–22	<10	<10–17
IL-13	10	<3–18	<3	<3–8	<3	<3	<3	<3–7	<3	<3
IL-15	<100	<100	<100	<100–311	<100	<100–520	<100	<100	<100	<100–158
TNFα	17	12–75	12	<5–28	9	8–17	9	<5–13	<5	<5–20
IFNγ	65	<5–375	599	<5–16297	62	<5–189	393	128–997	214	45–702
GM-CSF	<10	<10–158	<10	<10–143	<10	<10–162	<10	<10	19	<10–39
CCL11	24	14–40	21	<5–153	19	<5–28	9	<5–29	<5	<5–19
CCL2	154	65–288	5057	132–35520	2343	457–9923	15484	2074–49909	7763	1251–29343
CCL5	15	<5–87	10	<5–618	24	7–34	57	6–101	6	<5–40
CXCL12	475	225–1380	90	<50–1235	339	175–1443	85	<50–416	109	<50–573
TGFβ2	353	<40–497	86	<40–667	209	170–332	<40	<40–344	<40	<40–104

* Results show the median (pg/mL) and range for each cytokine, and are representative of 2 separate experiments.

hours, followed by streptavidin-phycoerythrin (Upstate Biotechnology UK) at 40 μg/mL for 30 minutes. Samples were analyzed using a microbead analyzer (Luminex 100; Luminex Corporation). Standard curves of known concentrations of recombinant human cytokines (R&D Systems) were used to convert fluorescence units to cytokine concentration (pg/mL).

Statistical Analysis

Dunn's multiple comparison tests were used to compare the levels of each cytokine measured between the noninflammatory control, idiopathic, FHC, and herpes-viral and Behçet's uveitis groups. Spearman (two-tailed, nonparametric) correlations were used to assess the significance of correlations between each cytokine and between the cytokines and cellular composition of the AqH. This was only carried out for the idiopathic group. The level of confidence at which the results were judged significant was $P < 0.05$.

Cluster and Random Forest Analysis

Clustering was performed using average linkage on a similarity matrix derived using Pearson moment correlations. The analysis was performed using the Web-based toolset GEPAS. To develop classification models, we have used a recently developed tree classification algorithm, random forest.[33] This method grows a collection of decision trees constructed using a different bootstrap sample of the data. Each node is split, using the best among a subset of predictors randomly chosen at that node.

Importance of Individual Cytokines in the Classification

The random forest algorithm estimates the importance of a variable by looking at how much prediction error increases when data for that variable is permuted while all others are left unchanged. The necessary calculations are carried out tree by tree as the random forest is constructed. In this study we have determined the smallest number of cytokines that could be used in each classification.

Visualization of the Models

The models developed using random forest can be visualized graphically using a representation of the collection of trees. The first step is to produce a proximity matrix that represents the relative similarity between samples as represented in the trees. This is achieved as follows: the (i, j) element of the proximity matrix produced by random forest is the fraction of trees in which elements i and j fall in the same terminal node. The idea is that data points (patients, in our case) that are similar to one another will frequently end up in the same terminal node of a tree; exactly what is measured by the proximity matrix. Thus, the proximity matrix can be taken as a similarity measure, and multidimensional scaling using this similarity can be used to divide the original data points into groups for visual exploration.

RESULTS

Multiplex Bead Immunoassay Detection of Cytokines from Naïve and Primed $CD4^+$ T Lymphocytes

We first determined that multiplex bead immunoassays could efficiently detect changes in the profiles of cytokines and chemokines relevant to uveitis. $CD4^+CD45RA^+$ naïve and $CD4^+CD45RO^+$ primed T cells were purified from the peripheral blood of healthy control individuals (Fig. 1A). After nonspecific stimulation with anti-CD3/CD28, a range of cytokines was detected from a single 50 μL supernatant sample (Fig. 1B). Naïve $CD4^+$ T cells produced high levels of IL-2 but not a range of effector cytokines. By contrast, supernatants from highly purified $CD4^+CD45RO^+$ primed T cells contained only a very low level of IL-2, whereas T_H1 and T_H2 effector cytokines IL-5, -10, -13, TNFα, and IFNγ were all detectable at high levels (Fig. 1B). These cytokines were only detected after anti-CD3/28 stimulation. IL-8 and CCL5 could be detected from both naïve and primed $CD4^+$ T cells. The proliferation of the two subsets of cells was equivalent. These results demonstrate that multiplex bead immunoassays can be used to identify distinct cytokine profiles in single small sample volumes.

Alterations of Cytokines in Idiopathic Uveitis, Compared with Noninflammatory AqH

The concentrations of IL-1β, -2, -4, -6, -7, -8, -10, -12, -13, -15, TNFα, IFNγ, GM-CSF, CCL2, CCL5, CCL11, CXCL12, and

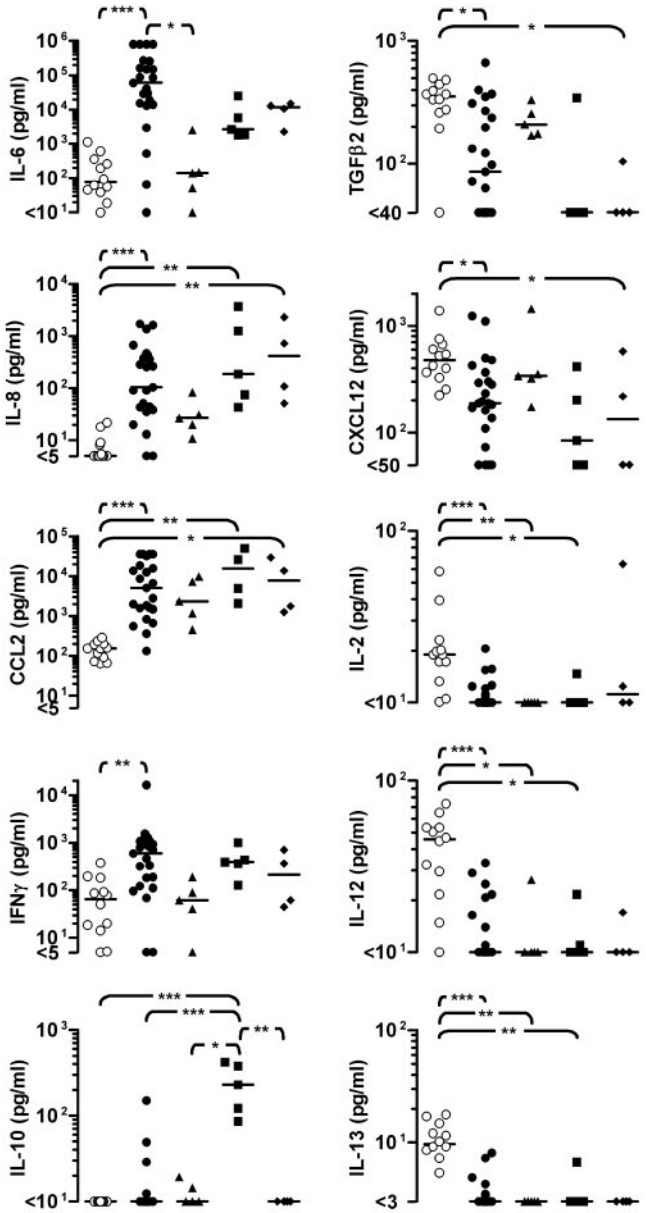

FIGURE 2. AqH cytokines detected by multiplex bead immunoassay. All cytokines were measured in each AqH from controls (*open circles*), idiopathic uveitis (*filled circles*), FHC (*triangles*), herpes-viral uveitis (*squares*), or Behçet's disease (*diamonds*). Results are representative of two separate experiments. All groups were compared for each cytokine using the Dunn's multiple comparison test. Where no *P* value is given, there was no significant difference between the groups ($P > 0.05$).

TGFβ2 were measured in each AqH sample from control patients undergoing routine cataract surgery and patients with idiopathic uveitis, FHC, herpes-viral and Behçet's uveitis (Table 1). Levels of IL-6, IL-8, IFNγ, and CCL2 were significantly increased in idiopathic uveitis AqH, compared with noninflammatory controls (Fig. 2, Table 1). Both TGFβ2 and CXCL12 were detectable in noninflammatory control AqH, but these levels were significantly reduced in idiopathic uveitis AqH. For IL-2, -12, and -13, there was a low level present in control AqH which decreased to be undetectable in the majority of idiopathic uveitis AqH. For all the other cytokines and chemokines tested, levels were either below detection level or did not significantly differ between idiopathic uveitis and control AqH. When comparing treated and untreated idiopathic uveitis AqH, the only significant differences were elevated IL-8 (untreated 248, treated 787 pg/mL; $P < 0.05$) and decreased TGFβ2 (untreated 182, treated 27 pg/mL; $P < 0.05$) in the treated group.

We were also able to compare the levels of cytokines between uveitis of specific uveitis entities (FHC, Behçet's disease, or herpes-viral uveitis) and idiopathic uveitis or control AqH. Despite the limited number of samples in each group, a number of significant differences were detected. IL-6 levels in FHC AqH did not differ from those in noninflammatory AqH, in contrast to idiopathic uveitis (Fig. 2, Table 1). However, FHC AqH showed significant decreases in the very low levels of IL-2, -12, and -13 found in noninflammatory AqH. The increased levels of IL-8 and CCL2 did not reach statistical significance. For both Behçet's and herpes-viral uveitis, there were significant elevations above the control group for CCL2 and IL-8. In Behçet's AqH, both TGFβ2 and CXCL12 were significantly lower than control AqH. Again, elevations in some cytokines were evident but did not reach statistical significance, probably due to the low number of samples available for analysis. One of the most striking observations was the presence of high levels of IL-10 in all herpes-viral uveitis AqH. Intriguingly, IL-10 was also found at similar levels in 3/23 idiopathic uveitis patients, although analysis of other individual cytokines for these patients failed to reveal any other significant differences.

Cluster Analysis of Cytokine Profiles in Idiopathic, FHC, Behçet's and Herpes-Viral Uveitis, and Noninflammatory Control Aqueous Humor

Traditional univariate statistical analysis allows the determination of cytokines that are significantly altered between two groups of samples. However, to understand the complex relationships between cytokines and to predict which cytokines might allow discrimination of sample populations, some form of multivariate analysis is required. Cluster analysis was performed on the levels of cytokines and chemokines for all AqH, and showed that noninflammatory control and idiopathic uveitis AqH tended to cluster on different branches of the dendrogram (Fig. 3). IL-6, IL-8 and CCL2, cytokines that were significantly elevated in uveitis AqH, clustered at one end of the dendrogram, with TGFβ2 and CXCL12, which were both decreased, at the other end. We applied random forest analysis to the data. This resulted in a 100% classification accuracy between noninflammatory control and idiopathic uveitis AqH (Fig. 4A). The importance of individual cytokines in classifying controls from idiopathic uveitis AqH was also calculated. Only IL-6, IL-8, CCL2, IL-13, IL-2, and TNFα were required for maximum classification accuracy (Fig. 4B). Clustering was also observed for 2/4 herpes-viral, 4/5 FHC, and 3/4 Behçet's uveitis AqH. The numbers of samples would have to be increased for each group to observe any significant clustering or to apply random forest analysis.

Correlations Between Cytokines and with the Cellular Infiltrate in Idiopathic Uveitis AqH

One of the major advantages of multiplex immunoassays is the ability to measure all the cytokines studied in each individual sample at the same time, reducing variability and allowing the determination of precise correlations between molecules. For idiopathic uveitis AqH, numerous positive correlations were observed, the most significant of which were between IL-8 and IFNγ ($r = 0.75$, $P < 0.0001$) and between TGFβ2 and CXCL12 ($r = 0.87$, $P < 0.0001$; Fig. 5, Table 2). By contrast, the levels

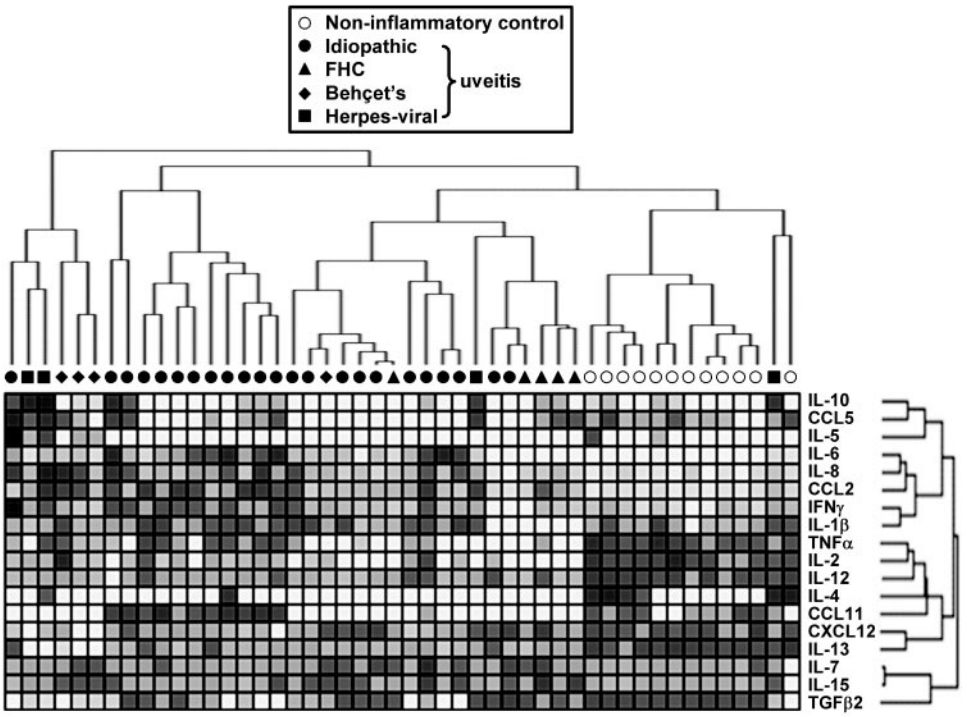

FIGURE 3. Two-dimensional cluster analysis of the cytokine/chemokine dataset. The similarity between profiles of different patients is represented by the *top* dendrogram, whereas the relationship between cytokines is represented by the dendrogram on the *right*. The expression level of the individual cytokines is represented by shades of *gray* in the central heatmap. The highest values are indicated in *white*, with negative values in *black*.

of TGFβ2 (or CXCL12) inversely correlated with IL-6 ($r = -0.74$, $P < 0.0001$) and IL-8 ($r = -0.53$, $P < 0.01$).

The relative proportions and the absolute numbers of lymphocytes, macrophages, and neutrophils were determined from cytocentrifuge preparations of each uveitis AqH. There were a number of positive correlations between IL-6, -8, -10, CCL2, IFNγ, and the cellular infiltrate in idiopathic uveitis AqH (Fig. 5, Table 3). The most significant were IL-8 with the total number of cells and IL-6 with the number of neutrophils. There was an inverse correlation between levels of TGFβ2 and the number of leukocytes and neutrophils. CXCL12 also showed an inverse correlation with neutrophil numbers. These data show close associations between cytokines in uveitis AqH and with the cellular infiltrate present.

DISCUSSION

Uveitis is a complex disease classified into specific syndromes, diseases of known etiology, and the remainder labeled as idiopathic. Despite the many analyses of cytokines and chemokines present in the inflammatory ocular environment, both aqueous and vitreous, there is little consensus on the patterns of molecules that may classify the etiology and the pathology of each disease. Despite idiopathic uveitis representing a heterogeneous group of conditions, our results using multiplex bead immunoassays and bioinformatic analysis have identified the specific cytokines that classify idiopathic uveitis AqH from noninflammatory control AqH. In addition many molecules, whose levels differ significantly in inflammatory AqH, correlate with the cellular infiltrate present.

Multiplexed bead-based immunoassays provide a powerful technique to identify cytokines and chemokines that are likely to play significant roles in the pathology of uveitis. However, although the use of multiplexed bead-based immunoassays is now well established,[27–30] there are a number of issues that merit discussion. With any new technology, it is important to establish that the results are consistent with previous technologies. A direct comparison of ELISA and bead assays revealed that the patterns of cytokines detected were the same in each case.[34] The authors detected variation between suppliers, but this applied to both bead-based and ELISA assays. Therefore, the bead-based assays do not appear to suffer from any greater variation than that found with more conventional analysis. As with conventional analysis, it is also necessary to ensure that measurements are reproducible. We performed repeated analyses for IL-6, IL-8, and IFNγ on a few samples, and, although the levels were not always identical, there was an excellent correlation ($r = 0.91$, $P < 0.0001$; data not shown). The data from in vitro–stimulated T cells also shows the low level of error for triplicate samples (Fig. 1B). We do not consider this to be any greater a problem than for conventional assays, and multiplexed assays have the advantage of reduced variability between different cytokines, because they are all measured in the same sample.

Once the reliability of multiplexed bead immunoassays is established, the next challenge is to analyze the large amount of data generated. We performed standard statistical analysis by using Dunn's multiple comparison, because these tests correct for multiple comparisons. It is important to appreciate that if sufficient comparisons are performed, then, by chance, a significant difference is likely to be detected. Only by using statistical tests that correct for multiple comparisons can this issue be circumvented, and, even under these conditions, considerable caution must be exercised when drawing conclusions. In addition to traditional statistical analysis, we performed both cluster and random forest analysis. Cluster analysis allows a visual profile to be established, giving an indication of the relationship between samples and cytokines. Although there is no quantitative statistical power to these profiles, they are often very useful. To classify the samples into control noninflammatory and uveitis AqH, we used random forest analysis. This method has the advantage of determining which molecules are required for maximal classification of the sample groups, therefore indicating which cytokines might play key roles in pathogenesis of the disease.

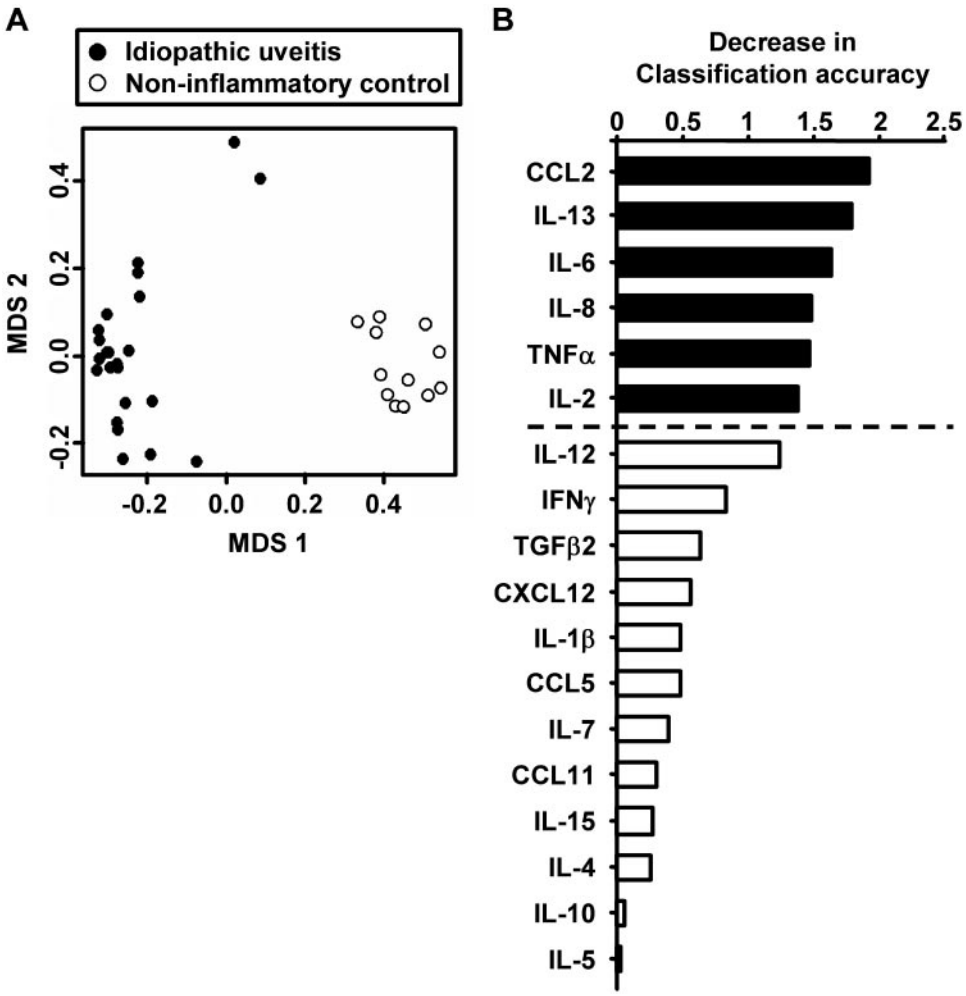

FIGURE 4. Classification of aqueous humor from noninflammatory controls and idiopathic uveitis. (**A**) Multidimensional scaling (MDS) representation of the proximity matrices of the random forest models demonstrating the relationship between individual patients in the models. The two axes represent the first and second MDS axes. (**B**) The relative importance of individual cytokines in classifying aqueous humor from controls and idiopathic uveitis. The individual plots represent the relative importance of the cytokines in the overall classification. The *vertical axes* represent individual cytokines sorted by importance. The *horizontal axes* represent the average decrease in classification accuracy. The *horizontal dotted line* shows the minimum number of cytokines required for maximum classification accuracy.

$CD4^+$ T lymphocytes are able to induce disease in experimental models and are found at high frequencies in uveitis AqH.[10] The cytokine responses of $CD4^+$ T lymphocytes can be polarized to a T_H1 (IFNγ) or T_H2 (IL-4) phenotype. Although the majority of experimental uveitis models induce a strong T_H1 response that correlates with susceptibility to disease,[35] T_H2 cells are also able to induce experimental uveitis.[36] Studies of human uveitis have favored a T_H1 dominated response; however, this issue remains unresolved.[37] Our results definitively show that uveitis AqH is dominated by T_H1 cytokines. We could not detect any of the T_H2 cytokines IL-4, -5, or -13, whereas IFNγ was significantly elevated in idiopathic uveitis and levels correlated with the numbers of all infiltrating leukocyte populations. The highest levels of IL-13 were actually found in noninflammatory control AqH and decreased in uveitis.

IL-12 is a heterodimeric complex made up of p35 and p40 subunits, able to polarize toward T_H1-type T cell responses. We were unable to detect significant elevations in the level of IL-12, instead, the very low levels found in noninflammatory controls decreased in uveitis AqH. The levels of uveitis AqH and vitreous IL-12 reported in the literature are inconsistent. Studies by el Shabrawi et al.[8] showed elevated IL-12 in the AqH and vitreous from active uveitis patients in the range 72 to 293 pg/mL. However, other studies of vitreous[38] and AqH[9] have failed to find these elevations. It is unclear why these discrepancies exist. Our assay detects the active p70 subunit of IL-12, whereas other assays may detect the p40 subunit that can be found in excess. It is also possible that IL-12 has not reached a peak in our samples, all of which were of very recent onset.

The etiology of uveitis is unknown but is assumed to be (auto)immune in nature. However, it is clear that a number of proinflammatory cytokines are elevated in the local ocular environment during episodes of the disease. We confirmed that high levels of IL-6, IL-8, CCL2, and IFNγ can be found in idiopathic uveitis AqH. Additionally, there were correlations between the levels of IL-6, IL-8, and IFNγ, suggesting that these cytokines are interdependent or co-regulated. IL-6 has been reported to antagonize the anti-inflammatory actions of TGFβ2,[39] and we have recently reported that this cytokine protects uveitis AqH lymphocytes from apoptosis.[40] In contrast to IL-6, the anti-inflammatory cytokine TGFβ2, measured here in its active form, decreased as the proinflammatory cytokines increased, consistent with the loss of immune regulation seen in experimental uveitis.[21,39] The same pattern was seen for the constitutive chemokine CXCL12, which has been shown to be downregulated by inflammatory cytokines.[41,42]

By applying cluster and random forest analysis to the cytokine and chemokine AqH data, we were able to classify idiopathic uveitis AqH from noninflammatory AqH with 100% efficiency. This classification only required the measurement of IL-6, IL-8, CCL2, IL-13, IL-2, and TNFα. This suggests that these cytokines are key molecules in the pathogenesis of idiopathic uveitis. Interestingly, IL-13, IL-2, and TNFα decreased in uveitis and were only found at very low levels in noninflammatory controls. The biological significance of this is unclear. It may

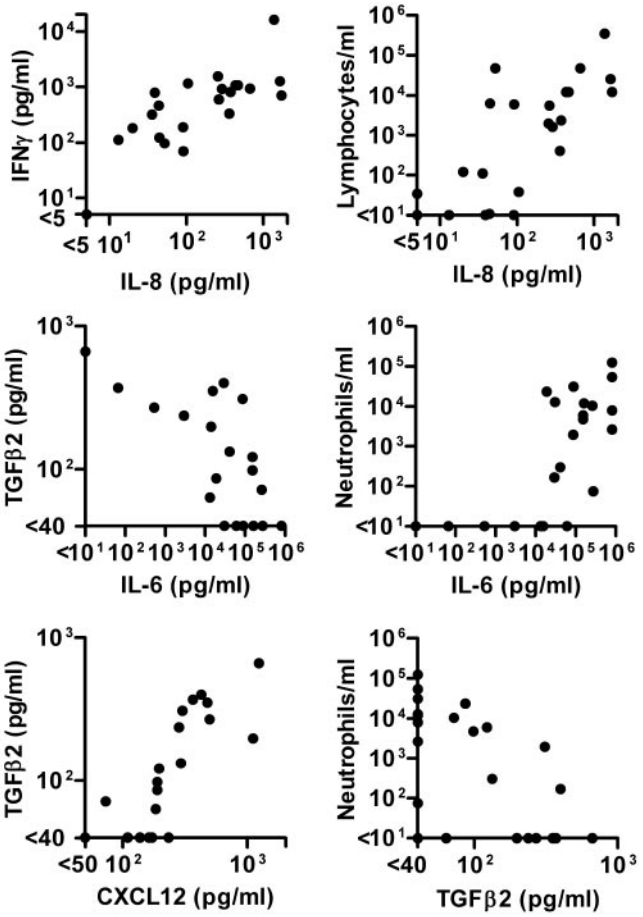

FIGURE 5. Correlation between cytokines and with the cellular infiltrate in idiopathic uveitis AqH. The number and the percentage of neutrophils, lymphocytes, and macrophages were determined from cytocentrifuge preparations of AqH. The cytokines were measured by multiplexed bead immunoassay from the same samples. The data represent some of the associations between cytokines and with the cellular infiltrate where significant correlations were observed. For details of all correlations see Tables 2 and 3.

reflect a true decrease in production or increased consumption of cytokine during inflammation. It is important to realize that AqH cytokines will only provide a reflection of the processes occurring within the ocular tissues, where local levels of some cytokines may be much higher. We performed all studies from patients with active disease and did not consider it ethical to take samples as inflammation resolves. Although we did not have the opportunity in this study, it would be very interesting to study the longitudinal profile of cytokines in patients with persistent disease.

As well as idiopathic uveitis, we analyzed the patterns of cytokines in AqH from FHC, herpes-viral uveitis, and Behçet's disease. Due to the small numbers of samples, it was not possible to draw firm conclusions or to perform random forest analysis. However, there were clear differences for some cytokines, and a degree of clustering was observed, suggesting that future studies with larger numbers of samples might reveal distinct cytokine/chemokine profiles in these groups of samples.

IL-10 is considered to be an immunoregulatory cytokine able to limit pathologic damage and is produced by some regulatory T cell subsets.[43] The relative concentration of IL-10 to IL-6 has been suggested as a useful marker to distinguish intraocular lymphoma from uveitis, although this issue is still controversial.[25,38] Our own data, in conjunction with published data on IL-10 in infectious uveitis,[12] clearly shows that in uveitis IL-10 is only present in combination with high levels of IL-6. Hence, our ratios of IL-10:IL-6 were 0.003–0.21 (median 0.03), in contrast to the ratios reported for intraocular lymphoma, which are in excess of 1.0. In a study by Ongkosuwito et al., [12] IL-10 was found in the AqH from 10/17 herpes-viral uveitis patients with a range of 29 to 3927 pg/mL but only 3/51 control samples that included 21 idiopathic uveitis cases, as well as cases of *Toxoplasma gondii* uveitis. Therefore, it seems likely that the majority of infectious uveitis will induce significant IL-10 production. The presence of IL-10 could reflect a host antiviral response, an attempt to regulate inflammatory damage and/or be an attempt by the pathogen to escape immune intervention. The γ-Herpes virus family carry many homologues of cytokines, chemokines, and receptors to deviate the host immune response, and these include IL-10.[44] It is possible that the α-Herpes-viruses (VZV, HSV-1 and -2), which do not carry this array of homologues, might instead induce a deviant host immune response, possibly involving IL-10. During the course of experimental autoimmune uveitis, IL-10 appears late in the disease[45] and has been proposed to be involved in the resolution of inflammation. Recently, peripheral blood lymphocytes from patients receiving anti-TNFα therapy were shown to secrete increased levels of IL-10,[46] though the significance of these results is currently unclear. IL-10 secreting lymphocytes have also been identified in both idiopathic and FHC uveitis.[47] We detected IL-10 in only 3/22 idiopathic uveitis AqH, which may reflect the immunoregulatory role proposed for this molecule. The absence of any clustering of these

TABLE 2. Correlations between Cytokines Significantly Altered in Idiopathic Uveitis AqH*

	IL-8	CCL2	IL-6	IL-10	IFNγ	TGFβ2	CXCL12
IL-8		NS	0.61 ($P < 0.01$)	0.49 ($P < 0.05$)	0.75 ($P < 0.0001$)	−0.53 ($P < 0.01$)	−0.50 ($P < 0.05$)
CCL2			NS	NS	NS	NS	NS
IL-6				NS	0.59 ($P < 0.01$)	−0.74 ($P < 0.0001$)	−0.65 ($P < 0.001$)
IL-10					0.63 ($P < 0.01$)	NS	NS
IFNγ						NS	−0.42 ($P < 0.05$)
TGFβ2							0.87 ($P < 0.0001$)

* The data represent the Spearman correlations (r) and significance between cytokines in idiopathic uveitis AqH, measured by multiplexed bead immunoassay. NS, not significant ($P > 0.05$).

TABLE 3. Correlations between the Cellular Infiltrate and Cytokine Levels in Idiopathic Uveitis AqH*

	IL-8	CCL2	IL-6	IL-10	IFNγ	TGFβ2	CXCL12
Total cells (per mL)	0.66 ($P < 0.001$)	NS	0.58 ($P < 0.01$)	0.45 ($P < 0.05$)	0.51 ($P < 0.05$)	−0.52 ($P < 0.05$)	−0.51 ($P < 0.05$)
Neutrophils (per mL)	0.61 ($P < 0.01$)	NS	0.71 ($P < 0.0001$)	NS	0.52 ($P < 0.05$)	−0.59 ($P < 0.001$)	−0.64 ($P < 0.01$)
(%)	NS	NS	0.60 ($P < 0.01$)	NS	NS	NS	NS
Lymphocytes (per mL)	0.75 ($P < 0.0001$)	NS	NS	0.47 ($P < 0.05$)	0.44 ($P < 0.05$)	−0.45 ($P < 0.05$)	NS
(%)	NS	NS	NS	NS	NS	NS	NS
Macrophages (per mL)	0.56 ($P < 0.01$)	NS	0.54 ($P < 0.01$)	NS	0.47 ($P < 0.05$)	NS	−0.42 ($P < 0.05$)
%	NS	NS	NS	NS	NS	NS	NS

* Spearman correlations (r) and significance between the the cellular infiltrate and cytokines in idiopathic uveitis AqH, measured by multiplexed bead immunoassay. NS, not significant ($P > 0.05$).

samples with the herpes-viral group suggests that these idiopathic cases do not represent undiagnosed herpes-viral uveitis.

Multiplex bead immunoassay analysis is a powerful tool with the ability to measure many different cytokines in a single sample, requiring only a 50-μL volume. This allows the quantification of each cytokine, and the examination of their relationship to each other and to the clinical characteristics of the disease. The application of multiplex bead immunoassays to uveitis AqH has revealed a close association between cytokines and the cellular infiltrate, as well as distinctive cytokine profiles in certain clinical uveitis entities. These studies will add to our understanding of the pathogenesis of uveitis and will be useful in determining the effects of the current range of biological therapies.

References

1. Forrester JV, McMenamin PG. Immunopathogenic mechanisms in intraocular inflammation. *Chem Immunol*. 1999;73:159–185.
2. Boyd SR, Young S, Lightman S. Immunopathology of the noninfectious posterior and intermediate uveitides. *Surv Ophthalmol.* 2001;46:209–233.
3. Murray PI, Hoekzema R, Van Haren MA, et al. Aqueous humour analysis in Fuchs' heterochromic cyclitis. *Curr Eye Res.* 1990; 9(suppl):53–57.
4. Hoekzema R, Murray PI, Kijlstra A. Cytokines and intraocular inflammation. *Curr Eye Res.* 1990;9(suppl):207–211.
5. Murray PI, Clay CD, Mappin C, et al. Molecular analysis of resolving immune responses in uveitis. *Clin Exp Immunol*. 1999;117:455–461.
6. Verma MJ, Lloyd A, Rager H, et al. Chemokines in acute anterior uveitis. *Curr Eye Res*. 1997;16:1202–1208.
7. Wakefield D, Cuello C, Di Girolamo N, et al. The role of cytokines and chemokines in uveitis. *Dev Ophthalmol*. 1999;31:53–66.
8. el Shabrawi Y, Livir-Rallatos C, Christen W, et al. High levels of interleukin-12 in the aqueous humor and vitreous of patients with uveitis. *Ophthalmology*. 1998;105:1659–1663.
9. Muhaya M, Calder V, Towler HM, et al. Characterization of T cells and cytokines in the aqueous humour (AH) in patients with Fuchs' heterochromic cyclitis (FHC) and idiopathic anterior uveitis (IAU). *Clin Exp Immunol*. 1998;111:123–128.
10. Calder VL, Shaer B, Muhaya M, et al. Increased CD4+ expression and decreased IL-10 in the anterior chamber in idiopathic uveitis. *Invest Ophthalmol Vis Sci*. 1999;40:2019–2024.
11. Lacomba MS, Martin CM, Chamond RR, et al. Aqueous and serum interferon gamma, interleukin (IL) 2, IL-4, and IL-10 in patients with uveitis. *Arch Ophthalmol*. 2000;118:768–772.
12. Ongkosuwito JV, Feron EJ, van Doornik CE, et al. Analysis of immunoregulatory cytokines in ocular fluid samples from patients with uveitis. *Invest Ophthalmol Vis Sci*. 1998;39:2659–2665.
13. Hamzaoui K, Hamzaoui A, Guemira F, et al. Cytokine profile in Behcet's disease patients. Relationship with disease activity. *Scand J Rheumatol*. 2002;31:205–210.
14. de Boer JH, Hack CE, Verhoeven AJ, et al. Chemoattractant and neutrophil degranulation activities related to interleukin-8 in vitreous fluid in uveitis and vitreoretinal disorders. *Invest Ophthalmol Vis Sci*. 1993;34:3376–3385.
15. El Shabrawi YG, Christen WG, Foster SC. Correlation of metalloproteinase-2 and -9 with proinflammatory cytokines interleukin-1b, interleukin-12 and the interleukin-1 receptor antagonist in patients with chronic uveitis. *Curr Eye Res.* 2000;20:211–214.
16. Muhaya M, Calder VL, Towler HM, et al. Characterization of phenotype and cytokine profiles of T cell lines derived from vitreous humour in ocular inflammation in man. *Clin Exp Immunol*. 1999; 116:410–414.
17. Murray PI, Hoekzema R, Van Haren MA, et al. Aqueous humor interleukin-6 levels in uveitis. *Invest Ophthalmol Vis Sci*. 1990;31: 917–920.
18. Petrinovic-Doresic J, Mazuran R, Henc-Petrinovic L, et al. Interleukin 6 and its soluble receptor are elevated in aqueous humor of patients with uveitis. *Ocul Immunol Inflamm*. 1999;7:75–84.
19. Santos LM, Marcos MC, Gallardo Galera JM, et al. Aqueous humor and serum tumor necrosis factor-alpha in clinical uveitis. *Ophthalmic Res*. 2001;33:251–255.
20. Crane IJ, McKillop-Smith S, Wallace CA, et al. Expression of the chemokines MIP-1alpha, MCP-1, and RANTES in experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci*. 2001;42:1547–1552.
21. Ohta K, Wiggert B, Yamagami S, et al. Analysis of immunomodulatory activities of aqueous humor from eyes of mice with experimental autoimmune uveitis. *J Immunol*. 2000;164:1185–1192.
22. Tuaillon N, Shen DF, Berger RB, et al. MCP-1 expression in endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 2002;43:1493–1498.
23. de Vos AF, Van Haren MA, Verhagen C, et al. Kinetics of intraocular tumor necrosis factor and interleukin-6 in endotoxin-induced uveitis in the rat. *Invest Ophthalmol Vis Sci*. 1994;35:1100–1106.
24. Mo JS, Matsukawa A, Ohkawara S, et al. Involvement of TNF alpha, IL-1 beta and IL-1 receptor antagonist in LPS-induced rabbit uveitis. *Exp Eye Res*. 1998;66:547–557.
25. Buggage RR, Whitcup SM, Nussenblatt RB, et al. Using interleukin 10 to interleukin 6 ratio to distinguish primary intraocular lymphoma and uveitis. *Invest Ophthalmol Vis Sci*. 1999;40:2462–2463.

26. Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem.* 1998;44: 2057–2060.
27. Vignali, DA. Multiplexed particle-based flow cytometric assays. *J Immunol Methods*. 2000;243:243–255.
28. Cook EB, Stahl JL, Lowe L, et al. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. *J Immunol Methods*. 2001;254:109–118.
29. De Jager W, Te VH, Prakken BJ, et al. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol*. 2003;10:133–139.
30. Kellar KL, Kalwar RR, Dubois KA, et al. Multiplexed fluorescent bead-based immunoassays for quantitation of human cytokines in serum and culture supernatants. *Cytometry*. 2001;45:27–36.
31. Prabhakar U, Eirikis E, Davis HM. Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP assay. *J Immunol Methods*. 2002;260:207–218.
32. Hitchon CA, Alex P, Erdile LB, et al. A distinct multicytokine profile is associated with anti-cyclical citrullinated peptide antibodies in patients with early untreated inflammatory arthritis. *J Rheumatol*. 2004;31:2336–2346.
33. Breiman L. Random forests. *Machine Learning*. 2001;45:5–32.
34. Khan, SS, Smith MS, Reda D, et al. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry B Clin Cytom*. 2004;61:35–39.
35. Caspi RR, Sun B, Agarwal RK, et al. T cell mechanisms in experimental autoimmune uveoretinitis: susceptibility is a function of the cytokine response profile. *Eye*. 1997;11:209–212.
36. Kim SJ, Zhang M, Vistica BP, et al. Induction of ocular inflammation by T-helper lymphocytes type 2. *Invest Ophthalmol Vis Sci*. 2002;43:758–765.
37. de Smet MD, Chan CC. Regulation of ocular inflammation–what experimental and human studies have taught us. *Prog Retin Eye Res*. 2001;20:761–797.
38. Akpek EK, Maca SM, Christen WG, et al. Elevated vitreous interleukin-10 level is not diagnostic of intraocular-central nervous system lymphoma. *Ophthalmology*. 1999;106:2291–2295.
39. Ohta K, Yamagami S, Taylor AW, et al. IL-6 antagonizes TGF-beta and abolishes immune privilege in eyes with endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 2000;41:2591–2599.
40. Curnow SJ, Scheel-Toellner D, Jenkinson W, et al. Inhibition of T cell apoptosis in the aqueous humor of patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. *J Immunol*. 2004;173: 5290–5297.
41. Fedyk ER, Jones D, Critchley HO, et al. Expression of stromal-derived factor-1 is decreased by IL-1 and TNF and in dermal wound healing. *J Immunol*. 2001;166:5749–5754.
42. Ueda Y, Yang K, Foster SJ, et al. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. *J Exp Med*. 2004;199:47–58.
43. Battaglia M, Roncarolo MG. The role of cytokines (and not only) in inducing and expanding T regulatory type 1 cells. *Transplantation*. 2004;77:S16–S18.
44. Alcami A. Viral mimicry of cytokines, chemokines and their receptors. *Nat Rev Immunol*. 2003;3:36–50.
45. Li Q, Sun B, Matteson DM, et al. Cytokines and apoptotic molecules in experimental melanin-protein induced uveitis (EMIU) and experimental autoimmune uveoretinitis (EAU). *Autoimmunity*. 1999;30:171–182.
46. Greiner K, Murphy CC, Willermain F, et al. Anti-TNFalpha therapy modulates the phenotype of peripheral blood CD4+ T cells in patients with posterior segment intraocular inflammation. *Invest Ophthalmol Vis Sci*. 2004;45:170–176.
47. Hill T, Galatowicz G, Akerele T, et al. Intracellular T lymphocyte cytokine profiles in the aqueous humour of patients with uveitis and correlation with clinical phenotype. *Clin Exp Immunol*. 2005; 139:132–137.