Aqueous Humor Suppression of Dendritic Cell Function Helps Maintain Immune Regulation in the Eye during Human Uveitis

Alastair K. Denniston,^{1,2} Paul Tomlins,^{1,2} Geraint P. Williams,^{1,2} Sherine Kottoor,^{1,2} Imran Khan,² Kadambari Oswal,² Mike Salmon,¹ Graham R. Wallace,^{1,2} Saaeba Rauz,^{1,2} Philip I. Murray,^{1,2} and S. John Curnow^{1,2}

PURPOSE. Noninfectious uveitis is characterized by a dysregulated inflammatory or immune response in the eye. It is unclear whether this represents a failure of immune privilege or an overwhelming inflammatory drive that has exceeded the capacity of regulatory mechanisms that are still functioning. The authors investigated immune regulation in the human eye during intraocular inflammation (uveitis) and its impact on dendritic cell (DC) function and subsequent T-cell responses.

METHODS. Myeloid DCs were isolated from the aqueous humor (AqH) and peripheral blood of patients with active uveitis and characterized by flow cytometry. The effect of uveitis AqH was interrogated in an in vitro model of peripheral blood monocyte-derived DCs from healthy controls.

RESULTS. Myeloid DCs isolated from uveitic AqH were characterized by elevated major histocompatibility complex classes I and II (MHC I/II), but reduced CD86 compared with matched peripheral blood DCs. Exposure of peripheral blood monocyte-derived DCs from healthy controls to the inflammatory AqH supernatant recapitulated this phenotype. Despite interferon gamma (IFN γ)-dependent upregulation of MHC I, inflammatory AqH was overall suppressive to DC function, with reduced CD86 expression and diminished T-cell responses. This suppressive effect was equal to or greater than that induced by noninflammatory AqH, but was glucocorticoid independent (in contrast to noninflammatory AqH).

CONCLUSIONS. These data indicate that the ocular microenvironment continues to regulate DC function during uveitis, despite IFN γ -driven upregulation of MHC expression, supporting the hypothesis that immune regulation within the eye is maintained during inflammation. (*Invest Ophthalmol Vis Sci.* 2012; 53:888–896) DOI:10.1167/iows.11-8802

The eye is one of a number of sites within the body demonstrating a highly regulated relationship with the immune system.^{1–4} This phenomenon, known as immune privilege, appears to be important in protecting these vital structures from immune-mediated inflammatory damage that would result in critical loss of function. The key contributors to immune privilege in the eye appear to be ocular sequestration (the blood–ocular barriers, limited lymphatic drainage),³ an immunosuppressive ocular microenvironment (due to regulatory molecules such as transforming growth factor-beta [TGF- β], α -melanocyte stimulating hormone [α -MSH], and cortisol^{5–9}), Fas-FasL-induced apoptosis,^{10,11} and active immune deviation (such as described in anterior chamber-associated immune deviation [ACAID]¹²). During uveitis, a condition characterized by intraocular inflammation involving the uveal tract, these natural protective mechanisms either fail or are overwhelmed, with resultant leakage of cells and proteins into the optically clear aqueous humor (AqH) that circulates within the front of the eye. Subsequent damage to the delicate intraocular structures results in sight loss, with uveitis overall representing 15% of total blindness in the developed world.¹³ The most common form of uveitis is anterior uveitis that, although carrying a lower rate of acute visual loss than that of those types affecting the posterior segment of the eye (intermediate, posterior, and panuveitis), is of importance on account of its incidence and the long-term sight-threatening complications experienced by a significant minority of sufferers.¹³ Fundamentally it remains unclear whether uveitis represents the “failure” of immune privilege due to a reduction in the efficacy of one or more of the protective mechanisms, or the “overpowering” of immune privilege due to an inflammatory drive that exceeds the capacity of otherwise normally functioning regulatory mechanisms.^{2,14–19}

Dendritic cells (DCs) are bone-marrow–derived leukocytes that have a pivotal role in presenting antigen, and link innate and adaptive immune responses. They respond to the presence of pathogens and inflammation at peripheral tissue sites, migrating to secondary lymphoid organs where they present antigen to either naïve or memory T cells, leading to T-cell proliferation and differentiation toward effector and memory cells.^{20–23} More recently it has been recognized that, depending on the signals they have received, DCs may adopt a number of different phenotypes capable of inducing a range of broadly

From the ¹Centre for Translational Inflammation Research, College of Medical and Dental Sciences, University of Birmingham Research Laboratories, Queen Elizabeth Hospital Birmingham, Birmingham, United Kingdom; and the ²Academic Unit of Ophthalmology, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham and Midland Eye Centre, Birmingham, United Kingdom.

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Corresponding author: S. John Curnow, Centre for Translational Inflammation Research, College of Medical and Dental Sciences, University of Birmingham Research Laboratories, Queen Elizabeth Hospital Birmingham, Mindelsohn Way, Edgbaston, Birmingham, B15 2WB, UK; s.j.curnow@bham.ac.uk.

stimulatory or regulatory responses, according to their expression of key surface molecules (such as the costimulatory molecules CD80/86) and altered cytokine production.^{22,24} It is thought that these DC phenotypes may be plastic, enabling appropriate responses to a changing environment.²²

In the ocular microenvironment, and in particular the AqH, DCs or any other potential antigen-presenting cells (APCs) are exposed to a number of molecules that are either stimulatory or suppressive. Animal studies have suggested that the dominant regulatory molecules are TGF β ^{25,6} and α -MSH,^{7,8} with effects on macrophage inflammatory activity and on the generation of Th1 responses. We have recently shown that in humans, under resting conditions, the endogenous glucocorticoid cortisol (together with TGF β) significantly contributes to AqH inhibition of DC function.⁹ During uveitis there are significant changes in the ocular microenvironment. Analysis of the cytokine profile of uveitis AqH has identified increased levels of a number of proinflammatory cytokines (such as IL-6 and IFN γ ^{25,26}). This, coupled with a fall in TGF β levels,²⁵ pointed to the likelihood that uveitis AqH would be stimulatory. Alternatively, it was possible that AqH might continue to be immunosuppressive due to the persistence, influx, or production of immunoregulatory molecules in response to the inflammation. In this study, we aimed to interrogate whether the ocular microenvironment retains its regulatory function during uveitis, specifically with regard to the role and function of dendritic cells and consequent T-cell responses.

MATERIALS AND METHODS

Patient Samples

Patients with active uveitis involving the anterior segment of the eye were recruited for this study. Local ethical committee approval was granted and, after informed consent, all samples were collected and stored according to the Human Tissue Act 2004 (United Kingdom). These studies conform to the Declaration of Helsinki. The uveitis cohort comprised 80 patients with noninfectious uveitis: 70 patients with anterior uveitis and 10 with panuveitis. Of the 70 patients with anterior uveitis, 52 were idiopathic and 18 were HLA-B27 related. Of the 10 patients with panuveitis, 7 had Fuchs' heterochromic uveitis, 2 were idiopathic, and 1 had Vogt-Koyanagi-Harada syndrome. Disease was classified as acute first episode ($n = 17$), recurrent ($n = 62$), or chronic ($n = 1$). At the time of sampling 54/80 patients were receiving no treatment. In the remaining patients treatment comprised topical corticosteroid alone ($n = 22$), oral prednisolone and topical corticosteroid ($n = 2$), and intravenous methylprednisolone and topical corticosteroid ($n = 2$).

Duration of clinical symptoms at the time of sampling was 5.9 ± 4.9 days (mean $\pm$ SD). Disease activity in the anterior chamber of the eye was measured clinically with a biomicroscope and scored in accordance with the Standardization of Uveitis Nomenclature criteria, resulting in a value between 0 and 4.²⁷ AqH sampling was performed according to our published protocol.^{28,29} AqH was centrifuged at 300g for 5 minutes, after which the supernatant was removed and frozen in aliquots at -80°C , whereas the cellular component was stained for flow cytometry. Peripheral blood was taken by venipuncture into preservative-free heparin.

Control Samples

Peripheral blood was taken from normal healthy volunteer donors with the exclusion of any individuals with a history of inflammatory disease, infection at the time of sampling, or systemic immunosuppression. AqH was obtained from otherwise healthy patients by paracentesis before routine cataract surgery (noninflammatory AqH), excluding any individuals with a history of inflammatory disease (ocular or systemic), as well as any taking ocular medication. AqH was centrifuged at 300g

for 5 minutes, after which the supernatant was removed and frozen in aliquots at -80°C .

Generation of Monocyte-Derived DCs and Isolation of Myeloid DCs

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using a commercial density-gradient media (Ficoll-Paque; GE Healthcare [formerly Amersham Biosciences], Buckinghamshire, UK) according to the manufacturer's instructions, and were washed three times with RPMI 1640 to remove platelets. Monocytes were isolated from PBMC using a commercial cell-separation reagent (MACS CD14 MicroBeads; Miltenyi Biotec, Surrey, UK) as described by the manufacturer ($>98\%$ purity), and cultured for 6 days (37°C , 5% CO_2 , humidified) in RPMI, 10% pooled human AB+ male serum (Biosera, Ringmer, UK), 500 U/mL recombinant human IL-4 (ImmunoTools GmbH, Friesoythe, Germany), and 1000 U/mL GM-CSF (ImmunoTools) at 2.5×10^6 cells/T25 flask (Sarstedt Ltd, Leicester, UK). At day 3, 2 mL medium was removed and 2.5 mL fresh medium was added. At day 6 the nonadherent monocyte-derived DCs were harvested.

Culture of DCs in the Presence of AqH

DCs were washed and resuspended in serum-free medium: RPMI 1640 medium, 1% liquid media supplement (Sigma-ITS+3), 1% nonessential amino acid solution, and 1 mM sodium pyruvate (all Sigma-Aldrich, Gillingham, UK). DCs were placed in triplicate in a round-bottom 96-well plate (Greiner, Gloucester, UK) at $20\,000$ cells per well, and cultured in the presence or the absence of 50% human AqH. AqH was used at 50% , as previously described, to ensure greater precision when resuspending cells that could otherwise be variably contaminated with culture medium.⁹

The role of cortisol was tested with 10^{-7} M $>98\%$ HPLC cortisol (hydrocortisone; Sigma-Aldrich) and the role of dexamethasone with 10^{-7} M water-soluble dexamethasone (Sigma-Aldrich); both were inhibited with 10^{-7} M of the glucocorticoid inhibitor RU486 (Mifepristone; Sigma-Aldrich). The role of IFN γ was tested with recombinant human IFN γ at 0.1 – 100 ng/mL (ImmunoTools) inhibited with an IFN γ blocking antibody at $10 \text{ }\mu\text{g/mL}$ (R&D Systems, Abingdon, UK). The role of IL-6 was tested with recombinant human IL-6 at 0.01 – $1 \text{ }\mu\text{g/mL}$ (ImmunoTools) inhibited with an IL-6 receptor blocking antibody at $1 \text{ }\mu\text{g/mL}$ (R&D Systems). After a 48-hour culture, DC supernatants were harvested, and the cells were either stained for flow cytometry or washed three times in RPMI and 10% heat-inactivated fetal calf serum (HI-FCS) for use in an allogeneic proliferation assay.

Allogeneic Proliferation Assay

Naïve CD4^{+} T cells, memory CD4^{+} T cells, and CD8^{+} T cells were isolated from PBMC using a cell-separation reagent T-cell isolation kit (MACS; Miltenyi Biotec) as described by the manufacturer and labeled with $1 \text{ }\mu\text{M}$ long-term stain (CFSE [carboxyfluorescein diacetate succinimidyl ester]; Invitrogen, Paisley, UK) for 10 minutes at 37°C . Cells were then washed three times in RPMI, 10% HI-FCS, rested overnight, and washed once further before being placed in triplicate in the washed DC-culture plates at $100,000$ cells per well in RPMI and 10% HI-FCS. After a 4-day culture, supernatants were harvested, cells were stained with the dead cell exclusion dye propidium iodide, and proliferation of live cells was analyzed with a flow cytometer. The number of cells that had proliferated was calculated by gating on cells with a lower level of positive CFSE staining than unstimulated cells and assessing total numbers using counting beads (Invitrogen).

Multiplex Bead Immunoassay

Culture supernatants were analyzed using a multiplex bead immunoassay (Human Cytokine Twenty-Five-Plex; Biosource, Nivelles, Belgium) detecting (range, pg/mL) IL-2 (0.8 – $2,500$), IL-10 (4.5 – $15,000$), IL-13 (0.8 – $3,500$), IL-17 (1.4 – 921), tumor necrosis factor- α (TNF α ; 2.4 – 600), and IFN γ (2.5 – $3,500$). The procedure was carried out ac-

cording to the manufacturer's instructions. Samples were analyzed using a microbead analyzer (Luminex 100; Luminex Corp., Austin, TX).

Flow Cytometry

Identification of myeloid DCs and other populations of interest in AqH and peripheral blood was achieved on the basis of forward scatter/side scatter profile, and labeling with a combination of APC-anti-BDCA-1 (CD1c; AD5-8E7; Miltenyi Biotec), APCCy7-anti-CD14 (MφP9; BD Pharmingen), PE-anti-CD86 (2331; BD Biosciences, Oxford, UK), ECD-HLA-DR (Immu-357; Beckman Coulter, High Wycombe, UK), PECy7-CCR5 (2D7/CCR5; BD Pharmingen), anti-HLA-A,B,C (Pacific Blue, W6/32; BioLegend), FITC-CD19 (LT19; ImmunoTools) and FITC-CD56 (MEM-188; ImmunoTools). Monocyte-derived DCs were labeled with a combination of FITC-anti-CD80 (2D10; Biolegend, San Diego), PE-anti-CD83 (HB15e; AbD Serotec, Oxford, UK), PE-Cy5 anti-CD86 (2331; BD Biosciences), anti-HLA-A,B,C (Pacific Blue, W6/32; BioLegend), PE-Texas Red-anti-HLA-DR (Immu-357; Beckman Coulter), PECy7-CCR5 (2D7; BD Pharmingen), FITC-CCR7 (150,503; R&D Systems) for 20 minutes at 4°C, in PBS 2% bovine serum albumin (Sigma-Aldrich). The extent of positive staining was determined using an isotype-matched negative control antibody (data not shown). All antibodies were used at predetermined optimal dilutions. Dead cells were stained with propidium iodide (Sigma-Aldrich) according to the manufacturer's instructions, and excluded from further analysis. Cells were analyzed using a Cyan 3 laser nine-color flow cytometer with commercial software (Summit; Beckman Coulter).

Cortisol ELISA

Human AqH was diluted 25-fold before testing in a cortisol acetylcholinesterase competitive EIA assay (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instruction. Absorbance was measured with a multiwell plate reader (Anthos HT 111; Anthos Labtec Instruments, Salzburg, Austria). Standard curves were plotted using a four-parameter logistic equation fitted to the logarithmic transformation of the standard concentrations versus the percentage cortisol bound.

Statistical Analysis

All data were analyzed using a commercial software package (GraphPad Prism 4; GraphPad Software Inc., San Diego, CA). The figure legends provide details of the specific statistical tests used.

RESULTS

Myeloid Dendritic Cells Are Found in Human AqH during Active Uveitis with a Distinct MHC^{hi}CD86^{lo} Phenotype

Myeloid DCs were identified by flow cytometry in both the PBMC fraction and the AqH. Myeloid DCs were defined according to forward/side light scatter profile and characteristic expression of BDCA-1(CD1c)⁺CD19[−]CD56[−]CD14^{−/low} (Fig. 1). Myeloid DCs comprised $0.6 \pm 0.7\%$ (mean $\pm$ SD) of total AqH cells. This compares with the peripheral blood, where $0.3 \pm 0.6\%$ (mean $\pm$ SD) of the PBMC fraction were myeloid DCs. Myeloid DCs in uveitic AqH expressed significantly higher levels of major histocompatibility complex classes I and II (MHC I/II), but lower levels of the costimulatory molecule CD86 compared with the matched peripheral blood samples (Fig. 1). CD14⁺ cells (monocytes/macrophages) were also present ($3.3 \pm 3.9\%$ [mean $\pm$ SD] of AqH cells; $7.9 \pm 5.5\%$ [mean $\pm$ SD] of the PBMC fraction).

Treatment of Monocyte-Derived DCs with Uveitis AqH Induces an MHC^{hi}CD86^{lo} Phenotype

Having identified that DC in the uveitic AqH appeared to have a distinct phenotype, we sought to identify whether this was due

to differential recruitment of MHC^{hi}CD86^{lo} expressing DCs or a consequence of exposure to the intraocular microenvironment. To investigate the role of the intraocular microenvironment we tested the effects of uveitis AqH on the phenotype of monocyte-derived DC in vitro. Treatment of monocyte-derived DCs with uveitis AqH supernatant resulted in significant upregulation in class I MHC and downregulation of class II MHC (specifically HLA-DR) and the costimulatory molecule CD86 (Fig. 2). CD80 and CD83 were expressed at low levels in all cases, showing no significant increase or decrease on exposure to uveitis AqH (data not shown).

Uveitis AqH-Induced Upregulation of MHC I on DCs Is IFN γ Dependent

To identify the mechanism by which uveitis AqH-induced upregulation of class I MHC, we investigated the role of a number of molecules that had previously been noted to be elevated in active uveitis, notably IFN γ and IL-6^{25,26}; additionally, IFN γ is known to be capable of MHC upregulation.³⁰ We therefore tested the effects of the recombinant molecule and the consequence of blocking its effects within AqH.

Recombinant IFN γ caused a dose-dependent increase in MHC (class I and class II) and CD86 (Fig. 3A). There was differential sensitivity of molecular expression to IFN γ with upregulation of class I MHC occurring from concentrations of IFN γ of 0.1 ng/mL, HLA-DR from 1 ng/mL, and CD86 upregulation, being present only at the higher concentrations of 10 and 100 ng/mL. AqH induction of class I MHC was shown to be IFN γ dependent, with significant reversal with the addition of an IFN γ blocking antibody. As previously observed, AqH treatment downregulated HLA-DR but HLA-DR expression was further reduced in the presence of IFN γ blockade. CD86 levels that were reduced in the presence of AqH (as previously noted) were unaffected by IFN γ blockade (Fig. 3B).

In a similar set of experiments investigating the potential contribution of IL-6, the effects of uveitis AqH on DC phenotype were not recapitulated by the addition of recombinant IL-6, and were not reversed (nor augmented) by IL-6 blockade (data not shown).

Cortisol Levels Are Elevated in Uveitis AqH

Despite the proinflammatory IFN γ -dependent upregulation of class I MHC, the predominant effect of uveitis AqH appeared to be inhibitory, with downregulation of class II MHC and CD86, which we have previously shown to be due to cortisol and TGF β for noninflammatory AqH.⁹ To our knowledge the presence of cortisol in human AqH during uveitis has not previously been determined. In a series of 13 untreated uveitis AqH samples we observed the concentration of cortisol in uveitis AqH to be significantly elevated above noninflammatory AqH levels. Cortisol levels in uveitis AqH ranged from 1884 to 25,536 pg/mL, with a median (interquartile range [IQR]) of 5468 (3905–12,300) pg/mL, compared with a median (IQR) of 2820 (1650–4297) pg/mL for noninflammatory AqH (Fig. 4A). Cortisol levels in AqH increased with severity of uveitis as measured by the standard clinical parameter of anterior chamber activity (an estimate of the number of cells in the AqH, graded from 0 [no inflammation] to 4 [most severe] as described earlier²⁷) (Fig. 4B; linear trend test, $P < 0.01$).

Control of DC Expression of CD86 by Glucocorticoids Present in AqH

The observation that cortisol levels were elevated during active uveitis (Fig. 4), coupled with our earlier observations regarding the inhibitory role of cortisol within AqH on DC expression of CD86 and induction of T-cell responses,⁹ led to the hypothesis that the ability of AqH to regulate DC function during uveitis

FIGURE 1. Myeloid DCs can be identified in AqH during active uveitis and have a distinct MHC^{hi}CD86^{lo} phenotype. Myeloid DCs were identified in the peripheral blood and aqueous humor of patients with active anterior uveitis. The gating strategy comprised identifying cells of appropriate scatter profile that were BDCA-1(CD1c)⁺CD19⁻CD56⁻ and CD14^{-/lo}. (A) Representative histograms and (B) median fluorescence intensity (MFI) scans of MHC and CD86 from matched PBMC and AqH of six or more patients with acute anterior uveitis. Wilcoxon matched-pairs analysis; **P* < 0.05; ***P* < 0.01; NS, not significant.

FIGURE 2. Treatment of naïve monocyte-derived DCs with uveitis AqH (UvAqH) supernatant in vitro induces an MHC^{hi}CD86^{lo} profile similar to that seen in UvAqH myeloid DCs. Monocyte-derived DCs were cultured for 48 hours in the presence or the absence of 50% uveitic or noninflammatory AqH. (A) Representative histograms and (B) MFI scans of MHC and CD86 for nine uveitis AqH (acute anterior; open circles) and six noninflammatory AqH (filled circles), representative of three separate experiments; for medium alone the mean $\pm$ SD of MFI scans for triplicate cultures are given. One-way ANOVA with Bonferroni post hoc test for multiple comparisons; data passed a Kolmogorov-Smirnov test for normality; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

was also linked to endogenous intraocular cortisol, augmented by exogenous (i.e., therapeutic) glucocorticoids in the case of samples from patients who were being treated with corticosteroid drops. We therefore tested the inhibitory effects of uveitis AqH in the presence or the absence of a glucocorticoid inhibitor (RU486), recognizing that for these “treated uveitis” AqH samples, glucocorticoid inhibition would block the combined effect of endogenous cortisol and the exogenous glucocorticoid treatment, such as dexamethasone (Figs. 5A–C). Both untreated and treated uveitis AqH caused significant reduction in CD86 expression (Fig. 5C). As previously shown,⁹ we confirmed that the inhibitory effects of noninflammatory AqH were reversed by RU486, but similar reversal was not seen for the uveitis groups; there was no effect for untreated uveitis AqH and only a partial reversal for treated uveitis AqH (Fig. 5C). Uveitis AqH-induced changes in class I and class II MHC were not affected by the addition of RU486 (Figs. 5A, 5B).

Uveitis AqH Inhibits DC Capacity to Induce Proliferation of CD4⁺ and CD8⁺ T Cells

Having identified that the inflamed ocular microenvironment induced both a “stimulatory” upregulation of MHC (class I and class II in vivo; class I only in vitro) and an “inhibitory” downregulation of CD86, we sought to investigate the functional consequences of this altered phenotype and the impact of treatment on DC function.

The addition of uveitis AqH significantly inhibited DC capacity to induce T-cell proliferation, whether naïve or memory

CD4⁺ or CD8⁺ (Fig. 6). Inhibition with uveitis AqH was of a magnitude similar to that seen with noninflammatory AqH.

Effect of In Vivo Glucocorticoid Treatment on DC-Induced T-Cell Responses

Having noted that exposure to uveitis AqH caused similar inhibition of DC function to noninflammatory AqH, we sought to observe whether the inhibitory effects of uveitis AqH were affected by the presence of glucocorticoid treatment. In addition, we sought to determine whether T-cell cytokine production was also affected.

Exposure of DCs to uveitis AqH resulted in lower levels of proliferation regardless of whether the AqH was a “treated” or “untreated” sample (Fig. 7A), although this was not statistically significant ($P = 0.18$). Similarly, exposure of DCs to uveitis AqH, whether “untreated” or “treated,” resulted in lower levels of IL-2, IL-10, IL-13, IFN γ , and TNF α (Fig. 7B); correlation with proliferation was high for all these cytokines (Fig. 7C). IL-17 was near baseline from all cultures and showed no significant effect of AqH treatment.

DISCUSSION

This study addresses an important question regarding immune regulation in an immune privileged site under inflammatory conditions. Specifically, we have investigated whether DC regulation in the human eye is maintained during intraocular inflammation. During active uveitis, we found that myeloid

FIGURE 3. Increasing IFN γ levels in AqH during uveitis promote up-regulation of MHC, but are insufficient to overcome AqH-induced regulation of HLA-DR or CD86. Monocyte-derived DCs were cultured in the presence or the absence of (A) 0.1–100 ng/mL IFN γ or (B) 50% UvAqH, with or without an IFN γ -blocking antibody. (A) and (B) are each representative of three separate experiments. (B) comprises nine patients with uveitis (acute anterior; open circles); mean $\pm$ SD of MFI scans for triplicate cultures are given except for individual AqH samples. Wilcoxon matched-pairs analysis; * P < 0.05; NS, not significant.

DCs from the anterior chamber expressed high levels of MHC but low levels of CD86. This phenotype was recapitulated in vitro by exposure of monocyte-derived DCs to uveitis AqH supernatant and this resulted in a suppressed function of these cells. Furthermore, the regulatory effects of uveitis AqH appeared to be distinct from the cortisol and TGF β 2-dependent mechanism we have previously demonstrated for noninflammatory AqH.⁹ Maintenance of these regulatory mechanisms suggests that, for DC function at least, immune privilege within the eye is maintained in the presence of inflammation.

Although numerous leukocyte populations may be modified by the unusual microenvironment of an immune privileged site, DCs are of particular interest due to their pivotal role in the adaptive immune response. Previous animal and cadaveric studies^{31,32} have identified ocular APC with DC-like properties, but to our knowledge this is the first study to identify BDCA-1⁺ myeloid DCs in the AqH of human patients with uveitis. Our study parallels the findings in rodents in which both DCs and macrophage-like populations are identified in uveal tissue.^{33–35} We have similarly identified both a CD14⁺ monocyte/macrophage population and a separate CD14^{low} BDCA-1⁺ myeloid DC population. DCs were found to be less abundant than CD14⁺ monocyte/macrophages in uveitic aqueous humor, paralleling the findings in rodent uveal tissue,³⁶ but their role

should not be underestimated because DCs are far more potent than macrophages in their antigen presentation capacity, can fulfill both regulatory and stimulatory roles, and are unique in their ability to induce naïve T-cell responses.³⁷ One of the key factors for DCs controlling the activation of naïve T cells is the expression of coreceptors CD80/CD86. It is clear that not only is CD86 not upregulated by uveitis AqH, but that the levels are well below those induced on normal maturation of these dendritic cells.⁹

Our finding that uveitis AqH continues to be inhibitory to DC function is an important addition to the predominantly murine literature regarding the retention or otherwise of immune privilege during uveitis. Rodent models of uveitis such as experimental autoimmune uveitis (EAU), endotoxin-induced uveitis (EIU), and *Mycobacterium tuberculosis* adjuvant-induced uveitis (MTU) vary widely in disease severity, time course, and eventual outcome, reflecting very different immunologic processes. In both EAU and EIU the ability of the ocular microenvironment to suppress anti-CD3-driven T-cell proliferation in vitro was lost at the onset of disease but recovered at the peak of disease.^{15,16} In contrast, a variant of the EIU model suggests that severe intraocular inflammation is compatible with maintenance of immune privilege. When LPS is injected, not systemically but into the vitreous cavity of BALB/c mice, it was observed that even at the peak of intraocular inflammation these eyes permit the proliferation of allogeneic tumor cells and support ACAID in vivo, and their AqH would still strongly inhibit T-cell activation in vitro.¹⁹ Furthermore, in the MTU model, intravitreal injection of *M. tuberculosis* adjuvant into the eyes of BALB/c mice resulted in an intense anterior uveitis but similar preservation of immune privilege behavior.¹⁸ Our study provides novel evidence in humans that retention of an immune privileged microenvironment (or at least some components thereof) is consistent with active inflammation.

It is noteworthy that although uveitis AqH was overall suppressive on human DCs, the presence of significant levels of IFN γ was associated with a limited proinflammatory activity resulting in elevated MHC class I expression by DCs. We, and others, have previously demonstrated elevated IFN γ levels in human uveitic AqH that correlate with the severity of inflammation.²⁵ In our study we observed a median (IQR) IFN γ concentration of 0.6 (0.005–16.3) ng/mL IFN γ in idiopathic uveitic AqH compared with 0.065 (0.005–0.375) ng/mL in noninflammatory AqH.²⁵ It is interesting to note that there are differences between DC expression of MHC class II observed

FIGURE 4. Cortisol levels are elevated in UvAqH versus noninflammatory AqH. Cortisol levels in noninflammatory (CAqH) and uveitic AqH (untreated; UvAqH) were measured by ELISA. (A) Cortisol levels in noninflammatory AqH versus UvAqH and (B) cortisol levels according to the cellular activity of an AqH sample; cellular activity is measured clinically, as discussed. (A) and (B) comprise 13 patients with uveitis (acute anterior; open circles) and 17 control patients (noninflammatory AqH; filled circles); Mann-Whitney U test (A) and linear trend test for all four columns (B); ** P < 0.01.

FIGURE 5. Control of DC expression of CD86 by glucocorticoids present in AqH. Monocyte-derived DCs were cultured in the presence or the absence of 50% noninflammatory (CAQH) or uveitis AqH (UvAqH), with or without the glucocorticoid blocker, RU486. (A), (B), and (C) comprise seven patients with untreated and seven patients with treated uveitis (acute anterior or panuveitis; *open circles*) and seven control patients (noninflammatory AqH; *filled circles*), cortisol or dexamethasone, were used as positive controls. Mean $\pm$ SD of MFI scans for triplicate cultures are given except for individual AqH samples. Wilcoxon matched-pairs analysis; * $P < 0.05$; NS, not significant; Dex, dexamethasone; Med, medium alone.

in vivo versus in vitro. The reasons for this are unclear, but possible explanations include a differential sensitivity of cultured cells to the balance of IFN γ and inhibitory factors present in AqH or, less likely, loss of IFN γ activity from the uveitis AqH specimen during preparation. In rodent models of uveitis, more attention has been given to IL-6. Development of disease is associated with increasing IL-6 levels and is implicated in the loss of regulation seen in both the EAU and EIU models, where IL-6 blockade resulted in restoration of the normal regulatory properties of AqH in vitro.^{15,16} Although we have also noted elevated IL-6 levels in human uveitis AqH, and that these inhibit T-cell apoptosis within the uveitic eye, blockade of IL-6 in uveitis AqH did not significantly affect DC phenotype.³⁸

Our observation of the persistence of the regulatory properties of human AqH during uveitis is also intriguing since it appears to be operating by an alternative mechanism to the dominant cortisol/TGF β 2 pathway of noninflammatory AqH.⁹ In mice the predominant regulatory molecules are neuropeptides such as α -MSH and vasoactive intestinal peptide (VIP), with TGF β 2 becoming increasingly dominant once inflammation triggers its activation from the predomi-

nant latent form.^{15,16} We have previously observed that in humans, at least in our in vitro DC model, α -MSH and VIP were not found to be significantly inhibitory at physiological levels.⁹ In humans active TGF β 2 levels decrease in uveitis, falling from a median of 353 (range, <40 to 497) pg/mL to a median of 86 (<40 to 667) pg/mL.²⁵ Since this lower uveitis level is significantly below the concentration at which we have found TGF β to inhibit DC function,⁹ TGF β was not considered further in this context. Conversely, cortisol levels in human AqH increased during uveitis (Fig. 4), although the suppressive effects of uveitis AqH on DC function were not reversed by glucocorticoid blockade. Nevertheless, given the sensitivity of DCs to these levels of cortisol,⁹ it is likely that this continues to be a significant regulator of DC function, but that the presence of novel alternative regulatory molecules provides redundancy in the system.

As part of this study we have sought to optimally model DC function in the uveitic human eye by the use of human DCs cultured in the presence of human uveitis AqH; however, this brings with it a number of limitations. We recognize that AqH

FIGURE 6. UvAqH inhibits DC capacity to induce T-cell proliferation for CD4⁺ and CD8⁺ T cells. CFSE-labeled allogeneic naïve CD4⁺ T cells were cultured for 4 days with monocyte-derived DCs, which had been pretreated with medium or 50% AqH (noninflammatory [CAQH] or uveitis [UvAqH]). (A) CFSE versus side scatter (SS) for live cells and (B) number of divided cells; each plot is representative of three separate experiments with the mean $\pm$ SD of triplicate cultures for cultures without AqH (i.e., T-cell cultures with iDC or medium only). Twenty-four patients with uveitis (acute anterior or panuveitis; untreated or treated; *open circles*) and six control patients (noninflammatory AqH; *filled circles*) shown. One-way ANOVA with Bonferroni post hoc test for multiple comparisons (B). Normality of distribution demonstrated by Kolmogorov-Smirnov test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

FIGURE 7. UvAqH is suppressive for DC function regardless of glucocorticoid treatment and without skewing T-cell cytokine production. CFSE-labeled allogeneic naïve CD4⁺ T cells were cultured with pretreated monocyte-derived DCs as described previously. Supernatants were harvested after 4 days and analyzed by multiplex-bead immunoassay. (A) Proliferation (number of divided cells) or (B) cytokine concentration or (C) correlation of proliferation versus cytokine concentration shown; representative of three separate experiments with the mean $\pm$ SD of triplicate cultures for medium alone. Twelve patients with uveitis (acute anterior or panuveitis; untreated shown as *open circles*; treated shown as *open triangles*) and six control patients (noninflammatory AqH; *filled circles*) shown. Kruskal-Wallis with Dunn's post hoc test for multiple comparisons (B); * $P < 0.05$; all other differences were nonsignificant. Spearman's r for correlation (C).

primarily reflects the ocular microenvironment of the anterior segment and, thus, one should be cautious of extrapolating our findings to posterior segment disease; also that the behavior of resident or newly recruited DCs to the anterior segment in vivo may be significantly more complex than modeled by our in vitro studies of human AqH. It should be noted that our model seeks to establish the behavior of DCs that have been conditioned within the ocular microenvironment before engaging with a naïve T cell, for example in a draining lymph node. It is interesting to speculate whether the presence of AqH at the time of engagement of DCs with either memory (or possibly even naïve) T cells within the eye would continue to cause inhibition or skewing of T-cell function (as discussed earlier), although the scarcity of human uveitis AqH samples has so far limited these additional studies.

Our finding that, in humans, uveitis AqH continues to be inhibitory to DC function is an important addition to the predominantly murine literature regarding the retention or otherwise of immune privilege during uveitis. Even in the presence of clinically severe inflammation, AqH is capable of significantly suppressing DC maturation, retaining this regulatory role despite IFN γ -driven upregulation of MHC expression, and appearing to globally establish DC capacity to induce adaptive T-cell responses. Importantly, these data from the uveitic eye support the hypothesis that many of the mechanisms that constitute immune privilege are maintained during inflammation (at least with regard to DC function) and may have implications for our understanding of the relationship of immune privilege and inflammation in other sites.

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