Inhibition of T Cell Apoptosis in the Aqueous Humor of Patients with Uveitis by IL-6/Soluble IL-6 Receptor *trans*-Signaling

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A fundamental mechanism of immune privilege in the eye is the induction of T lymphocyte apoptosis. Intraocular inflammation in uveitis implies compromise of immune privilege. This study sought to determine whether apoptosis of T cells is actively inhibited in patients with uveitis and by what pathways this may occur. Apoptotic lymphocytes were found to be absent from aqueous humor (AqH) of virtually all patients with recent-onset uveitis. However, T cells removed from the eye were highly susceptible to both spontaneous and Fas ligand-induced apoptosis in vitro. AqH from patients with uveitis had no modulatory effect on Fas ligand-induced apoptosis, but strongly suppressed survival factor deprivation-induced apoptosis. In contrast, noninflammatory AqH from patients undergoing cataract surgery had no modulatory effects on apoptosis at all. These data suggest that triggering of the Fas pathway is diminished in uveitis, and also that homeostatic resolution through survival factor deprivation-induced apoptosis is inhibited by factors present in AqH. The most widely recognized pathways, common γ-chain cytokines and type I IFNs, did not contribute to AqH-mediated T cell survival. High levels of both IL-6 and soluble IL-6R were found in AqH. IL-6 alone did not induce T cell survival, because IL-6R expression on T cells in AqH was too low to facilitate signaling. However, combinations of IL-6 and soluble IL-6R were highly effective inhibitors of T cell apoptosis, suggesting that the *trans*-signaling pathway is likely to be a key mediator of T cell apoptosis inhibition mediated by uveitis AqH. *The Journal of Immunology,* 2004, 173: 5290–5297.

Uveitis is a group of diseases characterized by intraocular inflammation (1). Many cases resolve rapidly, but a significant number of patients develop persistent disease, with damage to ocular structures resulting in severe visual impairment. The eye is a site of immune privilege where both passive and active mechanisms restrict inflammation (2). These include cytokines such as TGFβ2 and α-melanocyte-stimulating hormone (3), but also processes such as anterior-chamber-associated immune deviation (4), and the induction of apoptosis of infiltrating activated T lymphocytes, mediated by Fas ligand (FasL)[3] expressed on intraocular surfaces (5, 6). Intraocular inflammation appears to represent a breach in immune privilege, but it is currently unclear how this may operate, or indeed whether uveitis is a distinct form of inflammation, representing the action of immune privilege under specific circumstances.

The accumulation of lymphocytes at any site is controlled by their rates of recruitment to and emigration from the site, but also their rates of proliferation and apoptosis. Lymphocyte apoptosis has been shown to play a key role in maintaining immune privilege in the eye and inhibiting inflammation (6). Lymphocyte apoptosis can be induced by two pathways: engagement of death receptors such as Fas (CD95) or through the withdrawal of essential growth factors. Fas-mediated apoptosis appears to be important for regulating the extent of clonal expansion during immune responses, but resolution requires survival factor deprivation (SFD)-induced apoptosis (7–10). The constitutive expression of FasL in healthy eyes induces apoptosis of activated lymphocytes, leading to rapid resolution of immune responses. The absence of either FasL from the eye, or Fas from the lymphocytes, results in a destructive inflammatory response (6, 11). This suggests that uveitis might result from insufficient Fas-mediated apoptosis, although paradoxically, both Fas and FasL are required for the induction of experimental autoimmune uveoretinitis (12).

Many studies have focused on Fas-mediated apoptosis during intraocular inflammation (13–16), but the failure of many cases of uveitis to resolve suggests that SFD-induced apoptosis may be diminished. T lymphocytes can be protected from SFD-induced apoptosis by members of the common γ-chain cytokine family, including IL-2, IL-4, IL-7, IL-9, and IL-15 (17), and type I IFNs (18). IL-6 has also been shown to protect T cells from SFD-induced apoptosis (19, 20). Intriguingly, this may operate through two distinct mechanisms. First, IL-6 can signal directly, by binding to IL-6Rα (CD126) expressed on the cell surface. This in turn links

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[3] Abbreviations used in this paper: FasL, Fas ligand; SFD, survival factor deprivation; s, soluble; AqH, aqueous humor; RA, rheumatoid arthritis; SF, synovial fluid; SFM, serum-free medium; $DiOC_6$, dihexyloxacarbocyanine iodide; DAPI, 4′,6′-diamidino-2-phenylindole.

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to gp130 (CD130) for signal transmission. Second, in the absence of IL-6Rα expressed on the cell surface, IL-6 can bind to soluble (s) IL-6Rα, and the resulting complex then binds to gp130 expressed on the cell surface, in a process termed *trans*-signaling (21). IL-6/sIL-6R *trans*-signaling is involved in a number of inflammatory processes, including the switch from neutrophil to mononuclear cell recruitment during inflammation (22), and has been suggested to play a role in several pathological conditions (23, 24). Both IL-6/sIL-6R *trans*-signaling and IFN-β have been shown to inhibit lymphocyte apoptosis in inflammatory diseases such as rheumatoid arthritis (RA) and Crohn's (25, 26).

The presence of large numbers of lymphocytes in the anterior chamber in uveitis may reflect an extremely high rate of recruitment or proliferation that effectively overwhelms the capacity to induce apoptosis through the Fas pathway, but may also reflect active inhibition of apoptosis. Persistent uveitis, like other chronic inflammatory diseases, may show inhibition of SFD-induced apoptosis as a mechanism for maintaining a persistent infiltrate (25). In this study, we tested the hypothesis that, in uveitis, insufficient apoptosis contributes to the accumulation of lymphocytes through deficient Fas-mediated and/or SFD-induced apoptosis.

Materials and Methods

Patients, diagnosis, and aqueous humor (AqH) samples

AqH samples (~100 μl) were collected from 33 patients with recent-onset acute uveitis 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. Using slit lamp biomicroscopy, the degree of anterior chamber inflammatory activity was assessed using the Hogan clinical grading system (27). The majority of AqH samples (23 of 33) were from patients with idiopathic uveitis. The remainder comprised one *Candida albicans*, three confirmed HLA-B27$^+$, one varicella zoster, two Behçet's disease, two Fuchs' heterochromic cyclitis, and one patient with RA. Uveitis was classified as anterior in 22 and panuveitis in the remainder. The majority of patients were on no treatment at the time of sampling (22 of 33) with the remainder on topical (9 of 33) or systemic (2 of 33) 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).

AqH was centrifuged at 300 $\times$ *g* for 5 min; the cell-free supernatant (subsequently referred to as AqH) was removed and frozen in aliquots at −70°C. Cell pellets were resuspended, counted, cytocentrifuged (Cytospin; Shandon, Pittsburgh, PA), stained with Diff-Quik (Dade Behring, Marburg, Germany), and viewed by light microscopy. The percentage of apoptotic lymphocytes with characteristic nuclear condensation was also calculated. Synovial fluid (SF) samples from RA and self-limiting arthritis patients were similarly processed for the determination of apoptotic lymphocyte frequencies. These samples were used, because previous reports have identified inhibition of lymphocyte apoptosis in RA but not self-limiting arthritis (25).

Maintenance of short-term CD4$^+$ T cell lines

CD4$^+$ short-term T cell lines were derived from the peripheral blood of normal healthy volunteers as previously described (17). These cells share many features of the CD4$^+$ lymphocytes observed in uveitis, including Fas and HLA-DR expression (14, 28). Cells were maintained in RPMI 1640 (Sigma-Aldrich, Irvine, U.K.), streptomycin (100 μg/ml), penicillin (100 U/ml), glutamine (10 mM), and 10% heat-inactivated FCS with stimulation every 2 wks with PHA-H15 (9 μg/ml; Murex Biotech, Dartford, U.K.) and gamma-irradiated (30 Gy) autologous EBV-transformed B cells. IL-2 (Chiron, Harefield, U.K.) at 25 U/ml was added every 3–4 days. Cells were used 7 days after restimulation.

Induction of apoptosis in vitro

CD4$^+$ T cells were washed three times with RPMI 1640 to remove IL-2 and serum factors. Cells were cultured in serum-free medium (SFM) containing RPMI 1640 with 1% low endotoxin BSA (Sigma-Aldrich) at 25,000 cells per well in Terasaki plates (Nunc, Roskilde, Denmark). For SFD-induced apoptosis cells were cultured in SFM alone or with 50% AqH for 20 h at 37°C. IL-2, IFN-α (PeproTech, London, U.K.), and IFN-β (Serotec, Oxford, U.K.) were used at 25 U/ml, 20 ng/ml, and 10 ng/ml, respectively, for inhibition of SFD-induced apoptosis. Blocking Abs to IFN-α and IL-6R (R&D Systems, Abingdon, U.K.), and IFN-β (Serotec) were added at 20 μg/ml in SFM and were preincubated with AqH, IFN-α, or IFN-β for 30 min before addition of CD4$^+$ T cells. For Fas-induced apoptosis, cells were cultured in SFM containing 400 ng/ml sFasL (Super sFasL; Alexis Corporation, Nottingham, U.K.) alone or with 50% AqH, and cultured for 4 h at 37°C. An Ab to Fas (CH-11; Upstate Biotechnology, Buckingham, U.K.) was used at 20 ng/ml, and cells were incubated for 2 h to detect active caspase-3, or 8 h for morphology.

Analysis of apoptosis

Mitochondrial depolarization was routinely used as an indicator of T cell apoptosis and was measured using dihexyloxacarbocyanine iodide ($DiOC_6$), a fluorescent dye that accumulates inside active mitochondria. When mitochondria depolarize during apoptosis, the dye no longer accumulates, and the cells appear as a $DiOC_6^{low}$ fraction (29). Following culture, cells were labeled with $DiOC_6$ (Molecular Probes, Eugene, OR) at 23 ng/ml for 20 min at 37°C, washed in ice-cold PBS, and immediately analyzed by flow cytometry on a Coulter EPICS XL cytometer (Coulter Electronics, Hialeah, FL). For Fas-induced apoptosis, results are expressed as follows: percentage of $DiOC_6^{low}$ with sFasL − percentage of $DiOC_6^{low}$ in SFM alone, accounting for any effect of AqH on spontaneous apoptosis. For apoptosis of cultured peripheral blood and AqH, CD4$^+$ lymphocytes were identified with CD4-Tricolor (Caltag Laboratories, Towcester, U.K.), and apoptosis was measured after 12 h with $DiOC_6$.

To confirm analysis of apoptosis, both caspase-3 activation and nuclear morphology were analyzed. Cytocentrifuge preparations of cultured T cells were air-dried and fixed with dry acetone for 10 min, and nonspecific binding was blocked with PBS containing 2% BSA for 15 min. Control rabbit IgG (DakoCytomation, Ely, U.K.) or anti-active caspase-3 (BD Pharmingen, San Diego, CA) at 0.5 μg/ml was added for 1 h, followed by biotin goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) at 10 μg/ml for 30 min, streptavidin Texas Red (Southern Biotechnology Associates) at 10 μg/ml for 30 min, and 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at 0.2 μg/ml for 2 min. Between each step, slides were washed twice for 5 min with PBS.

Simultaneous analysis of active caspase-3 together with CD3 required modifications to the fixation and blocking steps in the above protocol. Cytocentrifuged cells were air-dried, fixed with 1% paraformaldehyde in PBS for 30 min, and permeabilized with 0.1% Nonidet P-40 in PBS for 30 min. Nonspecific binding was blocked with 2.5% human Ig (Flebogamma, 5%; Grifols, Cambridge, U.K.) for 30 min, followed by 20% goat serum (DakoCytomation) and 0.2% cold-water fish skin gelatin (Sigma-Aldrich) in PBS/0.5% casein (Fisher Scientific, Loughborough, U.K.) for 60 min. Primary and secondary Abs were diluted in 20% goat serum, 0.2% cold-water fish skin gelatin in PBS/0.5% casein. Control rabbit IgG (DakoCytomation) or anti-active caspase-3 (BD Pharmingen) at 1 μg/ml, and mouse anti-CD3 (DakoCytomation) at 12 μg/ml were added for 60 min followed by biotin goat anti-rabbit IgG (Southern Biotechnology Associates) at 10 μg/ml and goat anti-mouse IgG Texas Red (Southern Biotechnology Associates) at 20 μg/ml for 30 min, streptavidin-FITC (Invitrogen Life Technologies, Paisley, U.K.) for 30 min, and DAPI (40 ng/ml) for 2 min. Between each step, slides were washed twice for 5 min with PBS. Images were captured with a SPOT-2 (Diagnostic Instruments, Sterling Heights, MI) digital camera and Image-Pro (Media Cybernetics, Silver Spring, MD) software.

Cytokine measurement in AqH

AqH samples (50-μl volumes) were analyzed for the presence of IL-2, IL-4, IL-6, sIL-6R, IL-7, IL-10, and IL-15 by multiplex bead analysis using microspheres as the solid support for immunoassays. This facilitated the analysis of all of these molecules from each sample (30). IL-2, IL-4, and IL-10 were measured according to the manufacturer's instructions (Upstate Biotechnology, Buckingham, U.K.). IL-6, sIL-6R, IL-7, and IL-15 were measured using pairs of ELISA Abs (R&D Systems) in a multiplex bead immunoassay. Monoclonal capture Abs were coupled to fluorescent microspheres (Luminex Corporation, Austin, TX) according to the manufacturer's protocol. For cytokine measurements, AqH was incubated with mAb-coated capture beads for 2 h at 20°C. Washed beads were further incubated with biotin-labeled polyclonal anti-human cytokine Ab for 2 h followed by streptavidin-PE (Upstate Biotechnology) at 40 μg/ml for 30 min. Samples were analyzed using a Luminex 100. Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units to cytokine concentration (picograms per milliliter).

For each sample, 100 beads were analyzed, and each assay was sufficiently sensitive to detect <10 pg/ml.

Statistical analysis

The Mann-Whitney *U* test was used to determine significant differences in apoptosis between groups. Correlations were calculated using the Spearman correlation. The Wilcoxon test was used to test for the effect of anti-IFN-α and -IFN-β blocking Abs. The level of confidence at which the results were judged significant was $p < 0.05$.

Results

Few apoptotic lymphocytes are detectable in uveitis AqH

Induction of lymphocyte apoptosis is a characteristic mechanism of immune privilege in the eye, leading to suppression of intraocular inflammation. However, analysis of AqH from patients with recent-onset uveitis showed that apoptotic lymphocytes were virtually absent, despite the presence of large numbers of cells (Fig. 1). This suggests suppression of apoptosis induction, rather than a large number of cells exceeding the capacity of the eye to induce apoptosis. To ensure that all stages of apoptosis were detected, both early and late markers were studied; these included the activation of caspase-3, an early event and also nuclear condensation and fragmentation, which are late markers of apoptosis. Where apoptotic cells were detected, they showed all of these features (Fig. 1*A*). A short-term $CD4^+$ T cell line treated with anti-Fas was used as an apoptosis control. The proportion of apoptotic lymphocytes in AqH (Fig. 1*B*; median, 0.15%; range, 0.0–0.8%; $n = 9$) was similar to that observed in SF from chronic RA patients (*B*; median, 0.75%; range, 0.0–3.7%; $n = 6$) where active inhibition of apoptosis had been previously documented (25). In contrast, significantly increased numbers of apoptotic lymphocytes were detected in the SF of patients with self-limiting arthritis (Fig. 1*B*; median, 3.4%; range, 0.0–11.5%; $n = 6$) compared with uveitis AqH lymphocytes ($p < 0.05$). Thus, despite the immune-privileged nature of the eye, there was virtually no detectable apoptosis of infiltrating lymphocytes, suggesting that induction of apoptosis may be deficient in uveitis, or it may be actively suppressed.

Fas-induced apoptosis is not affected by AqH

We tested the possibility that Fas-mediated apoptosis may be compromised in uveitis by the inflammatory ocular microenvironment. Cells from a short-term $CD4^+$ T cell line, which shares many features of the $CD4^+$ lymphocytes observed in uveitis (13, 28, 31), were treated with a range of concentrations of aggregated sFasL (Fig. 2*A*). The primary measure of apoptosis shown in this and all subsequent experiments was mitochondrial depolarization assessed with $DiOC_6$. For experiments to assess the modulatory capacity of AqH, a dose of 400 ng/ml sFasL was used. This intermediate level of sFasL facilitated detection of either pro- or antiapoptotic effects of AqH. However, no significant modulatory action was observed with either uveitis or control AqH (Fig. 2*B*). Nevertheless, sFasL was able to induce significant levels of apoptosis of $CD4^+$ T cells isolated from the AqH of patients with uveitis (Fig. 2*C*), indicating that these cells were highly susceptible to Fas-mediated death. Peripheral blood lymphocytes, the majority of which are resting cells, remained relatively resistant to the induction of apoptosis by this route.

SFD-induced apoptosis is inhibited in uveitis

Fas-mediated apoptosis is frequently detectable during the early stages of an immune response. However, for resolution of immune responses, SFD-induced apoptosis plays a key role (9). $CD4^+$ T cells isolated from the anterior chamber of patients with uveitis entered apoptosis spontaneously when cultured in the absence of AqH (Fig. 2*C*). This suggested active inhibition of apoptosis by the ocular microenvironment. To test this, SFD-induced apoptosis was initiated by withdrawal of serum and IL-2 from a short-term $CD4^+$ T cell line (17). Significant apoptosis was observed after 20 h of culture, which was inhibited by the addition of either IL-2, IFN-β, or uveitis AqH, but not AqH from patients undergoing cataract surgery (Fig. 3). Survival of T cells in the presence of uveitis AqH was significantly greater than that observed with noninflammatory AqH from cataract patients ($p < 0.0001$). The primary measure of apoptosis in these experiments was mitochondrial depolarization assessed using $DiOC_6$, but results were confirmed by analysis of caspase-3 activation and nuclear morphology (data not shown). Patients with severe disease, assessed using the Hogan clinical grading system, showed significantly greater inhibition of apoptosis than those with mild disease (Fig. 3*C*). However, no significant differences were observed in apoptosis inhibition between patients who had received topical or systemic glucocorticoid therapy and those who were studied before treatment. There was no correlation between age and inhibition of apoptosis, and segregation of patients into those suffering from anterior uveitis or panuveitis also showed no significant difference (data not shown). This suggests that inhibition of SFD-induced apoptosis is a characteristic of active uveitis, relating to the degree of ocular inflammation.

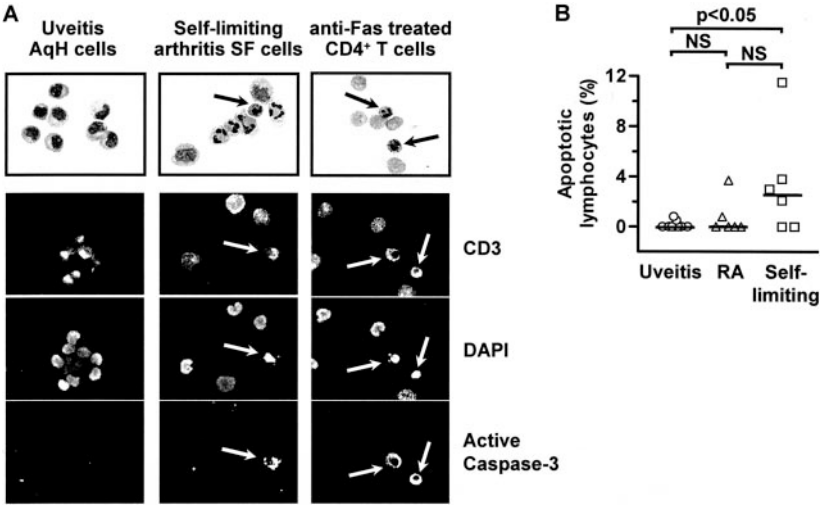

FIGURE 1. Apoptotic lymphocytes are rarely detected in uveitis AqH. Cytocentrifuged cell pellets from untreated idiopathic uveitis AqH, self-limiting arthritis SF, and anti-Fas-treated $CD4^+$ T cells were stained to differentiate lymphocytes and identify any apoptotic cells (arrows) with cell shrinkage and condensed nuclei. Slides were also stained with anti-CD3, anti-active caspase-3, and DAPI. *A*, Images are representative of at least four different samples. Magnification, ×400. *B*, The percentage of lymphocytes with apoptotic morphology was calculated from uveitis AqH-, and RA and self-limiting arthritis SF-cytocentrifuged slides. Bars represent the median value for each group. NS, Not significant ($p > 0.05$).

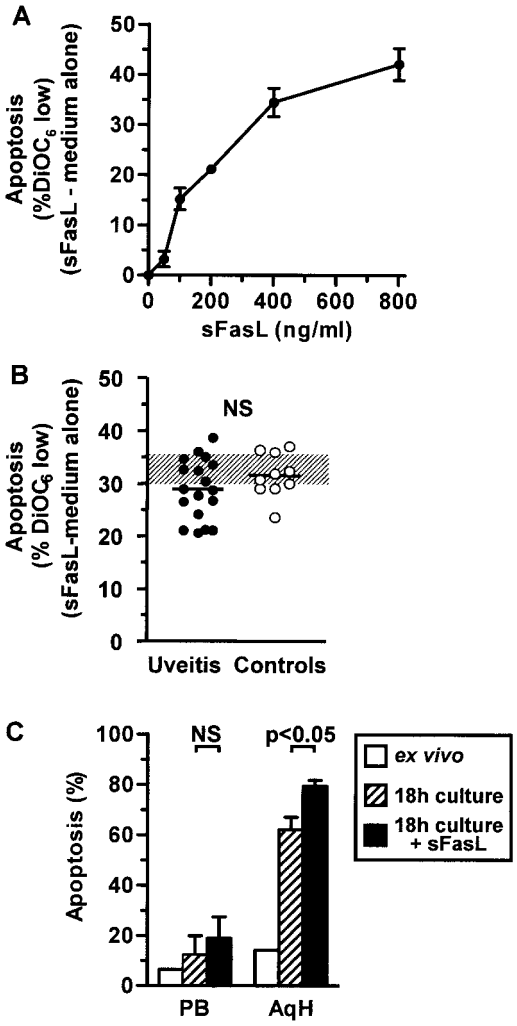

FIGURE 2. Fas-mediated T cell apoptosis is unaffected by the ocular microenvironment. *A* and *B*, Apoptosis of a short-term $CD4^+$ T cell line was induced by culture for 4 h with the indicated concentration (*A*) or 400 ng/ml (*B*) of sFasL, and measured as cells staining $DiOC_6^{low}$. Results are expressed as follows: percentage of $DiOC_6^{low}$ with sFasL − percentage of $DiOC_6^{low}$ in SFM alone, accounting for any effect of AqH on SFD-induced apoptosis. Uveitis and control AqH were added at 50% (*B*). The shaded area indicates the range of triplicate values for apoptosis induced by sFasL in the absence of AqH. Results are representative of three separate experiments. *C*, Apoptosis of peripheral blood (PB) and AqH $CD4^+$ lymphocytes was analyzed ex vivo (□) or following an 18-h culture in medium alone (▨), or sFasL (■), using staining with CD4-Tricolor to identify $CD4^+$ lymphocytes and $DiOC_6$ to measure apoptosis. NS, Not significant ($p > 0.05$).

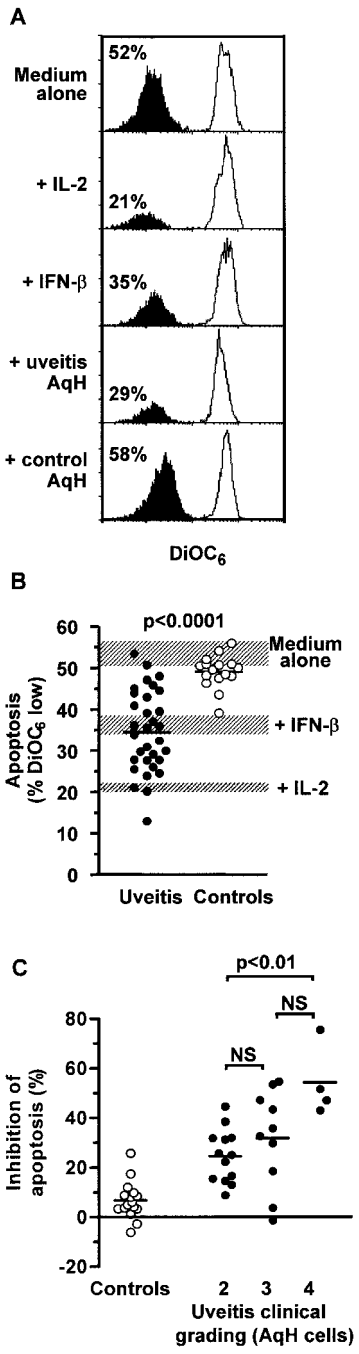

FIGURE 3. Uveitis but not control AqH inhibits SFD-induced apoptosis. SFD-induced apoptosis of a short-term $CD4^+$ T cell line was induced by a 20-h culture in the absence of serum and IL-2. *A*, Representative histograms of $DiOC_6$ fluorescence are shown where the $DiOC_6^{low}$ population (black) represents apoptotic cells. *B*, The percentage of apoptotic cells was measured as cells staining $DiOC_6^{low}$. Uveitis and control AqH were added at 50%. The shaded areas indicate the ranges of triplicate values for apoptosis with medium alone, IL-2, and IFN-β. Results are representative of three separate experiments. *C*, Inhibition of apoptosis was calculated as follows: ((medium alone − AqH)/medium alone) × 100%, and is shown with the degree of anterior chamber inflammatory activity as assessed by slit lamp biomicroscopy using the Hogan clinical grading system. NS, Not significant ($p > 0.05$).

Common γ-chain cytokines and type I IFNs are not responsible for the inhibition of SFD-induced apoptosis by uveitis AqH

Cytokines that signal through the common γ-chain and also type I IFNs (IFN-α and IFN-β) are able to inhibit SFD-induced T cell apoptosis. We tested the possible role of IFN-α and IFN-β in the inhibition of apoptosis mediated by uveitis AqH, by using specific blocking Abs. These Abs prevented the inhibition of apoptosis by recombinant IFN-α and IFN-β in control experiments (Fig. 4*A*), but had no significant effect on the inhibition of apoptosis by uveitis AqH (*B*). The levels of common γ-chain cytokines in uveitis AqH were invariably very low, although some samples contained detectable quantities of IL-2 and IL-15 (Fig. 4*C*). No significant correlation was observed between the capacity of samples of AqH to inhibit apoptosis and the presence of any common γ-chain cytokines (Fig. 4*D*).

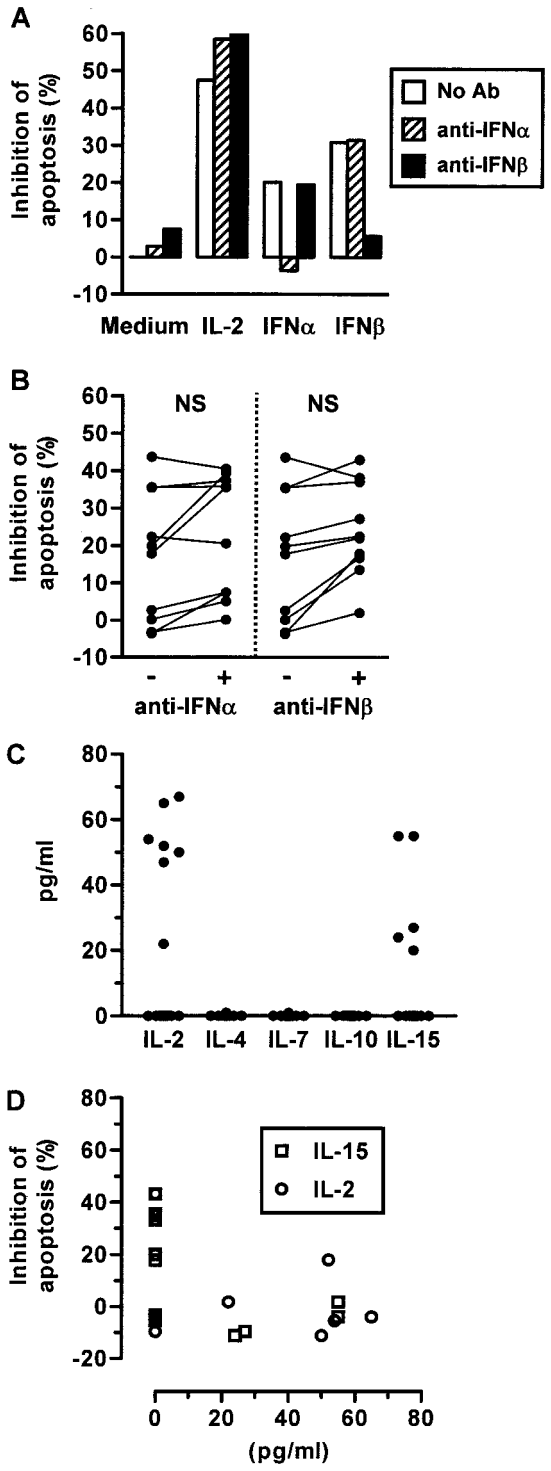

FIGURE 4. Inhibition of apoptosis by uveitis AqH is not due to type I IFNs, IL-2, or IL-15. SFD-induced apoptosis of a $CD4^+$ T cell line was induced by a 20-h culture in the absence of serum and IL-2. Cells were cultured in medium alone or with the addition of IL-2, IFN-α, IFN-β, or uveitis AqH as indicated. *A*, Medium alone (□), or optimal concentrations of specific blocking Abs for IFN-α (▨) or IFN-β (■) were added. *B*, There was no significant effect of either Ab on the inhibition of apoptosis by nine acute anterior uveitis AqH. Uveitis and control AqH were added at 50%. NS, Not significant ($p > 0.05$). Results are representative of three separate experiments. *C*, IL-2, IL-4, IL-7, IL-10, and IL-15 were measured using a multiplex bead immunoassay in a range of uveitis AqH samples. Results are expressed as picograms per milliliter. *D*, There was no significant correlation between the inhibition of apoptosis by uveitis AqH and the concentrations of IL-2 ($r = -0.43$; $p > 0.05$) or IL-15 ($r = -0.48$; $p > 0.05$) in those AqH.

IL-6/sIL-6R trans*-signaling by uveitis AqH inhibits SFD-induced apoptosis*

Primed T cells ($CD4^+CD45RO^+$) isolated from the peripheral blood of patients with uveitis expressed IL-6R on their surface, but $CD4^+CD45RO^+$ T cells isolated from AqH of the same patients expressed significantly lower levels (Fig. 5, *A* and *B*). The levels of IL-6R observed on AqH T cells were similar to those found on short-term $CD4^+$ T cell lines. However, both IL-6 and sIL-6R were present in uveitis AqH (Fig. 5, *C* and *D*) and correlated with the inhibition of SFD-induced apoptosis (*C* and *D*). These results suggested that IL-6/sIL-6R *trans*-signals might be responsible for the observed inhibition of apoptosis.

IL-6 inhibited apoptosis of the $CD4^+$ T cell line at high concentrations, significantly above those found in the majority of uveitis AqH (Fig. 6*A*). However, apoptosis was very effectively inhibited by *trans*-signaling with either a combination of rIL-6 and sIL-6R (Fig. 6*A*), or the fusion protein Hyper IL-6 (*B*), which is a construct of IL-6 linked to sIL-6R (32). An IL-6R-specific blocking Ab markedly diminished the inhibition of SFD-induced apoptosis of $CD4^+$ T cell lines mediated by Hyper IL-6 (Fig. 6*B*). This Ab had no effect on the inhibition of apoptosis mediated by the common γ-chain cytokine IL-2 (Fig. 6*C*), but significantly diminished the inhibition of apoptosis mediated by uveitis AqH (*D*).

Discussion

The presence of intraocular inflammation in uveitis suggests that the mechanisms that maintain immune privilege are breached. A key characteristic of immune privilege in the eye is the induction of T cell apoptosis (6, 33). This study reports a deficiency of T cell apoptosis in recent-onset uveitis and also a propensity for ocular fluid from uveitis patients, but not controls, to inhibit SFD-induced apoptosis. IL-6/sIL-6R *trans*-signaling appeared to be a significant pathway in the inhibition of SFD-induced T cell apoptosis in uveitis.

The constitutive expression of FasL on ocular tissues has been suggested to play a major role in maintaining immune privilege in the eye, through the induction of T cell apoptosis (6, 33, 34). However, the absence of detectable apoptotic lymphocytes in the majority of patients with recent-onset uveitis in the present study, suggested that induction of Fas-induced apoptosis is limited during the active stage of disease. Furthermore, ocular fluid had no modulatory effect on in vitro Fas-mediated apoptosis assays. However, murine experiments strongly support the role of Fas-induced apoptosis in ocular immune privilege (6). In humans, functional mutations in the *Fas* gene leads to a range of autoimmune diseases, including uveitis (35). Previous reports have suggested that AqH from healthy subjects contains proapoptotic factors (36), and that Fas-induced apoptosis might be involved in the spontaneous resolution of inflammation observed in patients with acute anterior uveitis (13, 15, 16). The present report suggests that the ocular microenvironment in uveitis has no modulatory effect on Fas-induced apoptosis. The absence of significant apoptosis in these samples therefore suggests insufficient triggering of the Fas pathway during inflammation, particularly because the ocular T cells were highly susceptible to Fas-induced apoptosis in vitro. There is evidence of reduced expression of FasL in the eye during inflammation, which would accord with this observation (16). In experimental models of uveitis, T cell apoptosis usually occurs within 2–3 days of the development of disease (37–40). This is approximately the same time frame that we have studied in recent-onset patients. However, it is possible that Fas-mediated apoptosis may occur later in the course of disease (13, 41). We were unable to study the resolution phase in the current study, because repeated

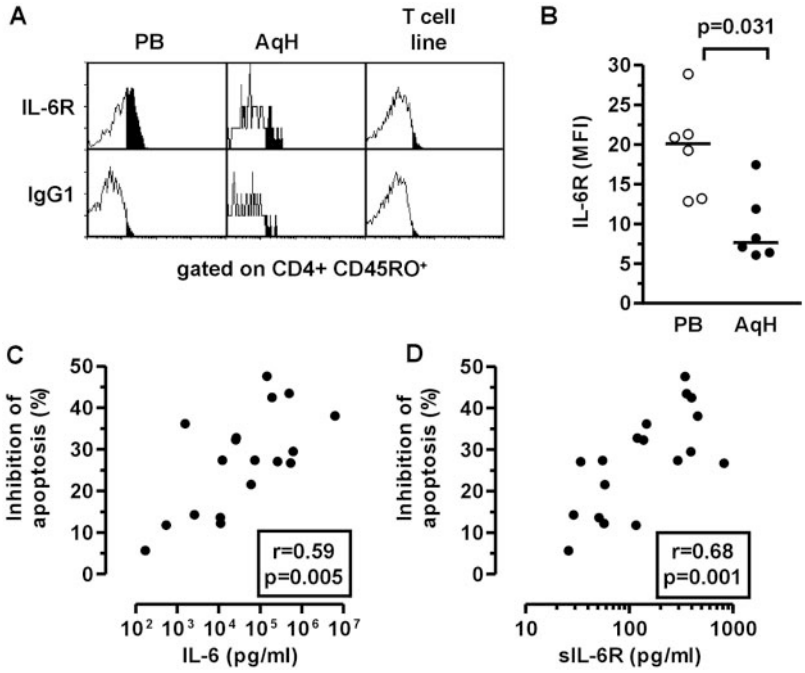

FIGURE 5. IL-6R is down-regulated on AqH lymphocytes, but both IL-6 and sIL-6R are present in uveitis AqH, and levels correlate with the inhibition of SFD-induced apoptosis. PBMC (PB), AqH cells, and the $CD4^+$ T cell line were stained with Abs specific for IL-6R, CD4, and CD45RO. *A*, Histograms are shown for IL-6R and an isotype-matched irrelevant control Ab (IgG1), following gating for CD4 and CD45RO expression. *B*, The median fluorescence intensity (MFI) values obtained from six acute anterior uveitis patients are shown, with bars indicating the median IL-6R expression. The concentrations of IL-6 and sIL-6R in uveitis AqH were determined by multiplex bead immunoassay, along with the inhibition of apoptosis (as for Fig. 3) by the same samples. SFD-induced apoptosis of a $CD4^+$ T cell line was induced by a 20-h culture in the absence of serum and IL-2. *C* and *D*, The inhibition of SFD-induced apoptosis correlated with the levels of IL-6 (*C*) and sIL-6R (*D*).

sampling of AqH, or aspiration during resolution was not justified ethically.

Adaptive immune responses lead to a rapid expansion of lymphocyte clones required for the effector response. The maintenance of these cells requires a number of survival factors that regulate the transcription of antiapoptotic proteins, including bcl-x_L and bcl-2 (9). When the pathogenic stimulus is removed, the levels of survival factors fall and extensive SFD-induced apoptosis occurs, resulting in restoration of T cell homeostasis (8, 9, 42). Inhibition of SFD-induced apoptosis contributes to the abnormal persistence of inflammation in many conditions (25, 26, 43). The absence of detectable apoptotic lymphocytes in virtually all uveitis AqH not only implies deficiency of Fas-mediated apoptosis, but also suggests that SFD-induced apoptosis is inhibited as well. Strikingly, when lymphocytes were removed from the inflamed eye, they rapidly entered apoptosis, suggesting that they are highly susceptible to spontaneous SFD-induced apoptosis. AqH from patients with uveitis strongly inhibited SFD-induced apoptosis in vitro, suggesting that inhibition of this pathway may contribute to intraocular inflammation. The central role played by T cells in the pathogenesis of uveitis suggests that their extended survival may significantly enhance tissue damage.

Common γ-chain cytokines and type I IFNs inhibit apoptosis in many situations (17, 18), but did not account for the inhibition of SFD-induced T cell apoptosis mediated by uveitis AqH. The levels of common γ-chain cytokines were extremely low, well below the levels required for induction of survival (17), and did not correlate with the potential of those AqH to inhibit apoptosis in vitro. Blocking Abs have previously demonstrated the importance of type I IFNs in the inhibition of T cell apoptosis in the rheumatoid synovium (18). However, in the present study, similar experiments failed to support a role for type I IFNs in the inhibition of intraocular T cell apoptosis in patients with uveitis. In Crohn's disease, IL-6 has been shown to play a role in the inhibition of SFD-induced T cell apoptosis (26). Data presented in this study show that IL-6 might also be involved in the inhibition of SFD-induced apoptosis in uveitis. IL-6 can signal directly, or by ligating sIL-6R and subsequently binding surface gp130 (*trans*-signaling) (21). Very low levels of IL-6R were detected on the surface of ocular T cells, suggesting that direct IL-6 signaling was unlikely. The number of T cells that could be recovered from AqH precluded direct analysis of the effects of IL-6 signaling pathways on these cells. However, short-term $CD4^+$ T cell lines closely resembled ocular T cells in their expression of IL-6R and were consequently used to study the efficacy of IL-6 signaling pathways in vitro. Apoptosis of short-term T cell lines was not inhibited by IL-6 alone, except for very high concentrations above those found in the majority of uveitis AqH. However, combinations of IL-6 and sIL-6R strongly inhibited SFD-induced apoptosis. Both molecules were present in uveitis AqH at levels sufficient to inhibit apoptosis. The hybrid molecule Hyper IL-6, which is a covalently linked composite of IL-6 coupled to sIL-6R (32), also profoundly suppressed SFD-induced apoptosis. Furthermore, Abs to the IL-6R that block function were able to significantly reduce the inhibition of apoptosis by uveitis AqH.

IL-6 has previously been suggested to act in experimental models of uveitis, by antagonizing the actions of TGFβ2, leading to reduced suppression of T cell proliferation (44, 45). However, these results might also have reflected enhanced T cell survival mediated by IL-6 *trans*-signals. The source of sIL-6R in the inflamed eye has not yet been identified. The soluble molecule can be generated by alternative mRNA splicing, or by cleavage of the full-length molecule from the cell surface (24). In inflammatory microenvironments, cleavage from neutrophils appears to be the major source of sIL-6R (24, 46) and is likely to be a key contributor in recent-onset uveitis, where neutrophils are a major intraocular cell population.

In patients with idiopathic uveitis, which does not have an infective etiology, the inhibition of apoptosis is likely to play a role in exacerbating or prolonging the disease process. However, where inflammation of the eye results from infection, the inhibition of T cell apoptosis may facilitate a functional effector response. The balance between immune protection from pathogens and immune-mediated bystander damage is of particular importance for immune-privileged sites such as the eye, where any damage is likely

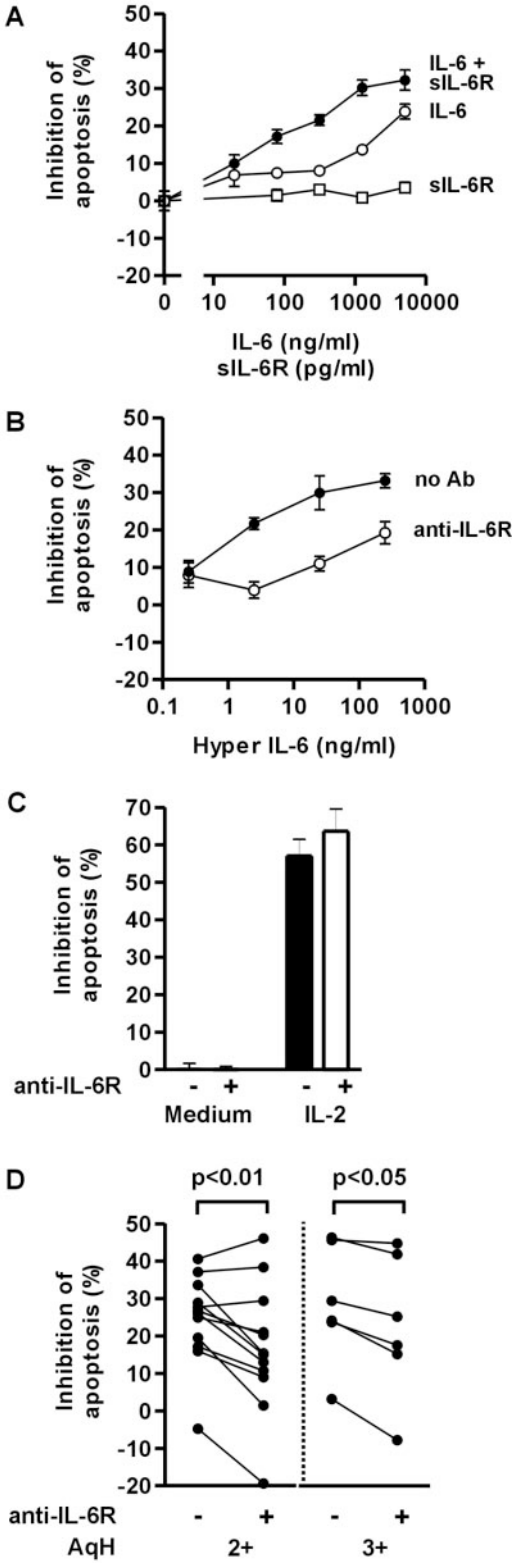

FIGURE 6. Blocking IL-6/sIL-6R *trans*-signaling decreases the inhibition of apoptosis by uveitis AqH. SFD-induced apoptosis of a $CD4^+$ T cell line was induced by a 20-h culture in the absence of serum and IL-2. *A*, IL-6 (○), sIL-6R (□), or a combination of IL-6 and sIL-6R (1000 pg/ml) (●) were added at the indicated concentrations. *B*, Hyper IL-6, a fusion protein of IL-6 and sIL-6R, was added at the indicated concentration alone (●) or with an optimal concentration of anti-IL-6R Ab (○). Results are the mean ± SD of three separate experiments. *C* and *D*, IL-2 at 25 U/ml (*C*) (mean ± SD of triplicate values) or AqH from patients with 2+ or 3+ disease at 50% were added (*D*), with the addition of an anti-IL-6R Ab as indicated. Results are representative of three separate experiments.

to be severe and irreversible. The role of IL-6/sIL-6R *trans*-signaling in the inhibition of apoptosis in uveitis suggests that therapeutic intervention may prove beneficial in patients with idiopathic disease.

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