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*J Immunol* 2003; 171:2789-2796; doi: 10.4049/jimmunol.171.6.2789

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Role of TRAIL and IFN-γ in CD4⁺ T Cell-Dependent Tumor Rejection in the Anterior Chamber of the Eye

Shixuan Wang,* Zita F. H. M. Boonman,† Hao-Chuan Li,* YuGuang He,* Martine J. Jager,† Rene E. M. Toes,§§ and Jerry Y. Niederkorn*‡

Although the anterior chamber of the eye expresses immune privilege, some ocular tumors succumb to immune rejection. Previous studies demonstrated that adenovirus-induced tumors, adenovirus type 5 early region 1 (Ad5E1), underwent immune rejection following transplantation into the anterior chamber of syngeneic mice. Intraocular tumor rejection required CD4⁺ T cells, but did not require the following: 1) CD8⁺ T cells, 2) B cells, 3) TNF, 4) perforin, 5) Fas ligand, or 6) NK cells. This study demonstrates that CD4⁺ T cell-dependent tumor rejection does not occur in IFN-γ-deficient mice. Ad5E1 tumor cells expressed DR5 receptor for TRAIL and were susceptible to TRAIL-induced apoptosis. Although IFN-γ did not directly induce apoptosis of the tumor cells, it rendered them 3-fold more susceptible to TRAIL-induced apoptosis. Both CD4⁺ T cells and corneal endothelial cells expressed TRAIL and induced apoptosis of Ad5E1 tumor cells. The results suggest that Ad5E1 tumor rejection occurs via TRAIL-induced apoptosis as follows: 1) tumor cells express TRAIL-R2 and are susceptible to TRAIL-induced apoptosis, 2) IFN-γ enhances TRAIL expression on CD4⁺ T cells and ocular cells, 3) IFN-γ enhances tumor cell susceptibility to TRAIL-induced apoptosis, 4) apoptotic tumor cells are found in the eyes of rejector mice, but not in the eyes of IFN-γ knockout mice that fail to reject intraocular tumors, 5) CD4⁺ T cells and corneal endothelial cells express TRAIL and induce apoptosis of tumor cells, and 6) apoptosis induced by either CD4⁺ T cells or corneal cells can be blocked with anti-TRAIL Ab. The Journal of Immunology, 2003, 171: 2789–2796.

It has been recognized for more than a century that many inflammatory and immune processes are excluded from the eye (1). The anterior chamber (AC) in particular is endowed with a remarkable array of mechanisms that either inhibit or diminish the impact of immune inflammation in the eye. The reduced expression, or frank absence, of MHC class I Ags on the corneal endothelium lessens the likelihood of injury by class I-restricted, CD8⁺ CTL (2–5). The aqueous humor contains a myriad of anti-inflammatory and immunosuppressive factors that restrict the induction and expression of immune-mediated inflammation (1). Moreover, Ags introduced into the AC elicit Ag-specific down-regulation of Th1 immune responses, a phenomenon termed AC-associated immune deviation (1). AC-associated immune deviation acts to prevent the expression of delayed-type hypersensitivity (DTH) responses in the eye, thereby circumventing collateral injury to innocent bystander tissues that frequently occurs in DTH lesions. Thus, the ocular immune apparatus appears to be designed to either inhibit or minimize immune-mediated injury to eye tissues that are notoriously incapable of regeneration—a phenomenon termed immune privilege.

The immune privilege of the eye would seem to favor the development of intraocular neoplasms. However, uveal melanoma, the most common intraocular malignancy in adults, represents <1% of the annual cancer registrations and occurs with an incidence of only six cases per million in the Caucasian population (6). Several factors might influence the incidence of intraocular neoplasms, including immune surveillance—ocular immune privilege not withstanding. That is, ocular immune privilege can be circumvented, leading to T cell-dependent immune rejection of intraocular tumors (7–9). Another potential mechanism for limiting intraocular tumor progression has recently come to light. Lee and colleagues (10) have demonstrated that TRAIL is constitutively expressed in mouse and human eyes and exerts a tumor surveillance function. DBA/2 tumors engineered to express TRAIL-R2 undergo TRAIL-induced resolution in the eyes of BALB/c mice (10). Thus, the eye appears to possess both T cell-dependent and T cell-independent mechanisms for controlling the growth of intraocular neoplasms.

Recently we described a CD4⁺ T cell-dependent form of intraocular tumor rejection that did not require CD8⁺ T cells, perforin, TNF-α, NK cells, B cells, or Fas ligand (FasL) (11). Tumor rejection occurred without inflicting damage to innocent bystander tissues in the eye that often occurs in DTH-mediated intraocular tumor rejection (7–9). The nonphthisical nature of the intraocular tumor resolution was consistent with an apoptotic mechanism of tumor resolution. In the present study we examined the hypothesis that the CD4⁺ T cell-dependent rejection of intraocular tumors was mediated by apoptosis induced by either IFN-γ or TRAIL. The rationale for considering IFN-γ is based on the observation that this cytokine is produced by CD4⁺ T cells and is known to induce apoptosis in a wide variety of cells. The notion that TRAIL-mediated apoptosis

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Received for publication March 4, 2003. Accepted for publication July 7, 2003.

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1 This work was supported by National Institutes of Health Grants EY05631 and CA90276 and an unrestricted grant from Research to Prevent Blindness, Inc. (New York, NY).

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3 Abbreviations used in this paper: AC, anterior chamber; Ad5E1, adenovirus type 5 early region 1; DTH, delayed-type hypersensitivity; KO, knockout; FasL, Fas ligand.
might be involved in intraocular tumor resolution arises from previous observations indicating that some intraocular tumors can be eliminated by TRAIL expressed on ocular cells (10). In addition, CD4+ T cells express TRAIL (12–14) and can kill melanoma cells by a TRAIL-dependent process (15). With this in mind, we examined the roles of IFN-γ and TRAIL in CD4+ T cell-dependent resolution of syngeneic intraocular tumors.

Materials and Methods

Animals

C57BL/6 (H-2b) and IFN-γ knockout (KO) mice on a C57BL/6 (H-2b) background (B6.129S7-Il6tm1Sor) were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, National Institutes of Health Guidelines on laboratory animal welfare, and the Association for Research in Vision and Ophthalmology statement about the Use of Animals in Ophthalmic and Vision Research.

Tumor cells

C57BL/6 mouse embryo cells were transformed with the human adenovirus type 5 early region 1 (Ad5E1) adenovirus and maintained as previously described (16, 17). Single-cell suspensions of Ad5E1 tumor cells were washed in HBSS and suspended for AC injections.

AC injections

Tumor cell suspensions were injected into the AC as previously described (18). Mice were anesthetized with 0.66 mg/kg of ketamine hydrochloride (Vetalar; Parke-Davis and Co., Detroit, MI) given i.m. A glass micropipette (diameter ~80 μm) was fitted onto a sterile infant feeding tube (5 French; Professional Medical Products, Greenwood, SC) and mounted onto a 0.1-ml Hamilton syringe (Hamilton, Whittier, CA). A Hamilton automatic dispensing apparatus was used to inject 5 μl of a monocellular suspension of Ad5E1 tumor cells (3 × 105 cells/5 μl). Eyes were examined three times per week, and the tumor volume was recorded as the percentage of AC occupied with tumor (18).

Cytokines, Abs, and reagents

Recombinant human TRAIL was obtained from R&D Systems (Minneapolis, MN). Recombinant murine IFN-γ and TNF-α were purchased from R&D Systems. Murine-specific Abs were purchased from the following commercial sources: anti-TRAIL (Santa Cruz Biotechnology, Santa Cruz, CA), anti-TRAILR2 (R&D Systems), anti-IFN-γ (BD PharMingen, San Diego, CA), rabbit anti-caspase-3 (BD PharMingen), and anti-CD4 (BD PharMingen).

Immunohistochemistry

Tumor-containing eyes were removed from euthanized mice, fixed in formalin, embedded in paraffin, and cut into 8-μm sections. Sections were deparaffinized, and endogenous peroxidases were quenched by incubating the slides in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for 12 h at room temperature. Slides were washed twice in PBS, incubated in blocking serum (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA), washed once in PBS, incubated overnight at 4°C in either anti-TRAIL Ab (0.25 μg/ml) or an isotype control Ab (0.25 μg/ml), and incubated for 30 min at 37°C. Slides were washed in PBS and incubated in biotinylated anti-rabbit IgG (Vectastain Elite ABC kit; Vector Laboratories). Slides were washed in PBS/0.1% Tween 20 and incubated in Vectastain ABC reagent for 30 min at 37°C. Slides were washed as before and developed in peroxidase substrate (DAB Peroxidase Substrate kit; Vector Laboratories) for 2–10 min at room temperature. Slides were washed and counterstained in methyl green.

The following two techniques were used for in situ detection of apoptosis: 1) anti-active caspase-3, and 2) TUNEL. It has been suggested that anti-active caspase-3 staining is more sensitive than TUNEL staining because it detects apoptosis at an early stage, before DNA fragmentation occurs (19, 20). Paraffin-embedded ocular tumor sections were incubated with rabbit anti-mouse/human active caspase-3 (R&D Systems) or normal rabbit IgG diluted to 0.25 μg/ml (BD PharMingen) and processed for in situ staining using the same immunohistochemical protocol described above. Specimens were also evaluated for apoptosis by TUNEL using an in situ cell death detection kit according to the manufacturer’s instructions (Roche Diagnostic Systems, Indianapolis, IN). Two independent investigators evaluated and scored all of the immunohistochemical specimens.

Ocular cell cultures

C57BL/6 corneal endothelial cells were established from freshly dissected corneal explants as previously described and propagated in MEM supplemented with 10% FCS (21, 22). After the primary cultures were established, the cells were immortalized with human papilloma virus genes E6 and E7 using the disabled recombinant retroviral vector pLXSN16E6/E7 (23). These cells proliferate indefinitely while maintaining their original morphologic characteristics. Furthermore, the cells express the same histocompatibility Ags as their nontransformed counterparts (21, 22). Iris/ciliary body cell cultures were established and propagated in complete RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer solution (JRH Biosciences, Lenexa, KS), 1% penicillin, streptomycin, Fungizone solution, 1% nonessential amino acids solution (BioWhittaker, Walkersville, MD), and 5 × 10−3 M 2-ME (Sigma-Aldrich) (24, 25).

CD4+ T cell preparation

For CD4+ enrichment, splenocytes were collected and erythrocytes were lysed. The splenocytes were enriched for T cells by passing the cells over a nylon wool column. This enriched population was then incubated with CD4-specific microbeads (10-μl beads/107 cells; Miltenyi Biotec, Auburn, CA) in 0.5% BSA in PBS for 15 min in the refrigerator. The cells were washed with 0.5% BSA in PBS followed by magnetic separation using MS+ columns as described by the manufacturer. The retained cells were eluted from the column and shown by flow cytometric analysis to be enriched CD4+ T cells (94% positive).

FIGURE 1. Intraocular tumor growth in C57BL/6 mice and IFN-γ KO mice. Ad5E1 tumor cells (3 × 105 cells/5 μl) were injected into the AC on day 0. A, Tumor growth was scored as the percentage of AC occupied by tumor (mean ± SD) in normal C57BL/6 (n = 20) and IFN-γ KO mice (n = 15). B, Percent of mice with intraocular tumors on day 40. Tumors resolved in 18 of 20 normal mice, but in none of the 15 IFN-γ KO mice. The tumors that did not resolve in two of the normal mice occupied <5% of the AC at the conclusion of the experiment on day 40.
Three-color apoptosis assay

Tumor cell apoptosis was evaluated using a three-color assay. Single-cell suspensions of Ad5E1 tumor cells (1 × 10^6 cells/ml) were added to 24-well plates (3526; Corning Glass, Corning, NY) and allowed to adhere overnight. Either CD4^+ T cells or corneal endothelial cells (1 × 10^6 cells/ml) were incubated for 1.5 h with Cyto-59 Red Nucleic Acid Stain (Molecular Probes, Eugene, OR) diluted 1:1000 in complete RPMI 1640 medium at room temperature with constant shaking. Cyto-Red-labeled cells were washed in complete RPMI 1640 medium, resuspended (1 × 10^6 cells/ml), and added to tumor cell cultures to give a range of effector:target cell ratios. Staurosporine (3 μg/ml; Sigma-Aldrich) was used as a positive control for inducing apoptosis (26). Cultures were incubated for 24 h at 37°C. Cells were removed by trypsinization and resuspended in complete RPMI 1640 medium. Tumor cells and effector cells (CD4^+ T cells or corneal endothelial cells) were stained with a TACS Annexin V FITC kit (R&D Systems). Cell suspensions were evaluated using a FACScan flow cytometer (BD Biosciences, Palo Alto, CA), and the results were analyzed using

FIGURE 2. In situ detection of apoptosis of intraocular tumors 17 days after injection. A, Diffuse staining of active caspase-3 in an intraocular tumor in a C57BL/6 mouse. B, TUNEL staining of an intraocular tumor in a C57BL/6 mouse. C, Active caspase-3 staining of individual tumor cells in a C57BL/6 mouse. D, TUNEL staining of individual tumor cells in a C57BL/6 mouse. E, Absence of staining for active caspase-3 in intraocular tumor cells in a C57BL/6 IFN-γ KO mouse. F, Absence of TUNEL staining in intraocular tumor cells in a C57BL/6 IFN-γ KO mouse. Photographs were taken at either ×250 (A, B, and E) or ×800 (C, D and F).

FIGURE 3. Effect of recombinant murine IFN-γ on apoptosis of Ad5E1 tumor cells in vitro. Tumor cells were exposed to recombinant murine IFN-γ for 24, 48, and 72 h at 37°C. Apoptosis was determined by flow cytometry using the caspase-3 assay. Staurosporine (3 μg/ml) served as a positive control for apoptosis. This experiment was performed twice with similar results. Values of p > 0.05 for all of the IFN-γ groups compared with the negative control.

FIGURE 4. Murine TRAILR2 expression on Ad5E1 tumor cells and TRAIL-induced apoptosis. A, TRAILR2 expression on Ad5E1 tumor cells. Tumor cells were stained with either anti-TRAILR2 (bold line) or an isotype control Ab (thin line) and evaluated by flow cytometry. B, Ad5E1 tumor cells were incubated for 24, 48, and 72 h at 37°C in the presence of IFN-γ (50 or 200 ng/ml) and examined by flow cytometry for TRAILR2 expression. C, Ad5E1 tumor cells were incubated for 24 h at 37°C in the presence of IFN-γ (50 or 200 ng/ml), TRAIL (50 ng/ml), or a combination of the two. Apoptosis was determined by flow cytometry using the caspase-3 assay. Mean ± SD. * p = 0.0003 compared with IFN-γ (50 ng/ml) + TRAIL and IFN-γ (200 ng/ml) + TRAIL.
CellQuest version 3.1f software (BD Biosciences). Cyto-Red-staining effector cells and propidium iodide-positive cells were independently removed by gating. Propidium iodide-negative cells that stained positively with annexin V were considered to be apoptotic.

Flow cytometric analysis

Surface expression of TRAIL was assessed by flow cytometry as previously described (27). Single-cell suspensions of C57BL/6 CD4⁺ T cells and C57BL/6 corneal endothelial cells were prepared and washed in fluorescence-activated cell sorter buffer consisting of PBS containing 1% BSA and 0.02% sodium azide. Cells (1 × 10⁶) were incubated with anti-murine TRAIL mAb (1 µg/ml) for 30 min on ice, washed three times, incubated with FITC-labeled secondary Ab for 20 min at 4°C, washed three additional times in PBS, fixed in 1% paraformaldehyde, and assessed for fluorescence in a FACScan flow cytometer (BD Biosciences). Cytoplasmic expression of IFN-γ was assessed by incubating purified CD4⁺ T cells in RPMI 1640 medium containing brefeldin A (10 µg/ml; Sigma-Aldrich) for 6 h at 37°C. Cells were washed in HBSS and incubated (30 min at 4°C) in FITC-labeled goat anti-mouse CD4 (BD PharMingen) at a concentration of 1 µg/ml. Cells were washed three times in HBSS and resuspended in cytofix/cytoperm solution (BD PharMingen). Cells were washed in Perm-Wash buffer (BD PharMingen) and resuspended in PE-labeled anti-mouse IFN-γ (BD PharMingen) at a concentration of 5 µg/ml. Controls consisted of CD4⁺ T cells incubated with PE-labeled normal rat IgG. Cell suspensions were evaluated using a FACScan flow cytometer (BD Biosciences), and the results were analyzed using CellQuest version 3.1f software (BD Biosciences).

Caspase-3 assay for apoptosis

Tumor cells were incubated in either IFN-γ, TRAIL, or a combination of the two cytokines. Tumor cells were removed from cultures by gentle trypsinization, suspended in HBSS, and washed twice in HBSS. Cells were suspended in PE-conjugated rabbit anti-caspase-3 or PE-conjugated normal rabbit IgG and incubated for 30 min at 4°C in the dark. Cells were washed
once with 1 ml of 5% FCS in HBSS, resuspended in 1× binding buffer, and examined by flow cytometry.

Statistics
Student’s t test was used to assess the statistical significance of the differences between experimental and control groups. A p value of <0.05 was considered significant.

Results
Progressive growth of intraocular tumors in IFN-γ KO mice
We have previously reported that Ad5E1 tumors undergo CD4+ T cell-dependent rejection in the eyes of syngeneic C57BL/6 mice. Intraocular tumor resolution does not culminate in collateral nonspecific damage to innocent bystander tissues that can often accompany DTH-mediated rejection of intraocular tumors (7–9, 28).

Ad5E1 tumor resolution was mediated by apoptosis induced by CD4+ T cells. CD4+ T cells express FasL and are known to induce apoptosis of Fas-bearing cells (29, 30). However, Ad5E1 tumor resolution occurs in FasL-defective gld/gld mice, thereby ruling out a major role for Fas-induced apoptosis in the CD4+ T cell-dependent rejection of intraocular Ad5E1 tumors (11). CD4+ T cells produce TNF-α and IFN-γ, each of which is capable of inducing apoptosis of a wide variety of target cells (12, 31, 32). Because rejection of Ad5E1 tumor cells is not impaired in TNF-α KO mice (11), we considered the possibility that intraocular tumor resolution was mediated by apoptosis induced by IFN-γ elaborated by CD4+ T cells. This hypothesis was tested by transplanting Ad5E1 tumor cells into the AC of normal C57BL/6 mice and IFN-γ KO mice. The results of a typical experiment are shown in Fig. 1, and demonstrate that intraocular tumors grew in both wild-type and IFN-γ KO mice for ∼3 wk. However, the intraocular tumors disappeared by 5 wk in the wild-type C57BL/6 mice. By contrast, the intraocular tumors never underwent rejection in IFN-γ KO mice.

Separate experiments were performed to confirm our suspicion that apoptosis contributed to tumor resolution. Eyes were enucleated at the early onset of intraocular tumor resolution in wild-type mice (day 17) and examined by either active caspase-3 staining or TUNEL for the presence of apoptotic tumor cells. Tumor cell apoptosis was clearly demonstrated in wild-type mice, but not in the eyes removed from IFN-γ KO mice (Fig. 2). These results indicate that IFN-γ is necessary for CD4+ T cell-dependent rejection of Ad5E1 tumors and that apoptosis is a major feature of tumor resolution in normal hosts, but apoptosis is conspicuously absent in hosts that are incapable of generating IFN-γ.

Role of IFN-γ in tumor cell apoptosis
The simplest explanation for the observed progressive tumor growth in IFN-γ KO mice and CD4 KO mice is that CD4+ T cells elaborate IFN-γ, which induces apoptosis of the intraocular tumors. Accordingly, Ad5E1 tumor cells were tested for their in vitro susceptibility to IFN-γ-induced apoptosis. The results of a typical experiment are shown in Fig. 3, and they demonstrate that Ad5E1 tumor cells were not susceptible to a wide range of doses of IFN-γ.

Adenovirus-induced tumors are exceptionally susceptible to TRAIL-induced apoptosis (33), and IFN-γ is known to up-regulate TRAIL-R2 (DR5) on mouse cells and increases their susceptibility to TRAIL-induced apoptosis (34). Accordingly, we examined Ad5E1 tumor cells for the expression of TRAIL-R2 and their susceptibility to TRAIL-induced apoptosis. Flow cytometric analysis revealed that Ad5E1 tumor cells expressed TRAIL-R2 (Fig. 4). The tumor cells were only mildly susceptible to apoptosis when incubated with either recombinant human TRAIL or IFN-γ (Fig. 4). However, tumor cell susceptibility to TRAIL-induced apoptosis was increased 3-fold by pretreatment with IFN-γ (Fig. 4).

CD4+ T cell-mediated apoptosis of Ad5E1 tumor cells
TRAIL is expressed on some CD4+ T cells and can contribute to CD4+ T cell-induced apoptosis of tumor cells (15). Accordingly, flow cytometry was used to examine the expression of cytoplasmic IFN-γ and cell membrane TRAIL on CD4+ T cells from wild-type C57BL/6 mice and IFN-γ KO mice. As expected, IFN-γ was undetectable in CD4+ T cells from IFN-γ KO mice, but was strongly expressed in CD4+ T cells from normal C57BL/6 mice (Fig. 5). Moreover, TRAIL expression on CD4+ T cells was approximately 5 times greater in normal C57BL/6 mice compared with IFN-γ KO mice (Fig. 5). An in vitro assay was used to determine whether CD4+ T cells from mice immunized with Ad5E1 tumor cells were capable of inducing apoptosis of Ad5E1 tumor cells. The results of a typical experiment are shown in Fig. 6 and demonstrate that CD4+ T cells from mice immunized with Ad5E1 tumor cells were capable of inducing apoptosis of Ad5E1 tumor cells. More than half of the CD4+ T cell-induced apoptosis was blocked with anti-TRAIL Ab (Fig. 6). Thus, CD4+ T cells can directly induce TRAIL-mediated apoptosis of Ad5E1 tumor cells.
TRAIL expression on ocular cells and induction of tumor cell apoptosis

TRAIL is expressed on the surface of numerous cells that line the AC and induces significant apoptosis of TRAIL-R2\(^*\) tumor cells in vitro (10). Thus, the possibility exists that ocular cells might induce apoptosis of TRAIL-R2\(^*\) AD5E1 tumor cells and thereby mediate intraocular tumor resolution, as has been proposed in a recent study using TRAIL-R2 transfected allogeneic P815 mastocytomas (10).

Immunohistochemical staining of eyes from wild-type and IFN-\(\gamma\) KO mice revealed the expression of TRAIL on multiple ocular tissues. As previously reported, TRAIL was expressed on corneal endothelial cells and iris/ciliary body epithelial cells—two epithelial layers that line the AC of the eye and would be in direct

FIGURE 8. Apoptosis of Ad5E1 tumor cells by C57BL/6 ocular cells. A, Corneal endothelial cells. B, Iris/ciliary body cells. Apoptosis of Ad5E1 tumor cells was determined by flow cytometry using a three-color annexin V apoptosis assay using C57BL/6 corneal endothelial cells and iris/ciliary body cells in the presence or absence of anti-murine TRAIL mAb or normal IgG. Mean ± SD. *, \(p = 0.02\) compared with endothelial cells without Ab and endothelial cells plus normal IgG; **, \(p = 0.0001\) iris/ciliary body cells incubated with anti-TRAIL compared with normal IgG.
contact with intraocular tumors. TRAIL staining was also present on all layers of the retina in wild-type mice, but conspicuously absent in the retinas of the IFN-γ KO mice. In all cases TRAIL staining was markedly more intense on ocular cells of the wild-type mice compared with IFN-γ KO mice (Fig. 7).

The capacity of ocular cells to induce apoptosis of Ad5E1 tumor cells was tested in vitro. C57BL/6 corneal endothelial cells and iris ciliary body cells were labeled with nucleic acid red stain and cocultured with Ad5E1 tumor cells for 48 h. Cell cultures were subjected to flow cytometric analysis in which the corneal cells were gated out based on their expression of red nucleic acid. Apoptosis of the remaining tumor cells was assessed using annexin V staining. The results indicated that both corneal cells and iris ciliary body cells induced extensive apoptosis of the tumor cells (Fig. 8). Moreover, the ocular cell-induced apoptosis could be blocked with anti-TRAIL Ab.

**Discussion**

Immune-mediated rejection of intraocular tumors can follow two fundamental pathways. The first pathway is DTH mediated and culminates in extensive collateral damage to innocent bystander cells in the eye (7–9, 35, 36). Extensive DTH-related necrosis of ocular tissues leads to phthisis of the affected eye and blindness. By contrast, a second pathway of intraocular tumor rejection involves CTL-mediated piecemeal necrosis of intraocular tumors and leaves the eye morphologically intact (36–38). In the present study, we report a third pattern of intraocular tumor rejection that is CD4+ T cell dependent, but does not require TNF-α, FasL, B cells, Ab, perforin, CD8+ T cells, or NK cells (11). However, this nonphthisical form of tumor rejection is IFN-γ-dependent and appears to be mediated by TRAIL.

On first blush, one might expect IFN-γ to act by directly inducing apoptosis of the tumor cells. However, tumor cells did not undergo apoptosis when exposed to a range of IFN-γ doses over a 72-h period. Therefore, we entertained the hypothesis that IFN-γ acted indirectly through a TRAIL-dependent pathway. This proposition was based on the previous observation that TRAIL expression is reduced on NK cells in IFN-γ KO mice (39), and that TRAIL can act in the surveillance of intraocular tumors (10). Our results indicated that IFN-γ was indeed produced by CD4+ T cells, and that exposure to IFN-γ resulted in a 3-fold increase of TRAIL-induced apoptosis of tumor cells.

The detection of caspase-3-positive tumor cells in the eyes of normal C57BL/6 mice explains the absence of innocent bystander injury to ocular tissues and supports the hypothesis that tumor rejection occurs via apoptosis. Multiple mechanisms could be invoked to induce apoptosis of intraocular Ad5E1 tumor cells. We have found that the Ad5E1 tumor cells express Fas and are susceptible to Fas-induced apoptosis (data not shown). However, intraocular Ad5E1 tumors undergo rejection in syngeneic gldgld mice that are incapable of mediating Fas-induced apoptosis (11). Ad5E1 tumor cells are also susceptible to TNF-α-induced apoptosis (data not shown), yet intraocular tumor rejection proceeds unabated in TNF KO mice (11). The absence of apoptotic tumor cells in the eyes of IFN-γ KO mice, and the progressive growth of intraocular tumors in CD4 KO mice and IFN-γ KO mice, suggests that tumor rejection relies on IFN-γ produced by CD4+ T cells. The possibility that CD4-derived IFN-γ functions indirectly by activating NK cells, CTL, or DTH is not supported by previous findings that indicate that tumor rejection occurs in CD8 KO mice, perforin KO mice, and NK-depleted mice (11).

The weight of evidence suggests that CD4+ T cell-dependent rejection of intraocular Ad5E1 tumors is mediated by TRAIL. In support of this are the following observations: 1) tumor cells express TRAIL-R2 and are susceptible to TRAIL-induced apoptosis, 2) IFN-γ enhances TRAIL expression on CD4+ T cells and ocular cells, 3) IFN-γ enhances tumor cell susceptibility to TRAIL-induced apoptosis, 4) apoptotic tumor cells are found in the eyes of rejector mice, but not in the eyes of IFN-γ KO mice that fail to reject their intraocular tumors, 5) CD4+ T cells, corneal endothelial cells, and iris ciliary body cells express TRAIL and are capable of inducing apoptosis of tumor cells, and 6) apoptosis induced by either CD4+ T cells or corneal cells can be blocked with anti-TRAIL Ab.

We propose that following their introduction into the AC, Ad5E1 tumor cells circumvent immune privilege and induce the generation of CD4+ T cells that produce IFN-γ and express TRAIL. The production of IFN-γ up-regulates TRAIL expression on ocular cells and CD4+ T cells. Upon entering the intraocular milieu, CD4+ T cells elaborate IFN-γ, which up-regulates TRAIL expression on ocular cells and increases the susceptibility of tumor cells to TRAIL-induced apoptosis. Other tumor-infiltrating cells, including macrophages, might be induced to express TRAIL and might act as ancillary effector cells for inducing apoptosis of intraocular tumor cells. It is conceivable that TRAIL protein is present in the aqueous humor that bathes the intraocular tumors and acts to further accentuate TRAIL expressed on ocular cells and the infiltrating CD4+ T cells. We are currently exploring these hypotheses, and if possible, the fate of these tumors in TRAIL KO mice.

**Acknowledgments**

The technical assistance of Elizabeth Mayhew is greatly appreciated.

**References**

1. Niederkorn, J. Y. 2002. Immune privilege in the anterior chamber of the eye. Crit. Rev. Immunol. 22:13.
2. Williams, K. A., J. K. Ash, and D. J. Coster. 1985. Histocompatibility antigen and passenger cell content of normal and diseased human cornea. Transplantation 39:265.
3. Whitsett, C. F., and R. D. Stulting. 1984. The distribution of HLA antigens on human corneal tissue. Invest. Ophthalmol. Visual Sci. 25:519.
4. Fujiwara, L. S., R. B. Colvin, A. K. Bhan, T. C. Fuller, and S. Foster. 1982. Expression of HLA-A/B/C and DR loci antigens on epithelial, stromal, and endothelial cells from the human cornea. Cornea 1:213.
5. Newsome, D. A., M. Takasaki, K. R. Kenyon, W. F. Stark, and G. Opelz. 1974. Human corneal cells in vitro: morphology and histocompatibility (HL-A) antigens of pure cell populations. Invest. Ophthalmol. 15:23.
6. Osterlund, A. 1987. Trends in incidence of ocular malignant melanoma in Denmark. 1943–1982. Int. J. Cancer 40:161.
7. Niederkorn, J. Y. 1995. Immunopathogenesis of intraocular tumors. Prog. Retinal and Eye Res. 14:505.
8. Niederkorn, J. Y. 1991. The immunopathology of intraocular tumor rejection. Eye 5:186.
9. Niederkorn, J. Y. 1997. Immunoregulation of intraocular tumours. Eye 11:249.
10. Lee, H. O., J. M. Herndon, R. Barreiro, T. S. Griffith, and T. A. Ferguson. 2002. TRAIL: a mechanism of tumor surveillance in an immune privileged site. J. Immunol. 169:4739.
11. Schurmans, L. R., L. Diehl, A. T. den Boer, R. P. Sutmuller, Z. F. Boonman, J. P. Medema, E. I. van der Voort, J. Laman, C. J. Melief, M. J. Jager, and R. E. Toes. 2001. Rejection of intraocular tumors by CD4+ T cells without induction of phthisis. J. Immunol. 167:5832.
12. Senik, A., I. Gresser, C. Maury, M. Gidlund, A. Orn, and H. Wigzell. 1979. Enhancement by interferon of natural killer cell activity in mice. Cell. Immunol. 44:186.
13. Kaplan, M. J., D. Ray, R. R. Mo, R. L. Yung, and B. C. Richardson. 2000. TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages. J. Immunol. 164:2897.
14. Kayagaki, N., N. Yamaguchi, M. Nakamura, A. Kawasaki, H. Akiba, K. Okumura, and H. Yagita. 1999. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. J. Immunol. 162:2639.
15. Thomas, W. D., and P. Hersey. 1998. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4+ T cell killing of target cells. J. Immunol. 161:2195.
16. Toes, R. E., R. Offringa, R. J. Blom, R. M. Brandt, A. J. van der Eb, C. J. Melief, and W. M. Kast. 1995. An adenovirus type 5 early region 1B-encoded CTL epitope-mediating tumor eradication by CTL clones is down-modulated by an activated ras oncogene. J. Immunol. 154:3396.
17. Kast, W. M., R. Offringa, P. J. Peters, A. C. Voorhout, R. H. Meloen, A. J. van der Eb, and C. J. Melief. 1989. Eradication of adenovirus E1-induced tumors by EIA-specific cytotoxic T lymphocytes. Cell 59:603.

18. Niederkorn, J., J. W. Streilein, and J. A. Shadduck. 1981. Deviant immune responses to allogeneic tumors injected intracamerally and subcutaneously in mice. Invest. Ophthalmol. Visual Sci. 20:355.

19. Bantel, H., A. Lugering, C. Poremba, N. Lugering, J. Held, W. Domschke, and K. Schulze-Osthoff. 2001. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc. Natl. Acad. Sci. USA 98:4185.

20. Rajalingam, K., H. Al-Younes, A. Muller, T. F. Meyer, A. J. Szczepek, and T. Rudel. 2001. Epithelial cells infected with Chlamydophila pneumoniae (Chlamydia pneumoniae) are resistant to apoptosis. Infect. Immun. 69:7880.

21. He, Y. G., J. Mellon, and J. Y. Niederkorn. 1996. The effect of oral immunization on corneal allograft survival. Transplantation 61:920.

22. Ma, D., X. Y. Li, J. Mellon, and J. Y. Niederkorn. 1998. Immunologic phenotype of hosts orally immunized with corneal alloantigens. Invest. Ophthalmol. Visual Sci. 39:744.

23. Wilson, S. E., J. Weng, S. Blair, Y. G. He, and S. Lloyd. 1995. Expression of E6/E7 or SV40 large T antigen-coding oncogenes in human corneal endothelial cells indicates regulated high-proliferative capacity. Invest. Ophthalmol. Visual Sci. 36:32.

24. Streilein, J. W., and D. Bradley. 1991. Analysis of immunosuppressive properties of iris and ciliary body cells and their secretory products. Invest. Ophthalmol. Visual Sci. 32:2700.

25. Niederkorn, J. Y., E. Y. Chiang, T. Ungchusri, and I. Stroynowski. 1999. Expression of a nonclassical MHC class Ib molecule in the eye. Transplantation 68:1790.

26. Rajalingam, K., H. Al-Younes, A. Muller, T. F. Meyer, A. J. Szczepak, and T. Rudel. 2001. Epithelial cells infected with Chlamydophila pneumoniae (Chlamydia pneumoniae) are resistant to apoptosis. Infect. Immun. 69:7880.

27. Smyth, M. J., E. Cretney, K. Takeda, R. H. Wiltrout, L. M. Sedger, N. Kayagaki, H. Yagita, and K. Okumura. 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon γ-dependent natural killer cell protection from tumor metastasis. J. Exp. Med. 193:661.

28. Thornton, A. M., and E. M. Shevach. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J. Immunol. 164:183.

29. Ju, S. T., H. Cui, D. J. Panka, R. Ettinger, and A. Marshak-Rothstein. 1994. Cytokines and the Th1/Th2 paradigm in transplantation. Curr. Opin. Immunol. 6:757.

30. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. J. Immunol. 152:1127.

31. Nickerson, P., W. Steurer, J. Steiger, X. Zheng, A. W. Steele, and T. B. Strom. 1994. Cytokines and the Th1/Th2 paradigm in transplantation. Curr. Opin. Immunol. 6:757.

32. Dallman, M. J. 1995. Cytokines and transplantation: Th1/Th2 regulation of the immune response to solid organ transplants in the adult. Curr. Opin. Immunol. 7:632.

33. Routes, J. M., S. Ryan, A. Clare, T. Muir, A. Kuhl, T. A. Potter, and J. L. Cook. 2000. Adenovirus E1A oncogene expression in tumor cells enhances killing by TNF-related apoptosis-inducing ligand (TRAIL). J. Immunol. 165:4522.

34. Meng, R. D., and W. S. El-Deiry. 2001. p53-independent up-regulation of KILLER/DR5 TRAIL receptor expression by glucocorticoids and interferon-γ. Exp. Cell Res. 262:154.

35. Niederkorn, J. Y., and T. L. Knisely. 1988. Immunological analysis of a destructive pattern of intraocular tumor resolution. Curr. Eye Res. 7:515.

36. Knisely, T. L., M. W. Lackenbach, B. J. Fischer, and J. Y. Niederkorn. 1987. Destructive and nondestructive patterns of immune rejection of syngeneic intraocular tumors. J. Immunol. 138:4515.

37. Ma, D., H. Alizadeh, S. A. Comerford, M. J. Gething, R. Anand, and J. Y. Niederkorn. 1994. Rejection of intraocular tumors from transgenic mice by tumor-infiltrating lymphocytes. Curr. Eye Res. 13:361.

38. Knisely, T. L., and J. Y. Niederkorn. 1990. Emergence of a dominant cytotoxic T lymphocyte antitumor effector from tumor-infiltrating cells in the anterior chamber of the eye. Cancer Immunol. Immunother. 30:323.

39. Takeda, K., Y. Hayakawa, M. J. Smyth, N. Kayagaki, K. Okumura, S. Yakuta, Y. Iwakura, H. Yagita, and K. Okumura. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med. 7:94.