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Regulation of Fas Ligand-Induced Apoptosis by TNF

Bennett D. Elzey,* Thomas S. Griffith,2* John M. Herndon,* Ramon Barreiro,* Jurg Tschopp,‡ and Thomas A. Ferguson3*†

Fas ligand (FasL, CD95L) expression helps control inflammatory reactions in immune privileged sites such as the eye. Cellular activation is normally required to render lymphoid cells sensitive to FasL-induced death; however, both activated and freshly isolated Fas+ lymphoid cells are efficiently killed in the eye. Thus, we examined factors that might regulate cell death in the eye. TNF levels rapidly increased in the eye after the injection of lymphoid cells, and these cells underwent apoptosis within 24 h. Coinjection of anti-TNF Ab with the lymphoid cells blocked this cell death. Furthermore, TNFR2−/− T cells did not undergo apoptosis in the eyes of normal mice, while normal and TNFR1−/− T cells were killed by apoptosis. In vitro, TNF enhanced the Fas-mediated apoptosis of unactivated T cells through decreased intracellular levels of FLIP and increased production of the pro-apoptotic molecule Bax. This effect was mediated through the TNFR2 receptor. In vivo, intracameral injection of normal or TNFR1−/− 2,4,6-trinitrophenyl-coupled T cells into normal mice induced immune deviation, but TNFR2−/− 2,4,6-trinitrophenyl-coupled T cells were ineffective. Collectively, our results provide evidence of a role for the p75 TNFR in cell death in that TNF signaling through TNFR2 sensitizes lymphoid cells for Fas-mediated apoptosis. We conclude that there is complicity between apoptosis and elements of the inflammatory response in controlling lymphocyte function in immune privileged sites. The Journal of Immunology, 2001, 167: 3049–3056.

Immune privileged sites are areas that maintain a unique relationship with the immune system. Whereas most organ systems can tolerate inflammatory and immune reactions, areas such as the eye, brain, and reproductive organs tend to prohibit immunity to prevent the damaging sequelae associated with immune responses to foreign invaders (1, 2). In the eye, constitutive Fas ligand (FasL)+ expression helps maintain immune privilege by inducing apoptotic cell death of Fas+ lymphoid cells that enter in response to infection (3). Studies from our laboratory have also linked this Fas-mediated apoptosis to the induction of immune deviation (i.e., a preferential induction of a Th2-like response over what would normally be Th1 dominated) (4). In this setting, apoptosis directly leads to the activation of a systemic Th2-type response through the interaction between APCs and dead cells, an effect mediated by the production of the anti-inflammatory cytokine IL-10 in the apoptotic cells before death (5). Thus, the elimination of potentially damaging inflammatory cells and the induction of immune deviation prevent the activation of dangerous cellular immune reactions that could damage vision or lead to autoimmunity.

The Fas/FasL system is not only critical for immune privilege (3, 6, 7), but also is important in the development of the immune response, homeostasis of the immune system, and maintenance of self-tolerance (8, 9). Additionally, Fas-mediated apoptosis is involved in T cell cytotoxicity, tumorigenesis, and liver disease (10–12). Fas is a type I membrane protein belonging to the TNFR superfamily, which includes the p55 (TNFR1) and p75 (TNFR2) TNFRs, CD40, 4-1BB, and the family of TNF-related apoptosis-inducing ligand receptors, among others (13, 14). Cross-linking of Fas by FasL or specific Ab initiates the cell death cascade, with the binding of Fas-associated death domain protein (FADD) to the cytoplasmic domain of Fas being the first molecular event (15, 16). This facilitates the binding and activation of caspase-8 (FADD-like IL-1β-converting enzyme), followed by the activation of other downstream caspases, degradation of DNA, and ultimately cell death (17, 18). This process is tightly regulated and depends, in the case of T cells, on prior cellular activation.

Interestingly, elements of the immune response (such as IL-2; Ref. 19) potentiate Fas-mediated death through the modulation of the apoptosis regulatory protein FADD-like IL-1β-converting enzyme inhibitory protein (FLIP) (20).

FLIP was originally described as a viral product (vFLIP) (21, 22), but has since been shown to exist in mammalian cells (cFLIP). cFLIP mRNA exists as multiple splice variants, but protein expression is limited to a long and short form (cFLIPL and cFLIPS) (21). cFLIPL contains two death effector domains (DED) and is structurally related to vFLIP, whereas cFLIPS contains an additional caspase-like domain lacking catalytic activity. The DED of both FLIPL and FLIPS enable them to bind to the DED of FADD and caspase-8, preventing their recruitment to the death domain of Fas. This prevents formation of the death-inducing signaling complex and inhibits apoptosis (23, 24). The resistance of naïve T cells to Fas-mediated death has been attributed to high intracellular levels of FLIP, which decrease following cellular activation and IL-2 production, thereby making the T cells susceptible to Fas-mediated...
death (20, 23). In addition to protecting cells from Fas-induced apoptosis, FLIP can protect cells from apoptosis induced by TNF. NF receptor-related apoptosis-mediated protein, and TNF-related apoptosis-inducing ligand. FLIP has also been found in heart, skeletal muscle, and kidney (23) where it is presumed to function as an antiapoptotic factor to help maintain organ homeostasis. Expression in tumors promotes tumor growth presumably by blocking the cell death pathway (25, 26). The importance of FLIP was recently demonstrated by the embryonic lethality of targeted deletion of this molecule (27). In our studies of FasL-induced death in the eye, we have observed that apoptotic cell death occurs rapidly in both activated and freshly isolated lymphoid cells (3, 4). Since Fas-mediated apoptosis is tightly regulated, we suspected that other factors might contribute to this process. In the present study, we examined the role of the proinflammatory cytokine TNF in the regulation of Fas-mediated apoptosis in the eye. Our results show that TNF, via binding to TNFR2, increases the sensitivity of T cells to Fas-mediated death by modulating the expression of a molecule that regulates apoptosis.

Materials and Methods

Mice

BALB/c and C57BL/6 were purchased from the National Cancer Institute. TNFR1−/−, TNFR2−/−, and normal littermate mice were purchased from The Jackson Laboratory (Bar Harbor, ME). In all in vivo experiments, groups consisted of five or more animals and were used at 6–10 wk of age. Experiments were repeated at least three times with similar results.

Reagents

2,4,6-Trinitro-1-chlorobenzene (TNCB) was purchased from Eastern Chemical (Smithtown, NY). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma (St. Louis, MO). Recombinant murine TNF was purchased from Genzyme (Cambridge, MA). Jo2 (anti-Fas/CD95), anti-CD3-FITC, and streptavidin–peroxidase were purchased from Becton Dickinson (Mountain View, CA). 2,4,6-Trinitrophenyl (TNP) coupling of T cells was performed with TNP3 19.12 mAb (30) diluted in coating buffer (0.1 M NaHCO3/0.1 M Na2CO3, pH 9.8) at room temperature. Wells were washed four times, followed by the addition of 100 μl/well peroxidase-conjugated rabbit anti-goat IgG (diluted 1:2000 in DMEM-10% FCS; provided by Dr. K. Sheehan, Washington University) was added for 1 h at room temperature. Wells were washed four times, followed by the addition of 100 μl/well peroxidase-conjugated rabbit anti-goat IgG (diluted 1:2000 in DMEM-10% FCS; provided by Dr. K. Sheehan). After development of diaminobenzidine substrate, the plate was analyzed on an ELISA plate reader at 492 nm.

In situ TUNEL staining

At various times following AC injection of TNP-coupled cells, eyes were removed, fixed in Formalin, and processed for paraffin sectioning. Ten-micrometer sections were mounted onto microscope slides and incubated overnight at 55°C. Sections were then deparaffinized by washing for 5 min in xylene twice, 5 min in absolute ethanol twice, 3 min in 95% ethanol, 3 min in 70% ethanol, and 5 min in PBS. Protein present in the sections was digested with 20 μg/ml proteinase K for 20 min at room temperature. Following four washes in distilled water, endogenous peroxidase was quenched with 2.0% H2O2 for 5 min at room temperature and sections were washed twice in PBS. Labeling of 3'-OH fragmented DNA ends was performed with an in situ apoptosis detection kit (ApopTag; Oncor, Gaithersburg, MD) following package instructions. Detection of labeled ends was done with the kit-supplied antidigoxigenin-peroxidase Ab and development of diaminobenzidine substrate (Vector Laboratories, Burlingame, CA).

RT-PCR

Total RNA was isolated with TRIzol reagent (Life Technologies, Gaithersburg, MD) as per the manufacturer’s instructions. RNA samples (1 μg each) were tested for DNA contamination by 30 cycles of PCR with mouse β-actin primers. After it was shown there was no DNA contamination, cDNA synthesis was performed using an RNA PCR kit (PerkinElmer, Norwalk, CT) with the supplied oligo(dT)16 primer. Reverse transcription was performed using a thermal program of 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min. PCR were performed using the following primers: β-actin (forward, 5'-TGGAAATCCTGTGGCATCCATGAAAC-3'; reverse, 5'-TAAACAGCAGCTCAGTAACAGTCCG-3'); and FLIP (forward, 5'-CAAGATAGCCAAGGACAAGAG-3'; reverse, 5'-GATGGAAGTGACCTACCTGGACATGAAAC-3'). PCR cycle conditions were 95°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min for 30 cycles. FLIP PCR cycle conditions were 95°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min for 35 cycles. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide.

Western blot for FLIP

Cellular proteins from 4 × 106 cells were separated by SDS-PAGE on a 10% polyacrylamide gel under reducing conditions and then transferred to nitrocellulose membranes by semidy electroblotting. Ponceau S (Sigma) staining of the nitrocellulose confirmed the equal loading of proteins. Membranes were blocked overnight with 5% nonfat dry milk in PBS and incubated with anti-FLIP (AL 109) (20, 23) at a dilution of 1/1400 for 1 h. Membranes were washed three times and then incubated with peroxidase-conjugated goat anti-rat IgG (Amersham, Arlington Heights, IL) for 1 h. The membranes were washed thoroughly with PBS/0.05% Tween 20 and developed with chemiluminescence according to the manufacturer’s protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Induction of immune deviation

Immune deviation was induced as described elsewhere (29). Briefly, TNP-coupled spleen cells were injected into the AC, and then the mice were immunized 48 h after AC injection with 0.050 ml of 1% TNCB in acetone/olive oil (3:1) applied to shaved abdominal skin. Five days later, mice were challenged with 0.033 ml of 10 mM TNBS in PBS in the right footpad and 0.033 ml of PBS in the left footpad. Values are expressed in micrometers ±SE and represent the difference between the right footpad (Ag challenge) and the left footpad (PBS challenge). Background values represent the difference between the challenged and unchallenged foot in uninununized mice.
**Isolation of ocular inflammatory cells**

Twenty-four or 48 h following AC injection of HSV-1, mice were enucleated and eyes were minced between the ends of two frosted glass slides in HBSS without Ca²⁺/Mg²⁺ containing 10 mM EDTA. Large pieces were removed by filtration through 70-μm cell strainers and mononuclear cells were washed three times with HBSS. Following wash, cells were counted and yields were typically $1 \times 10^7$/injected eye. Cells were then analyzed by flow cytometry.

**Flow cytometry (FACS)**

FACS staining was performed by standard protocols. Briefly, for surface labeling, $1 \times 10^7$ spleen cells/ml were incubated with 20 μl of 10 μg/ml goat anti-TNFR1 or TNFR2 for 30 min at 4°C. Cells were washed twice in staining buffer (2% FCS in PBS plus 0.01% NaN₃) and resuspended in 400 μl of 2% formaldehyde, and collected on a BD Biosciences FACSCalibur (Mountain View, CA). In some cases, cells were also stained with anti-CD3-PE and analyzed for CD3 and TNFR expression. To measure TUNEL-positive lymphocytes in the eye, cells were isolated and surface stained as described above. Cells were then fixed, permeabilized, incubated with TdT and biotinylated dNTP per kit instructions (R&D Systems) and stained with streptavidin-PE. All events were analyzed using CellQuest software.

**Cell death assay (DO11.10 T cell hybridoma)**

DO11.10 cells ($4 \times 10^5$/ml) were labeled for 2 h at 37°C with 5 μCi/ml [³H]thymidine. Following three washes in culture medium, cells ($2 \times 10^5$/well of 96-well plate) were incubated with medium alone, murine TNF, Jo2, or TNF plus Jo2 for 16 h at 37°C. Unfragmented DNA was collected by filtration through glass fiber filters (Packard Instrument, Meriden, CT) using a Filtermate 96 cell harvester (Packard Instrument) and counted on a Microplate scintillation counter (Packard Instrument). Percent DNA fragmentation was determined by the formula (cpm without RPE cells – cpm with RPE cells) divided by the cpm without RPE cells ($\times 100$). Treatments were done in triplicate.

**Cell death assays (spleen cells)**

Spleen cells ($1 \times 10^7$/ml) were cultured with TNF or goat anti-mouse TNFR (R1 or R2) for 6 h at 37°C in 1 ml of RPMI 1640 with 0.5% normal mouse serum. Cells were collected, concentrated by centrifugation, and added to 96-well plates (Immunlon II; Dynatech Laboratories) containing immobilized Jo2. Cells were incubated for an additional 16 h and cell death was determined by trypan blue exclusion. Immobilized Jo2 was prepared by first coating plates with 5 μg/ml rabbit anti-goat Ab for 2 h at 37°C. Following three washes with PBS, 5 μg/ml Jo2 was added for 2 h at 37°C. Cells were then washed following a three times wash with PBS. Death was assessed by trypan blue exclusion.

For T cell killing by sFasL and TNF, CD4⁺ T cells were obtained from C57BL/6 mice by purification over antismouse Ig columns. Typical yields were 85% CD4⁺ T cells (data not shown). Cells were placed in 96-well flat-bottom culture plates in triplicate wells ($2 \times 10^5$/well) in 100 μl of complete RPMI 1640 (10% FCS, 2 mM l-glutamine, 0.05 mM 2-ME, 10 mM HEPES, 1 mM sodium pyruvate, and antibiotics) and incubated overnight with 100 ng/ml murine TNF in a 5% CO₂ humidified 37°C incubator. Wells were then given FLAG-tagged sFasL (100 ng/ml) for $\frac{1}{2}$ h before adding aggregating anti-FLAG Ab (M2, 2 μg/ml) and incubating for 24 h at 37°C. To measure apoptotic cell death, subdiploid DNA analysis was performed. Cells were collected and washed in buffer (2% FCS in PBS plus 0.01% NaN₃) and fixed at room temperature for 30 min in EtOH. Cells were then resuspended in 500 μl of buffer plus 50 μg/ml propidium iodide. Apoptosis was determined by gating on the population containing subdiploid DNA using CellQuest software after data acquisition on a BD Biosciences FACSCalibur.

**Statistical analysis**

Significant differences between groups were evaluated using a two-tailed Student’s t test ($p < 0.01$).

**Results**

**TNF and lymphocyte apoptosis within the eye**

Studies from our laboratory have shown activated and freshly isolated lymphoid cells entering the AC of the eye rapidly undergo apoptotic cell death (3, 4). This death is induced by the FasL expression on ocular tissues and is a prerequisite for the induction of immune deviation to the Ags encountered in the AC (4, 5). The production of TNF is also an important event in this phenomenon since TNF mRNA levels peak shortly after AC injection and the coinjection of a neutralizing mAb to TNF with the Ag blocked immune deviation (32). Since members of the TNF family cooperate in the induction of apoptosis (33, 34), we tested for a link between TNF production and FasL-induced death.

Following AC injection of TNP-T cells, TNF protein levels increased rapidly over the first 2–3 h before falling to near normal levels by 9 h (Fig. 1). In situ TUNEL staining 24 h following AC injection showed numerous apoptotic cells (Fig. 2A), confirming previous observations (4). However, when a neutralizing anti-TNF mAb was coinjected with the TNP-T cells, apoptosis was prevented (Fig. 2B). The role of TNF in cell death was then examined using TNP-T cells from TNFR1⁻/⁻ or TNFR2⁻/⁻ mice. Although TNFR1⁻/⁻ TNP-T cells underwent apoptosis in the AC much like the normal TNP-T cells, TNFR2⁻/⁻ TNP-T cells did not (Fig. 2, C and D, respectively).

Immune deviation following lymphoid cell injection into the eye requires both cell death (4) and TNF (32). Since TNFR2⁻/⁻ TNP-T cells do not undergo apoptosis in the AC of the eye (Fig. 2D), a prediction is that immune deviation cannot be induced with these cells. To test this, we compared the ability of normal, TNFR1⁻/⁻ and TNFR2⁻/⁻ TNP-T cells to induce immune deviation in normal B6 mice. Upon AC injection, we found that TNP-T cells from the normal and TNFR1⁻/⁻ mice induced immune deviation, whereas those from TNFR2⁻/⁻ or TNFR1/2⁻/⁻ mice did not (Fig. 3).

Previous studies showed that activated T cells do not express detectable TNFR1 (35). Thus, one possible explanation for our results is that only TNFR2 is expressed on the cells we inject, thus the dominance of TNFR2. When we examined freshly isolated spleen cells for expression of TNFRs, only low levels of TNFR2 were detected (Fig. 4A). Therefore, the apparent preference for TNFR2 may be because TNFR1 is simply absent from the lymphoid cell population.

To determine whether TNFR2 signaling is crucial for inducing apoptosis in cells infiltrating the eye, we needed to examine a situation where both receptors are expressed on the inflammatory infiltrate. Following ocular injection of the HSV-1, we have shown...
that infiltrating cells are apoptotic within 48 h (3) and this apoptosis is required for the induction of immune deviation to viral Ags (4). Data in Fig. 4, B and C, show that wild-type cells entering the eye in response to HSV-1 express both TNFR1 and TNFR2 at significant levels. We then examined apoptosis in TNFR1−/− and TNFR2−/− mice and found that only cells invading the TNFR1−/− eye were TUNEL+, whereas there was little or no apoptosis in the eyes of TNFR2−/− mice (Fig. 5). Thus, FasL-induced cell death is facilitated only in the presence of an active TNFR2. Collectively, results in Figs. 1–5 suggest that TNF signaling through TNFR2 is an integral part of FasL-induced apoptosis of cells in the eye. When cells lack TNFR2, they do not undergo apoptosis and they do not induce immune deviation because they fail to receive a “sensitizing” signal from TNF.

**TNF and Fas cooperate to induce cell death**

To further explore the role of TNF in regulating Fas-mediated apoptosis, we tested whether TNF could alter the sensitivity of cells in vitro to Fas-mediated death. When T cell hybridoma DO11.10 cells (high expressers of both TNFRs by FACS analysis; data not shown) were cultured with TNF, minimal apoptosis was observed (Fig. 6A). Similarly, culturing the cells with anti-Fas Ab (Jo2) resulted in a modest level of killing only at the highest concentration tested. However, when noncytotoxic concentrations of TNF and anti-Fas were used together, a substantial increase in cell death was observed. This suggests that TNF and Fas can cooperate to induce cell death in this hybridoma.

Results in Fig. 7 extend these observations to splenic cells using TNF or agonistic Abs to the TNFRs. In Fig. 7A, only the combination of TNF and anti-Fas resulted in measurable cell death, while data in Fig. 7B show that inclusion of anti-TNFRI (but not anti-TNFRII) at 0.1 and 1.0 μg/ml sensitized cells for death on anti-Fas Ab. Although 1.0 μg/ml anti-TNFRI with anti-Fas appears to protect cells from death, the data did not reach statistical significance. Data in Table I extend these observations to the natural ligand for Fas and show that only in the presence of TNF and sFasL do CD4+ T cells undergo significant apoptosis as measured by subdiploid DNA analysis. We conclude from these data that FasL-induced apoptosis of lymphoid cells in the eye is assisted by TNF via the TNFR2 receptor.
Effect of TNF on cell death molecules

Next, we explored the potential mechanisms by which TNF signaling through TNFR2 could facilitate apoptosis in lymphoid cells via Fas. Possible mechanisms for this effect could be increased Fas expression, increased expression of pro-apoptotic molecules, and/or down regulation of antiapoptotic molecules. When we examined Fas expression, we observed that TNF did not significantly increase the cell surface expression of Fas measured by flow cytometry (data not shown).

We then considered molecules that regulate apoptosis. Bcl-2 family members are known to regulate cell death by repressing (i.e., Bcl-2, Bcl-xL, Mcl-1, and A1) or promoting (i.e., Bax, Bcl-xS, Bad, and Bak) the apoptotic process (36). These molecules form homodimers and heterodimers that are key to the induction of apoptosis (37, 38). Another potent regulator of cell death is FLIP, which is a crucial repressor of apoptosis (23). Fig. 6B shows that incubation of DO11.10 hybridoma T cells with TNF significantly decreased the levels of FLIP. In addition, mRNA levels for the pro-apoptotic molecule Bax were elevated following treatment of cells with TNF (Fig. 6C) (mRNA levels for Bcl-2 and Bcl-xL were unchanged; data not shown).

Treatment of normal spleen cells with TNF or agonistic anti-TNFR Abs also revealed modulation of the expression of Bax and FLIP. Treatment of B6 spleen cells with anti-TNFR2 increased the level of Bax in these cells (Fig. 8A). Whereas FLIP transcripts were detectable in untreated B6, TNFR1⁻/⁻ and TNFR2⁻/⁻ spleen cells, incubation with TNF resulted in the disappearance of FLIP mRNA only in B6 or TNFR1⁻/⁻ spleen cells (Fig. 8B). FLIP mRNA was still detectable in the TNFR2⁻/⁻ spleen cells after incubation with TNF, further supporting the hypothesis that signaling through TNFR2 is necessary for sensitization to Fas-mediated apoptosis. (Note: levels of Bcl-2 or Bcl-xL mRNA remained unaltered after incubation with agonistic TNF or anti-TNFR Abs (data not shown).)

Discussion

Recently, we demonstrated that Fas-FasL interactions were critical for maintaining immune privilege in the eye. The FasL expressed throughout the eye kills infiltrating inflammatory cells before they can damage the eye, thereby helping to preserve vision (3). Moreover, cell death induced by FasL is important for the induction of immune deviation following Ag encountered in the eye (4, 5).
damage can be done. Constitutive FasL rapidly kills TNF-sensitized lymphocytes before the immune reaction; however, in the immune privileged site, TNF induces TNF-sensitized lymphocytes to produce TNF. Under normal circumstances, this cytokine would promote tissue (32). Thus, a possible scenario might be that ocular cells produce TNF in response to lymphoid cell invasion leading to increased sensitivity of the lymphocytes to killing by constitutive FasL. Another possibility is the production of TNF by ocular cells for death in the eye. An effect mediated through a high-affinity receptor would also provide a way to sensitize cells that are unactivated, cells we asked whether other molecules produced in the ocular microenvironment might affect sensitivity to apoptosis. The studies presented here focused on the effect of TNF and TNFR signaling. We demonstrate that TNF, acting through the TNFR2, increases the susceptibility of T cells to FasL-induced death. These results further demonstrate that noncytotoxic levels of TNF may regulate Fas-mediated apoptosis through the modulation of two important intracellular regulators of apoptosis. The sensitizing signal that TNF transmits to the cell down-regulates an antiapoptotic protein (FLIP), while up-regulating a pro-apoptotic one (Bax), enabling a signal through Fas to activate the cellular apoptotic pathway.

Previous studies from our laboratory have revealed that TNF mRNA levels rapidly increase over the first 2–3 h after AC injection of TNF-spleen (32). In the present studies, TNF protein levels were found to follow similar kinetics after AC injection. Cell death induced by TNF was detected within 0.5–1 h following Ag application (39). It is interesting that TNF protein levels increase almost immediately after cell injection into the AC. We have previously observed that TNF mRNA production was the result of the interaction of T cells with ocular tissue (32). Thus, a possible scenario might be that ocular cells produce TNF in response to lymphoid cell invasion leading to increased sensitivity of the lymphocytes to killing by constitutive FasL. Another possibility is that the lymphoid cells make TNF in response to other elements in the ocular milieu, leading to their destruction by FasL. A third situation would be relevant to inflammatory reactions in the eye where lymphoid cells entering the eye in response to infection produce TNF. Under normal circumstances, this cytokine would promote the immune reaction (39); however, in the immune privileged site constitutive FasL rapidly kills TNF-sensitized lymphocytes before damage can be done.

Since the cell death in the eye is rapid and takes place in activated, as well as unactivated, cells we asked whether other molecules produced in the ocular microenvironment might affect sensitivity to apoptosis. The studies presented here focused on the effect of TNF and TNFR signaling. We demonstrate that TNF, acting through the TNFR2, increases the susceptibility of T cells to FasL-induced death. These results further demonstrate that noncytotoxic levels of TNF may regulate Fas-mediated apoptosis through the modulation of two important intracellular regulators of apoptosis. The sensitizing signal that TNF transmits to the cell down-regulates an antiapoptotic protein (FLIP), while up-regulating a pro-apoptotic one (Bax), enabling a signal through Fas to activate the cellular apoptotic pathway.

Previous studies from our laboratory have revealed that TNF mRNA levels rapidly increase over the first 2–3 h after AC injection of TNF-spleen (32). In the present studies, TNF protein levels were found to follow similar kinetics after AC injection, with intracellular levels peaking 2 h after injection before returning to background levels by 8–9 h. These kinetics were similar to those found in the skin during the initiation of a contact sensitivity reaction, where TNF can be detected within 0.5–1 h following Ag application (39). It is interesting that TNF protein levels increase almost immediately after cell injection into the AC. We have previously observed that TNF mRNA production was the result of the interaction of T cells with ocular tissue (32). Thus, a possible scenario might be that ocular cells produce TNF in response to lymphoid cell invasion leading to increased sensitivity of the lymphocytes to killing by constitutive FasL. Another possibility is that the lymphoid cells make TNF in response to other elements in the ocular milieu, leading to their destruction by FasL. A third situation would be relevant to inflammatory reactions in the eye where lymphoid cells entering the eye in response to infection produce TNF. Under normal circumstances, this cytokine would promote the immune reaction (39); however, in the immune privileged site constitutive FasL rapidly kills TNF-sensitized lymphocytes before damage can be done.

In both splenic T cells and inflammatory cells responding to HSV-1, the activity of TNF observed was mediated through the p75 (or TNFR2) receptor. Although the exclusive expression of TNFR2 may explain the results using splenic T cells, this explanation is not sufficient to account for apoptosis of inflammatory cells responding to HSV-1. These cells express both receptors but are killed by FasL following the interaction of TNF with TNFR2. The dominance of TNFR2 may reflect the higher affinity of TNFR2 for the ligand (40). This may have relevance to immune privilege by allowing lower levels of TNF to sensitize lymphoid cells for death in the eye. An effect mediated through a high-affinity receptor would also provide a way to sensitize cells that are early in activation with the relatively low levels of TNF available.
Our results show that TNF affects several components of the cell death machinery. TNF-TNFR2 interaction alters the expression of the intracellular regulators of apoptosis, Bax and FLIP. Bax is a pro-apoptotic member of the Bcl-2 family of proteins that regulates cell death through the formation of homodimers and heterodimers with Bcl-2. It has been suggested that the ratio of Bcl-2:Bax determines cell survival or death following an apoptotic stimulus (36–38, 53). Following incubation with TNF, Bax mRNA levels rapidly increase, suggesting that the amount of Bcl-2 within the cell could no longer prevent Bax from forming homodimers and promoting cell death. We have previously demonstrated that Bcl-xL overexpression in T cells inhibits apoptosis of these cells in the eye (4). In this instance, excess Bcl-xL probably inhibited Bax homodimer formation and blocked cell death.

We also observed that TNF, acting through the TNFR2, caused a decrease in the intracellular levels of FLIP mRNA and protein. The expression of FLIP in T cells depends on the degree of cellular activation, with high levels present in naive T cells as well as T cells early in the activation process (23). FLIP levels decline with prolonged activation, in direct correlation with the T cell’s increased sensitivity to Fas-mediated apoptosis. FLIP has also been found to play a role in AICD of lymphocytes (20). AICD is an important mechanism of self-tolerance mediated by Fas-FasL interactions and enhanced by IL-2 (19). Interestingly, analysis of the biochemical mechanisms of IL-2-enhanced Fas-mediated T cell apoptosis revealed that IL-2 induces expression of FasL and simultaneously suppresses the transcription and expression of FLIP (20). Our observation that FLIP transcription decreased with TNF signaling through TNFR2 is similar to these observations with IL-2. Thus, these cytokines may play parallel roles in terminating an immune response. In the periphery, the growth-promoting cytokine IL-2 sensitizes T cells for death through suicide and fratricide. This permits an immune response but prevents excessive immune reactions that could lead to autoimmunity. In immune privileged sites, the proinflammatory cytokine TNF sensitizes cells entering the eye for death by constitutive FasL expression. This prevents the evolution of the immune response to protect the function of the visual axis.

When considering the mechanisms of immune privilege, it is important to realize that multiple factors are responsible for this biological phenomenon, including immunosuppressive cytokines (54, 55), neuropeptides (56), proinflammatory cytokines (32), and FasL (3). We initially examined a number of these factors (including TGF-β and vasoactive intestinal peptide) to determine their complicity with FasL, for the induction of cell death in the eye. These mediators had no effect on the induction of Fas-mediated death in our system (data not shown). The antiproliferative properties of these molecules (particularly TGF-β) are likely the underlying reason for their contribution to immune privilege (54). The only factor we have found thus far that promotes FasL-induced death in the eye is the proinflammatory cytokine TNF.
where it functions with FasL to protect the eye from the damaging effects of immune and inflammatory reactions.

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