Lack of TNF- α Promotes Caspase-3–Independent Apoptosis during Murine Cytomegalovirus Retinitis

Ming Zhang, Jason Covar, Brendan Marshall, Zheng Dong, and Sally S. Atherton

PURPOSE. Both caspase-dependent and caspase-independent apoptosis contribute to retinal damage during murine cytomegalovirus (MCMV) retinitis, and TNF- α is among the inducers of apoptosis. The aim of this study was to determine the contribution of TNF- α by studying virus replication and apoptosis in immunosuppressed (IS) TNF- $\alpha^{-/-}$ mice.

METHODS. IS TNF- $\alpha^{-/-}$ mice or wild-type mice were inoculated with MCMV by the supraciliary route. Injected eyes were examined by plaque assay, electron microscopy, Western blot analysis (caspase-3, caspase-8, caspase-12, Bid, NF- κ B, cFlip, XIAP), staining for MCMV early antigen, and TUNEL assay.

RESULTS. Although the titer of MCMV was similar in both groups, significantly more apoptotic cells were observed in the retinas of IS TNF- $\alpha^{-/-}$ mice than in those of wild-type mice. The level of active caspase-3 was similar in both groups; however, more activated proteins for genes involved in the mitochondrial pathway (cleaved caspase-8, tBid) and endoplasmic reticulum (ER) stress (cleaved caspase-12) and, though less active, NF- κ B subunits and antiapoptotic proteins (XIAP and cFlip) were detected in the TNF- $\alpha^{-/-}$ eyes compared with wild-type mice.

CONCLUSIONS. Although TNF- α is an inducer of apoptosis, the results of this study suggest that TNF- α is also antiapoptotic by the following mechanism: TNF- α activation of NF- κ B promotes the production of the antiapoptosis genes, c-flip or XIAP, which, in turn, inhibit the activation of caspase-8 and the mitochondrial pathway or the activation of caspase-12 and ER stress. (*Invest Ophthalmol Vis Sci.* 2011;52:1800–1808) DOI: 10.1167/iops.10.6904

Cytomegalovirus (CMV) retinitis is a serious ocular complication in patients who are immunosuppressed (IS) because of AIDS, chemotherapy, or malignancy and in newborns who are congenitally infected.^{1–3} Left untreated, the infection may result in retinal damage and blindness. Although highly active antiretroviral therapy (HAART) has resulted in a significant decrease in the number of new cases of AIDS-related human CMV (HCMV) retinitis,^{2,4} HCMV retinitis continues to be a chronic sight-threatening ophthalmologic problem among HIV-1-infected patients who do not respond to HAART^{4–6} or who discontinue therapy.^{7–9}

Although retinal necrosis is one of the hallmarks of CMV retinitis, apoptotic cells have been observed during micro-

scopic examination of biopsy specimens of eyes from patients with HCMV retinitis.^{10,11} In the mouse model of CMV retinitis used in our laboratory, apoptotic cells and necrotic cells are observed in the retina during the evolution of murine cytomegalovirus (MCMV) retinitis.^{12–14} As shown by immunohistochemistry and electron microscopy, most apoptotic cells are not infected by virus, and apoptosis of uninfected bystander neuronal cells appears to be an important component of the pathogenesis of CMV retinitis.^{12–14} However, the apoptosis-inducing factor or factors remain to be identified.

TNF- α has been detected in macrophages and astrocytes in the retina of AIDS patients with CMV retinitis,¹⁵ and an increase in the level of intraocular TNF- α was observed in MCMV-inoculated eyes of MAIDS mice with MCMV retinitis.¹⁶ Our previous results also showed that more apoptotic cells were observed in the retinas of MCMV-infected wild-type C57BL/6 mice than in the retinas of TNF- α receptor 1 (R1)^{-/-} mice.¹⁴ Therefore, TNF- α is among the factors that induce apoptosis during MCMV infection of the retina. However, TNF- α can exert opposing effects at the cellular level: induction of apoptosis through the TNF-R1-death domains and adapter proteins that couple the receptor complex to the activation of caspase-8 on the one hand, increased cell survival through the activation of nuclear factor- κ B (NF- κ B)-dependent genes on the other.¹⁷ Therefore, whether the net effect of TNF- α is proapoptotic, antiapoptotic, or both during MCMV retinitis is still unclear. The aim of this study was to determine the role of TNF- α by studying viral replication and apoptosis in IS TNF- $\alpha^{-/-}$ mice.

METHODS

Virus and Virus Titration

The original stock of MCMV (K181 strain) was a generous gift of Edward S. Mocarski (Stanford University School of Medicine, Stanford, CA). Virus was prepared from the salivary glands of MCMV-infected BALB/c mice, as described previously.¹⁸ Virus stocks were titered by plaque assay on monolayers of mouse embryo fibroblast (MEF) cells and were stored at -70°C . A fresh aliquot of stock virus was thawed and diluted to the appropriate concentration immediately before each experiment.

Mice

Adult (6–8 weeks old) female TNF- $\alpha^{-/-}$ mice and C57BL/6 (wild-type) mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Mice were housed in accordance with National Institutes of Health guidelines. Mice were maintained on a 12-hour light cycle alternating with a 12-hour dark cycle and were given unrestricted access to food and water. All ocular injections were performed after the mice had been anesthetized with a mixture of 42.9 mg/mL ketamine, 8.57 mg/mL xylazine, and 1.43 mg/mL acepromazine at a dose of 0.5 to 0.7 mL/kg body weight. The treatment of animals in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

From the Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia.

Supported by National Institutes of Health Grant EY009169.

Submitted for publication November 16, 2010; revised December 20, 2010; accepted January 12, 2011.

Disclosure: M. Zhang, None; J. Covar, None; B. Marshall, None; Z. Dong, None; S.S. Atherton, None

Corresponding author: Sally S. Atherton, Department of Cellular Biology and Anatomy, Medical College of Georgia, R and E Building, Augusta, GA 30912; satherton@mail.mcg.edu.

FIGURE 1. Merged photomicrographs of staining for MCMV EA (red), TUNEL (green), and DAPI (blue) in MCMV-injected eyes of TNF- $\alpha^{-/-}$ mice and wild-type mice at day 3 p.i. and day 6 p.i. More TUNEL-positive apoptotic cells were observed in the retinas of MCMV-infected TNF- $\alpha^{-/-}$ mice than in the retinas of IS wild-type mice; most TUNEL-positive cells were MCMV EA-negative. Magnification, 316 $\times$.

Antibodies

Antibodies for cleaved caspase-3 (5A1), caspase-8, caspase-12, NF- κ B p65 (C22B4), cFlip (cellular FLICE inhibitory protein), XIAP (X-linked Inhibitor of Apoptosis Protein), c-IAP-1 (cellular inhibitor of apoptosis 1), and Bcl-xl (54H6) were obtained from Cell Signaling Technology (Danvers, MA). NF- κ B p50 and BCL-2 (B-cell leukemia/lymphoma 2) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Active caspase-8 antibody was obtained from Acris Antibodies GmbH (Herford, Germany). Anti-mouse Bid cleavage site (59/60) antibody was purchased from Chemicon International (Temecula, CA). Antibody against β -actin (AC15) was from Sigma-Aldrich (St. Louis, MO). Both Texas Red-labeled avidin and Texas Red-labeled anti-rabbit IgG were from Vector Laboratories (Burlingame, CA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were from BD-PharMingen (San Jose, CA).

Experimental Plan

Mice were immunosuppressed by intramuscular injection of 2 mg sterile methylprednisolone acetate suspension every 3 days beginning on day -2. As determined by flow cytometry of splenocytes, this treatment typically depletes 93% of the CD4 $^{+}$ and CD8 $^{+}$ T cells as well as macrophages from MCMV-infected mice.¹⁹ Mice were injected with 5×10^4 PFU of MCMV contained in a volume of 2 μ L by the supraciliary route on day 0; several rounds of euthanatization were performed several times postinoculation (p.i.). Injected eyes of TNF- $\alpha^{-/-}$ or wild-type mice (four eyes in each type at each time point) were removed, homogenized in serum-free tissue culture medium using a handheld tissue homogenizer (Biospec Products, Inc., Racine, WI), and plated on MEF cells for the detection of replicating virus. Eyes of additional mice were removed and prepared for immunohistochemistry, electron microscopy, or Western blot analysis, as described below.

Immunohistochemistry

Monoclonal antibody to an MCMV early gene product was biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. The injected eyes (four eyes in each type at each time point) were embedded in OCT compound (Tissue-Tek; VWR Scientific, Houston, TX), snap frozen, and sectioned on a cryostat. Frozen sections were fixed with 4% paraformaldehyde for 15 minutes

and stained first with TUNEL (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. After washing and blocking, biotinylated anti-EA was applied to the section. Immunohistochemistry was conducted using Texas Red-labeled avidin. The slides were then mounted with antifade medium containing DAPI (Vectashield; Vector Laboratories) and examined microscopically.

Western Blot Analysis

Western blot analysis was performed as previously described.²⁰ Briefly, proteins were extracted from normal eyes (pool of four eyes in each type), from MCMV-injected eyes (pool of five eyes in each type), or from medium-injected control eyes (pool of four eyes in each type). Equal concentrations of protein were loaded for SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ). After blocking with 5% nonfat dry milk for 1 hour at room temperature, the membrane was incubated overnight at 4°C with primary antibody. The next day, binding of HRP-conjugated secondary

FIGURE 2. Electron micrographs showing virus particles (arrows) in the injected eye of an immunosuppressed TNF- $\alpha^{-/-}$ mouse on day 6 p.i.

FIGURE 3. Electron micrographs showing apoptotic cells in the outer nuclear layer (A), inner nuclear layer (B), and ganglion cell layer (C) of the MCMV-injected eye of IS $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice. More apoptotic cells were observed in all layers of the inner retina in $\text{TNF-}\alpha^{-/-}$ mice than in the wild-type mice. Most apoptotic retinal cells were characterized by nuclear shrinkage and strong chromatin condensation (arrows). Advanced chromatin compaction and nuclear fragmentation were only occasionally seen in apoptotic cells (arrowhead).

antibody was performed for 1 hour at room temperature. The immune complex was visualized by a chemiluminescence detection system (ECL; GE Healthcare) and exposure to x-ray film. The membrane was stained for β -actin to verify equal loading among lanes. Each experiment was repeated, and most were repeated at least three times.

Electron Microscopy

Eyes of experimental and control mice were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer overnight at 4°C, washed in cacodylate buffer, postfixed with 4% osmium tetroxide for 1 hour at room temperature, dehydrated in a graded ethanol series, and embedded in resin (Pure Embed 812 mixture; Electron Microscopy Science, Hatfield, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and examined (Jem 1230; JEOL, Tokyo, Japan). Quantification of apoptotic cells was made by counting the number of apoptotic cells in a $180\ \mu\text{m} \times 180\ \mu\text{m}$ area of the electron micrographs, and the results were expressed as mean $\pm$ SD.

RESULTS

Lack of $\text{TNF-}\alpha$ Increases Apoptosis but Not Viral Spread or Replication in the Retina

After immunosuppression with methylprednisolone, which depletes T cells as well as macrophages,^{19,21} and inoculation of

MCMV into the supraciliary space, virus-infected cells were observed in the anterior segment and the RPE layer of both groups of mice at day 3 p.i. (Fig. 1). At this time, although the titer of MCMV was higher in the injected eyes of $\text{TNF-}\alpha^{-/-}$ mice (average PFU $\log_{10} \pm$ SEM: 3.16 ± 0.15) than in the injected eyes of wild-type mice (2.39 ± 0.09), the difference was not significant. At day 6 p.i., a similar number of virus-infected cells was noted mainly in the inner retina of the injected eyes in both animal models (Fig. 1). At this time, the difference in virus titer between $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice was also not significant (average PFU $\log_{10} \pm$ SEM: 3.85 ± 0.38 vs. 3.49 ± 0.17 , respectively). Apoptotic cells were located in the retina of the injected eye in both $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice. Most TUNEL-positive cells were uninfected. At day 3 p.i., most apoptotic cells were uninfected photoreceptor cells, whereas virus-infected cells were observed mainly in the choroid and RPE. At day 6 p.i., apoptotic cells were present in all layers of the retina, and virus-infected cells were also observed throughout the retina. However, more apoptotic cells were always observed in the retinas of MCMV-infected $\text{TNF-}\alpha^{-/-}$ mice than in the retinas of IS wild-type mice (Fig. 1). To augment the light microscopy studies, sections of injected eyes were examined by electron microscopy. Virus particles (Fig. 2) and apoptotic cells (Fig. 3) were observed in the inner retina of $\text{TNF-}\alpha^{-/-}$ and wild-type mice at day 6 p.i. However, significantly more apoptotic cells were observed in the $\text{TNF-}\alpha^{-/-}$ mice than in the wild-type mice (29.71 ± 27.12 vs. 8.14 ± 4.19 ; $n = 14$; $P < 0.01$) in all layers of the inner retina, including photoreceptors (Fig. 3A), inner nuclear layer (Fig. 3B), and ganglion cell layer (Fig. 3C). The apoptotic retinal

FIGURE 4. (A) Western blot of cleaved caspase-3 in MCMV-injected eyes of $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice at day 6 p.i. Protein from medium-injected eyes was used as the control. The level of cleaved caspase-3 in MCMV-injected eyes of $\text{TNF-}\alpha^{-/-}$ mice and of wild-type mice was similar. β -Actin was used to verify loading. (B) Ratio of cleaved caspase-3 to β -actin.

cells exhibited nuclear shrinkage and strong chromatin condensation.²² Later stages of apoptosis, characterized by more homogeneous chromatin condensation and increased cell shrinkage, were also observed. Advanced chromatin compaction and nuclear fragmentation²³ were occasionally seen in apoptotic cells in both TNF- $\alpha^{-/-}$ mice and wild-type mice (Fig. 3).

Increased Apoptosis in the Retinas of MCMV-Infected TNF- $\alpha^{-/-}$ Mice Is Caspase-3 Independent

Both caspase-dependent and caspase-independent apoptosis are observed during MCMV infection of the retina.²⁰ To determine whether increased apoptosis in the retinas of MCMV-infected TNF- $\alpha^{-/-}$ mice was caspase-3 dependent or independent, cleaved caspase-3 levels were detected by Western blot analysis. As shown in Figure 4, the level of cleaved caspase-3 was similar in the injected eyes of both TNF- $\alpha^{-/-}$ mice and wild-type mice, which suggests that increased apoptosis in the retinas of MCMV-infected TNF- $\alpha^{-/-}$ mice is caspase-3 independent. Active caspase-3 was not detected in control eyes injected with medium alone (Fig. 4).

Lack of TNF- α Decreases NF- κ B Activation and Subsequent Production of Antiapoptotic Proteins

Previous reports have shown that TNF- α increases cell survival by NF- κ B activation and subsequent production of NF- κ B-dependent antiapoptosis proteins, including cFlip,^{24,25} IAP family proteins (IAP-1, IAP-2, XIAP),^{26–28} and BCL-2 family proteins (Bcl-2 and Bcl-xL).^{29–31} However, the antiapoptosis response varies among different cells and tissues.^{24–31} Western blot analysis was used to compare the levels of NF- κ B precursor p105 and active subunits p50 and p65 in virus-injected eyes of wild-type mice with the levels in control eyes. A higher level of NF- κ B active form p50, p65 was detected in the MCMV-infected eyes of wild-type mice than in the control eyes (Fig. 5), which suggests that MCMV infection activates NF- κ B. How-

ever, both NF- κ B precursor p105 and active form p50, p65 were decreased in the MCMV-injected eyes of TNF- $\alpha^{-/-}$ mice (Fig. 5), suggesting that NF- κ B activation is impaired in the absence of TNF- α .

To determine whether MCMV infection and reduced activation of NF- κ B affected antiapoptosis proteins, the expression of antiapoptotic proteins in the eyes of TNF- $\alpha^{-/-}$ mice and wild-type mice was examined by Western blot analysis. In wild-type mice, the production of antiapoptotic proteins varied; short-form cFlip (cFlip_s) and BCL-2, which inhibit the mitochondrial pathway, were downregulated, whereas IAP family proteins, including XIAP and IAP-1, were upregulated (Fig. 6). Not surprisingly, the level of these four antiapoptotic proteins was lower in MCMV-infected TNF- $\alpha^{-/-}$ eyes than in MCMV-infected wild-type mice (Fig. 6). The reductions in cFlip_s ($>5\times$) and XIAP ($>9\times$) were particularly striking. In addition, Bcl-XL expression was not different among infected wild-type mice, TNF- $\alpha^{-/-}$ mice, or control mice (Fig. 6).

Activation of Mitochondrial Pathway and ER Stress Are Induced by MCMV Retinal Infection

Because of its structural homology with caspase-8, cFlip_s acts as a dominant-negative inhibitor of caspase-8 by preventing the processing and release of active caspase-8, which cleaves and activates downstream effector caspases, such as caspase-1, -3, -6, and -7. Active caspase-8 also cleaves Bid to generate the active form (tBid), which translocates to the mitochondria and induces cytochrome *c* or AIF release and mitochondrial damage.^{32,33} Western blot analysis was performed to determine whether caspase-8 activation was affected by the absence of TNF- α . As previously reported,^{14,20} active caspase-8 was detected in MCMV-infected eyes but not in control eyes of wild-type animals (Fig. 7). However, a higher level of active caspase-8 was detected in MCMV-infected TNF- $\alpha^{-/-}$ eyes than in MCMV-infected wild-type eyes (Fig. 7). Given that caspase-3

FIGURE 5. (A) Western blot of NF- κ B in MCMV-injected eyes of TNF- $\alpha^{-/-}$ mice and wild-type mice at day 6 p.i. Protein from medium-injected eyes was used as the control. A higher level of NF- κ B active form p50, p65 was detected in the MCMV-infected eyes of wild-type mice than in the control eyes, whereas both NF- κ B precursor p105 and active form p50, p65 were decreased in the TNF- $\alpha^{-/-}$ eyes after MCMV infection. β -Actin was used to verify loading. (B) Ratio of NF- κ B to β -actin.

FIGURE 6. (A) Western blot of antiapoptotic proteins in MCMV-injected eyes of $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice at day 6 p.i. Protein from medium-injected eyes was used as the control. Less cFLIP_s and BCL-2 and more XIAP and IAP-1 were detected in the MCMV-infected eyes of wild-type mice than in control eyes. A lower level of these four antiapoptotic proteins was observed in MCMV-infected $\text{TNF-}\alpha^{-/-}$ eyes than in MCMV-infected wild-type eyes. No difference in BCL-XL was observed in MCMV-infected or medium-injected wild-type or $\text{TNF-}\alpha^{-/-}$ animals. β -Actin was used to verify loading. (B) Ratio of antiapoptotic protein to β actin.

activation was not affected by $\text{TNF-}\alpha$ and our previous results showed that the mitochondrial pathway is activated during MCMV retinal infection,²⁰ we hypothesized that an increase in the level of cleaved caspase-8 in MCMV-infected $\text{TNF-}\alpha^{-/-}$ mice would correlate with increased activation of Bid. To test this hypothesis, Western blot analysis for Bid was performed. A higher level of tBid was detected in MCMV-infected $\text{TNF-}\alpha^{-/-}$ eyes than in MCMV-infected wild-type eyes (Fig. 7).

XIAP is a potent suppressor of apoptosis that directly inhibits its specific members of the caspase family of cysteine proteases, including caspase-3, caspase-7, and caspase-9.^{34–36} and recent results demonstrated that XIAP inhibits the cleavage of caspase-12 in the spinal cord of ALS mice.³⁷ Because caspase-12, a key indicator of ER stress, is reported to be related to

apoptosis in many neurologic diseases,^{37–39} we wanted to know whether caspase-12 participates in retinal apoptosis after MCMV infection and if XIAP plays an important role in inhibiting apoptosis induced by ER stress. Western blot analysis was used to determine the level of total and cleaved caspase-12. In MCMV-infected $\text{TNF-}\alpha^{-/-}$ mice and wild-type mice, the level of total caspase-12 was lower, whereas the level of cleaved caspase-12 was higher than in control eyes of both kinds of mice (Fig. 8), a finding that suggests ER stress and caspase-12 activation are induced by MCMV eye infection. The level of cleaved caspase-12 in MCMV-infected $\text{TNF-}\alpha^{-/-}$ eyes was higher than in MCMV eyes of wild-type mice (band density, 0.82–0.43), which supports the idea that during MCMV infection of the eye, the absence of $\text{TNF-}\alpha$ induces more ER stress than its presence.

FIGURE 7. (A) Western blot of Bid, precursor and active caspase-8 in MCMV-injected eyes of TNF- $\alpha^{-/-}$ mice and wild-type mice at day 6 p.i. Protein from medium-injected eyes was used as the control. A higher level of tBid was detected in MCMV-infected TNF- $\alpha^{-/-}$ eyes than in MCMV-infected TNF- $\alpha^{+/+}$ eyes. More precursor caspase-8 and active caspase-8 was detected in the MCMV-infected eyes than in the control eyes of both wild-type and TNF- $\alpha^{-/-}$ animals. A higher level of active caspase-8 was detected in MCMV-infected TNF- $\alpha^{-/-}$ eyes than in the eyes of wild-type mice. β -Actin was used to verify loading. (B) Ratio of apoptotic protein to β -actin.

DISCUSSION

TNF- α can exert opposing effects at the cellular level: induction of apoptosis by TNF-R1 death domains and adapter proteins that couple the receptor complex to the activation of caspase-8 on the one hand, increased cell survival by the activation of NF- κ B-dependent genes on the other.¹⁷ For example, as one of the potential neurotoxins, TNF- α (released by activated microglial cells) is associated with neuronal apoptosis in several diseases, such as AIDS, Alzheimer disease, and multiple sclerosis.^{40–43} On the other hand, TNF- α protects primary hippocampal neurons against hypoxia or nitric oxide-induced injury³⁰ and cultured mesencephalic neurons against glutamate neurotoxicity.⁴⁴ TNF- α also prevents secondary death of retinal ganglion cells after axotomy of the optic nerve in vivo.⁴⁵ TNF-R1 and TNF-R2 might exhibit different functions. During a comparison of ischemia reperfusion-induced retinal damage in mice deficient in TNF-R1 or TNF-R2, the absence of TNF-R1 correlated with a strong reduction of neurodegeneration whereas the lack of TNF-R2 led to an enhancement of neurodegeneration, indicative of both TNF-independent and TNF-depend-

ent processes in the retina, with TNF-R1 augmenting neuronal death and TNF-R2 promoting neuroprotection.⁴⁶

Although TNF- α is an important apoptotic inducer in cytomegalovirus retinitis,^{14,16,17} other factors, such as Fas ligand and iNOS, have also been detected in cytomegalovirus-infected retinal tissue or retinal cells and are players in retinal apoptosis during cytomegalovirus infection.^{13,47} The results of the studies described herein demonstrate that TNF- α plays both proapoptotic and antiapoptotic roles in the pathogenesis of MCMV retinitis. TNF- α induces the apoptosis of some retinal cells while it prevents apoptosis induced in retinal cells by other factors or by TNF- α itself through the activation of NF- κ B and the subsequent production of antiapoptotic proteins XIAP and cFlip, which inhibit apoptosis induced by caspase-12 activation and the mitochondrial pathway, respectively.

The ER is the cellular site of newly synthesized secretory and membrane proteins. Such proteins must be properly folded and posttranslationally modified before exiting the organelle. Proper protein folding and modification requires molecular chaperone proteins and an ER environment conducive for these reactions.^{48,49} When ER luminal conditions

FIGURE 8. (A) Western blot of caspase-12 in MCMV-injected eyes of TNF- $\alpha^{-/-}$ mice and wild-type mice at day 6 p.i. Protein from medium-injected eyes was used as the control. A lower level of total caspase-12 and a higher level of cleaved caspase-12 were observed in the MCMV-infected eyes than in the control eyes of both kinds of mice. The level of cleaved caspase-12 in MCMV-infected TNF- $\alpha^{-/-}$ eyes was higher than that in the eyes of MCMV-infected wild-type mice. β -Actin was used to verify loading. (B) Ratio of caspase-12 to β -actin.

are altered or chaperone capacity is overwhelmed, the cell activates signaling cascades that attempt to deal with the altered conditions and restore a favorable folding environment. Such alterations are referred to as ER stress, and the response activated is the unfolded protein response (UPR).^{48,49} When the UPR is perturbed or is insufficient to deal with the stress conditions, apoptotic cell death is initiated. Caspase-12 localized on the cytoplasmic side of the ER⁵⁰ is the central player in ER stress-induced apoptosis.³⁸ Activation of caspase-12 during apoptosis has been reported in mouse, rat, rabbit, cow, and human cells.³⁹ Several studies have examined the activation and function of caspase-12 in response to ER stress-mediated apoptosis in murine cells. Caspase-12^{-/-} mice were resistant to A β peptide-induced apoptosis in an Alzheimer's disease model.⁵¹ Morishima and coworkers⁵² described the caspase cascade initiated in UPR. This cascade is novel and does not depend on either mitochondria or death receptor activation. On activation, caspase-12 translocates from the ER to the cytosol, where it directly cleaves pro-caspase-9, which, in turn, activates the effector caspase, caspase-3.⁵² However, Michalak and coworkers⁵³ showed that the ER and the mitochondria are linked closely and that Ca²⁺ released from the ER eventually accumulates in the mitochondria. In addition, ER stress causes oxidative stress and mitochondrial changes that can be blocked by overexpressing Bcl-2.⁵⁰ Furthermore, in retinal degeneration, the inhibition of ER stress and caspase-12 activation decrease the cleavage and release of apoptosis-inducing factor (AIF) from the mitochondria,³⁸ demonstrating cross-talk between caspase-12 and AIF, which is a ubiquitously expressed flavoprotein that plays a critical role in caspase-independent apoptosis.⁵⁴⁻⁵⁶

Our results support the idea that ER stress and caspase-12 activation are induced by ocular MCMV infection. However, this activity was limited by elevated XIAP, which was stimulated by TNF- α -induced NF- κ B activation during the course of MCMV eye infection. Lack of TNF- α and subsequent lack of XIAP production in MCMV-infected TNF- $\alpha^{-/-}$ mice resulted in loss of inhibition of caspase-12 activation and, in turn, increased apoptosis induced by ER stress and caspase-12 activation.

As we have observed previously,¹³ the mitochondrial pathway is activated during MCMV retinal infection. The result that increased activation of the mitochondrial pathway was observed in TNF- $\alpha^{-/-}$ mice suggested that one function of TNF- α is to act as an inhibitor of the mitochondrial pathway. The probable mechanism of inhibition is related to TNF- α -induced NF- κ B activation and subsequent production of cFlip, which acts as a dominant-negative inhibitor of caspase-8 activation. Active caspase-8 cleaves Bid and generates the active form (tBid), which translocates to mitochondria and induces cytochrome *c* or AIF release and mitochondrial damage.

The results presented herein confirm that both caspase-3-dependent and caspase-3-independent apoptosis occur during MCMV infection of the retina.¹³ However, the result that the lack of TNF- α only increases caspase-3-independent apoptosis was surprising because less XIAP and more active caspase-8 and active caspase-12 in MCMV-infected TNF- $\alpha^{-/-}$ mice appeared to have no influence on caspase-3 activation, despite reports that XIAP directly inhibits specific members of the caspase-family of cysteine proteases, including caspase-3,³⁴⁻³⁶ and because cleaved caspase-8 and caspase-12 can activate the downstream executor, caspase-3, during apoptosis.⁵² There are at least three explanations for these results. First, some retinal cells underwent apoptosis before caspase-3 was activated, and these cells would not have required activation of caspase-3 to undergo apoptosis. The presence of more activated caspase-12 in TNF- $\alpha^{-/-}$ mice might have stimulated the release of AIF through cross-talk between caspase-12 and AIF³⁸ and might have contributed to apoptosis independently of caspase-3 activation. Second, caspase-3 activation might have been inhibited by other antiapoptotic proteins that are not regulated by NF- κ B activation. Third, lack of caspase-3-dependent apoptosis induced by the TNF- α death receptor pathway in TNF- $\alpha^{-/-}$ animals might have kept some retinal cells rich in TNF- α receptor 1 from undergoing apoptosis.

In summary, the role of TNF- α during MCMV infection of the retina is proving to be more complicated than either straightforward induction of apoptosis or prevention of apoptosis and likely depends on the extent and stage of infection.

Studies to decipher the interplay among and between all the factors in this complicated pathway are continuing.

Acknowledgments

The authors thank the Electron Microscopy/Histology core at the Medical College of Georgia and the core staff, especially Robert Smith and Libby Perry, for their assistance.

References

- Cohen J. AIDS therapy: new hope against blindness. *Science*. 1995; 268:368–369.
- Drew WL. Cytomegalovirus infection in patients with AIDS. *Clin Infect Dis*. 1992;14:608–615.
- Istas AS, Demmler GJ, Dobbins JG, Stewart JA. Surveillance for congenital cytomegalovirus disease: a report from the National Congenital Cytomegalovirus Disease Registry. *Clin Infect Dis*. 1995;20:665–670.
- Jabs DA, Bartlett JG. AIDS and ophthalmology: a period of transition. *Am J Ophthalmol*. 1997;124:227–233.
- Paella FJ Jr, Delaney KM, Moorman AC, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection: HIV Outpatient Study Investigators. *N Engl J Med*. 1998;338:853–860.
- Song MK, Karavellas MP, MacDonald JC, Plummer DJ, Freeman WR. Characterization of reactivation of cytomegalovirus retinitis in patients healed after treatment with highly active antiretroviral therapy. *Retina*. 2000;20:151–155.
- Torriani FJ, Freeman WR, Macdonald JC, et al. CMV retinitis recurs after stopping treatment in virological and immunological failures of potent antiretroviral therapy. *AIDS*. 2000;14:173–180.
- Cunningham ET Jr, Stalder A, Sanna PP, et al. Localization of tumor necrosis factor receptor messenger RNA in normal and herpes simplex virus-infected mouse eyes. *Invest Ophthalmol Vis Sci*. 1997;38:9–15.
- Jouan M, Saves M, Tubiana R, et al. Discontinuation of maintenance therapy for cytomegalovirus retinitis in HIV-infected patients receiving highly active antiretroviral therapy. *AIDS*. 2001; 15:23–31.
- Jaffe GJ, Van Le L, Valea F, et al. Expression of interleukin-1 alpha, interleukin-1 beta, and an interleukin-1 receptor antagonist in human retinal pigment epithelial cells. *Exp Eye Res*. 1992;55:325–335.
- Pfeffer BA, Flanders KC, Guerin CJ, Danielpour D, Anderson DH. Transforming growth factor beta 2 is the predominant isoform in the neural retina, retinal pigment epithelium-choroid and vitreous of the monkey eye. *Exp Eye Res*. 1994;59:323–333.
- Bigger JE, Tanigawa M, Zhang M, Atherton SS. Murine cytomegalovirus infection causes apoptosis of uninfected retinal cells. *Invest Ophthalmol Vis Sci*. 2000;41:2248–2254.
- Zhang M, Atherton SS. Apoptosis in the retina during MCMV retinitis in immunosuppressed BALB/c mice. *J Clin Virol*. 2002; 25(suppl 2):S137–S147.
- Zhou J, Zhang M, Atherton SS. TNF-alpha-induced apoptosis in murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci*. 2007; 48:1691–1700.
- Lin XH, Kashima Y, Khan M, Heller KB, Gu XZ, Sadun AA. An immunohistochemical study of TNF-alpha in optic nerves from AIDS patients. *Curr Eye Res*. 1997;16:1064–1068.
- Dix RD, Cousins SW. Susceptibility to murine cytomegalovirus retinitis during progression of MAIDS: correlation with intraocular levels of tumor necrosis factor-alpha and interferon-gamma. *Curr Eye Res*. 2004;29:173–180.
- Micheau O, Tschopp J. Induction of TNF- receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*. 2003;114: 181–190.
- Atherton SS, Newell CK, Kanter MY, Cousins SW. T cell depletion increases susceptibility to MCMV retinitis. *Invest Ophthalmol Vis Sci*. 1992;33:3353–3360.
- Duan Y, Ji Z, Atherton SS. Dissemination and replication of MCMV after supraciliary inoculation in immunosuppressed BALB/c mice. *Invest Ophthalmol Vis Sci*. 1994;35:1124–1131.
- Zhang M, Marshall B, Atherton SS. Murine cytomegalovirus infection and apoptosis in organotypic retinal cultures. *Invest Ophthalmol Vis Sci*. 2008;49:295–303.
- Duan Y, Atherton SS. Immunosuppression induces transcription of murine cytomegalovirus glycoprotein H in the eye and at non-ocular sites. *Arch Virol*. 1996;141:411–423.
- Zhang M, Xin H, Roon P, Atherton SS. Infection of retinal neurons during murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci*. 2005;46:2047–2055.
- Leist M, Jäättelä M. Four deaths and a funeral: from caspases alternative mechanisms. *Nat Rev. Mol Cell Biol*. 2001;2:589–598.
- Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF- κ B signals induce the expression of c-FLIP. *Mol Cell Biol*. 2001;21: 5299–5305.
- Kreuz S, Siegmund D, Scheurich P, Wajant H. NF- κ B inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol*. 2001;21:3964–3973.
- Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF- κ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*. 1998;281: 1680–1683.
- Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Dev*. 1999;13:239–252.
- Stehlik C, de Martin R, Kumabashiri I, et al. Nuclear factor (NF)- κ B-regulated X-chromosome-linked IAP gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med*. 1998;188:211–216.
- Sawada M, Nakashima S, Banno Y, et al. Influence of Bax or Bcl-2 overexpression on the ceramide-dependent apoptotic pathway in glioma cells. *Oncogene*. 2000;19:3508–3520.
- Tamatani M, Che YH, Matsuzaki H, et al. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NF κ B activation in primary hippocampal neurons. *J Biol Chem*. 1999;274:8531–8538.
- Chen C, Edelstein LC, Gelinas C. The Rel/NF- κ B family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol*. 2000;20:2687–2695.
- Lamkanfi M, Festjens N, Declercq W, et al. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ*. 2007;14: 44–55.
- Jellinger KA. Challenges in neuronal apoptosis. *Curr Alzheimer Res*. 2006;3:377–391.
- Chai J, Shiozaki E, Srinivasula SM, et al. Structural basis of caspase-7 inhibition by XIAP. *Cell*. 2001;104:769–780.
- Huang Y, Park YC, Rich RL, Segal D, Myszkowski DG, Wu H. Structural basis of caspase-inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell*. 2001;104:781–790.
- Riedl SJ, Renatus M, Schwarzenbacher, et al. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 2001;104:791–800.
- Wootz H, Hansson I, Korhonen L, Lindholm D. XIAP decreases caspase-12 cleavage and calpain activity in spinal cord of ALS transgenic mice. *Exp Cell Res*. 2006;312:1890–1898.
- Sanges D, Comitato A, Tammaro R, Marigo V. Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors. *Proc Natl Acad Sci USA*. 2006;103:17366–17371.
- Szegezdi E, Fitzgerald U, Samali A. Caspase-12 and ER-stress-mediated apoptosis: the story so far. *Ann NY Acad Sci*. 2003;1010:186–194.
- Dumuis A, Sebben M, Haynes L, Pin JP, Bockaert J. NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature*. 1988;336:68–70.
- Wilt SG, Milward E, Zhou JM, et al. In vitro evidence for a dual role of tumor necrosis factor-alpha in human immunodeficiency virus type 1 encephalopathy. *Ann Neurol*. 1995;37:381–394.
- Akassoglou K, Probert L, Kontogeorgos G, Kollias G. Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J Immunol*. 1997;158:438–445.
- Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. *Annu Rev Neurosci*. 1999;22: 219–240.

44. Shinpo K, Kikuchi S, Moriwaka F, Tashiro K. Protective effects of the TNF-ceramide pathway against glutamate neurotoxicity on cultured mesencephalic neurons. *Brain Res.* 1999;819:170-173.
45. Diem R, Meyer R, Weishaupt JH, Bahr M. Reduction of potassium currents and phosphatidylinositol 3-kinase-dependent AKT phosphorylation by tumor necrosis factor-(alpha) rescues axotomized retinal ganglion cells from retrograde cell death in vivo. *J Neurosci.* 2001;21:2058-2066.
46. Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U. Neurodegenerative and neuroprotective effects of tumor necrosis factor (TNF-) in retinal ischemia: opposite roles of TNF-receptor 1 and TNF- receptor 2. *J Neurosci.* 2002;22:1-7.
47. Chiou SH, Yang YP, Lin JC, et al. The immediate early 2 protein of human cytomegalovirus (HCMV) mediates the apoptotic control in HCMV retinitis through up-regulation of the cellular FLICE-inhibitory protein expression. *J Immunol.* 2006;177:6199-6206.
48. Bueter W, Dammann O, Leviton A. Endoplasmic reticulum stress, inflammation, and perinatal brain damage. *Pediatr Res.* 2009;66:487-494.
49. Lai E, Teodoro T, Volchuk A. Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology (Bethesda).* 2007;22:193-201.
50. Häcki J, Egger L, Monney L, et al. Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene.* 2000;19:2286-2295.
51. Nakagawa T, Zhu H, Morishima N, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature.* 2000;403:98-103.
52. Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase-cascade in apoptosis: cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem.* 2002;277:34287-34294.
53. Nakamura K, Bossy-Wetzel E, Burns K, et al. Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J Cell Biol.* 2000;150:731-740.
54. Daugas E, Nochy D, Ravagnan L, et al. Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett.* 2000;476:118-23.
55. Lipton SA, Bossy-Wetzel E. Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. *Cell.* 2002;111:147-150.
56. Candé C, Cecconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? *J Cell Sci.* 2002;115:4727-4734.