Tumor Necrosis Factor-α–Induced Apoptosis in Murine Cytomegalovirus Retinitis

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PURPOSE. Previous results suggest that apoptosis is involved in the pathogenesis of murine cytomegalovirus (MCMV) retinitis. To explore the mechanism underlying retinal apoptosis in MCMV retinitis, this study was initiated to determine whether the tumor necrosis factor receptor (TNFR1)-TNF pathway is involved in apoptosis during MCMV retinitis.

METHODS. The left eyes of nonimmunosuppressed (non-IS) BALB/c mice, immunosuppressed (IS) BALB/c mice, TNFR1+/−C57BL/6 mice, and wild-type C57BL/6 mice were inoculated with MCMV k181 by way of the supraciliary route. On postinoculation days 3, 7, and 10, injected eyes of non-IS control and IS experimental mice were removed for RT-PCR for TNF-α and TNFR1. Protein expression of TNF-α, caspase-8, and caspase-3 was determined by staining frozen sections and performing Western blot analysis and quantitative ELISA. Apoptotic cells were identified by TUNEL labeling.

RESULTS. In IS BALB/c mice, TNF-α mRNA and protein were detected in MCMV-infected eyes throughout the infection. Activation of caspase-3 and caspase-8 was observed. Most of the TNF-α–expressing cells were MCMV-infected RPE cells or macrophages derived from RPE cells. TNF-α was observed in the area of apoptotic retinal cells, and the level of this cytokine corresponded to the extent of the retinal abnormality and to the number of apoptotic cells. In non-IS MCMV–infected BALB/c mice, TNF-α was expressed early in the retinas of MCMV-infected eyes, but its expression was decreased thereafter. TNFR1 mRNA was increased in IS and non-IS BALB/c after MCMV infection. More apoptotic cells were observed in the retinas of non-IS MCMV–infected wild-type C57BL/6 mice than in the retinas of non-IS TNFR1−/− mice.

CONCLUSIONS. These results suggest that the TNFR1-TNF pathway is involved in the induction of apoptosis and the exacerbation of retinal abnormality during MCMV retinitis. Furthermore, because TNF-α and TNFR1 were present in IS and non-IS mice, TNF-α–induced retinal apoptosis during MCMV infection is not T-cell dependent. (Invest Ophthalmol Vis Sci. 2007;48: 1691–1700) DOI:10.1167/iovs.06-1040

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. Left untreated, the infection may result in retinal damage and blindness.1–5 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,6–8 HCMV retinitis continues to be a chronic sight-threatening ophthalmologic problem among HIV-1–infected patients who do not respond to HAART9–12 or who discontinue therapy.7–9

It is well recognized that the development of HIV-1–related HCMV retinitis correlates with the degree of HIV-1–induced immunosuppression. However, the effector mechanism(s) by which HCMV infection causes retinal pathogenesis remains unclear. Because of the strict species specificity of the cytomegaloviruses, ocular infection with murine CMV (MCMV) has been used to study the pathogenesis of retinitis in the mouse.10 A mouse model of MCMV retinitis has been established and studied in our laboratory. In this model, inoculation of 5 × 103 to 5 × 104 plaque-forming units (PFUs) of MCMV into IS BALB/c mice through the supraciliary route results in progressive retinitis. In contrast, supraciliary injection of the same dose of MCMV into immunocompetent BALB/c mice results in minimal retinal involvement.11

Although retinal necrosis is one of the hallmarks of CMV retinitis, apoptotic cells have been observed during microscopic examination of biopsy specimens of eyes from patients with HCMV retinitis.12,13 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 MCMV retinitis. 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 retinitis14,15 However, the apoptosis-inducing factor(s) remains to be identified.

Potential neurotoxins, such as TNF-α, released by activated microglial cells are associated with neuronal apoptosis in several diseases, such as AIDS, Alzheimer disease, and multiple sclerosis.16–19 TNF-α has also been detected in macrophages and astrocytes of the retina of AIDS patients with CMV retinitis,20 and an increase in the level of intracellular TNF-α was observed in MCMV–inoculated eyes of MAIDS mice with MCMV retinitis.21 Therefore, TNF-α should be considered one of the factors that induce apoptosis during MCMV infection of the retina. The purpose of this study was to determine the relationship between TNF-α and retinal apoptosis during MCMV retinitis and to determine whether loss of the TNF-TNF receptor 1 pathway prevents or reduces apoptosis.

MATERIALS AND METHODS

Mice

Adult (6–8 weeks old) female BALB/c mice (Taconic, Germantown, NY), adult TNFR1−/− mice (Jackson Laboratory, Bar Harbor, ME), and wild-type C57BL/6 mice (Jackson Laboratory) were randomly grouped and assigned to a specific experiment. All mice were allowed unrestricted access to food and water and were maintained on a 12-hour...
light cycle alternating with a 12-hour dark cycle. All animal experiments were performed in accordance with the National Institutes of Health guidelines, and all procedures in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Immunosuppression was induced by intramuscular injection of 2.0 mg sterile methylprednisolone acetate suspension every 3 days beginning on day -1. This treatment typically depletes ≥95% of the CD4+ and CD8+ T cells from MCMV-infected mice, as assessed by flow cytometry of splenocytes.22,23

**Virus and Virus Titration**
The original stock of MCMV (k181 strain) was the generous gift of Edward S. Mocarski (Stanford University School of Medicine, Stanford, CA). Virus stocks were prepared from salivary gland homogenates of BALB/c mice (Taconic), as described previously.24 Briefly, mice were infected with 2 mg methylprednisolone acetate intramuscularly every 3 days. Two days after the first injection of methylprednisolone, mice were infected intraperitoneally with 5 × 103 plaque forming units (PFUs) of MCMV in a volume of 0.2 mL. Fourteen days after infection, the salivary glands were removed aseptically and homogenized (10%, wt/vol) in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum. Preparations were clarified by centrifugation (2500 g, 20 minutes), and 0.1-mL aliquots of the supernatants were stored at -80°C. An aliquot of virus stock was titrated in duplicate on monolayers of mouse embryo fibroblasts grown in DMEM, as described previously.24 Mock virus stock was prepared from salivary gland homogenates of uninfected mice. Before injection, the salivary gland homogenates from mock-infected and MCMV-infected mice were diluted identically in serum-free DMEM. A fresh aliquot of MCMV stock was thawed and used immediately for each experiment.

**Ocular Inoculation**
Mice were anesthetized by intramuscular injection of a mixture of 42.9 mg/mL ketamine, 8.57 mg/mL xylazine, and 1.43 mg/mL acepromazine at a dose of 0.5-0.7 mL/kg body weight. The left eyes of mice were injected with 5 × 104 PFUs of MCMV for BALB/c mice and 5 × 104 PFUs of MCMV for TNFR1-/- and wild-type C57BL/6 mice in a volume of 2 μL by way of the suprachoroidal route, as previously described.25 Briefly, a superficial transscleral entry wound was made parallel and 1 hour before sectioning. After trimming, serial frozen sections (8-μm thick) were made on a cryostat, mounted on positively charged slides (SuperFrost/Plus; Fisher Scientific, Pittsburgh, PA), and stored at –80°C before immunostaining or TUNEL assay.

**Measurement of Apoptosis**
Apoptosis was detected by TdT-dUTP terminal end labeling (TUNEL) with minor modification of a method described previously.15 Briefly, frozen sections were brought to room temperature and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), incubated in terminal transference (TdT) buffer containing 150 U/mL TdT (Life Technologies, Rockville, MD) and 8 μL/mL FITC-dUTP nucleotide labeling mixture (Roche Molecular Biochemicals, Indianapolis, IN) for 120 minutes at 37°C, stopped with 0.5 M EDTA, mounted with mounting medium with DAPI (Vectorshield; Vector Laboratories, Burlingame, CA), and examined with an inverted fluorescence microscope (Eclipse TE400; Nikon, Tokyo, Japan). Images were captured with a digital camera (SPOT Insight; Diagnostic Instruments, Inc., Sterling Heights, MI).

**Western blotting for Caspase-8**
Fresh postmortem samples were frozen in liquid nitrogen and pulverized (Bessman Tissue Pulverizer; Spectrum Laboratories, Rancho Dominguez, CA). Proteins were extracted from pulverized samples using modified radioimmunoprecipitation (RIPA) buffer with an inhibition cocktail (complete Mini Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Protein concentrations were determined (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA), and equal amounts of protein were separated by 12% SDS-PAGE with mini-ready gels (Bio-Rad Protein Assay). After separation, proteins in gels were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Amerham Biosciences, Amersham, UK) at 250 mA for 1 hour at 4°C. Membranes were then incubated overnight at 4°C with rabbit anti-mouse antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T buffer (pH 7.4) containing 5% nonfat milk. Binding of HRP-conjugated secondary antibody (goat anti-rabbit IgG-HRP, 1:200; BD PharMingen, San Diego, CA) was performed for 1 hour at room temperature. The immune complex was detected by the ECL chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) and exposure to x-ray film.

**Reverse Transcription–Polymerase Chain Reaction**
Posterior segments of five injected eyes were isolated and pooled at each time point. Fresh samples were cut into small pieces in a 3-cm dish with a no. 15 surgical blade (Feather, Osaka, Japan) and were immediately placed in extraction reagent (TRIZol; Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted according to the manufacturer’s instructions and was resuspended in 10 to 20 μL RNase-free water. The concentration of the resultant RNAs was determined with a spectrophotometer (MBA 2000; PerkinElmer Life and Analytical Sciences Inc., Boston, MA), and the concentration was normalized before amplification.

Specific DNA products were generated with an RT-PCR system (One Step; Invitrogen Life Technologies). Primer sets were as follows:

- TNF-α sense, 5′-TTCCT TCTACTG AAACCG GTGTA GCCGG CC-3′; TNF-α antisense, 5′-GTATG AGATAG GACAA CGCCGC TACG TGTGGG GC-3′ (354-bp fragment);
- TNFR1 sense, 5′-ATCTG CTGCA CCAG GCC-3′; TNFR1 antisense, 5′-TGCTG GGCAAG TTAC CACC-3′ (342-bp fragment).

Amplification of β-actin was used as the control. Primers used for β-actin were: sense, 5′-TCCCT CGTGT CCACG ACA-3′; antisense, 5′-CGTCT CGGGA GTCC ACA-3′ (508-bp fragment). Spleen samples from virus-infected non-IFN-γ mice were used as the positive control. RT-PCR procedures were carried out according to the manufacturer’s protocol, with modification of the annealing temperatures for TNF-α (55°C), β-actin (55°C), and TNFR1 (60°C). RT-PCR products were analyzed on 1.5% gels (Invitrogen Life Technologies). Gels were photographed and densities of the bands were determined with a computerized image analysis system (Alpha Inotech, San Leandro, CA). The area of each band was calculated as the integrated density value (IDV). Mean values and standard deviations were calculated from three separate experiments. The IDV ratio of TNF-α to β-actin was calculated for each sample.
ELISA Quantification of TNF-α

For ELISA assay, eye samples were homogenized with a rotator-stator-type homogenizer (Biospec, Bartlesville, OK). Specifically, samples were homogenized for 1 minute in 10 mM HEPES-KOH buffer (pH 7.9) containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P-40, and protease inhibitor (Roche, Mannheim, Germany). Homogenates were centrifuged at 12,000g for 4 minutes at 4°C. Supernatants were removed, and five samples were pooled. The total protein concentration (µg/ml) was determined from a standard curve established from a Bradford assay (Bio-Rad). Quantification of TNF-α (pg/200 µg total protein) was accomplished using a commercially available sandwich-type ELISA according to the manufacturer’s protocol (R&D Systems, Wiesbaden, Germany). Spectrophotometric analysis was carried out with biotechnology software (Revelation 3.2 system; Dynex, Chantilly, VA) set at 602 nm for protein assay and 450 nm for TNF-α determination, in accordance with assay guidelines. Absorbance of the colored product was measured at 450 nm.

Immunohistochemical Staining

Rat anti-mouse TNF-α antibody was purified from ascites of the hybridoma X3.1126 (generously provided by Gary Klimpel, University of Texas Medical Branch, Galveston, TX) by ammonium sulfate precipitation and biotinylated with agent (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) according to the manufacturer’s instructions. Rabbit anti–RPE 65 (generously provided by Michael Redmond, National Eye Institute, National Institutes of Health, Bethesda, MD) was used to stain the cells of the retinal pigment epithelium (RPE). Monoclonal antibody to an MCMV early gene product27 was labeled with FITC (Sigma-Aldrich, St. Louis, MO) or biotinylation agent (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) according to the manufacturer’s instructions. FITC-labeled anti-F4/80 was purchased (BD Pharmingen). Retinal glial cells (including activated Müller cells and astrocytes) were stained with rabbit anti–GFAP (Chemicon, Temecula, CA).

Before staining, all samples were fixed in 4% paraformaldehyde. For biotinylated anti-TNF-α or anti-MCMV EA staining, fixed slides were blocked with 3% normal goat serum (NGS) in PBS for 30 minutes at room temperature and then incubated overnight at 4°C in biotin-labeled anti–TNF-α (1:100) or anti-MCMV EA (1:100). After washing, the sections were reacted with Texas Red–labeled avidin (1:200 in PBS; Vector Laboratories) for 1 hour at room temperature, mounted with mounting medium with DAPI (Vectorshield; Vector Laboratories), and examined under a fluorescence microscope.

For FITC-labeled anti–MCMV EA (1:400) or FITC-labeled anti–F4/80 (1:200) staining, the slides were permeabilized, blocked in PBS containing 10% NGS, 1% BSA, and 0.5% Triton X-100, and incubated overnight at 4°C in the primary antibody. For cell identification using rabbit-derived antibodies including anti–RPE 65 (1:400) or anti–GFAP (1:200), the slides were permeabilized, blocked in PBS containing 10% NGS, 1% BSA, and 0.5% Triton X-100, and incubated overnight at 4°C in the primary antibody. After washing, the sections were reacted with AMCA-labeled anti–rabbit IgG (1:200) or FITC-labeled anti–rabbit antibody (Vector Laboratories).

For triple staining of TNF-α, RPE 65, and F4/80, sections were stained first with rabbit anti–RPE 65 (1:400), and the reaction was developed with AMCA-labeled anti–rabbit IgG. Sections were then stained with biotin-labeled anti–TNF-α, and immunolabeling was detected with Texas Red–labeled avidin. Finally, the slides were reacted with FITC anti–F4/80. Slides were mounted with antifade medium without DAPI (Vectorshield) and examined microscopically.

For triple staining of TNF-α, RPE 65, and MCMV EA, sections were stained first with rabbit anti–RPE 65, and the reaction was developed with AMCA-labeled anti–rabbit IgG. Sections were then stained with biotin-labeled anti–TNF-α, and immunolabeling was detected with Texas Red–labeled avidin. Finally, the slides were reacted with FITC anti–MCMV EA and then were mounted and examined microscopically.

RESULTS

TNF-α-Induced Apoptosis in MCMV Retinitis

For double staining of TUNEL and TNF-α or MCMV EA, the sections were stained first with TUNEL and then stained with biotinylated anti–TNF-α or biotinylated anti–MCMV EA. Immunolabeling of TNF-α or MCMV EA was detected with Texas Red–labeled avidin. The slides were mounted with mounting medium with DAPI (Vectorshield; Vector Laboratories) and were examined under a fluorescence microscope.

Determination of Caspase-3 Activity

Caspase-3 activity was measured by modified enzymatic assay with the fluorogenic peptide substrate Ac-Asp-Glu-Val-Asp-AMCA (Ac-DEVD-AMCA). Briefly, fresh posterior segment samples were frozen in liquid nitrogen, pulverized, and treated with 1% Triton X-100. Samples from five eyes in each group were pooled. Eighty micrograms triton lysate was added to enzymatic reactions containing 25 µM DEVD-AMCA. After 60 minutes at 37°C, fluorescence at excitation (360 nm) and emission (550 nm) was monitored (GENios plate-reader, Tecan US Inc., Research Triangle Park, NC). A standard curve was constructed using free AFC in each measurement. With the use of this standard curve, the amount of liberated AFC (nanomoles per milligram protein per hour) was determined in accordance with assay guidelines. Absorbance of the colored product was measured at 450 nm.

Statistical Analysis

All data were expressed as mean ± SEM. Significant differences (P < 0.05 or better) between two groups were determined with the Student’s t-test.

TNF-α in BALB/c Mouse Eyes during MCMV Infection

To determine whether MCMV infection upregulated TNF-α, the amount of TNF-α in the injected eyes (including inner retina, RPE, and choroid) was measured by ELISA of the supernatants from homogenized samples. As shown in Figure 1, a very low level of TNF-α was detected on day 3 and disappeared by day 7 after mock injection. TNF-α was not detected in the eyes of normal un.injected mice (not shown). However, after MCMV injection, TNF-α protein was detected in the injected
eyes of non-IS and IS mice. The amount of TNF-α peaked on day 7 after infection in both groups. On day 7 and day 10 after infection, the amount of TNF-α in MCMV-infected IS mice was significantly higher than the amount of TNF-α in MCMV-infected non-IS mice \((P < 0.05)\). Overall, TNF-α levels in the eyes of IS and non-IS BALB/c mice were elevated before the development of necrotizing retinitis.

Transcription of TNF-α mRNA in the posterior segments of MCMV-infected eyes was evaluated by RT-PCR. As shown in Figure 2A, TNF-α mRNA was barely detectable in normal BALB/c mice, mock-injected IS BALB/c mice, or non-IS BALB/c mice. However, increased levels of mRNA for TNF-α were observed at all time points in non-IS and IS MCMV-infected mice. Band-density analysis (Fig. 2B) suggested that the level of TNF-α mRNA peaked on day 7 in non-IS MCMV-infected mice and decreased thereafter, consistent with the protein expression profiles for TNF-α determined by ELISA. In contrast, the level of TNF-α mRNA in IS MCMV–infected BALB/c mice remained elevated on day 10 after infection.

**TNF-α–Producing Cells**

Immunohistochemistry was used to determine the location and identity of TNF-α–producing cells. After inoculation of MCMV into the supraciliary space, TNF-α was observed in the posterior segments on days 3 and 7 after infection in IS and non-IS BALB/c mice and on day 10 after infection in IS BALB/c mice (Fig. 3). In non-IS mice, TNF-α was not observed in the retina on day 10 after infection. TNF-α was not observed in the eyes of normal BALB/c mice or in the mock-injected eyes of IS and non-IS mice (Fig. 3). TNF-α–positive cells were also observed in the anterior segments of injected eyes in IS and non-IS mice (not shown). Therefore, it is possible that TNF-α detected by ELISA in the eyes of non-IS mice on day 10 after infection might have resulted from the presence of TNF-α in the anterior segment and ciliary body.

Double staining for TNF-α and retinal antigens, including RPE 65, F4/80, and GFAP, was used to reveal the source of TNF-α. As shown in Figure 4, RPE cells were the major source of TNF-α. In non-IS mice, most TNF-α–producing cells were RPE 65–positive cells located in the RPE layer or the photoreceptor layer, whereas MCMV-infected cells were only observed in the choroid and RPE layer but not in the inner retina (Fig. 4A, images A–D; Fig. 4B, images A–D). In IS mice, many TNF-α–positive, RPE 65–positive cells were also observed in the RPE and photoreceptor layers (Fig. 4A, image H; Fig. 4B, image I).

**Figure 2.** TNF-α mRNA in the posterior segments of eyes of MCMV-infected BALB/c mice 3, 7, and 10 days after infection (dpi) (A). Densities of the bands were determined using a computerized image analysis system. Relative band densities of TNF-α and β-actin mRNA are shown (B). Five ocular samples from each group were pooled at each time point. Samples from mock-injected mice were analyzed as shown (not shown). Therefore, it is possible that TNF-α detected by ELISA in the eyes of non-IS mice on day 10 after infection might have resulted from the presence of TNF-α in the anterior segment and ciliary body.

**Figure 3.** Photomicrographs of TNF-α staining in normal and MCMV-infected BALB/c mouse eyes. No TNF-α–positive cells were observed in normal eyes (A), mock-injected eyes of non-IS mice (B), or mock-injected eyes of IS mice (C). TNF-α–positive cells were observed in the retinas of the injected eyes of non-IS BALB/c mice on day 5 after infection (D, arrows), day 7 after infection (E, arrows), or day 10 after infection (F, arrows) of IS BALB/c mice on day 3 after infection (G, arrows) and day 7 after infection (H, arrow). TNF-α was not observed on day 10 after infection in the injected eye of IS BALB/c mice (I).
However, on days 7 and 10 after infection, after MCMV spread to the retina (Fig. 4A, image G), many TNF-α-positive non-RPE macrophages/microglia (F4/80 positive, RPE65 negative) were noted in the inner retina (Fig. 4B, image H, circles). Among TNF-α–producing RPE cells, some were TNF-α positive, RPE 65 positive, and F4/80 positive and had the morphologic appearance of macrophages (Fig. 4B, image H, arrowheads). Triple staining for MCMV EA, RPE 65, and TNF-α also showed that most TNF-α–producing cells in IS and non-IS mice were uninfected RPE cells (Fig. 4A, images D and H). Double staining for GFAP and TNF-α showed that only rare cells were TNF-α and GFAP double positive, suggesting that glia were not the source of TNF-α (not shown).

### TNFR1 mRNA Expression in the Posterior Segment of MCMV-Infected BALB/c Mice

TNF-α activates the apoptosis program by binding to the TNFR1 on the cell surface.29–31 Transcription of TNFR1 in the posterior segment of MCMV-infected eyes was evaluated by RT-PCR. TNFR1 mRNA transcription was observed in all groups (normal mice, IS MCMV-infected, non-IS MCMV-infected), as shown in Figure 5A. Changes in the expression of TNFR1 mRNA at different times after MCMV inoculation are shown in Figure 5B. mRNA levels were higher in all MCMV-infected eyes at all times than in normal mice. In IS mice, the highest level of TNFR1 transcription was observed on day 7 after infection. In non-IS mice, the highest level was observed on day 3 after infection, with lower levels noted on days 7 and 10 after infection.

### TNF-α–Positive Cells and Apoptotic Cells in the Retina

To associate TNF-α expression with apoptosis during MCMV infection of the retina, ocular sections were stained for TNF-α and TUNEL. As described for day 7 after infection, most TNF-α–producing cells were located in the RPE layer or the photoreceptor layer in non-IS mice, and TUNEL-positive cells were detected in the nearby outer nuclear layer of the retina.
activity was elevated in IS- and non-IS MCMV–infected mice. Constitutively expressed in the eye of normal BALB/c mice, and TNFR1 mRNA to a computerized image analysis system. Relative band densities of TNFR1 mRNA in the posterior segment of eyes of MCMV-infected BALB/c mice (Figs. 5A–D). In IS mice, after MCMV spread to the retina, TNF-α-positive cells were observed in the RPE layer and inner retina, and many TUNEL-positive cells were also seen in the same area (Figs. 6E–L [E, F, circles]). However, even though some TNF-α-positive cells were also TUNEL positive, most TUNEL-positive cells in the retina were TNF-α negative and were likely retinal neurons, as has been described. By day 10, the number of apoptotic cells had increased, and staining intensity increased coincident with the occurrence of necrotic retinitis involving all layers of the retina in MCMV-infected IS BALB/c mice (Figs. 6I–L). In the necrotic retina, there were large numbers of TNF-α positive cells as well as TUNEL positive cells, but only a few cells were both TNF-α positive and TUNEL positive.

### Caspase Activation in MCMV-Infected BALB/c Mice

Because activation of the TNF-TNFRI apoptotic pathway has been shown to contribute to pathogenesis in several inflammatory disease models, we hypothesized that TNF-α expression during MCMV infection activates the extracellular apoptotic pathway, which might, in turn, contribute to the pathogenesis of MCMV retinitis. Activation of caspase-8 and caspase-3, the major initiator caspase and effector caspase involved in TNF-α-induced apoptosis, respectively, was investigated in the posterior segment of MCMV-infected eyes. Activation of caspase-3 was measured using cleavage of the fluorogenic substrate DEVD-AFC. As shown in Figure 7, in non-IS MCMV-infected BALB/c mice, caspase-3 enzyme activity was slightly elevated on day 3 after infection, peaked on day 7 after infection (Figs. 6A–D). In IS mice, after MCMV spread to the retina, TNF-α-positive cells were observed in the RPE layer and inner retina, and many TUNEL-positive cells were also seen in the same area (Figs. 6E–L [E, F, circles]). However, even though some TNF-α-positive cells were also TUNEL positive, most TUNEL-positive cells in the retina were TNF-α negative and were likely retinal neurons, as has been described. By day 10, the number of apoptotic cells had increased, and staining intensity increased coincident with the occurrence of necrotic retinitis involving all layers of the retina in MCMV-infected IS BALB/c mice (Figs. 6I–L). In the necrotic retina, there were large numbers of TNF-α positive cells as well as TUNEL positive cells, but only a few cells were both TNF-α positive and TUNEL positive.

### Figure 5

TNFR1 mRNA in the posterior segment of eyes of MCMV-infected IS mice, non-IS mice, and normal BALB/c mice 3, 7, and 10 days after infection (A). Densities of the bands were determined using a computerized image analysis system. Relative band densities of TNFR1 mRNA to β-actin mRNA are shown (B). TNFR1 mRNA was constitutively expressed in the eye of normal BALB/c mice, and TNFR1 activity was elevated in IS- and non-IS MCMV-infected mice.

### Figure 6

Photomicrographs of staining of TUNEL (A, E, I), TNF-α (B, F, J), and DAPI (C, G, K) in the MCMV-infected eye of a non-IS mouse on day 7 after infection (A–D), an IS mouse on day 7 after infection (E–H), and an IS mouse on day 10 after infection (I–L). Triple images were merged (D, H, L). In non-IS mice (A–D), most TNF-α-producing cells were located in either the RPE layer or the photoreceptor layer (arrows, arrowheads). TUNEL-positive cells were detected in the outer nuclear layer of the retina. In IS mice, TNF-α-producing cells were observed in the RPE layer and the inner retina, and some of the TNF-α-positive cells were also TUNEL positive on day 7 after infection (E–H). On day 10 after infection, a large number of TUNEL-positive cells and TNF-α-positive cells were observed in areas of necrotizing retinitis, but few cells were both TUNEL positive and TNF-α positive (I–L).
infection, and returned to baseline by day 10 after infection. In IS MCMV-infected mice, activated caspase-3 showed a gradual increase between day 3 and day 7 after infection and then again between day 7 and day 10 after infection. Caspase-8 cleavage was measured with Western blotting (Fig. 8). In non-IS BALB/c mice, cleavage of caspase-8 was initiated in MCMV-infected eyes on day 5 after infection and then slightly decreased on day 7 after infection. Unlike the activation pattern of caspase-3, by day 10 after infection, the cleavage of caspase-8 in non-IS mice had decreased almost to the level in normal mice. In IS mice, cleaved caspase-8 was slightly elevated on day 3 after infection and remained elevated on days 7 and 10 after infection, similar to the pattern of caspase-3 activation.

### Discussion

Results of the studies described herein provide information about the role of TNF-α in the pathogenesis of MCMV retinitis. In this study, TNF-α was produced in the posterior segments of MCMV-infected eyes of IS and non-IS BALB/c mice. TNF-α expression was first observed when apoptosis began in the retina and increased commensurate with the progression of apoptosis and retinitis. Results of immunohistochemistry studies indicated that apoptotic areas were close to areas of the retina containing TNF-α-producing cells. TNFR1 was activated after virus infection, and the mRNA profiles of TNF-α and TNFR1 were consistent with the profiles of apoptosis and damage in the retina.

TNF-α is a cytokine with multiple physiological roles in cell proliferation, cell death, and cell inflammation and with pathologic roles in immunologic processes. This cytokine was originally identified as an antitumor agent that induced necrotic cell death in sarcomas. Most TNF-α is produced by activated macrophages, but smaller amounts of TNF-α are also produced by stimulated monocytes, fibroblasts, endothelial cells, and cells of the immune system. Within the central nervous system (CNS), TNF-α has been shown to have neuroprotective and neurodestructive effects through direct or indirect activities. In the eye, TNF-α has been shown to contribute to ocular damage during uveitis and glaucoma.

The variety of effects exerted by TNF-α are mediated by TNFR1 and TNFR2. For example, the apoptotic effects of TNF-α in neural tissue are primarily mediated by TNFR1, whereas TNFR2 may potentiate the effects of TNFR1 in promoting cell death or inflammation. TNFα has been reported in murine CNS neurons. mRNA for TNFR1 is constitutively present in blood vessels in the rat brain and in the myelin sheath of the optic nerve of normal mouse eyes. In the normal human CNS, TNFR1 is found in oligodendrocytes, microglia, and astrocytes.

In agreement with what has been previously reported, the degree of retinal damage late in MCMV infection was disproportionate to the amount of virus infection in the retina. Apoptotic cells were noted in the retina as early as day 3 after infection in non-IS BALB/c mice, when only the choroid and the RPE were virus positive. This finding suggests that TNF-α-induced apoptosis, rather than the extent of retinal MCMV infection, may be primarily responsible for the retinal abnormality observed in this model.
MCMV EA and TNF-α-positive cells in non-IS MCMV-infected mice were located mainly in the RPE layer and choroid, whereas apoptosis was usually observed in the nearby retina (mostly in the outer nuclear area). Non-IS mice did not develop retinitis. It is possible that MCMV infection of the choroid and RPE stimulated the release of TNF-α from infected or uninfected RPE cells, which then caused apoptosis in the overlying retina. Furthermore, in IS BALB/c mice, it is possible that TNF-α released from RPE cells and macrophages/microglia caused retinal cell death, which also contributed to retinal destruction. Previously, we reported that after supraciliary inoculation of MCMV, apoptosis of uninfected bystander retinal cells appeared to be involved in the pathogenesis of MCMV retinitis. In IS MCMV-infected BALB/c mice, the cleavage profiles of caspase-8 and caspase-3 were similar from day 3 after infection and continued to increase until day 10 after infection. This finding suggests that caspase-8–induced apoptosis may play a major role in MCMV retinitis in IS BALB/c mice, either through the extracellular pathway or through the mitochondrial pathway. In non-IS MCMV–infected mice, the cleavage pattern of caspase-8 was different from that of caspase-3, and the cleavage of caspase-8 was maximal early (day 3 after infection) and then decreased thereafter. Because caspase-3 is a common executer in most apoptotic pathways, this observation suggests that by day 7, caspase-8 is not the only initiator of retinal apoptosis in MCMV-infected non-IS BALB/c mice. The reduction in retinal apoptosis observed in non-IS MCMV–infected TNFR1 knockout mice further supports the idea that the TNF-TNFRI pathway plays a role in the apoptosis of retinal cells during MCMV infection.

Based on the caspase activity profiles and because apoptotic cells were observed in TNFR1−/− mice, TNF-α is not the only factor involved in retinal apoptosis in this model of MCMV retinitis. Although human RPE-induced apoptosis of T cells has been reported independently of its expression in TNF-related
apoptosis-inducing ligand (TRAIL) or Fas/FasL pathway. Other reports have shown that TRAIL produced by RPE cells can cause T-cell apoptosis or can stimulate the production of other cell survival factors. Although the results of these studies implicate the TNF-TNFRI1 pathway as a contributor to apoptosis of retinal cells during MCMV infection, they do not exclude the mitochondrial pathway nor do they exclude the activation of caspase-8 and caspase-3. Additional studies are needed to elucidate the contributions of each of these pathways to the apoptosis of retinal cells during MCMV infection.

In summary, the results in this report support the idea that TNF-α–induced apoptosis is involved in the pathogenesis of MCMV retinitis. By extrapolation, it is possible that TNF-α, which has been demonstrated in the eyes of human patients with CMV retinitis, may also play a role in the pathogenesis of CMV retinitis in human patients. Direct targeting of the TNF-α apoptosis signaling pathways may constitute a future therapeutic possibility. However, a therapeutic strategy of targeting TNF-α alone may not be sufficient because TNF-α probably does not have a single effect in apoptosis and in increasing retinal damage. The additional pathways of cell death in MCMV retinitis and the precise signaling mechanism(s) by which TNF-α is linked to retinal cell apoptosis remain to be deciphered.

References

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