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Evidence For Multiple Cell Death Pathways during Development of Experimental Cytomegalovirus Retinitis in Mice with Retrovirus-Induced Immunosuppression: Apoptosis, Necroptosis, and Pyroptosis

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AIDS-related human cytomegalovirus (HCMV) retinitis remains a major ophthalmologic problem worldwide. Although this sight-threatening disease is well characterized clinically, many pathogenic issues remain unresolved, among them a basic understanding of the relative roles of cell death pathways during development of retinal tissue destruction. Using an established model of experimental murine cytomegalovirus (MCMV) retinitis in mice with retrovirus-induced immunosuppression (MAIDS), we initially investigated MCMV-infected eyes for evidence of apoptosis-associated molecules in mice with MAIDS of 4 weeks’ (MAIDS-4) and 10 weeks’ (MAIDS-10) duration, which were resistant and susceptible to retinal disease, respectively, but which harbored equivalent amounts of infectious MCMV. Whereas MCMV-infected eyes of MAIDS-4 mice showed little evidence of apoptosis-associated molecules, MCMV-infected eyes of MAIDS-10 mice showed significant amounts of tumor necrosis factor alpha (TNF-α), TNF receptors 1 and 2, active caspase 8, active caspase 3, TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-R(DR5), Fas, and Fas ligand mRNAs and/or proteins, all detected at peak amounts prior to development of most severe retinal disease. Immunohistochemical staining showed macrophages, granulocytes (neutrophils), Müller cells, and microglial cells as TNF-α sources. Remarkably, quantification of apoptosis by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay suggested that apoptosis contributed minimally to retinal disease in MCMV-infected eyes of MAIDS-10 mice. Subsequent studies demonstrated that MCMV-infected eyes of MAIDS-10 mice, but not MAIDS-4 mice, showed evidence of significant increases in molecules associated with two additional cell death pathways, necroptosis (receptor-interacting protein 1 [RIP1] and RIP3 mRNAs) and pyroptosis (caspase 1, interleukin 1β [IL-1β], and IL-18 mRNAs). We conclude that apoptosis, necroptosis, and pyroptosis participate simultaneously during MAIDS-related MCMV retinitis, and all may play a role during AIDS-related HCMV retinitis.

AIDS-related human cytomegalovirus (HCMV) retinitis is a slowly progressive retinal disease of betaherpesvirus origin that historically caused vision loss and blindness in up to 30% of AIDS patients (48). However, with the development of active antiretroviral therapy (ART) to manage HIV infection directly, the incidence of AIDS-related HCMV retinitis has fallen significantly in recent years. Nonetheless, this sight-threatening disease remains an ophthalmologic problem worldwide, affecting HIV-infected patients who do not have access to ART or who fail to respond to ART (39). AIDS-related HCMV retinitis is also not limited to HIV/AIDS patients and can develop in patients who are immunosuppressed for solid-organ or bone marrow transplantation, albeit at a lower incidence (44).

We have therefore continued our investigations of the pathogenesis of AIDS-related HCMV retinitis using a well-characterized experimental mouse model of murine cytomegalovirus (MCMV) retinitis that develops in C57BL/6 mice with MAIDS (14), a murine retrovirus-induced immunodeficiency syndrome that remarkably mimics HIV-induced AIDS in humans (37, 56). During the course of a previous investigation on the pathogenesis of MAIDS-related MCMV retinitis (15), we reported that MCMV-infected eyes of mice with MAIDS of 4 weeks’ duration (MAIDS-4 mice) exhibited retinal folding and proliferation of retinal pigment epithelium (RPE) in response to subretinal virus infection, but without development of retinal necrosis. In sharp contrast, ~100% of the MCMV-infected eyes of mice with MAIDS of 10 weeks’ duration (MAIDS-10 mice) exhibited severe retinal necrosis. Further investigation revealed that MCMV-infected eyes of MAIDS-4 and MAIDS-10 animals harbored equivalent amounts of infectious MCMV. Since MCMV-infected eyes of MAIDS-4 mice failed to develop retinal necrosis despite large amounts of infectious virus also found to be associated with consistent development of retinal necrosis in MCMV-infected eyes of MAIDS-10 mice, we concluded that virus infection alone is not sufficient for the onset and progression of the severe retinal destruction observed in our experimental model of AIDS-related HCMV retinitis. We therefore hypothesized that cell death pathways might contribute to MAIDS-related MCMV retinitis and elected to focus initially on tumor necrosis factor alpha (TNF-α)-induced apoptosis as a pathogenic mechanism whereby retinal tissue destruction ensues following MCMV infection of mice with MAIDS of 10 weeks’ duration.

TNF-α is a proinflammatory cytokine that induces diverse cellular responses ranging from apoptosis to the activation of antiapoptotic genes involved in inflammation and acquired immune responses (7, 59). TNF-α signal transduction is accomplished...
through two distinct receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (3, 70). Binding of TNF-α to TNFR1 results in activation of a caspase cascade involving active (cleaved) caspase 8 and active (cleaved) caspase 3 that leads to apoptosis and cell death. In contrast, binding of TNF-α to TNFR2 more commonly results in activation of cellular genes involved in the inflammatory process (7, 59). More importantly, a subset of these genes may also act to suppress TNF-α-induced apoptosis, thereby giving TNF-α an antiapoptotic role that can lead to cell survival (7, 59). Produced mainly by activated macrophages, TNF-α can also be produced by a wide variety of cell types, including neutrophils and lymphoid cells (2, 13, 69).

Mondino and coworkers (55) initially provided evidence that TNF-α is expressed within the ocular compartment during AIDS-related HCMV retinitis. A similar observation was made later by Hofman and Hinton (45), who suggested infiltrating macrophages as a possible source for TNF-α. We subsequently reported significantly elevated levels of TNF-α within whole MCMV-infected eyes of mice with MAIDS which were susceptible to retinal necrosis compared with levels in MCMV-infected eyes of normal mice resistant to retinal necrosis (15). A more thorough investigation of the role of TNF-α during experimental MCMV retinitis in methylprednisolone acetate-immunosuppressed BALB/c mice was then provided by several studies from Atherton’s laboratory (6, 74, 76). Following an initial study by Bigger et al. (6), who demonstrated that apoptosis (as measured by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] assay) increased during MCMV-induced retinal disease, Zhou et al. (76) concluded that the TNFR1/TNF-α pathway is indeed involved in induction of apoptosis and onset of retinal disease but that TNF-α can also play an antiapoptotic role during disease progression as suggested by Zhang et al. (74). Given the availability of our mouse model of MAIDS-related MCMV retinitis (14) and our observation that MAIDS-4 mice and MAIDS-10 mice exhibit strikingly different outcomes following subretinal MCMV infection despite eyes of both groups harboring equivalent amounts of infectious virus (15), we elected to explore TNF-α-induced apoptosis relative to onset and evolution of cytomegalovirus retinal disease from a different experimental perspective. We report herein that while apoptosis induced by TNFR1/TNF-α as well as TNF-related apoptosis-inducing ligand (TRAIL) and Fas/Fas ligand (FasL) may collectively contribute to MAIDS-related MCMV retinitis, apoptosis overall surprisingly contributes minimally to MCMV-induced retinal pathology in the setting of retrovirus-induced immunosuppression. Additional investigation of MCMV-infected eyes collected from MAIDS-10 mice during progression of MAIDS-related MCMV retinitis revealed molecules unique to two additional cell death pathways, necroptosis (31, 43) and pyroptosis (5). Taken together, our results suggest that multiple cell death pathways, perhaps operating simultaneously, contribute to the pathogenesis of MAIDS-related MCMV retinitis and possibly AIDS-related HCMV retinitis.

MATERIALS AND METHODS

Animals. Adult female wild-type C37BL/6 mice, adult female mice deficient in TNF-α (B6.129S6-Tnfatm1Mak/J), adult female mice deficient in TNFR1 (B6.129Tnfrsf1atm1Mak/J), and adult female mice deficient in TNFR2 (B6.129S2-Tnfrsf1btm1Mwm/J) were purchased from Jackson Laboratory (Bar Harbor, ME). Adult female wild-type BALB/c mice were purchased from Taconic Farms (Germantown, NY). All mice were maintained on alternative 12-h light-dark cycles and allowed access to food and drink ad libitum. All experiments were performed with strict adherence to National Institutes of Health guidelines, and all procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Viruses. Stocks of the Smith strain of MCMV were prepared in mouse salivary glands of BALB/c mice as described previously (16). Mice were infected intraperitoneally with 102 to 103 PFU of MCMV contained within a 0.2-ml volume. Fourteen days later, the salivary glands were removed aseptically, homogenized (10% [wt/vol]) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, and clarified by centrifugation. Aliquots of the supernatants were stored in liquid N2. Quantification of virus stocks was determined on monolayers of mouse embryo fibroblasts grown in DMEM. A fresh aliquot of MCMV stock was thawed and used for a single experiment.

Stocks of murine retrovirus (LP-BM5 murine leukemia virus [MuLV]) were prepared by using SC-1/LP-BM5 MuLV cells obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Approximately 105 SC-1/LP-BM5 MuLV cells were mixed with approximately 106 uninfected SC-1 cells and maintained for 6 days in DMEM. The cells were then scraped into the medium, pelleted by centrifugation, resuspended in phosphate-buffered saline, and stored at −70°C. Prior to use, the suspension was thawed and clarified by centrifugation to remove cellular debris.

Induction of MAIDS. MAIDS was induced in wild-type C57BL/c mice and in various groups of knockout mice (B6.129S6-Tnfatm1Mak/J, B6.129-Tnfrsf1atm1Mak/J, and B6.129S2-Tnfrsf1btm1Mwm/J) by intraperitoneal injection of 1.0 ml of the LP-BM5 MuLV preparation (56). The inoculum contained approximately 5 × 106 to 5 × 107 infectious murine retroviruses. Mice with MAIDS of 4 weeks’ duration (MAIDS-4) and 10 weeks’ duration (MAIDS-10) were used in these studies.

Experimental mouse model of MCMV retinitis. AIDS-related HCMV retinitis is thought to originate from virus that invades the retina from the bloodstream during systemic infection (46). Intravenous or intraperitoneal injection of immunosuppressed mice with MCMV to produce systemic MCMV infection fails to infect the neurosensory retina and cause retinal disease, although virus infects the choroid, RPE, and ciliary body of the eye (13, 32). Consequently, several laboratories (1, 16, 32, 49) routinely have induced experimental MCMV retinitis in mice by supraciliary and subretinal MCMV injection, a procedure that induces reproducible retinal disease in mice with histopathologic features similar to those found in AIDS-related HCMV retinitis (1, 16). The left eyes of all mice in the present study were therefore subjected to subretinal injection with approximately 106 PFU of MCMV contained within a 2-μl volume of DMEM following dilation of both eyes and administration of anesthesia as described previously (16). The right (contralateral) eyes of all mice were injected subretinally with DMEM alone and served as controls.

Histopathologic analysis and evaluation of retinitis. At 3, 6, or 10 days following subretinal MCMV infection, eyes were carefully removed from euthanized animals, fixed in 10% buffered formalin, and subjected to frozen sectioning using a Shandon Cryotome (Thermo Scientific, Rochester, NY) to yield 8-μm sections. Sections were stained with hematoxylin and eosin, examined by light microscopy, and scored for frequency and severity of disease as described previously (16).

Recovery of infectious MCMV. Whole MCMV-infected eyes from MAIDS-4 and MAIDS-10 animals were collected at 10 days after subretinal injection and frozen at −70°C. At the time of quantitative plaque assay, eyes were thawed, homogenized individually in 1.0 ml of cold DMEM, and clarified by centrifugation. Tenfold dilutions of the resulting supernatant were inoculated in duplicate onto monolayers of mouse embryo fibroblasts contained within six-well plates, allowed to adsorb for 1 h at 37°C, overlaid with 2% methylcellulose-containing DMEM, and incubated for 6 days at 37°C in a humidified CO2 atmosphere. Individual
plaques were counted with an inverted light microscope. Results for whole eyes were reported as PFU/ml/eye.

**Quantitative real-time reverse transcriptase PCR (RT-PCR) assay.** Whole MCMV-infected eyes and whole contralateral mock-infected eyes (controls) collected at 3, 6, and 10 days after subretinal injection were stored in RNA later solution (Ambion, Austin, TX) at −70°C prior to analysis. Upon analysis, whole eyes were thawed at room temperature for 5 min and individually homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) using a 2-ml Ten Broeck tissue grinder (Wheaton, Millville, NJ). Total RNA was extracted from each eye homogenate using the PureLink total RNA purification system (Invitrogen Life Technologies) according to the manufacturer’s instructions, and total RNA amounts for each sample were determined using a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA). After the total RNA concentration was normalized for each sample, cDNA synthesis was performed using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Detection and quantification of gene expression for each gene of interest were performed using specific primers and the SYBR green PCR master mix (Applied Biosystems, Foster City, CA), and products were detected and quantified using an ABI Prism 7500 real-time PCR instrument coupled with sequence detection system software (Applied Biosystems). Primers used for detection and quantification of transcripts for TNF-α, TNFR1, TNFR2, active (cleaved) caspase 8, active (cleaved) caspase 3, TRAIL, TRAIL-R(DR5), Fas, Fasl, caspase 9, cytochrome c, apoptotic protease-activating factor 1 (Apaf-1), receptor-interacting protein 1 (RIP1), RIP3, interleukin 1β (IL-1β), IL-6, IL-18, caspase 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Qiagen Inc., Valencia, CA. Primers used for detection and quantification of transcripts for MCMV-specific genes for immediate-early 1 (IE1) protein and glycoprotein H (gH) have been described previously (17, 64).

**Western blot analysis.** Whole MCMV-infected eyes and whole contralateral mock-infected (control) eyes collected at −70°C were thawed and individually homogenized in a protease-inhibitor cocktail contained within phosphate-buffered saline (Sigma, St. Louis, MO). Standard Western blot analysis was performed for detection of TNF-α, active (cleaved) caspase 8, active (cleaved) caspase 3, and GAPDH proteins using rabbit anti-mouse TNF-α antibody (1:1,000) (Millipore, Temecula, CA), rabbit anti-mouse active caspase 8 antibody (1:1,000) (Sigma), rabbit anti-mouse active caspase 3 antibody (1:1,000) (Sigma), or rabbit anti-mouse GAPDH antibody (1:3,000) (Sigma) as primary antibodies, respectively. Following incubation with ImmunoPure goat anti-rabbit IgG antibody (heavy plus light chains [H+L]) (Thermo Scientific, Pittsburgh, PA) as secondary antibody, the resulting nitrocellulose membrane (Bio-Rad, Hercules, CA) was treated with enhanced chemiluminescence (ECL) Western blot detection reagents (GE Healthcare, Piscataway, NJ) and exposed to BioMax light film (Kodak, Rochester, NY).

**TUNEL assay.** TUNEL assay was used for detection and quantification of apoptotic cells (33, 57, 58) in tissue sections of whole MCMV-infected and contralateral mock-infected (control) eyes recovered from animals at 3, 6, and 10 days after subretinal MCMV injection. Briefly, tissue sections on 1-μm-thick glass microscope slides were treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate buffer) for 5 min on ice, washed in phosphate-buffered saline, and subjected to the TUNEL assay using the fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse TNF-α antibody (1:200) (e Bioscience, San Diego, CA) at 4°C overnight in a humidified atmosphere. Control sections were incubated with FITC-conjugated rabbit anti-mouse antibody of matched IgG1 isotype (1:500) (e Bioscience). Identification of TNF-α-producing retinal cells was accomplished by incubating sections with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse TNF-α antibody (1:200) (e Bioscience, San Diego, CA) at 4°C overnight in a humidified atmosphere. Control sections were incubated with FITC-conjugated rat anti-mouse antibody of matched IgG1 isotype (1:500) (e Bioscience). Identification of TNF-α-producing retinal cells was accomplished by incubation with a second cell-specific antibody. Following three 5-min washes with phosphate-buffered saline, retinal sections initially reacted with anti-TNF-α antibody were again incubated at 4°C overnight in a humidified atmosphere with chicken anti-mouse glial fibrillary acidic protein (GFAP) antibody (1:500) (Abcam, Cambridge, MA) for identification of Müller cells, goat anti-mouse Iba-1 antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) for identification of retinal microglial cells, or rabbit anti-mouse Ly-6G antibody (1:100) (Santa Cruz Biotechnology) for identification of infiltrating granulocytes (neutrophils). Following three additional 5-min washes with phosphate-buffered saline, the retinal sections subsequently were incubated at room temperature for 1 h in a humidified atmosphere with donkey anti-chicken DyLight-594-labeled antibody (1:100) (Jackson ImmunoResearch, West Grove, PA) for visualization of TNF-α-producing, GFAP-positive Müller cells, goat anti-rabbit DyLight-594-labeled antibody (1:100) (Jackson ImmunoResearch) for visualization of TNF-α-producing, Iba-1-positive retinal microglial cells, or goat anti-rabbit DyLight-594-labeled antibody (1:100) (Jackson ImmunoResearch) for visualization of TNF-α-producing, infiltrating granulocytes (neutrophils). For visualization of TNF-α-producing, infiltrating macrophages, retinal sections were incubated with phycoerythrin (PE)-Texas red-conjugated rat anti-mouse F4/80 antibody (1:100) (Invitrogen) and rabbit anti-mouse TNF-α antibody (1:500) (Abcam) at 4°C overnight in a humidified atmosphere, followed by incubation with goat anti-rabbit DyLight-488 (1:100) (Jackson ImmunoResearch) for 1 h at room temperature. All antibody-treated retinal sections were ultimately washed three times for 5 min each with phosphate-buffered saline, mounted with medium containing DAPI (Vectorshield; VectoMax, Inc., villa Park, CA).
and examined and photographed by fluorescence microscopy.

**Statistical analysis.** Morphometric data for individual lesions in each eye were averaged to provide one value per eye. The mean and standard error of the mean for each group was calculated, and *P* values were determined using Student’s *t* test and the Wilcoxon rank sum test. *P* values of ≤0.05 were considered significant for all forms of statistical analysis used.

**RESULTS**

MCMV-infected eyes of MAIDS-10 animals, but not those of MAIDS-4 animals, show significant activation of genes encoding TNF-α, TNF-α receptors, and related caspases. We reported previously that MCMV-infected eyes of MAIDS-4 and MAIDS-10 animals harbor equivalent amounts of infectious virus (~3.0 log_{10} PFU/eye) at 10 days after subretinal MCMV infection; yet, MAIDS-4 mice are resistant to MCMV retinitis, whereas MAIDS-10 mice are susceptible to MCMV retinitis (15). This observation was confirmed and extended in new experiments whose results are shown in Fig. 1. As expected, histopathologic analysis revealed a progressive development of retinitis in MCMV-infected eyes of MAIDS-10 mice at 3, 6, and 10 days after subretinal MCMV inoculation that culminated in severe retinal necrosis at 10 days after inoculation (Fig. 1A). In sharp contrast, MCMV-infected eyes of MAIDS-4 mice exhibited some retinal folding and mild RPE proliferation but an absolute absence of retinal necrosis throughout the course of virus infection (Fig. 1A), as reported previously (15). In comparison, MCMV-infected eyes collected at 3, 6, and 10 days after subretinal MCMV inoculation of both MAIDS-4 and MAIDS-10 animals showed high and equivalent amounts of infectious virus (Fig. 1B).

Such diverse pathogenic outcomes following MCMV infection provided us the opportunity to compare MCMV-infected eyes of MAIDS-4 and MAIDS-10 animals for detection and quantification of various molecules associated with the TNF-α-induced apoptotic and antiapoptotic pathways (7–10). Whole MCMV-infected eyes were collected from groups of MAIDS-4 and MAIDS-10 animals at 3, 6, and 10 days after subretinal MCMV inoculation and subjected to RT-PCR assay for detection and quantification of TNF-α mRNA. Results are shown in Fig. 2A. MCMV-infected eyes of MAIDS-4 mice (resistant to retinitis) and MAIDS-10 mice (susceptible to retinitis) exhibited profound differences in patterns of TNF-α mRNA production. Whereas significant amounts of TNF-α mRNA were detected within MCMV-infected eyes of MAIDS-10 mice at all times during the course of virus infection and peaked at 6 days postinfection prior to the appearance of severe retinal necrosis at 10 days postinfection (Fig. 1A), levels of TNF-α mRNA within MCMV-infected eyes of MAIDS-4 mice were relatively modest or not detectable at all times examined. A similar pattern was observed for production of TNF-α protein when compared by Western blot analysis (Fig. 2B).

It is noteworthy that the same samples of MCMV-infected eyes used for detection and quantification of TNF-α mRNA and protein also exhibited significantly large amounts of mRNAs to two MCMV-encoded proteins, nonstructural immediate-early 1 (IE1) protein and late structural glycoprotein H (gH), indicating active and continuous virus replication within MCMV-infected eyes of both MAIDS-4 and MAIDS-10 animals (data not shown). Thus, MCMV-infected eyes that developed severe retinal necrosis exhibited a concomitant and significant upregulation of intraocular TNF-α mRNA and protein, but MCMV-infected eyes that did not develop severe retinal necrosis failed to produce significant amounts of intraocular TNF-α mRNA and protein. Moreover,
Detection of key apoptotic molecules (TNF-α, TNFR1, caspase 8, and caspase 3) and antiapoptotic molecule (TNFR2) in MCMV-infected eyes collected from groups of MAIDS-4 mice (n = 5) and MAIDS-10 mice (n = 5) at 3, 6, and 10 days after subretinal MCMV injection compared with contralateral, mock-infected eyes. (A) Levels (fold change) of TNF-α mRNA, TNFR1 mRNA, TNFR2 mRNA, caspase 8 mRNA, and caspase 3 mRNA were determined by quantitative RT-PCR assay. (B) Detection of TNF-α protein, active caspase 8 protein, active caspase 3 protein, and GAPDH protein (control) by Western blot analysis. Results for all are averages of three independent experiments. Bars indicate standard errors (*, P < 0.05; **, P < 0.001).
outcome was independent of active and continuous intraocular virus replication.

Since TNF-α is known to drive apoptosis and to play an anti-apoptotic role via the distinct receptors TNFR1 and TNFR2, respectively (3, 70), we compared MCMV-infected eyes of MAIDS-4 and MAIDS-10 animals for detection and quantification of TNFRI and TNFR2 mRNA levels at 3, 6, and 10 days after subretinal MCMV inoculation. Results shown in Fig. 2A paralleled those found for TNF-α. Little to no TNFR1 mRNA or TNFR2 mRNA was detected within MCMV-infected eyes of MAIDS-4 mice at all times investigated after subretinal virus inoculation. In sharp contrast, MCMV-infected eyes of MAIDS-10 mice showed increased levels of mRNA to both TNFR1 and TNFR2 at day 3 postinfection that significantly increased and peaked at day 6 postinfection prior to the appearance of severe retinal necrosis at day 10 postinfection, when TNFR1 and TNFR2 mRNA levels decreased dramatically. Although a significant increase in apoptosis-related TNFR1 was expected concomitant with the appearance of retinal pathology, the 60-fold increase in TNFR2 mRNA was unexpected, suggesting that TNF-α-driven antiapoptotic forces also operate to a far greater extent than originally appreciated during MAIDS-related MCMV retinitis.

The TNF-α-induced apoptotic pathway involves key proteins that include active (cleaved) caspase 8 and active (cleaved) caspase 3 (3, 7, 59, 70). If TNF-α-induced apoptosis is involved in onset and progression of retinal disease during MAIDS-related MCMV retinitis, we should detect evidence for upregulation of active (cleaved) caspase 8 and active (cleaved) caspase 3 within MCMV-infected eyes during development of retinitis. To confirm this prediction, the eyes of MAIDS-10 mice were inoculated subretinally with MCMV and collected 3, 6, and 10 days later for quantification of active (cleaved) caspase 8 and active (cleaved) caspase 3 mRNAs. Both molecules were detected in significantly large amounts (Fig. 2A) within MCMV-infected eyes in a pattern relative to virus infection that mimicked production for TNF-α, TNFR1, and TNFR2 mRNAs. Specifically, peak levels of the active forms of caspase 8 and caspase 3 mRNAs were observed at day 6 after MCMV infection and prior to the appearance of severe retinal necrosis. Evidence that caspase 8 and caspase 3 mRNAs were translated into active (cleaved) caspase 8 and caspase 3 proteins was provided by Western blot analysis of MCMV-infected eyes of MAIDS-10 mice collected at days 3, 6, and 10 after subretinal virus inoculation (Fig. 2B). Since MCMV-infected eyes of MAIDS-4 mice resistant to retinal disease failed to show significant increases in TNF-α, TNFR1, and TNFR2 mRNAs, it was not surprising to find that these eyes also did not contain significantly large amounts of active caspase 8 and caspase 3 proteins.

Apoptosis may also be induced during MAIDS-related MCMV retinitis by TRAIL and Fas/FasL cell death pathways but not by the apoptosome complex. Other cell death pathways have been identified that culminate in apoptosis (19), among them TRAIL and its receptors TRAIL-R(DR4) and TRAIL-R(DR5) (20, 34), the Fas/FasL cell death pathway (10, 18, 20, 71), and the mitochondrion-associated apoptosome, a multimeric complex consisting of cytochrome c, Apaf-1, and caspase 9 (11, 42). Whereas the TRAIL cell death pathway is TNF-α dependent (and therefore caspase 8 dependent), the apoptosis-inducing Fas/FasL pathway and the apoptosome are TNF-α independent. Since we initially investigated the TNF-α/TNFRI-induced apoptotic pathway relative to the onset and progression of MAIDS-related MCMV retinitis (Fig. 2), we also compared MCMV-infected eyes from MAIDS-4 and MAIDS-10 animals for detection and quantification of transcripts specific for key molecules associated with these other apoptotic pathways. As expected, significant amounts of TRAIL and TRAIL-R(DR5) mRNAs as well as Fas and FasL mRNAs were detected in MCMV-infected eyes of MAIDS-10 mice (susceptible to retinitis), with peak amounts seen at 6 days after subretinal MCMV infection (Fig. 3A and B). In comparison, MCMV-infected eyes of MAIDS-4 mice (resistant to retinitis) failed to show production of significant amounts of mRNAs to TRAIL, TRAIL-R(DR5), Fas, and Fasl at all times examined. To our surprise, however, was the finding that MCMV-infected eyes of both MAIDS-4 mice and MAIDS-10 mice consistently showed no detectable production or less than a 2-fold production of mRNAs to cytochrome c, Apaf-1, and caspase 9 (Fig. 3C), all key molecules associated with apoptosis-driven apoptosis. Thus, whereas the TNF-α-associated TRAIL and Fas/FasL apoptotic cell death pathways appear to be stimulated during the pathogenesis of MAIDS-related MCMV retinitis, the apoptosome apparently plays no role in MCMV-induced retinal tissue destruction during MAIDS.

Several cell populations within retinal tissue of MCMV-infected eyes of MAIDS-10 animals serve as sources for TNF-α production. Previous clinical studies (45) and experimental studies (76) have suggested that infiltrating macrophages may serve as a potential source for TNF-α production during development of cytomegalovirus retinitis in immunosuppressed hosts. To confirm and extend these findings using our MAIDS model of MCMV retinitis, frozen sections of MCMV-infected eyes collected from MAIDS-10 mice at 10 days postinfection were investigated using antibodies against individual cell-specific markers to identify TNF-α-producing cells among candidate cell populations within areas of retinal tissues showing severe pathology. Specifically, retinal sections were assessed using antibodies against F4/80 antigen as a marker for infiltrating macrophages, LY-6G antigen as a marker for infiltrating granulocytes (neutrophils), GFAP antigen as a marker for resident Müller cells, and Iba-1 antigen as a marker for resident microglial cells. Results are shown in Fig. 4. In agreement with previous findings from other laboratories (45, 76), TNF-α production was detected in infiltrating macrophages (Fig. 4A) within retinal tissues of MCMV-infected eyes with retinitis. In addition, TNF-α production was also detected in infiltrating granulocytes (neutrophils) (Fig. 4B) as well as resident Muller cells (Fig. 4C) and resident microglial cells (Fig. 4D). Thus, many diverse cell types may serve as possible sources for TNF-α production during MAIDS-related MCMV retinitis. These include infiltrating proinflammatory cells (macrophages and neutrophils) as well as resident retinal cells (Müller cells and microglial cells).

MCMV retinitis is reduced, but detectable, in TNF-α−/− mice with MAIDS, TNFRI−/− mice with MAIDS, and TNFR2−/− mice with MAIDS. To define with greater precision the contribution of TNF-α-induced apoptosis toward onset and progression of MCMV retinitis during MAIDS, we included MAIDS of 10 weeks’ duration in C57BL/6 mice deficient in TNF-α (TNFKO MAIDS mice), TNFRI (TNFRIKO MAIDS mice), or TNFR2 (TNFR2KO MAIDS mice). All TNF-α−/− mice, TNFRI−/− mice, and TNFR2−/− mice infected with the immunosuppressive murine retrovirus mixture (LP-BM5 MuLV) exhibited physical and immunologic characteristics (16) consistent with the development of MAIDS (data not shown). The eyes of
FIG 3 Detection of key apoptotic molecules [TRAIL, TRAIL-R(DR5), Fas, Fasl, and those of the apoptosome (caspase 9, cytochrome c, and Apaf-1)] in MCMV-infected eyes collected from groups of MAIDS-4 mice (n = 5) and MAIDS-10 mice (n = 5) at 3, 6, and 10 days after subretinal MCMV injection compared with contralateral, mock-infected eyes. Levels (fold change) of TRAIL mRNA and TRAIL-R(DR5) mRNA (A), Fas mRNA and Fasl mRNA (B), and caspase 9 mRNA, cytochrome c mRNA, and Apaf-1 mRNA (C) were all determined by quantitative RT-PCR assay. Bars indicate standard errors (**, *P < 0.001).
groups of wild-type C57BL/6 mice with MAIDS and the eyes of groups of TNFKO MAIDS mice, TNFR1 MAIDS mice, and TNFR2 MAIDS mice were infected with MCMV by subretinal inoculation, collected 10 days later, and either scored after histopathologic analysis for frequency and severity of MCMV retinitis using criteria established by us previously (16) or compared for amounts of infectious virus by standard plaque assay. Whereas 9 of 10 (90%) MCMV-infected eyes of wild-type mice with MAIDS exhibited retinitis (full-thickness retinal necrosis) (Fig. 5A), we were surprised to detect full-thickness retinal necrosis in MCMV-infected eyes of 2 of 10 (20%) TNFKO MAIDS mice, 1 of 6 (17%) TNFR1KO MAIDS mice, and 2 of 6 (33%) TNFR2KO MAIDS mice. Moreover, scoring of all eyes with detectable MCMV retinitis for degree of severity (16) indicated moderate to severe retinal disease (data not shown). A comparison of MCMV-infected eyes of TNFKO MAIDS mice, TNFR1 MAIDS mice, and TNFR2 MAIDS mice with MCMV-infected eyes of wild-type mice with MAIDS showed no significant differences in intraocular amounts of infectious virus as detected by plaque assay (Fig. 4B). Thus, the frequency of retinal necrosis was reduced, but not abolished, in MCMV-infected eyes of mice with MAIDS deficient in TNF-α, TNFRI, or TNFR2 despite a level of virus replication equivalent to that observed in MCMV-infected eyes of wild-type mice with MAIDS that exhibited high (90%) susceptibility to retinitis. It is also noteworthy that the pattern of retinal pathology was consistent with that of classic MCMV retinitis (16) in all mice with MAIDS deficient in TNF-α, TNFRI, or TNFR2. A new pattern of retinal pathology did not emerge.

Given our unexpected finding of reduced yet detectable retinitis of ~23% (average frequency of moderate to severe retinitis) in MAIDS mice deficient in TNF-α (20% frequency of retinitis), TNFRI (17% frequency of retinitis), or TNFR2 (33% frequency of retinitis), we were curious to know the levels of production of TNF-α, TNFRI, and TNFR2 mRNAs as well as apoptosis-related caspase 8 and caspase 3 mRNAs within MCMV-infected eyes of these animal groups. An initial study therefore focused on a comparison of MCMV-infected eyes of wild-type mice with MAIDS versus MCMV-infected eyes of TNFKO MAIDS mice at 10 days after subretinal MCMV infection. Results are shown in Fig. 6A. MCMV-infected eyes of TNFKO MAIDS mice showed a predictable lack of detection of TNF-α mRNA as well as a significant reduction in TNFRI mRNA. We were surprised, however, by the observation of dampened yet continuous production of caspase 8 and caspase 3 mRNAs within MCMV-infected eyes of TNFKO MAIDS mice that showed 20% susceptibility to retinitis compared with production within MCMV-infected eyes of wild-type mice with MAIDS that showed 90% susceptibility to retinitis. Moreover, the level of TNFR2 mRNA within MCMV-infected eyes of TNFR2 MAIDS mice was equivalent to that observed in MCMV-infected eyes of wild-type mice with MAIDS.

A second study focused on a comparison of MCMV-infected eyes of wild-type mice with MAIDS versus MCMV-infected eyes of MAIDS mice deficient in either TNFRI or TNFR2. As shown in Fig. 6B, loss of TNFRI during MAIDS did not impact TNF-α mRNA production negatively within MCMV-infected eyes of TNFRI MAIDS mice but instead led to increased TNF-α mRNA production. Caspase 8 mRNA levels decreased significantly, but caspase 3 mRNA remained at levels equivalent to those found in
MCMV-infected eyes of wild-type mice with MAIDS. Loss of TNFR1 mRNA during MAIDS also resulted in a significant increase in antiapoptotic TNFR2 mRNA production within MCMV-infected eyes. In comparison, loss of TNFR2 mRNA during MAIDS resulted in a profound stimulation of TNF-α mRNA within MCMV-infected eyes, doubling levels found within MCMV-infected eyes of wild-type mice with MAIDS (Fig. 6B). However, despite significantly high levels of intraocular TNF-α mRNA, TNFR1 mRNA levels did not appreciably change compared with those intraocular levels found in wild-type mice with MAIDS, and mRNA levels of both proapoptotic proteins, caspase 8 and caspase 3, decreased significantly. Taken together, these results may explain a decreased yet detectable frequency and severity of MCMV retinitis in mice with MAIDS deficient in TNF-α, TNFR1, or TNFR2.

Apoptotic cells can be detected by TUNEL assay within retinal sections of MCMV-infected eyes of wild-type MAIDS mice, TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice. TUNEL assay is an established method for detecting cells that have undergone DNA fragmentation associated with apoptosis (33, 57, 58). We therefore initially employed the TUNEL assay to detect apoptotic cells and determine the pattern of their distribution within retinal sections of MCMV-infected eyes of wild-type MAIDS-10 mice at 3, 6, and 10 days after subretinal MCMV inoculation. While contralateral, mock-infected eyes showed no detectable TUNEL-positive cells at all times examined, TUNEL-positive cells were detected, albeit sparsely, within retinal sections of wild-type MAIDS-10 mice at 3 days postinfection that showed only retinal folding (Fig. 7D). The number of TUNEL-positive cells, however, increased thereafter during progression of retinal disease. A substantial increase in TUNEL-positive cells was observed within retinal sections at 6 days postinfection, with a distribution that suggested localization within the RPE and neurosensory retina (Fig. 7E). This was followed by diffuse distribution of TUNEL-positive cells within retinal sections at 10 days postinfection, when severe retinal disease was apparent (Fig. 7F). We therefore observed a pattern of detectable TUNEL-positive cells during progression of retinitis in MCMV-infected eyes of wild-type MAIDS-10 mice that correlated directly with our previous findings regarding appearance and amounts of various TNF-α-induced apoptosis-related molecules (Fig. 2).

Since we detected TUNEL-positive cells within retinal sections of MCMV-infected eyes of wild-type MAIDS-10 mice in a pattern that coincided with progression of retinal disease following subretinal MCMV inoculation, we subsequently elected to perform TUNEL assays on retinal sections of TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice, which all showed decreased yet detectable retinitis (Fig. 5A), to determine the extent of apoptosis in MCMV-infected eyes of these mice with MAIDS that were deficient in TNF-α, TNFR1, or TNFR2. While contralateral, mock-infected eyes showed no detectable TUNEL-positive cells, retinal sections from MCMV-infected mice collected from TNFKO MAIDS mice (Fig. 8D), TNFR1KO MAIDS mice (Fig. 8E), and TNFR2KO MAIDS mice (Fig. 8F) all showed detectable TUNEL-positive cells. These TUNEL-positive cells were diffusely distributed in numbers less than those found within retinal sections of MCMV-infected eyes of wild-type MAIDS-10 mice (Fig. 7F). Thus, we observed TUNEL-positive cells in MCMV-infected retinal sections of TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice in a pattern in agreement with our previous findings showing reduced yet detectable susceptibility to MCMV retinitis in these animal groups deficient in TNF-α, TNFR1, or TNFR2 (Fig. 5, 6, and 7), respectively.

Quantification of apoptotic cells by TUNEL assay suggests minimal contribution of apoptosis to retinal disease during MAIDS-related MCMV retinitis. Although our findings suggest that TNF-α-induced apoptosis as a pathogenic mechanism contributes to the progression of retinal disease during MAIDS-related MCMV retinitis, we were curious as to the extent of this involvement. TUNEL assay was therefore used to quantify the number of apoptotic cells at different times during development of retinitis within retinal sections of MCMV-infected eyes collected at 3, 6, and 10 days after subretinal MCMV inoculation of wild-type MAIDS-10 mice. For quantification, 3 retinal sections each from MCMV-infected eyes of 5 individual wild-type mice with MAIDS-10 were scored and averaged for TUNEL-positive cells. Compared with those in TUNEL-negative and TUNEL-positive controls (see Materials and Methods), the number of TUNEL-positive cells within retinal sections of MCMV-infected eyes of wild-type MAIDS-10 mice was relatively low at 3 days postinfection but increased significantly and peaked at 6 days

**FIG 5** Analysis of MCMV-infected eyes collected from groups of wild-type MAIDS mice, TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice at 10 days after subretinal MCMV injection. All animals were analyzed at 10 weeks after injection with the immunosuppressive retrovirus mixture LP-BM5 MuLV. (A) Frequency of eyes showing MCMV retinitis (full-thickness retinal necrosis) following histopathologic analysis (16). Number of eyes positive versus total number of eyes examined per animal group is indicated. (B) Amounts of infectious MCMV detected in whole eyes as determined by standard plaque assay (see Materials and Methods). Bars indicate standard errors.
postinfection, subsequently decreasing at 10 days postinfection (Fig. 9B), the time of development of the most severe retinal disease. Importantly, however, the percentage of TUNEL-positive retinal cells within MCMV-infected eyes of wild-type MAIDS-10 mice was remarkably low, even at its peak. Only ~8% of retinal cells were found to be TUNEL positive at 6 days after MCMV infection, and this number decreased to ~4% at 10 days postinfection. This extraordinary observation suggests that while apoptosis contributes to retinal tissue destruction during the evolution of MAIDS-related MCMV retinitis, its pathogenic contribution is relatively minimal.

Since our previous studies showed reduced susceptibility of TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice to MCMV retinitis (Fig. 5A), it was of interest to quantify the number of TUNEL-positive cells within retinal sections of MCMV-infected eyes of these animal groups at day 10 postinfection. Results are shown in Fig. 9B. While retinal sections of MCMV-infected eyes of wild-type MAIDS-10 mice showed TUNEL positivity in 4.4% of retinal cells at 10 days postinfection, the quantities of TUNEL-positive cells in MAIDS mice deficient in TNF-α, TNFR1, or TNFR2 at 10 days postinfection were 2.7%, 2.1%, and 1.7%, respectively. Thus, the number of TUNEL-positive cells was significantly lower within retinal sections of MCMV-infected eyes of TNFKO MAIDS mice, TNFR1KO MAIDS mice, and TNFR2KO MAIDS mice, which all showed a significant decrease in frequency of retinitis compared with wild-type MAIDS-10 mice. A direct correlation was therefore observed between frequency of retinitis and percentage of TUNEL-positive retinal cells, an observation that also suggests a contribution of apoptosis toward retinal tissue destruction during MAIDS-related MCMV retinitis, albeit minimally.

Necroptosis-related molecules are detectable within MCMV-infected eyes of mice with MAIDS during progression of retinal tissue destruction. Since we showed evidence for operation of the TNF-α-induced apoptotic pathway during MAIDS-related MCMV retinitis, but also evidence that apoptosis as measured by TUNEL assay contributed minimally to retinal tissue destruction during the disease process, we explored the possibility that other cell death mechanisms in addition to apoptosis might also contribute to the pathogenesis of MCMV retinitis during MAIDS. This was accomplished by performance of experiments designed to detect and quantify key molecules unique to two other cell death pathways, necroptosis and pyroptosis.

Necroptosis is a form of TNF-α-induced programmed necro-
Evidence that necroptosis might operate during development of MAIDS-related MCMV retinitis was provided by detection of significant levels of RIP1 mRNA within MCMV-infected eyes of MAIDS-10 mice that peaked at 6 days after subretinal infection (Fig. 10A). In comparison, RIP1 mRNA was either not detectable or detected at very small amounts within MCMV-infected eyes of MAIDS-4 mice. A very different pattern of RIP3 mRNA synthesis was observed, however, within MCMV-infected eyes of the same MAIDS-4 and MAIDS-10 animals. As expected, MCMV-infected eyes of MAIDS-4 mice (resistant to retinitis) showed relatively no detectable intraocular levels of RIP3 mRNA at all times examined after subretinal MCMV infection (Fig. 10B). Surprisingly, however, MCMV-infected eyes of MAIDS-10 mice (susceptible to retinitis) exhibited a relatively modest yet detectable increase in intraocular RIP3 mRNA levels at day 6 postinfection, but not at the high levels anticipated for a key molecule expected to play a major role in necroptosis. Thus, the patterns of intraocular expression of necroptosis-dependent RIP1 and RIP3 mRNAs within MCMV-infected eyes were unexpected. Whereas the pattern of RIP1 mRNA production within MCMV-infected eyes resistant or susceptible to retinitis resembled that for mRNAs of apoptosis-related proteins, the patterns of RIP3 mRNA production within these two eye groups were remarkably different and distinct, suggesting involvement of RIP1-dependent, but not RIP3-dependent, necroptosis in development of MAIDS-related MCMV retinitis.

Pyroptosis-related molecules are detectable within MCMV-infected eyes of mice with MAIDS during progression of retinal tissue destruction. To assess a possible role for pyroptosis during development of MAIDS-related MCMV retinitis, we compared MCMV-infected eyes of MAIDS-4 mice and MAIDS-10 mice for detection and quantification of mRNAs to caspase 1, IL-1β, and IL-18, all markers for the pyroptotic pathway (5). Results are shown in Fig. 11. Whereas MCMV-infected eyes collected from MAIDS-4 mice (resistant to retinitis) showed only modest levels of mRNAs to caspase 1, IL-1β, and IL-18, MCMV-infected eyes collected from MAIDS-10 mice (susceptible to retinitis) showed significantly higher levels of mRNAs to caspase 1, IL-1β, and IL-18 that collectively peaked at 6 days postinfection. Of these, the most impressive increase in mRNA level was observed for that of IL-1β, which showed a 786-fold increase in mRNA compared with the IL-1β mRNA levels of contralateral, mock-infected eyes (Fig. 11C).

DISCUSSION

Since its appearance as a major cause of vision loss and blindness within the United States nearly 30 years ago, AIDS-related HCMV retinitis has become well characterized clinically and histologically (48). Despite many years of extensive clinical and laboratory investigation, however, a number of basic issues related to the virology, immunology, and pathogenesis of this sight-threatening disease within the unique immunosuppressive environment of HIV infection and disease remain unresolved. Among these is a crisp and comprehensive understanding of the basic pathogenic mechanisms and cell death pathways that operate during development of the retinal disease known as AIDS-related HCMV retinitis. This disease is also referred to as a necrotizing retinitis or retinal necrosis; these descriptors may be not only naïve but also perhaps incomplete when describing the severe retinal tissue destruction caused by HCMV infection of the retina during HIV/AIDS. While
virus-induced cytopathology no doubt contributes to retinal tissue destruction as evidenced by the appearance of cytomegalic cells during AIDS (46), programmed cell death pathways should also be considered. These include apoptosis, necroptosis, and pyroptosis.

Apoptosis is the best understood of the cell death pathways. Unlike the passive cell death of general necrosis, which involves progressive degradation and digestion of the injured cell (including its DNA) by the unregulated action of a number of cellular enzymes (50), apoptosis is an active, tightly regulated pathway of cell death that involves activation of several members of the caspase family of cysteine proteases and ultimate breakdown of DNA into oligonucleosomes of defined size by endonucleases (41). Moreover, apoptosis can be induced by a number of signaling proteins in concert with their specific death receptors (19). For example, TRAIL induces apoptosis via a caspase 8-dependent pathway after binding to the death receptors TRAIL-R(DR4) and TRAIL-R(DR5) (20, 34). FasL, a member of the TNF family, can also induce apoptosis via a caspase-independent pathway after binding to the Fas receptor (10, 18, 20, 71). Importantly, Fas-induced apoptosis as well as apoptosis induced by the perforin and granzyme B pathway are two mechanisms by which cytotoxic T cells induce death in cells expressing foreign antigens (10, 51).

New evidence for the participation of both the TRAIL/TRAIL-R(DR5) and Fas/FasL pathways toward induction of apoptosis during onset and progression of retinitis in MCMV-infected eyes of MAIDS-10 mice (susceptible to retinitis), but not in those of MAIDS-4 mice (resistant to retinitis), is presented in our investigation. In comparison, key molecules that participate in apoptosis induced by the apoptosome (11, 42), i.e., cytochrome c, Apaf-1, and caspase 9, were not upregulated in MCMV-infected eyes of either MAIDS-10 or MAIDS-4 animals, a finding that suggests no significant role for this mitochondrion-associated mechanism of apoptosis induction during the pathogenesis of MAIDS-related...
MCMV retinitis. It is noteworthy that previous work by Fleck and coworkers (26) using C57BL/6 mice deficient in both Fas (lpr mice) and TNFR1 exhibited greater susceptibility to systemic MCMV infection but that loss of Fas alone seemed more important in a chronic inflammatory response. While the role of Fas has not been investigated in the context of MCMV retinitis using lpr mice, we have previously investigated the role of FasL in MCMV retinitis using gld mice without MAIDS and reported that these animals were resistant to MCMV retinitis following subretinal MCMV injection (14). The precise contributions of the TRAIL/TRAIL-R(DR5) and Fas/FasL cell death pathways toward induction of apoptosis during the pathogenesis of MAIDS-related MCMV retinitis await further investigation by us and others.

Until our investigation, TNF-α-induced apoptosis received exclusive attention with respect to AIDS-related HCMV retinal disease. Following initial discoveries that TNF-α is expressed at elevated levels within the eyes of patients with AIDS-related HCMV retinitis (45, 55) and within the eyes of mice with MAIDS-related MCMV retinitis (15), Zhou and coworkers (76) used methylprednisolone acetate-immunosuppressed mice to show that TNF-α can induce apoptosis during progression of experimental MCMV retinal disease via the TNFR1 pathway. We also demonstrated herein a role for this apoptotic pathway during MAIDS by comparing MCMV-infected eyes of MAIDS-4 mice (resistant to retinitis) with MCMV-infected eyes of MAIDS-10 mice (susceptible to retinitis) for key TNFRI/TNF-α apoptosis-related molecules. For all key molecules examined (TNFR1, TNF-α, caspase 8, and caspase 3 mRNAs as well as their respective active proteins), significant amounts of these molecules were consistently observed in MCMV-infected eyes of MAIDS-10 mice but not in those of MAIDS-4 mice, despite the fact that equivalent amounts of infectious virus were observed in MCMV-infected eyes of both animal groups. Importantly, levels of transcripts of all key TNFRI/TNF-α apoptosis-related molecules in MCMV-infected eyes of MAIDS-10 mice exhibited a unique and consistent pattern of synthesis characterized by a significant increase in production from 3 to 6 days after infection, followed by a significant decrease in production from 6 to 10 days after infection to levels found in control eyes. Thus, levels of transcripts of all key TNFRI/TNF-α apoptosis-related molecules in MCMV-infected eyes of MAIDS-10 mice peaked at day 6 after infection and prior to development of severe retinal disease that was observed at 10 days after infection.

It is noteworthy that this pattern of mRNA production was not observed by Zhou et al. (76) during development of retinal disease in MCMV-infected eyes of mice immunosuppressed by methylprednisolone acetate. In their animals, TNF-α, TNFR1, and caspase 8 mRNA levels increased sharply at day 6 but consistently remained elevated without reduction at day 10 after infection. One interpretation of these discrepant findings is that the temporal pattern by which key TNFRI/TNF-α-induced apoptotic molecules are produced during development of retinal disease in MCMV-infected eyes may vary depending on different conditions of immunosuppression. Systemic treatment of mice with methylprednisolone acetate induces within days a global immunosuppressive environment that favors Th2 CD4+ T cells, CD8+ T cells, and macrophages (74, 76). In contrast, systemic treatment with murine retroviruses to induce MAIDS results in a gradual progression over weeks toward an immunosuppressive environment that favors Th2 CD4+ T cells over Th1 CD4+ T cells and reduction in function of CD8+ T cells, but
without loss of macrophages (56). These profoundly different protocols for immunosuppression might also explain our differences regarding sources for TNF-α production in MCMV-infected retinal tissues. Although there is agreement that infiltrating macrophages (F4/80-positive cells) are a source of TNF-α during progression of MCMV retinal disease, we also frequently detected TNF-α production by GFAP-positive glial cells (Müller cells) during MAIDS-related MCMV retinitis, whereas Zhou et al. (76) rarely observed GFAP-positive cells as a source of TNF-α in MCMV-infected eyes of mice immunosuppressed by methylprednisolone acetate treatment. Using our MAIDS model of MCMV retinitis, we also extended sources for TNF-α production to include infiltrating neutrophils (LY-6G-positive cells) as well as resident microglial cells (Iba-1-positive cells). We did not investigate RPE as a possible source for TNF-α, although Zhou et al. (76) reported positive staining in these cells.

That apoptosis contributes to the evolution of experimental MCMV retinitis was first demonstrated by Bigger et al. (6), who detected apoptotic cells by TUNEL assay in retinal tissues of MCMV-infected eyes collected from immunocompetent BALB/c mice as well as methylprednisolone acetate-immunosuppressed BALB/c mice. Quantification was not performed. Apoptosis was therefore considered by default to be the predominant, if not exclusive, mechanism by which retinal pathology develops following onset of AIDS-related HCMV retinitis. Our findings using the MAIDS model of MCMV retinitis challenge this assumption by providing new quantitative data that suggest that apoptosis as measured by TUNEL assay actually contributes minimally to MCMV-induced retinal disease during retrovirus-induced immunosuppression. Quantification of TUNEL-positive cells in retinal tissues of MCMV-infected eyes of MAIDS-10 mice at day 10 after infection, when retinal pathology is most severe and 90% of mice were susceptible to retinal disease, revealed only ~4% of retinal cells being TUNEL positive. Even at day 6 after infection, when all key TNFR1/TNF-α apoptosis-related molecules are at peak amounts, only ~11% of retinal cells were TUNEL positive. These findings therefore differ remarkably from those of Zhang et al. (74). While deficient in TNF-α, the MCMV-infected eyes of our retrovirus-immunosuppressed animals nonetheless harbored infectious virus in amounts equivalent to those of wild-type mice with MAIDS. Although frequency of retinal disease was not addressed, Zhang et al. (74) reported
more TUNEL-positive cells in retinal tissues of MCMV-infected TNF-α−/− mice immunosuppressed by methylprednisolone acetate treatment than in MCMV-infected eyes of wild-type mice treated with the immunosuppressive drug. Once again, differences in immunosuppressive protocols may account for this difference in findings.

At least two events, possibly working in concert, could explain our observation that apoptosis contributes minimally to retinal disease during MAIDS-related MCMV retinitis. First, while the signaling pathway initiated by TNFR1 is stimulated during development of MCMV retinal disease during systemic immunosuppression, whether it is drug-induced or retrovirus-induced, the signaling pathway(s) initiated by TNFR2 may also be stimulated during MCMV infection of eyes of mice with MAIDS as evidenced by significant upregulation of TNFR2 mRNA levels in a pattern identical to that observed for TNFR1 mRNA. The precise consequence(s) of TNFR2 stimulation on the pathogenesis of MAIDS-related MCMV retinal disease remains uncertain since the signaling pathway(s) initiated by TNFR2 is less well characterized than the TNFR1-initiated signaling pathway that stimulates apoptosis. Nevertheless, there is general agreement that TNFR2 appears to signal outcomes both shared with and opposite to that of TNFR1 and, unlike TNFR1, may independently mediate signals that promote tissue repair and angiogenesis (7, 59). Thus, intracellular increase in TNF-α production during the course of MCMV retinal disease development may place into motion two opposing effects, one promoting the death of retinal cells by apoptosis and the other promoting survival of retinal cells despite stimulation of TNFR1/TNF-α-induced apoptosis. A similar antiapoptotic role for TNF-α was suggested by Zhang et al. (74), although these workers did not investigate TNFR2 directly during the course of experimental MCMV retinal disease development.

Thus, TNF-α might promote retinal cell survival during MAIDS-related MCMV retinitis via the TNFR2 signaling pathway(s) and counteract retinal cell death by apoptosis induced by the TNFR1 signaling pathway. Second, several genes encoding suppressors of apoptosis have been identified in the genomes of HCMV and MCMV (reviewed by Brune) (8). Of these, the most attention has been given to the HCMV gene open reading frame (ORF) UL37x1, encoding vMIA (29), and the MCMV gene m38.5, encoding vB0 (52). Both gene products are thought to inhibit apoptosis at the mitochondrial level, possibly by functioning as cellular antiapoptotic proteins Bcl-x and Bax, respectively (8). Zhang et al. (74) suggested a similar mitochondrion-targeting antiapoptotic role for TNF-α during development of MCMV retinal disease in methylprednisolone acetate-immunosuppressed mice. Thus, we envision two counteractive forces at play during the evolution of MCMV retinal infection, one promoting apoptosis through the TNFR1/TNF-α pathway and the other protecting against apoptosis through the combined efforts of the TNFR2/TNF-α pathway and MCMV-encoded antiapoptotic gene products. We further postulate that retinal tissue destruction is actually minimized by the apoptotic cell death pathway as antiapoptotic forces counteract and dampen TNFR1/TNF-α-induced apoptosis.

If TNFR1/TNF-α-induced apoptosis were the exclusive cell death pathway by which retinal tissue destruction evolves during MAIDS-related MCMV retinitis, we would predict that mice with MAIDS deficient in TNF-α or TNFRI would remain resistant to MCMV retinitis. This was not our finding. MCMV-infected eyes of mice with MAIDS deficient in TNF-α (TNFKO MAIDS mice) or deficient in TNFR1 (TNFR1KO MAIDS mice) continued to exhibit susceptibility to retinitis, albeit at a reduced frequency. This observation prompted us to investigate other cell death pathways that might also operate in combination with apoptosis during MAIDS-related MCMV retinitis, focusing our attention on necroptosis and pyroptosis.

Necroptosis (31, 43) is a specialized TNF-α-induced caspase-independent programmed cell death process that results in early membrane and organelle swelling, followed by cell lysis (necrosis) via regulated signal transduction pathways mediated by receptor-interacting protein (RIP) kinases, especially when caspase 8 activity is compromised (23, 35, 54). RIP1, a serine-threonine protein kinase, promotes necroptosis through mitochondrial membrane permeabilization that is mediated by a Bcl-2 family member protein, Bmf (43). During apoptosis, RIP1 is cleaved and inactivated by caspase 8 (22). However, when caspase 8 is inhibited or cannot be activated efficiently, RIP1 is thought to mediate necroptosis through expression of another receptor-interacting protein kinase, RIP3, that leads to reactive oxygen species production and necroptotic cell death (12, 38, 75). While the precise interaction between RIP1 and RIP3 during development of necroptosis remains unclear, RIP3, for now, appears to be a key regulator of RIP1 kinase activation (30), and its level of expression correlates with responsiveness to programmed necrosis (38). Necroptosis has been shown recently to operate in the eye during experimental retinal detachment-induced photoreceptor death in rats when apoptosis is prevented by caspase inhibition (65), and during neuronal death associated with experimental retinal ischemia in rats (62). In the present investigation, we obtained new evidence that necroptosis may also operate during progression of MAIDS-related MCMV retinitis. Specifically, we observed a significant and dramatic upregulation of RIP1 mRNA levels within MCMV-infected eyes of MAIDS-10 mice (susceptible to retinitis) but not within those of MAIDS-4 mice (resistant to retinitis). Moreover, the pattern of RIP1 mRNA synthesis was similar to that observed for key molecules involved in the TNFR1/TNF-α apoptotic pathway; i.e., peak amounts were observed at day 6 after infection and prior to the appearance of severe retinal disease at day 10. Surprisingly, however, a similar pattern of synthesis was not observed for mRNA of RIP3, another necroptosis-related molecule. MCMV-infected eyes of both MAIDS-10 and MAIDS-4 animals showed only minimal increases of RIP3 mRNA throughout the course of disease development. This remarkable finding suggests that if RIP1-mediated programmed necrosis contributes to retinal tissue destruction during MAIDS-related MCMV retinitis, it does so without the need for significant upregulation of RIP3 mRNA. Present studies are oriented toward further understanding this unexpected observation.

As with apoptosis (8, 29, 52), MCMV also encodes a gene product that serves to modulate necroptosis during infection. Upton and coworkers (66) initially demonstrated that the M45 protein of MCMV suppresses necroptotic cell death in vitro by interaction with RIP1 via a RIP homotypic interaction motif (RHIP). Suppression of necroptosis is thought to take place soon after infection since M45 is a structural tegument protein that is delivered to cells upon virion entry, and therefore, it would be available for interaction with the RIPH modulator of RIP1 for immediate suppression of necroptosis. Moreover, since M45 is required for protection from MCMV-induced cell death in endothelial cells and macrophages (9), M45-mediated suppression of necroptosis may
also represent an important cell tropism determinant. UL45 (36), the HCMV homologue to MCMV M45, awaits investigation to determine if it, too, possesses an analogous function with respect to necrotic suppression. More recent in vitro work by Upton et al. (67) has suggested that induction of necroptosis following MCMV infection may be RIP3 mediated and proceed independently of RIP1 but that MCMV can nonetheless inhibit RIP3-mediated necroptosis through a virus M45-encoded inhibitor of RIP activation, vIRA. DNA-dependent activator of interferon regulatory factors (DAI), a cytosolic double-stranded DNA sensor that activates beta interferon expression associated with the innate immune response (63, 72), appears to interact with RIP3 to mediate MCMV-induced necroptosis (68). Whether these in vitro findings regarding MCMV infection and necroptosis can be duplicated in vivo using our MAIDS model of MCMV retinitis remains to be determined.

Pyroptosis (5) is an inflammatory process of caspase 1-dependent cell death that is morphologically and mechanistically distinct from apoptosis and necroptosis. Cells undergoing pyroptosis develop pores within their plasma membranes of sufficient size to allow a net increase in osmotic pressure, water influx, cell swelling, osmotic lysis, and eventually the release of intracellular contents that promote inflammation, including release of the inflammatory cytokines IL-18 and IL-1β. While pyroptosis results in cleavage of chromosomal DNA, this cleavage does not result in the oligonucleosomal fragments observed during apoptosis (4, 25, 73), and unlike the nuclear fragmentation observed during apoptosis, nuclear integrity is maintained during pyroptosis. Evidence for involvement of pyroptosis during development of MAIDS-related MCMV retinitis was provided by detection of significant amounts of mRNAs to three key molecules involved in the pyroptosis pathway, i.e., caspase 1, IL-18, and IL-1β. Importantly, these key pyroptosis-related molecules were detected in large amounts within the MCMV-infected eyes of MAIDS-10 mice but not within those of MAIDS-4 mice, and their patterns of synthesis during the course of MCMV retinal disease were identical to those of key molecules associated with apoptosis or necroptosis. Remarkably, levels of IL-1β mRNA within eyes of MCMV-infected MAIDS-10 mice were increased nearly 800-fold compared with those in mock-infected control eyes from MAIDS-10 mice, although the precise contribution of this IL-1β release to pyroptosis-associated inflammation compared with IL-1β release during caspase 1-independent inflammation (53) remains unclear.

Caspase 1 activity and the maturation of IL-1β and IL-18 are regulated by a multiprotein complex known as the AIM2 inflammasome (21, 27, 28, 40, 47, 61). Rathinam and coworkers (60, 61) recently showed that the AIM2 inflammasome regulates caspase 1-dependent processing of pro-IL-1β and pro-IL-18 in response to double-stranded DNA in dendritic cells and macrophages but does not mediate type 1 interferon responses to double-stranded DNA, instead providing a negative regulation. Using Aim2-deficient mice, these investigators subsequently demonstrated a central role for AIM2 in regulation of caspase 1-dependent maturation of IL-1β and IL-18 as well as pyroptosis in response to systemic MCMV infection (60). Since pilot studies by us detected AIM2-associated inflammasome molecules in MCMV-infected eyes of MAIDS-10 mice but not MAIDS-4 mice (data not shown), we therefore predict that the AIM2 inflammasome plays a central role in regulating pyroptosis during development of MAIDS-related MCMV retinitis.

In summary, our findings provide new evidence for the participation of at least three cell death pathways during the onset and progression of MAIDS-related MCMV retinitis: apoptosis, necroptosis, and pyroptosis (Table 1). Our study therefore serves to broaden our understanding of the pathogenesis of AIDS-related HCMV retinitis. In fact, our findings suggest that multiple programmed cell death pathways are involved in the development of this sight-threatening retinal disease in patients with HIV/AIDS, perhaps operating simultaneously within individual retinal cells to cause intermediate forms of cell death. Whether the TUNEL assay used by us and others (6, 76) as an in situ marker for apoptosis during experimental MCMV retinitis serves also as a marker for necroptosis and/or pyroptosis remains unclear and awaits further investigation. We suggest, however, that the TUNEL assay will no longer serve as a reliable in situ marker for apoptosis exclusively, and future investigations to detect and

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<td>MCMV m38.3 (vIBO)</td>
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<td>Fas, FasL</td>
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<td>HCMV UL37x1 (vMIA)</td>
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<td>Protein kinases</td>
<td>Release of intracellular contents (inflammation)</td>
<td>Yes</td>
<td>MCMV M45</td>
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<tr>
<td>Pyroptosis</td>
<td>Caspase 1</td>
<td>Release of cytokines (inflammation)</td>
<td>Yes</td>
<td>MCMV gene (?)</td>
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<td>AIM2 inflammasome</td>
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quantify cell death pathways within virus-infected tissues will therefore use immunostaining strategies that target and visualize key pathway-dependent molecules.

ACKNOWLEDGMENTS

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REFERENCES


