Depletion of the Receptor-Interacting Protein Kinase 3 (RIP3) Decreases Photoreceptor Cell Death During the Early Stages of Ocular Murine Cytomegalovirus Infection

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Purpose. The purpose of this study was to determine if the receptor-interacting protein kinase 3 (RIP3) plays a significant role in innate immune responses and death of bystander retinal neurons during murine cytomegalovirus (MCMV) retinal infection, by comparing the innate immune response and cell death in RIP3-depleted mice (Rip3−/−) and Rip3+/− control mice.

Methods. Rip3−/− and Rip3+/− mice were immunosuppressed (IS) and inoculated with MCMV via the supraciliary route. Virus-injected and mock-injected control eyes were removed at days 4, 7, and 10 post infection (p.i.) and markers of innate immunity and cell death were analyzed.

Results. Compared to Rip3+/− mice, significantly more MCMV was recovered and more MCMV-infected RPE cells were observed in injected eyes of Rip3−/− mice at days 4 and 7 p.i. In contrast, fewer TUNEL-stained photoreceptors were observed in Rip3−/− eyes than in Rip3+/− eyes at these times. Electron microscopy showed that significantly more apoptotic photoreceptor cells were present in Rip3−/− mice than in Rip3+/− mice. Immunohistochemistry showed that the majority of TUNEL-stained photoreceptors died via mitochondrial flavoprotein apoptosis-inducing factor (AIF)-mediated, caspase 3–independent apoptosis. The majority of RIP3-expressing cells in infected eyes were RPE cells, microglia/macrophages, and glia, whereas retinal neurons contained much lower amounts of RIP3. Western blots showed significantly higher levels of activated nuclear factor–κB and caspase 1 were present in Rip3+/− eyes compared to Rip3−/− eyes.

Conclusions. Our results suggest that RIP3 enhances innate immune responses against ocular MCMV infection via activation of the inflammasome and nuclear factor–κB, which also leads to inflammation and death of bystander cells by multiple pathways including apoptosis and necroptosis.

Keywords: apoptosis, murine cytomegalovirus, innate immune response, retinitis

Human cytomegalovirus (HCMV) is a ubiquitous virus, which is normally kept in check by a healthy immune system. However, compromise of the immune system, either deliberately, such as in immunosuppressed organ transplant patients, or naturally, such as in individuals infected with human immunodeficiency virus (HIV), may result in HCMV morbidity due to virus activation, leading to various clinical sequelae, including retinitis. HCMV retinitis is a serious ocular complication of HIV infection, and prior to development of combination antiretroviral therapy (cART),1–5 occurred in ~30% of HIV/AIDS patients. Although its incidence has diminished as a result of cART, it nevertheless remains a significant problem. In recent years, numerous reports have also linked HCMV retinitis with HIV-negative patients6 while the occurrence of HCMV retinitis in immunosuppressed (IS) patients who were undergoing solid-organ or bone marrow transplantation has also been accumulating.6,8 Microscopic analysis of human ocular tissue obtained postmortem, combined with close clinical analysis of patients, has provided some information on the pathogenesis of HCMV in the retina. However, since the human retina is not amenable to experimental manipulation, animal models have been invaluable in dissecting the progression of viral destruction of retinal tissue. The murine counterpart of HCMV, murine cytomegalovirus (MCMV), has been widely studied in this regard, both by ourselves and others, and the murine model has provided considerable data that have led to a deeper understanding of virus-induced cell death.9,10

The model utilized in our laboratory involves intraocular injection of MCMV via the supraciliary route following steroid-induced immunosuppression. This results in a retinal infection with similar pathogenic characteristics to those noted in human ocular specimens infected with HCMV.11–13 Our model has demonstrated that early in infection, MCMV is localized to various interstitial and intracellular compartments of the retina, resulting in focal areas of photoreceptor cell death. The model has also demonstrated that MCMV infection leads to the activation of the inflammasome and nuclear factor–κB, which is known to play a role in the pathogenesis of retinal disease. The inflammasome is a multiprotein complex that mediates the processing and release of interleukin-1β (IL-1β) and IL-18 from macrophages and dendritic cells. The inflammasome is activated by pathogens, including MCMV, and its activation leads to the release of inflammatory cytokines, which can contribute to the development of retinal disease. In our model, MCMV infection leads to the activation of the inflammasome and production of inflammatory cytokines, which can contribute to the development of retinal disease. The inflammasome is also activated by MCMV infection, leading to the release of inflammatory cytokines, which can contribute to the development of retinal disease. The inflammasome is a multiprotein complex that mediates the processing and release of interleukin-1β (IL-1β) and IL-18 from macrophages and dendritic cells. The inflammasome is activated by pathogens, including MCMV, and its activation leads to the release of inflammatory cytokines, which can contribute to the development of retinal disease. In our model, MCMV infection leads to the activation of the inflammasome and production of inflammatory cytokines, which can contribute to the development of retinal disease. The inflammasome is also activated by MCMV infection, leading to the release of inflammatory cytokines, which can contribute to the development of retinal disease. The inflammasome is a multiprotein complex that mediates the processing and release of interleukin-1β (IL-1β) and IL-18 from macrophages and dendritic cells. The inflammasome is activated by pathogens, including MCMV, and its activation leads to the release of inflammatory cytokines, which can contribute to the development of retinal disease. In our model, MCMV infection leads to the activation of the inflammasome and production of inflammatory cytokines, which can contribute to the development of retinal disease.

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and necroptosis. As a result, viral progeny are produced and infection spreads to surrounding cells. In contrast, nearby uninfected cells undergo cell death leading to drastic disruption of retinal structure, and substantial evidence now supports the hypothesis that death of uninfected bystander neuronal cells plays an important role in the pathogenesis of CMV retinitis. The receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-containing signaling adaptors, RIP1 and RIP3, are key decision makers in cell death and innate immunity and since production of RIP3 is greatly increased post ocular MCMV infection, we hypothesized that RIP3 might play an important role in both the innate immune response and the death of uninfected retinal cells. Therefore, the purpose of this study was to test this hypothesis by comparing the pathogenesis of MCMV retinal infection in Rip3+/− and wild-type control mice.

MATERIALS AND METHODS

Virus and Virus Titration

MCMV strain K181 was kindly supplied by Edward Morcarski, Emory University, Atlanta, Georgia, USA. Virus preparation from salivary glands of MCMV-infected BALB/c mice has been previously described together with methods of viral titer determination by plaque assay and virus storage. Mice

Breeding pairs of Rip3−/− mice and control C57BL/6 (Rip3+/+/+) mice were purchased from Genentech Corp. (San Francisco, CA, USA). All the mice tested negative by PCR for the rd8 mutation. National Institutes of Health guidelines for the care and maintenance of mice and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed in all experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Augusta University. Typically, 6- to 8-week-old adult mice were used in all experiments. Anesthesia protocols have been described previously.

Antibodies

Anti-MCMV early antigen (EA) was used to identify MCMV-infected cells. The preparation and use of FITC-labeled anti-MCMV EA has been described elsewhere. Rat anti-glycine antibody (specific for amacrine cells) was kindly provided by David V. Pow, University of Queensland, Australia. Mouse anti-IBA1 (specific for macrophages/microglia) was from Wako, Inc. (Richmond, VA, USA). Mouse anti-rhodopsin (specific for photoreceptors) was from Abcam (Cambridge, MA, USA). Mouse anti-GFAP (glial fibrillary acidic protein, specific for glia/Müller cells), FITC-labeled anti-CD11b (specific for macrophages/microglia), FITC-labeled anti-CD3 (specific for CD3 T cells), and FITC-labeled anti-Gr-1 (specific for neutrophils) were all from BD Biosciences (San Jose, CA, USA). Anti-cleaved caspase 3, anti-AIF (apoptosis-inducing factor), anti-caspase 1, anti-poly(ADP-ribose)polymerase 1 (PARP1), anti-nuclear factor-κB (NF-κB), anti-RIP3, goat anti-rabbit IgG-horseshadish peroxidase (HRP), and goat anti-mouse IgG-HRP were all from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA, USA). Anti-β-actin was from Sigma-Aldrich Corp. (St. Louis, MO, USA). Biotin-labeled anti-rat IgG (mouse serum absorbed), anti-mouse Alexa 488, anti-rabbit Alexa 594, AMCA-labeled avidin, FITC-labeled avidin, and Texas red-labeled avidin were from Vector Laboratories, Inc. (Burlingame, CA, USA).

Experimental Plan

Mice were IS by intramuscular injection of 2.0 mg sterile methylprednisolone acetate suspension every 3 days beginning on day -2 according to our usual protocol. This protocol typically depletes 93% of T cells (CD4, CD8) and macrophages from MCMV-infected mice as measured by flow cytometry of splenocytes. Ocular virus infection of mice was carried out with 1 × 10^6 plaque-forming unit (PFU) MCMV as previously described. Eyes, salivary gland, lung, and liver were removed and prepared for measurement of virus titer by plaque assay while electron microscopy, immunohistochemistry, and Western blots were performed on eyes of additional mice.

Immunofluorescence Staining

Injected eyes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 minutes, immersed in 25% sucrose overnight, snap frozen, and sectioned on a cryostat. At least four slides (each slide representing one injected eye) in each group were used for each type of staining. In single- and double-staining experiments, slides were mounted with antifade medium containing 4',6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories) to visualize nuclei and images were captured using a confocal microscope (Zeiss upright 780; Oberkochen, Germany).

For double staining with TUNEL and MCMV EA, sections were initially stained with TUNEL (in Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. After washing and blocking, biotinylated anti-EA (1:500) was applied to the section overnight, followed by Texas red-labeled avidin (1:600) for 1 hour at room temperature. For double staining with TUNEL and AIF, sections were initially stained with TUNEL as described above. After washing and blocking, sections were incubated overnight at 4°C in rabbit-derived anti-AIF; followed by Alexa 594-labeled anti-rabbit IgG (1:1000) for 1 hour at room temperature. For double staining of RIP3 and retinal antigens, slides were washed for 30 minutes in PBS followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. After blocking in PBS containing 10% normal goat serum, 2% BSA, and 0.5% Triton X-100 for 1 hour, sections were incubated overnight at 4°C in primary antibody, rabbit-derived anti-RIP3 (1:200). Following washing, sections were incubated with anti-rabbit Alexa 594 (1:1000) for 1 hour at room temperature, washed, and then stained for different retinal antigens; GFAP was used to identify glia, rhodopsin was used to stain photoreceptors, and IBA1 was used to identify microglia. For antibodies raised in mice including anti-GFAP (1:200), anti-rhodopsin (1:100), and anti-IBA1 (1:100), each mouse-derived primary antibody was mixed with biotinylated anti-mouse secondary antibody (ARK; Dako Corp., Carpinteria, CA, USA). After 15 minutes, the blocking reagent, containing normal mouse serum, was added to the mixture. Mouse immunoglobulin in the blocking reagent bound to the biotinylated anti-mouse antibody that was not bound to primary antibody, thus minimizing reactivity with endogenous immunoglobulin in the specimen. The biotin-labeled primary antibody was then applied to the section. Staining was completed by addition of FITC-avidin (1:600) for 1 hour at room temperature.

For double staining of RIP3 and cellular antigens (CD3, CD11b, and Gr-1) or viral EA for which the antibodies were directly labeled with FITC, sections were initially stained with RIP3 as described above, then incubated with the relevant

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FITC-labeled antibody (1:50) overnight at 4°C. For double staining with TUNEL and RIP3, sections were first stained by TUNEL assay, washed, and then stained with RIP3 as described above.

For triple staining with TUNEL, RIP3, and rhodopsin, sections were first stained with TUNEL and RIP3 as described above, then incubated with mouse anti-rhodopsin (1:100) overnight at 4°C, also as described above. Staining was completed by addition of AMCA-avidin (1:200) at room temperature for 1 hour.

For quantitation of RIP3 staining in each type of ocular cell, 40 fields were chosen randomly from four slides (each slide representing one eye) under a fluorescence microscope for each double staining, and two images were acquired from red and green fluorescence channels, respectively, in each field. The number of RIP3-stained red cells and green-stained specific retinal cells, as well as double-stained cells (yellow in merged images), was quantitated in all fields. The total number of each of these three types of stained cells was used to calculate the percentage of RIP3-stained retinal cell types.

**Western Blot Analysis**

Western blotting was performed as previously described. Briefly, proteins were extracted from MCMV-injected or mock-injected eyes and equal amounts of proteins were separated by SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). Following blocking with 5% nonfat dry milk for 1 hour at room temperature, membranes were incubated overnight at 4°C with primary antibodies (as shown in the “Antibody” section in Methods, at the dilution of 1:1000 with 5% BSA) and for 1 hour at room temperature with HRP-conjugated secondary antibody. Immune complexes were detected using chemiluminescence (ECL; GE Healthcare) and exposure to x-ray film. To verify equal loading among lanes, membranes were probed with anti-β-actin. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify signal intensity.

**Electron Microscopy (EM)**

Fixation of eyes from experimental and control mice (three eyes per group) was performed by immersion in a solution of 2% glutaraldehyde, 2% paraformaldehyde, and 0.1 M cacodylate at 4°C overnight. Eyes were then washed in 0.1 M cacodylate and postfixed with 4% osmium tetroxide for 1 hour at room temperature prior to dehydration in a graded mixture (Electron Microscopy Sciences), and 70-nm sections were stained with uranyl acetate and lead citrate prior to visualization in a JEM 12300 electron microscope (JEOL, Tokyo, Japan). Quantification of apoptotic cells was performed by counting the number of apoptotic cells in a 45 × 45-μm area of the electron micrographs, and values were expressed as mean ± SEM.

**Retinitis Scoring**

To analyze the extent of retinopathy at late-stage infection, additional frozen sections of injected eyes at day 10 post infection (p.i.) were fixed with 4% paraformaldehyde for 15 minutes and stained with hematoxylin and eosin (H&E). Changes in the posterior segment of each section were evaluated microscopically as previously described. The highest posterior segment score for each eye was the retinal score and was used to determine the mean retinal score. Data were analyzed as described below.

**Statistical Analysis**

All data were expressed as means ± SEM (standard error of mean), where n is the number of mice or number of fields used in each experimental group. Statistical significance was determined using either a 2-tailed t-test or ANOVA using the GraphPad Prism 6 analysis tool (GraphPad Software, Inc., San Diego, CA, USA). P values < 0.05 were deemed significant.

**Results**

**Depletion of RIP3 Increases Viral Spread and Replication but Reduces Death of Photoreceptor Cells at Early Stages of Infection**

Following immunosuppression and virus inoculation, MCMV-positive cells were found in the anterior segment and RPE layer of both Rip3+/− and Rip3+/+ mice at day 4 p.i. (Fig. 1, A1, A2). At this time, TUNEL-positive staining was observed in uninfected photoreceptor cells, especially in areas of the retina where the subjacent RPE cells were MCMV infected, as we have previously described. Compared to wild-type mice, significantly more MCMV was recovered (Fig. 1B) and more MCMV-infected RPE cells were present in injected eyes of Rip3+/− mice (Fig. 1, A1, A2). In contrast, fewer TUNEL-positive photoreceptors were observed in MCMV-infected eyes of Rip3+/− mice compared to injected eyes of Rip3+/− mice (Fig. 1, A1, A2). Previous data from our laboratory have shown that death of photoreceptor cells is temporally associated with the spread of MCMV from the initial site of infection in the RPE layer to Müller cells during progression of MCMV retinitis. Probably because of less photoreceptor cell death, widespread RPE infection in Rip3+/− eyes did not result in an earlier spread of MCMV from the RPE to inner retina, since at day 7 p.i. (Fig. 1, A3, A4), similar numbers of virus-infected cells were observed in the inner retinas of Rip3+/− and Rip3+/+ mice. TUNEL-positive cells were also observed in the inner retina, with the majority being uninfected bystander retinal cells. Fewer TUNEL-positive cells were observed in the inner retina of Rip3+/− (Fig. 1, A4) compared to Rip3+/+ eyes (Fig. 1, A3). However, by day 10 p.i., significantly more MCMV was recovered (Fig. 1B) and more infected retinal cells were noted in Rip3+/− injected eyes (Fig. 1, A6), compared to Rip3+/− injected eyes (Fig. 1, A5). Not surprisingly, many TUNEL-positive cells were observed in the inner retina of both Rip3+/− and Rip3+/+ eyes at that time point (Fig. 1, A5, A6). We also measured viral titers in extraocular tissues at day 10 post intraocular MCMV infection. In contrast to MCMV replication in the eyes (Fig. 1B), we observed no significant differences of viral titers in salivary glands, livers, or lungs between Rip3+/− and control Rip3+/+ mice (Fig. 1C).

Since more MCMV was recovered and more infected retinal cells were present in Rip3+/− infected eyes compared to Rip3+/− infected eyes at day 10 p.i., the extent of retinopathy might eventually be exacerbated in Rip3+/− mice. To test this hypothesis, sections of MCMV-infected eyes were prepared at day 10 p.i. and stained with H&E. Compared to Rip3+/− infected eyes (Fig. 2, A1), more cytomegalic cells and increased disruption of retinal architecture were observed in Rip3+/− infected eyes (Fig. 2, A2). The average retinitis score of eyes of IS Rip3+/− mice was higher than that of IS Rip3+/+ mice (Fig. 2, A3).

**Electron Microscopy**

To determine if depletion of RIP3 reduces photoreceptor death by apoptosis or necrosis at early stages of MCMV ocular...
infection, additional injected eyes were collected at day 7 p.i. and prepared for EM. Considerable numbers of apoptotic photoreceptor cells were present in the outer nuclear layer of MCMV-infected Rip3\(^{+/+}\) wild-type mice (Fig. 2, B1, indicated by arrow). These photoreceptor cells exhibited typical apoptotic features including condensation of chromatin and reduction of nuclear volume.\(^{13}\) Additionally, cells at a more advanced stage of apoptosis were also noted and typically exhibited more extensive chromatin condensation and increased cell shrinkage. A few necrotic photoreceptor cells that exhibited marked lytic changes in cytoplasm and organelles were also noted (Fig. 2, B1, indicated by arrowheads). In addition, a relatively small number of photoreceptor cells in MCMV-infected Rip3\(^{+/+}\) eyes exhibited nuclear shrinkage and strong chromatin condensation as well as cytoplasmic lysis (Fig. 2, B3, indicated by arrows) typical of AIF-mediated necroptotic death (also called parthanatos\(^{29}\)). Compared to MCMV-infected Rip3\(^{+/+}\) eyes, significantly fewer apoptotic photoreceptor cells were observed in the outer nuclear layer of Rip3\(^{-/-}\) mice (Fig. 2, B2, B4). In addition, necrotic and necroptotic cells were rarely observed in the outer nuclear layer of Rip3\(^{-/-}\) mice (Fig. 2, B2).

**AIF-Mediated Caspase-Independent Cell Death**

To determine if these apoptotic photoreceptor cells were caspase dependent or caspase independent, sections of MCMV-infected and mock-injected eyes were stained with anti-cleaved caspase 3 antibody and subjected to TUNEL assay. The results...
showed that no cleaved caspase 3– or TUNEL-stained cells were present in the retina of control uninfected eyes (Fig. 3A) while only a small number of cleaved caspase 3–stained cells were observed in Rip3−/− infected eyes (Fig. 3B), suggesting that the majority of TUNEL-stained photoreceptors died through a caspase-independent mechanism.

AIF mediates a caspase-independent programmed cell death pathway in which AIF is released from the mitochondrial intermembrane space following calpain activation or overactivation of PARP1, before being translocated to the nucleus where it induces apoptosis-like cell death. Since depletion of RIP3 greatly decreased caspase-independent apoptosis-like cell death in photoreceptors (Fig. 2, B4), we hypothesized that AIF might induce photoreceptor cell death during MCMV retinitis. To determine if AIF was translocated to the nucleus of photoreceptors during MCMV retinitis, sections of MCMV-injected eyes and mock-injected control eyes were stained by TUNEL assay and for AIF. As shown in Figure 3C, AIF was not colocalized with nuclear DAPI staining in mock-injected control eyes, whereas in MCMV-infected eyes, AIF was translocated to the nucleus.
colocalized with DAPI in the majority of TUNEL-positive apoptotic cells in the outer nuclear layer (Fig. 3D).

To determine if PARP1 activation was affected by RIP3 depletion, Western blots were performed to measure expression of PARP1. As shown in Figures 4A and 4B, less cleaved PARP1 was observed in MCMV-injected \( \text{Rip}^3/^- \) eyes compared to MCMV-injected \( \text{Rip}^3^+/^+ \) eyes. In particular, at day 4 p.i., cleaved PARP1 was almost undetectable in MCMV-injected \( \text{Rip}^3/^- \) eyes. To determine if calpain activation was also affected by \( \text{Rip}^3/^- \) depletion, Western blots were performed to measure expression of calpain S1, a small 28-kDa regulatory subunit common to both calpain 1 and calpain 2, and calpain degradation products (150 kDa) of \( \alpha \)-fodrin. The results showed that less calpain S1 and cleaved \( \alpha \)-fodrin were observed in MCMV-injected \( \text{Rip}^3/^- \) eyes compared to MCMV-injected \( \text{Rip}^3^+/^+ \) eyes (Figs. 4C–E).

### Caspase 3–Dependent Apoptosis

To determine if caspase 3–dependent apoptosis was also affected by RIP3 depletion, expression of cleaved caspase 3 was measured in infected eyes of \( \text{Rip}^3/^- \) and \( \text{Rip}^3^+/^+ \) mice by Western blot. Cleaved caspase 3 was not present in mock-injected eyes of \( \text{Rip}^3/^- \) and \( \text{Rip}^3^+/^+ \) mice, whereas in MCMV-injected eyes of both \( \text{Rip}^3/^- \) and \( \text{Rip}^3^+/^+ \) mice, we observed the presence of cleaved caspase 3 (Fig. 5A). Quantification of band intensity indicated significantly lower amounts of cleaved caspase 3 in injected eyes of \( \text{Rip}^3/^- \) mice than in injected eyes of \( \text{Rip}^3^+/^+ \) mice (Fig. 5B).
RIP3-Mediated Activation of Inflammasomes and NF-κB

RIP3 is an important factor for innate immune responses against bacteria and viruses through activation of the inflammasome or NF-κB.24,38–42 The results presented here together with the results of other investigators 15,43 have demonstrated that depletion of RIP3 did not affect viral replication in systemic organs/tissues, whereas significantly more MCMV was recovered from the injected eyes of Rip3+/−/− mice than from the injected eyes of Rip3+/+ mice. Based on these observations, we hypothesized that RIP3 might play an important role in the innate immune response against ocular MCMV infection by activation of caspase 1 or NF-κB.

Therefore, to test this hypothesis, relative levels of caspase 1 and NF-κB were measured in the injected eyes of Rip3+/− and Rip3−/− mice by Western blot. Expression of cleaved caspase 1 was increased after MCMV infection in Rip3−/− eyes as early as day 4 p.i. In contrast, increased levels of cleaved caspase 1 were found in Rip3+/− eyes only at day 10 p.i. (Fig. 5C). Quantification of band intensity indicated significantly lower amounts of cleaved caspase 1 in infected eyes of Rip3−/− mice compared to infected eyes of Rip3+/+ mice at all time points tested p.i. (Fig. 5D). Our results also showed that depletion of RIP3 reduces NF-κB activation since NF-κB was activated earlier in Rip3−/− eyes compared to Rip3+/+ eyes following MCMV infection (Fig. 5E). Significantly higher levels of active NF-κB were also observed in Rip3−/− eyes compared to Rip3+/− eyes at days 4, 7, and 10 p.i. (Fig. 5F).

Identification of Ocular Cells Exhibiting Increased RIP3 Expression Following MCMV Infection

To identify the cell types in which RIP3 was expressed, sections of MCMV-infected and uninfected control Rip3+/−
eyes were prepared at day 7 p.i. and double stained for RIP3 together with either MCMV EA, markers specific to various ocular and immune cells, or TUNEL assay. The results showed that retina neuronal antigens including rhodopsin (specific for photoreceptors) (Fig. 6A) and glycine (specific for amacrine cells) (Fig. 6B) were rarely colocalized with RIP3. Triple staining for RIP3, TUNEL, and rhodopsin (Fig. 6C) also showed that apoptotic photoreceptors were rarely colocalized with RIP3. RIP3 was almost undetectable in noninfected control eyes (Fig. 7A), whereas in contrast, many RIP3-expressing cells were observed in the choroid and pigmented RPE as well as in the inner retina of MCMV-injected eyes (Fig. 7B). IBA1 was expressed at high levels in infiltrating macrophages and microglia, and many IBA1-positive cells were present in the inner retina of MCMV-infected eyes. The majority of IBA1£ cells in MCMV-infected areas were also RIP3 positive while 40% of RIP3-positive cells in the inner retina of injected eyes were also IBA1 positive (Fig. 7C). Similarly, the majority of CD11b£ cells also stained positive for RIP3 (Fig. 7D). Since mice were IS, only a relatively small number of Gr-1£ neutrophils (Fig. 8A) and CD3£ T cells (Fig. 8B) were observed in the inner retina of MCMV-infected eyes, and therefore the majority of CD11b£ cells should be microglia/macrophages. In addition, no RIP3 staining was found among Gr-1£ positive cells (Fig. 8A). Double staining for CD3 and RIP3 showed that some CD3£ T cells were RIP3 positive (Fig. 8B). In addition, double staining for GFAP and RIP3 showed that more than 40% of RIP3-positive cells in the inner retina of injected eyes were GFAP-positive glia/Müller cells (Fig. 8C). Although some RIP3-producing cells were observed in the outer nuclear layer, these cells were either IBA1-positive microglia/macrophages (Figs. 7C, 7D, indicated by arrows) or GFAP+ glia (Fig. 8C, indicated by arrows), but not rhodopsin-stained photoreceptors (Fig. 6A). Although many RIP3-producing cells were pigmented RPE cells (Fig. 7B, indicated by circles) or GFAP+ glia (Fig. 8C), the majority of MCMV-infected RPE cells (Fig. 7B) or infected glia (not shown) were either RIP3 negative (Fig. 7B, indicated by arrowheads) or stained only weakly for RIP3 (Fig. 7B, indicated by arrows).

**DISCUSSION**

The experiments presented herein demonstrate that RIP3 plays an important role in the innate immune response against ocular MCMV infection via activation of the inflammasome and NF-κB. Although an increased innate immune response and inflammation limit viral spread and replication in the retina, RIP3 is also responsible for the death of bystander retinal neuronal cells via multiple cell death pathways, particularly AIF-mediated, caspase-independent apoptosis.

The innate immune system is equipped with germline-encoded pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) and thus act as a first line of defense against pathogenic infection. When engaged by relevant PAMPs, these PRRs initiate signaling...
pathways such as formation of the inflammasome and activation of the transcription factor NF-κB, resulting in the induction of immediate host defense mechanisms. RIP1 mediates activation of NF-κB in response to a number of receptor systems including TNF-R1, TRAIL, Fas, TLR3, TLR4. In contrast, RIP3 is not responsible for (or does not even negatively regulate) NF-κB signaling downstream of these receptor systems. However, RIP1 and RIP3 synergize with each other to potentiate activation of NF-κB via interaction with DNA-sensor protein DAI, which represents one important mechanism of anti-viral immunity. Inflammasomes are multiprotein complexes composed of caspase 1, the adaptor ASC, and an upstream sensor such as NLRP3, that can activate caspase 1 and ultimately lead to the processing and secretion of downstream effectors, which drives the innate immune response. RIP1 and RIP3 participate in activation of the inflammasome/caspase pathway, and several reports have also shown that RIP3 can facilitate inflammasome activation independently of necrosis.

However, RIP kinases have also been shown to play various tissue-specific roles and diverse models in the same organ have shown differing requirements for RIP1 and RIP3.

The MCMV encoded protein M45 inhibits RIP3 phosphorylation, thus blocking subsequent RIP3 signaling through DAI and thereby preventing NF-κB activation. Probably because of this RIP3 inhibition, the majority of MCMV-infected ocular cells including RPE cells and glia were RIP3 negative. However, RIP3 expression greatly increased in the nearby noninfected residential ocular cells including pigmented RPE cells, GFAP+ glia/Müller cells, and IBA1+ microglia/macrophages. RIP3 deficiency alone significantly reduced activation of caspase 1 and NF-kB and increased viral spread and replication in the retina. Interestingly, depletion of RIP3 does not affect viral spread and replication in extraocular organs/tissues including salivary glands, livers, or lungs, which is consistent with previous studies. In our model, the majority of systemic immune cells including T cells, macrophages, and neutrophils were deleted, and only a few neutrophils and T cells were observed in virus-infected eyes at early stages of infection. Taken together, we conclude from these results that (1) residential ocular cells including RPE cells, microglia, and glia/Müller cells play significant roles in the initial innate immune responses against virus infection in the retina and (2) RIP3 participates in activation of NF-κB and inflammasomes in these residential ocular cells.
RIP3 has emerged as a critical regulator of programmed necrosis/necroptosis, which is often associated with inflammation, either pathogen induced or sterile. Activation of RIP3 is known to contribute to cell death in many diseases, including several ocular diseases, via MLKL-mediated necroptosis or PARP1-AIF inducing necroptosis/apoptosis. It seems clear that death of uninfected retinal neurons contributes significantly to the pathogenesis of CMV retinitis, and previous experiments performed in our laboratory have shown that both caspase 3-dependent and caspase 3-independent apoptosis contribute to retinal damage during MCMV retinitis. Our results demonstrate that depletion of RIP3 not only decreases cell death via necrosis/necroptosis during MCMV retinitis, but also significantly reduces caspase 3-dependent and caspase 3-independent apoptosis, particularly AIF-mediated caspase 3-independent apoptotic cell death, a pathway traveled by the majority of bystander retinal neurons that subsequently died.
AIF-mediated cell death has previously been shown to play a role in the death of neurons following exposure to cytotoxic agents in vivo. AIF-mediated apoptotic-like cell death is associated with cleavage of AIF by calpains into a soluble form (tAIF) in response to endoplasmic reticulum (ER) stress, oxidative stress, or other specific lethal stimuli, leading to its release from mitochondria and translocation into the nucleus, where it operates as an endonuclease to promote large-scale chromatin degradation. AIF has also been implicated in PARP1-mediated necroptosis/apoptosis. PARP1 acts as a DNA nick sensor and upon activation, participates in DNA repair of single- or double-strand DNA breaks. Its hyperactivation by cellular insults can also trigger cell death. In PARP1-mediated necroptosis, PARP1 hyperactivation induces the depletion of NAD+, which has a dramatic bioenergetic impact including inhibition of mitochondrial ATP synthesis as well as glycolysis due to its impact on glyceraldehyde phosphate dehydrogenase. Such a potent inhibition of ATP synthesis has been thought to constitute a sufficient cause for necrotic cell death. PARP1-dependent necrosis relies on an additional molecular mechanism, in which PARP1 hyperactivation results in the release of AIF from the mitochondrial intermembrane space (through the binding of AIF to PARP moieties) followed by translocation to the nucleus, where its endonucleolytic activity results in profound degradation of chromatin. However, necroptosis is not the only outcome of hyperactivated PARP1, and depletion of NAD+ and energy reserves may not be enough for PARP1-mediated necrosis. Instead, depletion of NAD+ and energy reserves may be the trigger, which initiates apoptosis through AIF translocation. Previous studies suggested that RIP3 can activate metabolic enzymes such as glutamate dehydrogenase 1, and thereby increase mitochondrial ROS production and act upstream of PARP1/AIF-mediated necroptosis/apoptosis.

Interestingly, RIP3 was rarely detected in retinal neurons, including TUNEL-stained photoreceptors, during MCMV retinitis. In contrast, it was strongly expressed in ocular immune cells and was associated with activation of inflammasomes and NF-kB. This suggests that death of bystander retinal neurons may be triggered by activation of calpain or PARP1 due to oxidative or ER stress following an inflammatory response produced by ocular immune cells, rather than cell autonomous effects of RIP3.

Acknowledgments

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