DETECTION OF NECROPTOSIS AND PYROPTOSIS-ASSOCIATED MOLECULES DURING EXPERIMENTAL MAIDS-RELATED CYTOMEGALOVIRUS RETINITIS

LAUREN-ASHLEY DUNCAN
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by

LAUREN-ASHLEY DUNCAN

Under the Direction of Richard D. Dix, PhD

ABSTRACT

Sight threatening human cytomegalovirus (HCMV) retinitis still remains a cause for concern among AIDS patients who do not respond to or do not have access to current antiretroviral (ART) therapy [1]. However, little is currently known about the degenerative mechanisms behind HCMV retinal destruction during retroviral immunosuppression. The well-established murine AIDS (MAIDS) related murine cytomegalovirus (MCMV) retinitis model closely mimics disease progression seen in AIDS patients [4]. Previous work using this model has shown that while the cell death pathway apoptosis is involved in disease progression, it is not fully responsible [1]. It has been found that the mRNA of molecules associated with two other cell death pathways, necroptosis and pyroptosis, are also correlated with the pathophysiology of MAIDS-related MCMV retinitis [1]. We propose that retinal degeneration seen during CMV retinitis involves necroptosis/pyroptosis programmed cell death correlating with the increased mRNA/protein expression of associated molecules observed in this study.

INDEX WORDS: Necroptosis-associated molecules, Pyroptosis associated molecules, cytomegalovirus retinitis, HIV/AIDS, MAIDS, Cytomegalovirus (CMV), Cell death
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LAUREN-ASHLEY DUNCAN

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2018
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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2018
DEDICATION

Every step leading me up to this point has been driven by a goal to somehow help make the world a better place for my beloved family, human and non-human members alike. The faith placed in me by my family has simultaneously been a heavy burden and a duty I proudly follow; it is all the motivation I have needed.
ACKNOWLEDGEMENTS

It is with my sincerest appreciation that I thank my advisor, Dr. Richard Dix, for giving me a chance. I have learned far more than I imagined I would and it is thanks in no small part to his guidance. Additionally, I thank my committee members Dr. Julia Hilliard and Dr. John Houghton for their guidance. Furthermore, I could not have come so far without the help of members of our lab, past and future. I especially have to thank Jessica Carter who has been a wonderful mentor and friend. Finally, I can’t forget all the remarkable professors and classes I have had. The knowledge they provided has proven invaluable.
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<td>Receptor-interacting serine/threonine-protein kinase 1</td>
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<tr>
<td>RIPK3</td>
<td>Receptor-interacting serine/threonine-protein kinase</td>
</tr>
<tr>
<td>MLKL</td>
<td>Mixed lineage kinase domain like pseudokinase</td>
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<td>GSDMD</td>
<td>Gasdermin D</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>MCMV</td>
<td>Murine cytomegalovirus</td>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>MAIDS</td>
<td>Murine acquired immunodeficiency syndrome</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>cFLIP</td>
<td>Cellular FLICE (FADD-like IL-1β converting enzyme)-inhibitory protein</td>
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<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<td>TRADD</td>
<td>Tumor necrosis factor receptor type 1-associated DEATH domain protein</td>
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<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
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<tr>
<td>CYLD</td>
<td>Ubiquitin carboxyl-terminal hydrolase CYLD (cylindromatosis)</td>
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<td>RHIM</td>
<td>RIP homotypic interaction motif</td>
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<tr>
<td>DPI</td>
<td>Days post infection</td>
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<td>PFU</td>
<td>Plaque-forming unit</td>
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1 INTRODUCTION

Human cytomegalovirus (HCMV) is a large DNA betaherpesvirus that establishes lifelong myeloid lineage latency in individuals after initial infection of immunocompetent individuals [1][2]. These individuals usually remain asymptomatic carriers and by the age of 40, approximately 80% of the population is infected in this way [4][19][82]. In some individuals however, immune suppression can increase the risk of HCMV reactivation and subsequent inflammatory/degenerative pathologies respective to the type of immunosuppression [1][3]. Of concern to our lab is the HCMV induced retinal destruction (retinitis) that can occur during AIDS retroviral mediated immunosuppression [1].

HCMV exists under the Herpesviridae family of viruses along with Herpes simplex 1 and 2, Varicella-zoster and Epstein-Barr among others (Fig. 1)[20][21]. Herpesviridae viruses share a similar basic structure with a large double stranded (ds) genome contained by an icosahedral nucleocapsid, tegument proteins and then finally an envelope with associated glycoproteins needed for host cell binding and invasion (Fig. 2)[22]. Some of these glycoproteins needed for successful viral entry (gB, gH, gL) are also highly conserved between Herpesviridae viruses [57]. Additionally, other closely related species-specific CMV fall within the same Betaherpesvirinae subfamily (Fig. 1)(Fig. 3) and due to the stable nature of dsDNA viruses, there is a little possibility of viral change [23][24]. Thus, other CMV viruses can be of use in modeling human pathologies in non-human systems.
Figure 1. Taxonomy of human cytomegalovirus (HCMV) in relation to murine cytomegalovirus (MCMV).

Human herpesvirus (solid lines) in Family Herpesviridae as they relate to HCMV and MCMV (dotted lines). Information from [20][21].
Figure 2. Cytomegalovirus Structure.
Representation of the elements in an HCMV/MCMV virion. From [22].

Figure 3. Comparison of HCMV and MCMV genome.
Boxes: inverted repeats. TR_L: long terminal repeats. U_L: long unique sequence. IR_L: long internal repeats. IR_S: short internal repeats. TR_S: short terminal repeats. Modified from [23]. MCMV IR_S not shown [24].

Transmission of CMV infections is similar across affected species and can occur with direct contact of infected bodily fluids (saliva/urine), sexual activity, blood transfusions/organ transplants, breast feeding and in-utero transfer [58]. After viral transmission, glycoproteins initiate viral binding and entry in host cells [57]. Lytic replication, as reviewed in Figure 4, can then take place during initial infection. In immunocompetent individuals, the immune response is adequate to stop active infection and the production of virion progeny [59]. Subsequently the virus enters a period of latency in the genome of myeloid progenitor cells as provirus where it
continues to be able to replicate its genome during host cell replication [59]. During inflammatory events and the resulting differentiation of the infected progenitor cells into macrophages (MØ) and dendritic cells (DC), the viral genome is activated by the same regulatory signals acting on MØ/DC which leads to the production of certain viral replication promoters (such as immediate-early) and proteins that down-regulate antigen presenting cell (APC) activity [59][60]. In healthy individuals, robust APC activity is enough to overwhelm viral attempts of re-activation. In non-healthy individuals who have immunosuppression, the virus can take advantage of the reduced immune activity and overwhelm host cells defenses leading to re-activation of viral lytic activity [59].

Figure 4. Viral Replication of HCMV/MCMV. Viral coat proteins bind to cell membrane, virus invades, uncoating, entry into the nucleus, nuclear genetic replication machinery takeover, viral genome replication, capsid assembly, vesicle bound, exocytosis and recoating. From [25].

In humans, AIDS related HCMV retinitis results in retinal tissue destruction that ultimately leads to blindness. Before the advent of ART (anti-retroviral therapy), up to 30% of AIDS patients lost their vision this way; unfortunately this is still a significant risk for those who do not respond to or who do not have access to therapy [1]. Additionally, at lower rates, HCMV
retinitis can occur outside of AIDS in other immunosuppressed populations such as in donor organ/bone marrow recipients (15.2% out of non-AIDS HCMV retinitis cases) [1], those suffering from rheumatological conditions (19.1%), individuals of advanced age (33.1%) and even healthy individuals with localized immunosuppression (~5%) [3][61]. Regardless of the mode of immunosuppression, clinical presentation of HCMV retinitis appears to be similar. Not all cases are symptomatic but usually there are initial visual disturbances in the form of floaters and some loss of vision to the periphery or even more central areas of sight. Some reduction in overall visual acuity may also be reported. During examination, aside from retinal necrosis, other secondary pathologies may present such as macular oedema, optic nerve inflammation (papillitis) and retinal detachment [61][62]. As the retinitis progresses, the field of visual loss progressively increases until blindness results [1][62]. Even minimal inflammation and destruction of the retina proves problematic due to the relative permanency of damage and the consequent quality of life issues that may cause. For those individuals who are not under an effective treatment, the effects of HCMV retinitis are far more concerning.

The retina itself is located at the back of the eye, encompassing nearly the whole interior of the globe and creating a wide field of vision. It consists of several cellular and connecting layers as described in Figure 5 with the ganglion layer situated facing into the eye and the photoreceptors at the back. As light enters the eye, it passes to the back of the retina activating the rods and cones of the photoreceptor cells which then signal the bipolar cells in another layer (via synaptic activity). From there the signal is passed to the ganglion cells where they act as the neuronal interface between retinal visual signaling and the image processing of the brain [29]. Of note in regards to possible immune mediated activity during disease, two additional but non-
retina areas may be of interest. The RPE (retinal pigment epithelium) located behind the photoreceptors is responsible for nourishing the retinal cells and degrading damaged ones [63].

**Figure 5. The structure of the retina.**
Nuclear layers: Ganglion cell layer, inner nuclear layer (bipolar cells) and the outer nuclear layer (nucleus containing portion of the photoreceptor cells). These are the layers that are counterstained with DAPI. From [29].

Behind it, is the choroid which is the vasculature structure providing blood supply and bringing with it myeloid cells and their progenitors – potentially primed for inflammation and/or infected with virus [63]. During advanced HCMV retinitis, full-thickness retinal necrosis can be seen upon examination as large patchy white areas with interspersed hemorrhaging (Fig. 6D as compared to Fig. 6C). There may also be widespread secondary inflammation in other parts of the eye such as in the uvea [62].
Figure 6. Retinal folding and full retinitis.

A) Ocular histopathology of MAIDS related MCMV retinitis at 8 days post infection (dpi) showing both retinal folding and full retinal necrosis. We use retinal folding as a characteristic of 6 dpi however, there can still be some retinal folding by 10 dpi as retinitis is patchy. B) Non-diseased/healthy mouse retina C) Non-diseased/healthy human retina D) Similar ‘patchy’ disease pathology is seen in human patients with AIDS related HCMV retinitis. From Dr. Dix, Dr. Cousins, Bascom Palmer Eye Institute, Miami, FL and Dix Lab.
Understanding disease pathogenesis of HCMV induced retinitis during opportunistic pathogenicity may allow for better understanding of the cellular mechanisms at play in cell death and tissue destruction. In order to explore these concepts, a murine model was used to replicate AIDS-related HCMV retinitis in-lab. As previously discussed, HCMV is closely related to other species-specific CMV viruses including murine CMV (MCMV) (Fig. 1)(Fig. 3) [22][23][57]. To induce retroviral immunosuppression similar to AIDS, a murine leukemia cocktail is used (MAIDS) [1][4]. Mice are infected with MAIDS at 4 weeks of age and allowed to progress to systemic immunosuppression. Then 10 weeks later mice are directly injected subretinally with MCMV (Fig. 7) to prompt MCMV retinitis in this model. Lastly, in order to evaluate the progress of retinal destruction over time, groups of mice are euthanized at different time points (3, 6 and 10 days post infection - dpi) and eyes are harvested. MAIDS-related MCMV retinitis eyes are phenotypically characteristic and distinguishable between the different time points. This is especially clear when observing 6 dpi and 10 dpi. Retinal folding is associated with 6 dpi with full necrosis expected at 10dpi; although because retinitis is patchy in how it spreads, there can be untouched areas that still only exhibit retinal folding (Fig. 6A as compared to healthy retina.

Figure 7. Visual Representation of the site of delivery in subretinal injection.
2 µl injection containing MCMV in MAIDS mice in the subretinal space. Mice under full anesthesia during the procedure. Modified from [26].
Fig. 6B). In PCR work, mRNA was usually found to be more highly expressed at day 6 and dramatically decreased by day 10 [1], likely due to the drop in retinitis activity at the endpoint of full retinal necrosis. Day 3 mRNA and protein data was usually not highly stimulated. Ultimately, these time points were picked based on prior data in order to better define the steps of disease progression. In that same thought, MAIDS-10 (10 weeks MAIDS duration) mice are used because previous work showed that MCMV could not induce any retinitis without full immunosuppression being attained by MAIDS over the course of at least 10 weeks [1][12]. Viral titer remains the same when compared to mice unsusceptible to MCMV retinitis (MAIDS-4 mice) but only MAIDS-10 mice see progressively worsening retinitis at 6 dpi and 10 dpi (Fig. 8) [1]. All data shown going forward was collected from MAIDS-10 mice unless otherwise specified.

The mechanistic process leading to the dramatic destruction of the retina in both HCMV and MCMV retinitis is not yet fully elucidated. Findings using the MAIDS-related MCMV model in C57BL/6 mice seem to indicate that CMV infection and replication is not solely responsible for retinal tissue destruction [1] and that while cell death signaling can be variable it is a large purveyor of retinal degeneration [7][1]. In MAIDS-10 mice, it was seen that severe retinal destruction occurred in tandem with significant up-regulation of apoptotic molecules - tumor necrosis factor alpha (TNF-α), TNF receptor 1 and 2, cleaved caspase 8, cleaved caspase 3, TNF-related apoptosis-inducing ligand (TRAIL), TRAIL receptor (TRAIL-R), Fas, and Fas ligand [1]. However, TUNEL assay revealed only partial contribution by apoptosis to cell death (only ~8% and ~ 4% at 6 and 10 days post infection respectively); overall, apoptosis is associated with limited cell death activity compared to other cell death pathways in many retinal degenerative diseases [1][5]. Cross-talk between cell death pathways is an integral function in
Figure 8. Comparison of MAIDS 4 and MAIDS 10 MCMV infected mouse eyes. A) Severity of retinitis: MAIDS 4 eyes showed low levels or retinitis in comparison to the more fully developed MCMV disease pathophysiology in MAIDS 10 mouse eyes. B) There was no significant difference in MCMV titer levels between MAIDS 4 and MAIDS 10. These experiments were performed by Dr. Hsin Chien under the direction of Dr. Richard D. Dix, Georgia State University [1].

regulating specific coordinated cell death activity – turning off one pathway results in turning on another [2]. Identification of mRNA from other cell death pathway molecules seems to be indicative of both necroptosis (Fig. 9) and pyroptosis (Fig. 10) activity [1]. Given that the involvement of both has been documented in other retinal degenerative diseases [2][5][6], it could be postulated that they also play a role in MCMV retinitis alongside apoptosis.

Necroptosis is an alternative programmed pathway induced when apoptosis is inhibited by caspase blocking activity [8]. As a result, necroptosis shares the same receptor ligand binding activation and initiation of signal transduction as apoptosis. Extracellular ligand binding of
Figure 9. Necroptosis cell death signaling pathway.

Figure 10. Pyroptosis cell death signaling pathway.
Active caspase 1 and caspase 11 cleave GSDMD to initiate cellular death and inflammation by pyroptosis. From [27].

TNFR, Fas, TRAIL or TLRs induces the intracellular formation of a death complex with FADD, TRADD or TRIF adaptors and assorted accessory proteins and Receptor-interacting
serine/threonine-protein kinase 1 (RIPK1). Cellular stimuli that upregulate the deubiquitinating enzyme CYLD or the cellular inhibitor of apoptosis protein 1 (cIAP1) cause RIPK1 to dissociate from the first complex in order to form a second death complex with uncleaved caspase 8 and FADD \[10\]\[2\]. From this point on, the two pathways diverge dependent on caspase 8 activity.

Necroptosis signaling is prompted when Fas-associated protein with death domain (FADD) cleavage of pro-caspase 8 is inhibited via the competitive formation of uncleaved caspase 8 / cellular FLICE inhibitory protein (cFLIPSorR) heterodimers \[8\]. After which, RIPK1 recruits RIPK3 into the necrosome complex through their RIP homotypic interaction motif (RHIM) domains where autotransphosphorylation of both Ser227 also occurs via each other’s kinase domain \[10\]\[2\]\[64\]. Lastly, the now active RIPK3 then recruits and phosphorylates mixed lineage kinase domain-like (MLKL). Active MLKL forms an oligomer complex with other active MLKL before it migrates to the plasma membrane and in a process still not yet fully understood, ruptures the membrane via pore formation \[11\]\[64\]. This results in a necrotic cell death in which the release of damage-associated molecular patterns (DAMPs) trigger a highly pro-inflammatory response \[9\]. A brief schematic of this signaling pathway is shown in Figure 9. This is a well regulated form of necrosis controlled by a caspase independent signaling pathway – as opposed to passive necrosis, which occurs due to cell damage and a subsequent failure to maintain homeostasis with the result being cell swelling and eventual membrane disruption before death (Fig. 11)[5][28].

Pyroptosis on the other hand is similar to both apoptosis and necroptosis but exhibits its own specific pathway. Similarly to apoptosis, it is a caspase dependent mode of cell death that generally occurs in response to pathogen infection. Furthermore, it triggers an inflammatory response just as necroptosis does. The signal transduction pathway is activated when pathogen-
associated molecular patterns (PAMPs) are exhibited intracellularly due to cellular infection [2]. Pattern recognition receptors (PRRs) such as nod-like receptors (NLR) and absent in melanoma 2 (AIM2) protein detect these PAMPs and form oligomer complexes that become caspase activating inflammasomes upon the recruitment of apoptosis-associated speck-like adaptor protein containing a CARD (ASC) [2]. Once formed, pro-caspase 1 is bound to the
inflammasomes at the adaptor protein, ASC, and cleaved into active caspase 1. Final pyroptosis activity is then initiated by caspase 1 cleavage activity of both gasdermin D (GSDMD) and the inflammatory cytokines pro-interleukin (IL)-1β and pro-IL-18. Activated GSDMD proteins oligomerize at the cell membrane and form pores which serve as an avenue of cytokine release and ultimately cause cell swelling and lysis [27][2]. Thus pyroptosis is also an inflammatory form of cell death but unlike necroptosis, instigates a more specific immune response by means of extracellular cytokine signaling. A brief overview of both the canonical and non-canonical pathway can be seen in Figure 10. As can be seen in the non-canonical pathway, caspase 11 can also cleave and activate GSDMD but cannot stimulate cytokine release [27][2]. However, pyroptotic cell death can still occur due to GSDMD disruption of the plasma membrane [2].

Both necroptosis and pyroptosis pathways are potent forms of cell death and a pro-inflammatory state [2][6][9][56]. This might help explain the devastating levels of retinal destruction and the speed with which it spreads. In contrast, apoptosis cell death does not signal for cell death in surrounding cells nor does it seem capable of inducing inflammatory pathologies known to co-occur with CMV retinitis [1][2][8][10]. In past studies, this lab has already shown that necroptosis associated RIPK1 and RIPK3 mRNA are expressed in MAIDS-related MCMV retinitis eyes. RIPK1 mRNA is significantly upregulated with the greatest increase at 6 days post MCMV infection (dpi); RIPK3 by comparison exhibits minor but clear upregulation as well [1]. Additionally, the mRNA of several pyroptosis associated molecules were previously found to be upregulated in MAIDS-related MCMV retinitis as well [1]. Caspase 1, caspase 11, GSDMD, IL-1β and IL-18 all displayed significant upregulation by 6 dpi and dropped again in mRNA expression by 10 dpi [1][performed by Jessica Carter under the direction of Dr. Richard D. Dix, Georgia State University-manuscript in progress]. Following that data, it could be presumed that
programmed cell death via necroptosis and pyroptosis play roles in MCMV induced retinal cell death.

Therefore, we will expand on the central hypothesis that necroptosis-associated molecules RIP1, RIP3, and/or MLKL and pyroptosis-associated molecules caspase 1, caspase 11, GSDMD, IL-1β and/or IL-18 are stimulated intraocularly during the pathogenesis of MAIDS-related MCMV retinitis. Specific Aim 1 will investigate whether necroptosis cell death is involved in the retinal destruction observed in the experimental model. Although mRNA for necroptosis-associated molecules, RIPK1 and RIPK3, were found to be stimulated during MAIDS-related MCMV retinitis, the full extent of necroptosis in MCMV retinitis has yet to be elucidated. Therefore, the hypothesis of specific aim 1 postulates MLKL will be upregulated in MCMV infected eyes and that all tested necroptosis molecules RIPK1, RIPK3 and MLKL will be stimulated in retinal layers displaying MCMV retinitis in MAIDS-10 mice. This will be tested by assessment of MLKL mRNA via real-time RT-PCR quantitative analysis and RIPK1, RIPK3, MLKL protein via IHC staining and western blot in the murine model eyes. All data will be evaluated by comparison with non-infected controls. Specific Aim 2 will then follow the first in investigating the potential role of pyroptosis cell death in retinal tissue destruction in the experimental model. Multiple pyroptosis-associated molecules were found to have increased mRNA expression in MAIDS-related MCMV retinitis eyes but pyroptosis activity in the form of protein expression is still ongoing. Hypothesis of specific aim 2 postulates that pyroptosis molecules caspase 1, caspase 11, GSDMD, IL-1β and IL-18 will be stimulated in retinal layers displaying MCMV retinitis in MAIDS-10 mice. This will be tested by protein analysis of caspase 1, caspase 11, GSDMD, IL-1β and IL-18 via IHC staining in the murine model eyes. All data will be evaluated by comparison with non-infected controls.
2 MATERIALS AND METHODS

2.1 Cell Lines

All cell lines used were for the cultivation of viral stocks to be used for murine in vivo infection and disease initiation.

For MAIDS induction as a murine retroviral model, SC-1 fibroblast (ATCC #CRL-1404) and LP-BM5 – MuLV infected SC-1 - (AIDS Research and Reference Reagent Program, Germantown, MD) were utilized. SC-1 cells with and without murine leukemia viral (MuLV) infection were grown in Dulbecco’s modified eagle media (DMEM, Corning Life Sciences) with 10% fetal bovine serum (FBS)(Atlas Biologicals), 24 mM L-glutamine, 1% penicillin/streptomycin, 0.1 mg/mL gentomicin.

MCMV stocks for sub-retinal injections were grown and titered in mouse embryonic fibroblast (MEF) (ATCC #SCRC-1002) cells. Cells were maintained in DMEM with 15% FBS, 4 mM L-glutamine, 1% penicillin/streptomycin, and 0.1 mg/mL gentomicin.

2.2 Viruses

LP-BM5 (MuLV infected SC-1) and SC-1 cell lines were thawed and grown in SC-1 media until confluent. Established cultures of the two cell lines were mixed in a 1:1 ratio and separated in 16 T-150 flasks with 12 mL of SC-1 growth media. After 6 days, the cell monolayers in the flasks were gently scraped and transferred along with media into 150 mL conical tubes and stored at -80°C until use. For injection, MuLV retroviral cocktail was thawed and centrifuged to dispose of cellular debris.

MCMV stock, for use in subretinal injections, was propogated in BALB/c (Harlan/Envigo) mouse salivary glands [12]. 20 mice were injected intraperitoneally (IP) with 0.2 mL of Smith strain of MCMV (~10³ plaque forming unit (PFU)). Fourteen days post
infection (dpi), the mice were euthanized and the salivary glands harvested. Salivary glands were homogenized in 1.5 mL DMEM with 10% fetal bovine serum and centrifuged before aliquoting the supernatant into 0.5ml and storing in liquid nitrogen. Each viral stock was titered via plaque assay in advance of sub-retinal injections.

2.3 Animals

Adult female wild-type C57BL/6 (Jackson Laboratory) mice of 2 weeks of age and adult female wild-type BALB/c mice (Harlan/Envigo) of 8-12 weeks of age were acquired and acclimatized in Georgia State University’s Animal Facilities to the following husbandry conditions: 5 mice to a habitat, alternative 12-h light-dark cycles, an enrichment article and free access to food and water. Experimental procedures followed guidelines from the National Institutes of Health (NIH) and the Association for Research in Vision and Ophthalmology Resolution (ARVO) on the Use of Animals in Research. Experimental protocols received prior approval by The Institutional Animal Care and Use Committee (IACUC) at Georgia State University.

**Induction of MAIDS:** MAIDS was induced in groups of 4 week old wild-type C57BL/6 mice via intraperitoneal (IP) injection of 1 mL of MuLV retroviral cocktail (LP-BM5/SC-1 mixture at ~103 PFU). Infection was allowed to progress for 10 weeks (MAIDS-10 mice) post injection before the next experimental step (mice aged 14 weeks); previous work indicated such late stage MAIDS mice are more susceptible to retinitis than earlier stages (MAIDS 4 mice) [1][12].

**Subretinal MCMV Injections:** Systemic MCMV does not induce retinitis despite viral presence in the choroid and RPE [13]; however, it was found that directly injecting the viral load into the eye under the retina did in fact result in retinitis equivocal to disease pathologies of
AIDS related HCMV retinitis [12][14]. Therefore, a subretinal route of infection is a suitable murine model for representation of the human disease.

For subretinal MCMV infection, the previously described groups of MAIDS-10 mice underwent a series of steps to prepare them for subretinal injections. Both eyes for each mouse were dilated over the course of 3 rounds of atropine and tropicamide ophthalmic drops. Following the final round of eye drops, anesthesia procedures were initiated and mice were given their first injection of 0.1 mL xylazine (1.72 mg/mL) intramuscularly (IM) in a hind leg. After each mouse within a group received the first injection, a secondary injection of 0.1 mL acepromazine (0.28 mg/mL) was given in the alternative leg. Induction into full anesthesia was achieved with a final IP injection of 0.1–0.2 mL ketamine (8.58 mg/mL). Upon complete non-response to stimuli, mice were given a final round of phenylephrine ophthalmic drops before being subretinally (supraciliary) injected with 2µL of MCMV (prepared as previously discussed) in the left eye and 2µL of DMEM media in the right eye (contralateral injections). The media injected eye served as a control for the MCMV injected eye within one individual mouse due to the findings that MCMV does not pass from the infected to the non-infected eye in the durations under consideration here [15]. Groups of mice were euthanized at 3, 6 and 10 days post MCMV infection via a two step process: isoflurane induced anesthesia followed by cervical dislocation. Subsequently, the eyes were harvested and stored depending on how they would be later analyzed for necrotosis and pyroptosis associated molecules; RNAlater (whole eye mRNA analysis via real-time RT-PCR), frozen in liquid nitrogen (whole eye protein analysis via Western Blot), or stored in 10% buffered formalin solution at 4°C (eye section protein staining via immunohistochemistry).
2.4 Real-Time RT-PCR

Whole MCMV-infected eyes and contralateral media injected eyes (control) at 3, 6, and 10 days post infection were stored in RNAlater solution (Ambion) at −80°C. For analysis, each eye was thawed and homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies). RNA was extracted from each homogenized sample following the PureLink® RNA Mini Kit total RNA purification system (Invitrogen Life Technologies) protocol. RNA concentration for each sample was quantified via SmartSpec 3000 spectrometer (Bio-Rad Laboratories) and equalized between samples. Samples were then stored at -80°C until needed.

Samples were thawed prior to undergoing the steps involved in real-time reverse transcriptase polymerase chain reaction (RT-PCR). Extracted RNA was used to synthesize complementary DNC (cDNA) using the SuperScript™ III first-strand synthesis system (Invitrogen) and protocol. Mouse specific MLKL and GAPDH primers obtained from QIAGen (Valencia, CA) and SYBR green PCR master mix (Applied Biosystems) was used to help determine levels of MLKL mRNA transcripts in each sample during thermocycling using a ABI Prism 7500 real-time PCR instrument with sequence detection software (Applied Biosystems). System parameters for cycling was set up for 10 min at 95°C, followed by 40 cycles consisting of 15 s at 94°C, 31 s at 55°C, and 35 s at 70°C. MLKL mRNA transcript cycles to threshold (CT) were determined for MCMV infected and contralateral control eyes compared to constitutently expressed GAPDH.

2.5 Immunohistochemistry (IHC) Fluorescent Staining

Eyes were collected at 6 and 10 days post MCMV infection and immediately fixed with 10% neutralized formalin at 4°C for at least 5 days. Eyes were cut into 5-μm-thick transverse
sections and every 6th section embedded in paraffin on slides by the Pathology Department of the Emory Eye Center.

Prior to immunohistochemical staining, slides were soaked in Xylene substitute (Sigma-Aldrich, St. Louis, MO) for 3 5 minute intervals to remove paraffin. Then sections were rehydrated in decreasing 70% ethanol/PBS concentrations (95%, 75%, 50%) for 15 seconds each, washed in PBS for 30 seconds, treated with 10 mM sodium citrate retrieval solution for 10 minutes, washed with PBS for another 5 minutes, and finally blocked with 5% normal goat serum containing 0.2% Triton X-100 (Ambion/ThermoFisher) for 30 min at room temperature. Another wash in PBS for 3 intervals of 5 minutes prepares the sections for primary antibody application. Sections were incubated with primary antibodies specific for necroptosis and pyroptosis specific molecules overnight at 4°C. Necroptosis: rabbit anti-mouse RIPK1 (1:200) (Antibodies Online, Atlanta, GA), rabbit anti-mouse RIPK3 (1:200) (Antibodies Online, Atlanta, GA), rabbit anti-mouse MLKL (1:200) (Abcam, Cambridge, MA). Pyroptosis: rabbit anti-mouse caspase-1 (1:200) (Abcam, Cambridge, MA), rabbit anti-mouse caspase 11 (1:200) (Abcam, Cambridge, MA), rabbit anti-mouse GSDMD (1:200) (Abcam, Cambridge, MA), rabbit anti-mouse IL-1β (1:200) (Abcam, Cambridge, MA), rabbit anti-mouse IL-18 (1:200) (Abcam, Cambridge, MA). A matched isotype rabbit IgG was used as a negative (1:200) (Abcam, Cambridge, MA) for all primary antibodies. After incubation, three 5-min washes with PBS were done prior to secondary staining. Retinal sections subsequently were incubated at room temperature in the dark for 1 h with the secondary antibody conjugated goat anti-rabbit Cy3 (red) (1:100) (Jackson ImmunoResearch) for fluorescent visualization of each molecule in the retina. After secondary antibody incubation, sections were washed in PBS for three 5 minute and then mounted with medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vectorshield; Vector
Laboratories) to counterstain nuclear material. Sections were inspected and photographed by fluorescence microscopy (Nikon Eclipse). ImageJ was used to background correct images. Presence of each of the previously mentioned necroptosis and pyroptosis-associated molecules was qualitatively assessed by comparison of control (media injected) and MCMV infected eyes.

2.6 Western Blot

Western blot was used to evaluate levels of necroptosis-associated molecules present in MCMV infected whole eyes compared to contralateral media injected (control). Eyes were collected at 3, 6 and 10 days post infection (dpi), eyes harvested and stored in liquid nitrogen until needed. Eyes were used individually, representing one group each per blot.

In preparation for western blot, eyes were thawed before protein extraction. 2X extraction buffer was prepared using a protease inhibitor (complete Mini, EDTA-free) (Roche) in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO). Eyes were first homogenized in 0.5mL cold extraction buffer and then spun at 13,000 RPM for 1 minute. 130µl of the supernatant was transferred and added to equal amounts of cold DMEM for later viral quantification. Protein concentration of each sample was quantified following the “Microtitier Plate Protocols” of the protein assay kit used (Bio-Rad –Bradford). The spun down pellet of the remaining sample portion was resuspended and an equal volume of 2x extraction buffer was added before storing at -20℃ overnight. Samples were pulse sonicated following protein extraction steps and then centrifuged at 5,000xg for 5 minutes at 4℃. Protein concentration of each sample was equalized to one another via PBS dilution based on the protein quantification results. Finally, 5x sample buffer was added to each sample in a 1:4 ratio before boiling at 99℃ for 15 minutes (PCR Thermocycler). Samples were stored at -20℃ until needed.
A Western blot protocol was performed to evaluate MLKL protein levels. Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using a Power Blotter Transfer Stack (ThermoFisher Scientific, Waltham, MA, USA). Samples were blocked in 5% non-fat skim milk and probed for primary antibodies specific for rabbit anti-mouse-MLKL antibody (1:500, Abcam, Cambridge, MA) and rabbit anti-mouse GAPDH (1:1000, Sigma-Aldrich, St. Louis, MO). Goat-anti-rabbit IgG used (heavy plus light chains [H+L]) (1:2000, ThermoFisher) conjugated with horseradish peroxidase was used as a secondary antibody. An enhanced chemiluminescent (ECL) horseradish peroxidase (HRP) substrate (SuperSignal™ West Pico Plus - ThermoFisher) was then applied to the membrane for 5 minutes before exposure to HyBlot film (Denville, Holliston, MA).

2.7 Statistical Analysis

Statistical analysis of protein and mRNA levels between MCMV infected and media injected control eyes was performed where necessary with P values ≤0.05 considered significant. Quantitative data from real-time RT-PCR of MLKL mRNA values from each MCMV infected eye compared to the contralateral media injected eye from the same mouse was determined by the 2^{-\Delta\Delta CT} method. Statistical analysis was done using student t-test yielding P values for each group (* p<0.05, ** p<0.01). A standard error of deviation (values from at least 3 mice per group) was also calculated for each group and labeled with an error bar. Graphed results demonstrate mean fold change of mRNA levels in MCMV infected mice compared to that of the control eyes.

Protein levels of MLKL were determined via western blot. Band density was quantified using ImageJ and graphed to visualize protein level differences in MCMV infected eyes compared to control by calculating total % of GAPDH control for each sample.
3 MLKL EXPRESSION IN WHOLE EYES DURING MCMV RETINITIS

The final and critical determinant step of necroptosis signaling is the activation and oligomerization of MLKL [11][64][65]. As discussed earlier, real-time RT-PCR work was previously done for RIPK1 and RIPK3 to evaluate the potential involvement of these upstream necroptosis-associated molecules in MAIDS-related MCMV retinitis [1]. However MLKL was not investigated at that time because little was known then about MLKL and its role in the necroptosis signal transduction pathway [65]. Current literature has brought to light MLKL’s significant involvement in necroptosis as well as suggests other potential functions. Here the aim was to determine if MLKL was stimulated in MCMV-infected eyes of MAIDS mice and thus potentially confirm or refute the possibility of necroptotic cell death in MAIDS-related MCMV retinitis.

Real-time RT-PCR is widely used to determine mRNA levels of intra- and extracellular molecules in tissues and is generally done before protein assays. As an initial step in the production of cellular products (illustrated in Fig. 12), transcription is indicative of cellular driven regulatory activity aimed at promoting particular gene expression [16]. Quantification of mRNA thus allows for an estimation of downstream protein production activity. Through the process of mRNA amplification by RT-PCR (Fig. 13), even minute amounts of a given mRNA transcript can be accurately detected in real-time [16][18]. On the other hand, western blots and IHC provide important insights into final protein expression of a gene but are not as accurate or precise due to the potential for antibody and antigen variability [17][32]. They are also not necessarily able to gauge the total effect an experimental state has on gene expression [17] which may be integral for identifying the likelihood regulatory interplay by other molecules of interest [66]. More importantly, real-time RT-PCR has a faster turn-around than protein assay [16][17]
and prevents the wasting of valuable time on subsequent protein work should mRNA be absent. Hence why preliminary probing for necroptosis-associated RIPK1 and RIPK3 mRNA levels were previously done by Chien et al. (2012) (Fig. 14). With the postliminary identification of MLKL as a necroptosis participant by scientific literature, its mRNA expression in MAIDS-related MCMV infected eyes was therefore investigated.

*Figure 12. Central Dogma of molecular biology.*
Flow of genetic information from DNA to RNA to Protein.
Figure 13. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Schematic representation of the reverse transcriptase and polymerase chain reaction used to amplify mRNA (which is quantified via real-time methods for this study). Modified from [18].

Figure 14. RIPK1 and RIPK3 mRNA expression in MAIDS-10 subretinally infected eyes compared with media control eyes. Days 3, 6 and 10 post MCMV infection are shown. Values represent mRNA fold change of MCMV infected eyes compared to media injected control eyes. Expression of mRNA
quantified by real-time RT-PCR and statistically analyzed by student T test with P values ≤0.05 being significant **p≤0.001, *p≤0.05 (n=5). (A) RIPK1 mRNA shows a significant upregulation at 6 dpi and downregulation by 10 dpi. (B) RIPK3 mRNA shows downregulation from 6 dpi to 10 dpi. These experiments were performed by Dr. Hsin Chien under the direction of Dr. Richard D. Dix, Georgia State University [1].

In the case of mRNA expression, subsequent protein assays are necessary to assess true protein levels as mRNA does not always correlate with protein expression [37][68]. This is due in part to the adaptable nature of gene expression regulation; both mRNA and protein can be modified in a number of ways that may ultimately result in alternative products or premature degradation [66][67][104]. Therefore to confirm any mRNA based predictions of protein production, it is necessary to quantify the tissue’s protein product. In comparison to mRNA quantification via real-time RT-PCR and the localization of protein via IHC, westerns can quantify relative total protein through the use of specific antibody binding and signaling (Fig. 15 antibody schematic) [68]. Thus, it is a well-established method for providing an insight into overall cellular protein production/processing activity throughout the tissue of interest. Suggested as the key player in the necroptosis signaling pathway [65], overall MLKL activity in the eye was of interest. MLKL relative total protein in whole eyes as compared to mRNA expression also in whole eyes was done by western blot.

Figure 15. Schematic representation of an antibody.
Fc = receptor binding; Fab = antigen binding. Unconjugated primary antibodies bind to target antigen by the Fab region. Secondary antibodies are primed to target protein of the primary antibody host species (will bind to Fc region); the secondary antibody binds
‘antigen/primary Fc’ by its Fab and is conjugated by a fluorescent tag on its Fc. From [30].

3.1 MLKL mRNA Expression in MCMV Infected Eyes

Figure 16. MLKL mRNA expression in MAIDS-10 subretinally infected eyes compared with media control eyes. Days 3, 6 and 10 post MCMV infection are shown. Values represent mRNA fold change of MCMV infected eyes compared to media injected control eyes. Expression of mRNA quantified by real-time RT-PCR and statistically analyzed by student T test with P values ≤0.05 being significant **p≤0.001, *p≤0.05. n=3-5

As was previously mentioned, apoptosis was found to not be the sole progenitor of cell death in MCMV retinitis [1]. In evaluation of other possible cell death pathways, RIPK1 upregulation could be indicative of either apoptosis or necroptosis cell death signaling and the minor upregulation of RIPK3 alone was not enough to significantly confirm necroptosis (Fig. 14) [1]. MLKL involvement was thus investigated in order to clarify the suggestive but ambiguous nature of this previous RIPK1 and RIPK3 data. During programmed necroptotic cell death, MLKL acts as the ‘executioner’ in the final steps - initiating and ultimately causing cell death [2][64][65][79]. This final process involves the activation of MLKL by phosphorylation which induces oligomerization with other active MLKL and causes it to translocate to the plasma
membrane; resultant selective pore formation and eventual cell lysis is characteristic of necrosis-like death [43][11][64][65]. Therefore, in support of RIPK1/RIPK3 mRNA findings, upregulation of MLKL mRNA was expected to occur during MCMV retinitis.

To test this, eyes from MAIDS model mice were taken at days 3, 6 and 10 days after MCMV and contralateral mock (media) infection in accordance with earlier selected time points. Real-time RT-PCR was run on homogenized whole eyes with a commercial primer complementary for MLKL mRNA sequence; this was repeated three times with groups of three to five animals each time. Results from MCMV infected eyes were normalized using results from mock infected eyes and graphed as mRNA fold change; overall findings were significant and repeatable (Fig. 16). MLKL mRNA was increased 3 dpi by ~36 fold in MCMV infected eyes when compared to the media injected eyes, and was highly significant with little variability between samples. The increase in mRNA expression (~38 fold change) by day 6 was also found to be significant. The mRNA expression seen for day 10, while not significant due to sample variability, displays a noticeable decrease. This indicates a general trend of high day 6 expression that seems to greatly abrogate by day 10 and has been seen both in previous and subsequent data [1]. In comparison of mRNA data for the three necroptosis-associated molecules investigated (Fig. 14) (Fig. 16), it is pertinent to evaluate their combined results both in terms of regulation and sequential signaling along the necroptosis pathway.

Discussed beforehand, we know that RIPK1 is the first required molecule to initiate classical necroptosis signaling [2][11]. However it is also involved in several other pathways and thus highly regulated with specific responses likely being conserved between individuals [10][2][43][69][103]. Perhaps for that reason, mRNA values remain tightly clustered and significant at each time point. On the other hand, lack of significance in RIPK3 mRNA data may
result from a reduced need for strict RIPK3 regulation due to its limited roles and the unlikelihood of non-specific signaling [2]. Furthermore, it’s signaling activity is largely transient [79] and indirectly controlled by other activating/deactivating molecules already under tight/specific regulation such as caspase 8, RIPK1 and MLKL [43][65][72][75][80]. A proteasome system has been suggested as a method for direct RIPK3 regulation; variable poly-ubiquitination of different lysine residues by several non-specific ubiquitin ligases and the potential for proteasome degradation could result in differential outcomes that vary between cells [10][77][81]. Even at basal levels, necroptosis signal transduction can still occur as a few RIPK3 can transiently activate many more MLKL [2][10][79]. Even then, total absence of RIPK3 is relatively well tolerated as can be seen with RIPK3 knockout mice [76]. Overall RIPK3 may be an incredibly useful alternative for inducing necessary signaling in a compensatory but non-essential capacity; if so, this could indicate there is less selective pressure on RIPK3 expression between cells and individuals. Increased regulatory control is likely seen again though with the final effector of necroptotic cell death, MLKL [11][64]. It is crucial to necroptosis [65][78] and knocking it down/blocking it inhibits necrosis characterized death [65][77]. Current research also seems to indicate the possibility of alternative MLKL activation and functions [33][70][71]. Taken together it could be assumed that MLKL is integral to cellular function but potentially harmful if disregulation were to occur. Variation in expression however is still observed to increase with each successive time point with loss of significance by day 10. Unfortunately a lot is still unknown about MLKL [65], so there could be several reasons for the trend in its mRNA. It could simply be that the rate of necroptosis progression differs between individuals, perhaps in response to differential RIPK3 expression.
Despite any differences in mRNA expression between individuals, there is still a very clear overall trend between day 6 and 10. Initial mRNA upregulation differ between RIPK1, RIPK3 and MLKL but likely for some of the reasons discussed above. RIPK1 is expressed in healthy cells at higher amounts due to its multi-functionality [2][11][69][103]; during early disease there may not be a large change in expression between infected and non-infected eyes due to the abundance of readily available RIPK1. When infection and/or inflammation reach critical levels though, cells likely respond in kind in order to clear the infection such as seen at day 6 [2][43]. This spike in mRNA expression may be due to the combined effect of necroptosis and other RIPK1 mediated pathways sent into transcriptional overdrive in order to increase activity of certain pathways and to replenish the rapid turnover of RIPK1 [72]. In regards to RIPK3 initial upregulation, there is not yet a confirmed consensus on the possible expression of constitutive RIPK3 [77] or the lack thereof [70]. What is known is that low levels of RIPK3 are still able to efficiently signal for necroptosis [72] [2][10] and it plays a part in the cross-talk between cell death pathways, allowing for the cell to respond to different scenarios via alternative immune/cell death signaling [2][76]. Necroptosis is also well known as an anti-viral response [65]; transcriptional upregulation of RIPK3 mRNA at day 3 might be indicative of cellular prep for necroptosis upon apoptosis inhibition [1][2]. Expression decreases by day 6 but is still noticeably higher than control. MLKL on the other hand is constitutively expressed at basal levels [71] but is needed in greater amounts in order to oligomerize, form pores and successfully orchestrate cell death [2][11][64]. Again, likely in order to prep for potential necroptosis activity, MLKL mRNA expression is also increased at day 3 and even further at day 6. Overall, the increased mRNA expression of all three might suggest significant stimulation of necroptosis activity by day 6. Most notably, they show a noticeable decline in mRNA expression
from day 6 to 10 (Fig. 14 and 16). The interim between these two time points happens to be when the greatest changes to the retina occurs during MCMV retinitis. The decrease in transcription of these cell death pathway molecules correlates with the decrease in active retinitis once it has progressed to full retinal necrosis [1].

The assessment of MLKL expression via real-time RT-PCR quantitative analysis found that mRNA was upregulated in MAIDS-related MCMV infected eyes. The comparison with previous RIPK1/RIPK3 findings further links these necroptosis-associated molecules and known disease progression. Due to the integral nature of MLKL, its increased mRNA expression makes for the most definitive case of possible necroptosis so far. The translation of mRNA to actual protein levels must first be determined before the presence of MLKL can be fully confirmed.

3.2 MLKL Protein Expression in MCMV Infected Eyes

![MLKL protein expression graph]

Figure 17. MLKL protein is constitutively expressed in MAIDS-10 mice following subretinal injection of either MCMV or media control.
Western Blot. Top: bar graph of quantified total protein levels at days 3, 6 and 10 post MCMV infection (dpi) compared to contralateral mock non-infected eyes (media injected) with total % of ubiquitous housekeeping molecule GAPDH as a control for each sample. Bottom: blotted protein; band density measured in ImageJ to quantify. Statistical analysis via student T-test.

In necroptosis cell death signaling, MLKL is synonymous with the pathway more so than RIPK1 and RIPK3 [78][79]. It is also linked to RIPK3 activity in which one RIPK3 molecule can activate several MLKL via phosphorylation [2][11]. To function as the cell executioner, several active MLKL must form an oligomer [2][64]. MLKL mRNA must then correlate to an increased concentration of MLKL protein in order to support necroptosis cell death [64]. To further evaluate relevance of mRNA upregulation, total MLKL protein concentration was quantified via western blot to confirm the presence of this death determinant necroptosis-associated molecule in MAIDS-related MCMV retinitis eyes [11][64][65].

Whole eyes again were taken at the same time points, homogenized and prepped for western blot against a phospho S345 MLKL antibody. Each sample tested consisted of a pool of 5-10 animals. Results for MCMV eyes were compared directly to mock infected eyes by band density and graphed as a % of the control, GAPDH (Fig. 17). MLKL protein is constitutively expressed in mock injected eyes at all time points, and although not significantly different, there appears to be a trend of increased MLKL protein production in MCMV infected eyes when compared to the media injected controls. It is possible that MCMV infected eyes may not show substantially increased levels of MLKL protein due to alternative activation of MLKL or protein localization to specific areas of tissue. It is of interest to note that the MLKL antibody used is specific to phosphorylated MLKL at the 345 serine residue which is thought to signify its active state. This phosphorylation site is independent of two other phosphorylation sites along the activation loop of MLKL – Ser347, Th349 [74]. While there is disagreement on the necessity of
Th349 phosphorylation for activation [65][74], Ser347 has been found to be necessary for oligomerization [74][79]. Even with Ser347 phosphorylation, the ability to form oligomers is further blocked until a critical threshold of MILKL is reached [71]. Structurally, MLKL contains a c-terminal psuedokinase domain with an activation loop, a hinge and an attached N-terminal 4HB ‘killing’ domain [75][79]. When it is phosphorylated at its active loop, a conformational change moves the C-terminal and N-terminal apart [75][92]. The 4HB domain can then associate with the membrane through its two charged residue faces [70][75]. That being said, small inhibitory molecules can bind at the activation loop concurrently to phosphorylation activation [75][79]. These small molecules, such as ATP, can prevent conformational change and subsequent oligomerization/cell death [71][75]. MLKL can still translocate to the membrane but that alone is not sufficient to induce cell death [92]. Therefore, P345 MLKL might not always equate to active/functioning MLKL. Validation of other MLKL antibodies would be useful in elucidating the exact mechanism of MLKL involvement in MCMV infected eyes.

Constitutive expression of MLKL and other unknown effectors above threshold levels is maintained by constitutive IFN signaling [70][71]. This mechanism of cellular homeostasis is responsible for endosome activity and extracellular vesicle formation by MLKL [70]. Normally autocrine IFN signaling works through the cGAS – Sting DNA sensor pathway to respond to DNA damage, however, it can also respond to viral DNA and induce necroptosis augmentation [71]. This occurs commonly in autoimmune and viral mediated cases and primes cells for necroptosis [71]. In the case of MLKL activation, IFN signaling can either inhibit its activity through small molecule binding [79] or utilize it for enhanced extracellular vesicle formation [71]. For the latter, this enhanced activity helps maintain homeostasis in a stressed cell and/or facilitates the release of cytokines (ex: IL-1beta) – more interestingly, it also removes excess
activated MLKL in an effort to hold off necroptotic cell death [70][71]. Depending on cell type and the reason for necroptosis priming in the cell, some never succumb and instead are able to reestablish homeostasis [70]. The immunomodulation and suppression that results from MAIDS may be priming cells for heightened responses to normal cell housekeeping activity. The mock infected eyes could very well be displaying active MLKL without undergoing cell death in the retina.

While presence of MLKL can signify programmed necroptosis, total amount of protein should not be taken to equate to levels of necroptosis activity in a 1:1 ratio. Western blots for RIPK1 and RIPK3 (not shown) were indicative of increased total protein in MCMV infected whole eyes. RIPK1 showed only cleaved protein until day 10, at which time both a cleaved and full protein appeared. Cleavage is usually a sign of some sort of caspase 8 mediated activity such as apoptosis or NF-kB mediated cell survival/inflammation [43][69][72]. That considered together with the eventual shift to non-cleaved RIPK1 could be suggestive of either simultaneous apoptosis necroptosis or early apoptosis secondary necroptosis; regardless the ultimate outcome is necroptotic cell death [2][73][97]. If apoptosis was the main or sole cell death pathway at play, we would expect to see cleaved RIPK3 and MLKL [43][73][81]. RIPK3 also had two bands at different molecular weights. A band with a higher than normal RIPK3 weight was shown very prominently at day 3 and increased in size by day 10. The normal RIPK3 band was barely visible at day 3 but did increase in density by day 10. While it is difficult to accurately predict what the heavier RIPK3 protein is, there is at least some evidence for the existence of two RIPK3 isoforms [76]. Alternatively, post translational modifications are not uncommon during RIPK3 signaling and regulation; phosphorylation, ubiquitination or oligomerization may have resulted in a heavier protein [99]. While the presence of these necroptosis related proteins in MCMV
infected eyes indicate the possible stimulation of the necroptosis pathway, protein level alone fails to tell us if these proteins are functionally responsible for the observed MCMV retinitis pathology, nor does it actually localize them to the retina.

4 LOCALIZED EXPRESSION OF NECROPTOSIS-ASSOCIATED MOLECULES IN THE RETINA FOLLOWING MCMV INFECTION OF MAIDS-10 MICE

Immunohistochemical staining (IHC) allows for the localization of target molecules to specific areas of tissue [40]. Using fluorescently labeled antibodies (Fig. 18) differential patterning of molecules can be visualized across tissue and during different time points in disease pathogenesis [41]. The amount and location of a molecule of interest can thus be qualitatively assessed. This makes IHC an incredibly useful technique that is commonly used in disease pathology studies [41][52]. Therefore to confirm that mRNA and total protein findings associate with the retinitis disease model during MAIDS-related MCMV infection, localized staining of necroptosis-associated molecules at the retina was necessary.

*Figure 18. Indirect Immunofluorescence.*
IHC staining that uses a primary antibody to bind to the antigen and then a fluorescently labeled secondary antibody to bind to the primary antibody. In this study: antigen (mouse
tissue) > anti-antigen primary antibody (hosted in rabbit) > anti-rabbit secondary antibody labeled with Cy3 (hosted in goat). Modified from [36].

MCMV and contralateral-mock infected whole eyes were paraffin embedded eyes and sectioned then stained using specific antibodies for RIPK1, RIPK3 and MLKL respectively (Fig. 18). Day 6 and day 10 post-infected eye sections were examined with day 6 displaying retinal folding and day 10 displaying full retinal necrosis. These time points were selected in accordance with the observed largest change in disease state indicated by mRNA results (Fig. 14) (Fig. 16) and disease pathology in this model (Fig. 6). Results from MCMV eyes were normalized to mock infected (media injected) eyes and to an isotype negative control. It was expected that RIPK1, RIPK3 and MLKL expression would be upregulated in the retina during MAIDS-related MCMV infection.

While IHC is integral for confirmation of localization to the site of interest, it is not without its drawbacks. Due to antibody difficulty in binding effectively to antigens in tissue [17][32] there occasionally might be a loss of signal and the representation that certain molecules of interest are under-expressed. Additionally, issues can arise when diseased tissue is normalized to control tissue. Mock infected sections do not exhibit retinitis pathology however, they may be subject to inflammatory processes mediated by local tissue injury from the injection itself and/or can be subject to such problems as retinal detachment which is associated with necroptotic cell death [7][8]. Furthermore, in in-bred lab strains of mice, there could be possible inflammatory/immune related over-reactions triggered by this event or already in process due to the normal environmental stressors the eye undergoes [35]. This is due to the immunomodulatory nature of inbreeding-depression and other genetic effects [31]. Aside from any additional immune pressures mediated by this model, there is also a case to be made for normal levels of cell death undergone by healthy tissues and even constitutive or alternative expression of certain
molecules outside of active cell death signaling [2][33][45][65][69]. In part, healthy mock infected tissue could exhibit observable levels of RIPK1, RIPK3 and MLKL expression that has to be accounted for by normalizing and thus potentially reducing observable true expression in diseased tissue. IHC provides final confirmation of pathological association but is done with support from mRNA and total protein data in order to form a more complete picture of necroptosis associated cell death. Overall, it was indeed found that MCMV infected retinas expressed more RIPK1, RIPK3 and MLKL; especially at day 6 post-MCMV infection. Necroptosis signaling appears to be present during disease pathogenesis and could be inferred to be actively inducing cell death during the retinal destruction apparent in this model.

4.1 RIPK1 Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

Presence of RIPK1 protein in the retina was qualitatively evaluated via IHC to determine what differences, if any, there were in RIPK1 localized activity across MCMV infected and non-infected retina at different time points. As RIPK1 is also associated with apoptosis cell death signaling [2][42], its expression alone doesn’t determine what cellular death machinery is at work. However, RIPK1 is the branching off point of many cellular signaling pathways: apoptosis, necroptosis, cell survival and cytokine release through NFκβ [43][69]. Meaning cellular activity in response to different stimuli can have differential effects on RIPK1 that then determine its downstream signaling [2][8][10]. RIPK1 is an important molecule in understanding what other players may be at work or inhibited during certain cellular processes. It can also serve as a good indicator of overall initiation of programmed death signaling when tissue destruction is observed. Most importantly, as it is associated with necroptosis classical signaling [2][11],
RIPK1 expression in addition to RIPK3 and MLKL expression suggests necroptosis cell death signaling.

At day 6 post-MCMV infection, RIPK1 is expressed in all retinal layers in comparison to media injected eyes (Fig. 19). Of note is the expression of RIPK1 in the photoreceptor bodies (rods and cones) of the media injected eyes. Expression was high enough that it could not be normalized to black without losing all signaling in the MCMV infected retinas. This might indicate some increased constitutive expression, though what RIPK1 mediated activity, if any, is underway is unknown. However, photoreceptor bodies of MCMV infected retinas seem to have even higher levels of RIPK1 expression. This increased expression seems congruent with the dysregulated and diseased state observed in this layer, signifying active cell death. By day 10 (Fig. 20), full retinal destruction can be seen as the retina is no longer organized into discernable layers. RIPK1 is still shown to be expressed, although in decreased amounts due to general reduced cellular presence and activity consistent with end-stage disease. What little cell death still underway appears at least partly RIPK1 related. In regards to non-diseased tissue, photoreceptor bodies from media injected eyes again express RIPK1, further supporting potential constitutive expression. All together, these results show that RIPK1 is up-regulated during day 6 and 10 MCMV induced retinitis in support of mRNA findings (Fig. 14). This increased expression is clear despite possible issues discussed previously as well as other prospective difficulties arising from RIPK1 post translational modification [99], reduced antibody binding at the intracellular level [17][32][36] and/or protein degradation after cell lysis [93][99].
Figure 19. RIPK1 expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of RIPK1 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control. 3rd row: non-infected control (media injected) with primary antibody 4th row: media negative isotype control

Key: star labels the photoreceptor side of the retina for orientation where still intact. Retinal folding is characteristic of 6 days post MCMV infection (dpi).
Blue: Dapi  Red: RIPK1  Merge

Figure 20. RIPK1 expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of RIPK1 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control. 3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control
Key: star labels the photoreceptor side of the retina for orientation where still intact.
Retinal folding is characteristic of 6 days post MCMV infection (dpi).
4.2 RIPK3 Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

To further support the idea of necroptosis cell death signaling in MAIDS-related MCMV retinitis, RIPK3 localization was expected to follow RIPK1 IHC findings. RIPK3 is more associated with necroptosis and is often the alternative means of inducing cell death when apoptosis is otherwise inhibited [2][8][10]. It has also been found to modulate cellular activity in other ways before finally mediating cell death [43][76]. That being said, RIPK3 is not as widely used as RIPK1 [103] and appears to be dispensable for most cellular activity [76]. Even during necroptosis signaling it takes only a few active RIPK3 to transiently bind to and phosphorylate numerous MLKL [79]. So RIPK3 does not necessarily have to be greatly expressed to efficiently induce signal transduction along its pathway.

According to mRNA, RIPK3 is expressed in MCMV infected eyes even if at low amounts (Fig. 14). To determine if that translated into protein localized to the retina, IHC was done. At day 6 post-MCMV infection, RIPK3 is indeed expressed in the retina even if at minimal levels compared to media injected (Fig. 21). Its presence is far more disperse that that of RIPK1 which evenly presented itself throughout the retinal layers. By day 10, there is a dramatic decrease in expression but with some RIPK3 still present as compared to media injected (Fig. 22). It still occurs in a disperse manner whereas, at this time RIPK1 was strongly expressed in a few focused sites. The differential localization of RIPK1 and RIPK3 at day 10 may be related to simultaneous or early apoptosis, secondary necroptosis [2][73] and/or RIPK1 independent necroptosis coming more into play [72]. As discussed before, low RIPK3 expression in general could be due to the relatively efficient nature of RIPK3 in effecting downstream signal
transduction players; so less is needed. Or it may also be indicative of under-representation of
true levels because of post translational modification activity [99], rapid protein degradation after
use or cell lysis [77][81] and/or difficulty of intracellular antigen antibody binding [17][32][36].
In examination of media injected eyes, it was appeared that there may be some slight constituent
RIPK3 expressed in the photoreceptor bodies such as was found with RIPK1. Overall, results
indicate that RIPK3 is upregulated in the retina during MAIDS-related MCMV retinitis; a good
but not confirmatory predictor of potential necroptotic cell death activity.
Figure 21. RIPK3 expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of RIPK3 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control. 3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control
Key: star labels the photoreceptor side of the retina for orientation where still intact.
Blue: Dapi    Red: RIPK3    Merge

Figure 22. RIPK3 expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of RIPK3 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control. 3rd row: non-infected control (media injected) with primary antibody 4th row: media negative isotype control

Key: star labels the photoreceptor side of the retina for orientation where still intact
4.3 MLKL Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

Lastly, MLKL presence in the retina was investigated as a follow up to RIPK1 and RIPK3 data. The interactions between RIPK1, RIPK3, MLKL and subsequent signaling mechanisms are not yet fully understood; MLKL in particular is still a subject of much interest and new findings [65]. However, MLKL is currently known to be predominantly related to necroptosis and is the final step in initiating cell death [64][65][77]. In conjunction with RIPK1 and RIPK3 IHC findings, its presence could confirm cell death via necroptosis. Its absence on the other hand would definitively rule out necroptosis as a possible effector of retinal destruction [77]. Not only is MLKL integral to the final step in cell death but it also is responsible for the characteristic necroptosis manner of cellular death that is unique among programmed cell death pathways [5][43][65]. Overall, MLKL is the final clue in putting together a complete picture of necroptosis induced disease pathogenesis in MAIDS-related MCMV retinitis.

A potentially active form of MLKL was stained for via one of its phosphorylation sites (345). While this alone is not fully indicative of active MLKL [65][74], it can be valuable when taken into consideration along with cellular location of stained MLKL. Investigation by IHC at day 6 post-MCMV infection, found MLKL to be highly and evenly expressed in all nuclear retinal layers except in the ganglion layer where it was expressed at reduced levels (Fig. 23); this is suggestive of dominantly active necroptosis signaling and cell death in all but the ganglion cells. Of interest is the apparent association of MLKL with the plasma membrane of the retinal cells as exhibited by the circular periphery cell staining seen. As MLKL is translocated to the plasma membrane to form pores and effect final cell lysis [64], this is solid evidence of necroptosis activity in progress. MLKL was also expressed in the photoreceptor bodies of
MCMV infected retinas but not in healthy retinas unlike RIPK1 and RIPK3. By comparison, retina from media injected eyes do not exhibit MLKL expression in any amount or location. By day 10, at sites of full retinal necrosis, there is still clear expression of MLKL in focused locations and in a more widespread pattern than RIPK1 (Fig. 24). However, just as with RIPK1 and RIPK3, there is a general reduction in expression due to complete retinal destruction nearing. Again, there is no expression in retina of media injected eyes. MLKL retinal expression is apparently upregulated by MCMV infection. It appears to be more highly expressed than either RIPK1 or RIPK3; perhaps just as a matter of greater MLKL needed to efficiently participate in signaling and cell death. Alternatively it might be due to the aforementioned potential issues that could affect RIPK1/RIPK3 staining; in that case, MLKL would likely be easier to stain as it associates with and dysregulates the membrane [64]. As the last downstream signaling/effect protein involved, it is also possible that the timing of major changes in disease state coincide with MLKL activity at its peak as opposed to the earlier involvement of RIPK1 and RIPK3. Regardless of any such issues though, the abundant MLKL expressed in MAIDS-related MCMV infected retinas indicates necroptotic cell death involvement in MCMV retinitis.
Figure 23. MLKL expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of MLKL protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control. 3rd row: non-infected control (media injected) with primary antibody

4th row: media negative isotype control

Key: star labels the photoreceptor side of the retina for orientation where still intact.
**Figure 24. MLKL expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.**

Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of MLKL protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody.
2nd row: MCMV negative isotype control.
3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control

Key: star labels the photoreceptor side of the retina for orientation where still intact.
5 LOCALIZED EXPRESSION OF PYROPTOSIS-ASSOCIATED MOLECULES IN THE RETINA FOLLOWING MCMV INFECTION OF MAIDS-10 MICE

As addressed before, apoptosis and necroptosis were not the only programmed cell death pathways suspected to be involved in the pathogenesis of MAIDS-related MCMV retinitis [1]. The inflammatory disease state observed during MCMV retinitis suggests the additional involvement of pyroptosis cell death [2]. Similar to apoptosis, it is a caspase dependent signaling pathway; though the induction of cell death through membrane pore formation is more reminiscent of necroptosis [1][2][27]. That being said, pyroptosis is characterized by a distinctive style of cell death. Caspase 1, caspase 11 and GSDMD are widely recognized as significant intracellular pyroptosis facilitators [50] when coupled with, IL-1β and IL-18 activation and release [2][27][34][53][54]. Canonical pyroptosis is considered the classical pathway in which caspase 1 activation of GSDMD and IL-1β/IL-18 prompts a highly inflammatory form of programmed cell death [2]. Non-canonical pyroptosis on the other hand is affected by caspase 11 which is only capable of GSDMD activation and cell death that results in non-specific DAMPS inflammation [2]. Differential signaling permits a more adaptive response to pathogenic threats and is dependent on type of stimuli; thus both are of interest in disease pathology investigations [2][53][56].

Previous work found pyroptosis-associated mRNA and total protein to be upregulated [1] in MCMV infected eyes (Fig. 25) (Fig. 26) (the rest performed by Jessica Carter under the direction of Dr. Richard D. Dix, Georgia State University-manuscript in progress). This was supportive of pyroptosis activity during MCMV infection but was not definitive. [37][68]. Localization of protein to areas of disease pathology is necessary to assess involvement during disease pathogenesis [40][41][52]. Therefore, to confirm previous findings of pyroptosis signaling
during MAIDS-related MCMV infection, pyroptosis-associated proteins were evaluated by IHC in retinas of MCMV and mock infected eyes. It was expected that pyroptosis-associated proteins would be found stimulated in the retinas of MCMV infected eyes.

To qualitatively assess and localize caspase 1, caspase 11, GSDMD, IL-1β and IL-18 in retinal tissue, MCMV and contralateral-mock infected whole eyes were formalin fixed, paraffin embedded, sectioned and then stained as described. Primary antibodies specific to protein were used followed by a fluorescently labeled secondary antibody (Fig. 18) to visualize each protein of interest separately. Just as with prior IHC work, day 6 and day 10 post-infected eye sections were again examined. Results from MCMV eyes were normalized to mock infected (media injected) eyes and to an isotype negative control. For the most part, it was found that MCMV infected retinas expressed minimal amounts of investigated pyroptosis-associated molecules at day 6; expression of GSDMD, IL-1β and IL-18 appeared to increase by day 10 however. These low levels of expression could be true representations of pyroptosis intracellular signaling, signifying marginal involvement of pyroptosis cell death in MCMV retinitis. Alternatively, pyroptosis may be active at different time points; the increased expression at day 10 may indicate a different temporal expression than what was observed for necroptosis. Regardless, pyroptosis signaling does appear to be at least slightly active and thus may be responsible for some of the cell death activity observed in MAIDS-related MCMV retinitis.
Figure 25. Caspase 1 mRNA expression in MAIDS-10 subretinally infected eyes compared with media control eyes. Days 3, 6 and 10 post MCMV infection are shown. Values represent mRNA fold change of MCMV infected eyes compared to media injected control eyes. Expression of mRNA quantified by real-time RT-PCR and statistically analyzed by student T test with P values ≤0.05 being significant **p≤0.001, *p≤0.05 (n=5). Caspase 1 mRNA shows a significant upregulation at 6 dpi and down regulation by 10 dpi. These experiments were performed by Dr. Hsin Chien under the direction of Dr. Richard D. Dix, Georgia State University [1].

Figure 26. Cleaved caspase 11 protein in MAIDS-10 mice following subretinal injection of either MCMV or media control. Western Blot. Top: bar graph of quantified total protein levels at days 3, 6 and 10 post MCMV infection (dpi) compared to contralateral mock non-infected eyes (media injected) with total % of ubiquitous housekeeping molecule GAPDH as a control for each sample. MCMV infected eyes expressed only slightly more protein than the mock eyes. Bottom: blotted protein; band density measured in ImageJ to quantify. Statistical analysis via student T-test.
5.1 Caspase 1 Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

Qualitative examination of caspase 1 localization at the retina was done to assess potential pyroptosis activity in MCMV infected eyes compared to mock infected (media injected) eyes. Caspase 1 is not involved in either apoptosis or necroptosis [51]. Furthermore, canonical pyroptosis is caspase 1 dependent [51]. As such, it is considered a specific pyroptosis-associated molecule and its presence in diseased tissue is very telling. Caspase 1 is activated by autocatalytic cleavage of pro-caspase 1 after inflammasome recruitment; the inflammasome is itself assembled in response to a number of stimuli such as dsDNA, PAMPS, toxins, ATP and/or pathogen stimulation [2][27][50]. After caspase 1 is activated, it directly initiates pyroptosis activities through the cleavage and activation of GSDMD, IL-1β and IL-18 [34][2][27][56]. The end result of which is membrane pore formation and secretion of inflammatory cytokines before cell death [2][27]. This canonical pathway is essential for the cytokine specific inflammatory response often induced by pyroptosis [34][53]. However, a general inflammatory state is also induced with the fulfillment of cell death and DAMPS release [56][2][27]. Altogether, the heightened inflammatory state induced by caspase 1 canonical pyroptosis cell death is a potent means of defense against pathogens [27].

Previous caspase 1 mRNA demonstrated increased expression during MAIDS-related MCMV infection, especially at day 6 post infection (Fig. 25); this was further supported by total protein work (performed by Jessica Carter et al.). To confirm that this expression was at the retina, IHC staining was indispensable. IHC results at day 6 post-MCMV infection showed low levels of visible expression as compared to retina from media injected eyes (Fig. 27). Expression, while very minimal, was evenly distributed throughout the retina in all layers. By day 10,
expression was reduced throughout the retina but showed heightened expression in a few concentrated areas (Fig. 28). These observations indicate that MCMV infection stimulates caspase 1 production at low levels during retinitis progression. This could be indicative of true caspase 1 expression but alternatively might indicate staining issues arising from poor antibody availability, protein degradation and difficulty accessing intracellular proteins [17][32][37]. Further, it is entirely possible that the mRNA and total protein findings came from other areas of the eye; again supporting low expression in the retina. Day 6 results could more specifically imply that pro-caspase 1 is upregulated in retinal cells upon initial disease insult but otherwise remains inactive [106]. Meaning that they are not yet associated with inflammasomes or visibly concentrated around them [2][27][106]. Additionally, a single caspase 1 is able to activate multiple IL-1β during pyroptosis [34][53]; as such, this could explain why overall caspase 1 expression is low. Thus caspase 1 may remain relatively innocuous in its inactive state, giving rise to the even dispersal of expression throughout the retina seen at day 6. Overall, caspase 1 IHC staining seems to be characteristic of low but even expression across tissues in mild or non-diseased states [107]. Conversely, by day 10 it appears that caspase 1 exhibits some increased expression. On first consideration it appears that pyroptosis, as it relates to main initiator caspase 1, may occur later than apoptosis/necroptosis. Further thought however brings to mind the fact that caspase 1 activity can continue right up until cell death [2][11][34]; therefore it is not always an accurate indicator of initial pyroptosis induction. So while the idea of late onset pyroptosis could still be valid, it can’t be definitively confirmed from this data. That said, caspase 1 expression by day 10 could be the result of pro-caspase 1 inflammasome recruitment and activation as it serves to concentrate caspase 1 [2][34]. Taking a step back to day 6 caspase 1 expression, it is possible that many of the retinal cells degraded pro-caspase 1 in favor of other
cell death pathways more immediately available [2]. Those that didn’t may be the cells that exhibited increased caspase 1 by day 10. Even if day 6 caspase 1 is in fact expressed in the active form, it does not take away from day 10 changes but does suggest that caspase 1 mediated pyroptosis is minimally at play early on in disease pathogenesis. Given that canonical pyroptosis needs two separate stimuli to fully induce activity, it may be that the initial disease state provides for very few pyroptosis specific stimuli – enough to stimulate transcriptional activity and produce inactive forms of the proteins involved but not enough to induce catalytic activity in most of the cells [106]. Further inflammatory disease progression likely provides for more pyroptosis activating stimuli and thus increased levels of caspase 1 activation to facilitate further pyroptosis in what remains of the retina [2][27][43][50][53]. Of final interest is a proposed alternative mechanism for executing cell death within the canonical pathway through the use of IL-1b autocrine stimulation and subsequent pyroptosome formation, a poly ASC inflammasome-like complex that acts on caspase 1 [111]. Caspase 1 activity can be modulated a number of ways by pyroptosome transient recruitment and activation or complex formation [108][109][110]; most commonly it inhibits the ability of caspase 1 to mediate cytokine activation but accelerates its GSDMD cleavage and cell death associated activity [110][111]. The occurrence of which takes place after prior inflammasome caspase 1 pyroptosis activity and cytokine activation; the release and autocrine signaling of IL-1b potentiates this particular method of pyroptotic cell death [111]. Presence of and co-association with pyroptosome are usually observed as ‘specks’ [109].
Blue: Dapi  Red: caspase 1  Merge

Figure 27. Caspase 1 expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of caspase 1 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 6 dpi caspase 1, caspase 11 and GSDMD). 3rd row: non-infected control (media injected) with primary antibody.
4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD).
Key: star labels the photoreceptor side of the retina for orientation where still intact.
Blue: Dapi  Red: caspase 1  Merge

Figure 28. Caspase 1 expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of caspase 1 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 10 dpi caspase 1, caspase 11 and GSDMD). 3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD)
Key: star labels the photoreceptor side of the retina for orientation where still intact.
5.2 Caspase 11 Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

While caspase 1 is integral to canonical pyroptosis, caspase 11 is equally associated with the non-canonical pathway [2]. As discussed earlier, the non-canonical pathway only involves pyroptotic characterized cell death without the release of inflammatory cytokines [2][27]. Caspase 1 and 11 are thus both capable of executing pyroptosis but have also been known to work together [56]. Unlike caspase 1 though, caspase 11 can be cleaved and activated via bacterial LPS (lipopolysaccharide) without inflammasome involvement; the exact mechanism of which is currently unknown [2][27]. Consequently the sole presence of caspase 11 may be indicative of only a bacterial infection as opposed to other means of cellular insult [2][50]. In relation to MCMV mediated infection, caspase 11 would thus be considered a potential but not necessary player. Post activation, it can cleave GSDMD which leads to pore formation and eventual cell death [2][27][56]. It is important to note though that caspase 11 is currently under investigation for its potential to assist in caspase 1 activation and signaling [113]. Ultimately, caspase 11 signaling can act as an additional means to execute pyroptosis but without the specific inflammatory response achieved by cytokine activation and release [56].

Total protein findings showed increased protein levels at day 6 MCMV infection in whole eyes (Fig. 26) which was also supported by mRNA findings (performed by Jessica Carter et al.). Further work in IHC staining of the retina revealed several clues in the possible functional activity of caspase 11 in this disease model. To note though, due to a lack availability, the caspase 11 antibody was not specific to just the cleaved/active form. That in addition to potential issues already discussed ad nauseum for caspase 1 may misrepresent actual levels of active caspase 11. However, previous data and caspase 1 findings were integral in the formation of
reasonable explanations into caspase 11 expression and activity during disease progression. Protein localization revealed that caspase 11 overall shows even less upregulation than that of caspase 1. At day 6 MCMV infection, there was minimal protein exhibited in the retina except for a few of what appeared to be ‘specks’. While it has been seen that caspase 1 can be often associated with pyroptosome ‘specks’ [108][109], both can also be expressed without co-expression of the other [110]. Aside from the poly-ASC base complex, pyroptosome inclusion of certain inflammasome associated proteins and even other caspases can be variable and largely dependent on the type of cellular insult/stimuli [109][110]. It is suggested though that pyroptosome assembly can only occur with canonical pyroptosis associated stimulation and not in LPS stimulated caspase 11 non-canonical pyroptosis [108]. However, given that pyroptosis signaling can involve the interplay of both canonical and non-canonical pathways and that regardless it needs at least two stimuli to proceed to actual pyroptotic activity [106], early caspase 11 presence should not automatically preclude pyroptosome assembly. And while the mechanism and exact means of association is unknown, caspase 11 itself has additionally been implicated in inflammasome involvement [113]. This opens the door for its possible pyroptosome involvement as well; though it may still necessitate caspase 1 participation and/or alternative non-LPS cellular stimulation. Therefore the ‘speck’-like caspase 11 pattern observed in day 6 MCMV infected retinas may truly represent pyroptosome formation and co-expression as a functional indicator of pyroptosis activity. As briefly mentioned before, secondary upregulation of caspase 1 (Fig. 28) may be correlated with pyroptosome mediated activation through only a transient interaction which would explain the apparent lack of a ‘speck’ pattern. Alternatively, this ‘speck’ pattern may be another super-molecular formation altogether such as an active caspase 11 oligomer complex [112] which is supportive still of a rapid initial caspase
11 response. Regardless of the mode of expression however, caspase 11 protein is no longer seen by day 10 MCMV infection as compared to mock infected retinas (Fig. 30). This occurs again in contrast to caspase 1 activity, suggesting a possible sequential progression of non-canonical to canonical pyroptosis activity in this disease model or caspase 11 interference in canonical pyroptosis. More definitively the findings do show that caspase 11 is upregulated during the progression MCMV infection.
**Figure 29. Caspase 11 expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.**

Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of caspase 11 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody.
2nd row: MCMV negative isotype control (shared for 6 dpi caspase 1, caspase 11 and GSDMD).
3rd row: non-infected control (media injected) with primary antibody.
4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD).

Key: star labels the photoreceptor side of the retina for orientation where still intact.
Figure 30. Caspase 11 expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of caspase 11 protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody.
2nd row: MCMV negative isotype control (shared for 10 dpi caspase 1, caspase 11 and GSDMD).
3rd row: non-infected control (media injected) with primary antibody.
4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD).
Key: star labels the photoreceptor side of the retina for orientation where still intact.
5.3 GSDMD Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

GSDMD is critical to the pore forming mechanism needed to finally drive pyroptotic cells into death [2]. It was therefore qualitatively analyzed via IHC as its presence is characteristic of only pyroptosis [2][27]. It’s interactions with Caspase 1 and 11 make it of further interest as they both cleave and activate GSDMD separately [2][27][50]. Cleavage results in the loss of GSDMD’s C-terminal end, leaving the active N-terminus to initiate final pyroptosis activities. The N-terminus oligomerizes with other active GSDMD and translocates to the membrane where it can form pores and ultimately cause cell death [2][27]. In comparison to necroptosis, both it and MLKL fulfill the same end points in very similar ways [2]. However, classical necroptosis cell death releases danger associated molecular proteins (DAMPS)/debris from the cell [9]. Pyroptosis on the other hand relies on the GSDMD formed pores to release Caspase 1 activated cytokines before ultimate cell death and release of its own DAMPS [2][34][53][54]. Additionally, fewer GSDMD are needed to form pores to mediate cellular destruction; these pores are much larger than those formed by MLKL [105]. So while there are certainly similar processes that take place between the two cell death pathways, GSDMD is integral for more than one pyroptotic process [27].

Again, as with the other pyroptosis molecules under investigation here, total protein and mRNA findings GSDMD has been shown to be upregulated (performed by Jessica Carter et al.). Such findings are highly indicative of pyroptosis pathway signaling/activity due to the uniquely defining nature of GSDMD. However, its direct association with the actual disease state had yet to be shown, necessitating investigation by IHC in MCMV infected retinas. Results do indicate pyroptosis involvement as expressed by GSDMD protein localization in the retina during
MCMV retinitis. GSDMD expression seems to follow along a similar trend as Caspase 1, but with more apparent levels; this may be due to better antibody accessibility as GSDMD associates with the cellular membrane. Day 6 saw significant increase in GSDMD expression with an even presence throughout the diseased retina (Fig.31). By day 10, expression has decreased throughout with the exception of highly concentrated areas of retina still undergoing active retinitis progression (Fig. 32). Its presence is a clear indication MCMV induced pyroptosis involvement during retinal destruction.
Figure 31. GSDMD expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody.

2nd row: MCMV negative isotype control (shared for 6 dpi caspase 1, caspase 11 and GSDMD).

3rd row: non-infected control (media injected) with primary antibody.

4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD).

Key: star labels the photoreceptor side of the retina for orientation where still intact.
Figure 32. GSDMD expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 10 dpi caspase 1, caspase 11 and GSDMD). 3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control (shared for caspase 1, caspase 11 and GSDMD)
Key: star labels the photoreceptor side of the retina for orientation where still intact.
5.4 Interleukin 1β Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

In reference back to the main pyroptosis player - caspase 1 – and its primary role as an activator of cytokines, we looked next at two pyroptosis-associated inflammatory cytokines such as IL-1β and IL-18 [34][53]. As a key cytokines implicated in various immunomodulatory processes, it is indicative of further induction of inflammatory action [53]. IL-1β in particular is of interest due to its implications on inflammation induced or exacerbated diseases [54]. While it is associated with many different cell types and disease, the common denominator appears pyroptosis cell death activity [54]. Therefore, IL-1β is a good indicator of fully invested pyroptosis signaling in particular cells in areas of inflammation and tissue destruction. Further, it is currently thought that the pore forming GSDMD is integral for the release of IL-1β, at least in terms of inflammatory progression in a diseased state. And in the typical way cells do multi-functionality, the mechanism by which GSDMD is cleaved also plays a part in the cleavage of pro-IL-1β into its active form via caspase 1 (Fig. 10).

Following previous findings of increased total protein/mRNA in MCMV infected whole eyes, we sought again to investigate if protein localization at diseased tissue correlated with the other findings. IHC results at day 6 post MCMV infection in retinas show no significant expression (Fig. 33) while day 10 expression is increased throughout with pockets of apparent heightened activity (Fig. 34). This may be due to the necessity of a stimuli process cells use before fully committing to cell death by pyroptosis [106]. Cytokine release does not occur until after caspase 1 has cleaved and activated after the second stimuli [51]. These findings overall suggest that an inflammatory process is taking place and is stimulated by MCMV infection. This
further supports not only pyroptosis activity in the form of cell death but as a mediator of inflammation in the diseased state.
Blue: Dapi Red: Interleukin 1β Merge

Figure 33. Interleukin 1β expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody.
2nd row: MCMV negative isotype control (shared for 6 dpi IL-1β and IL-18).
3rd row: non-infected control (media injected) with primary antibody.
4th row: media negative isotype control (shared for 6 dpi IL-1β and IL-18).
Key: star labels the photoreceptor side of the retina for orientation where still intact.
Figure 34. Interleukin 1β expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 10 dpi IL-1β and IL-18). 3rd row: non-infected control (media injected) with primary antibody.

4th row: media negative isotype control (shared for 10 dpi IL-1β and IL-18)

Key: star labels the photoreceptor side of the retina for orientation where still intact.
5.5 Interleukin 18 Expression Following Ocular MCMV Infection at Days 6 and 10 Post-Infection

Lastly, IL-18 is the second inflammatory cytokine commonly associated with pyroptosis activity and was therefore of interest in the investigation of possible pyroptosis mediated tissue destruction during MCMV retinitis [56]. As before, IHC was used to localize protein at the site of disease in possible support of total protein and mRNA findings (not yet published). It, like IL-1β, is in the IL-1 family and activated by caspase 1. In contrast however, it is constitutively expressed in all cells thus making it a lesser target of interest in disease studies despite its role in inducing inflammatory responses [54]. This is because it is usually kept in balance by a binding protein (IL-18BP) and a resulting imbalance can lead to a severe inflammatory state, especially in chronic disease [54]. At this time though, it is far easier to evaluate IL-18 levels in conjunction with IL-1β rather than just alone. However in some diseases, it is being found that IL-18 is an important initiator of the inflammatory process which makes it both a potential acute and chronic disease cell death player as opposed to the slower to respond but seemingly potent IL-1β [55].

As with results for IL-1β, it too took a temporal expression pattern following increased expression at day 10 (Fig. 35) as opposed to the minimal expression seen at day 6 (Fig. 36). For the same reasons as seen with the expression of its co-cytokine, IL-18 is apparently released more abundantly later along in the diseased progression induced by MCMV. Therefore it too is also stimulated by MCMV infection of the retina where it mediates an inflammatory state as part of the overall activity of pyroptosis.
Figure 35. Interleukin 18 expression in the retina of MAIDS-10 mice Day 6 post-infection with MCMV or media control.

Whole eyes from MAIDS-10 mice were harvested at 6 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.

1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 6 dpi IL-1β and IL-18). 3rd row: non-infected control (media injected) with primary antibody.

4th row: media negative isotype control (shared for 6 dpi IL-1β and IL-18)

Key: star labels the photoreceptor side of the retina for orientation where still intact.
Blue: Dapi  Red: Interleukin 18  Merge

Figure 36. Interleukin 18 expression in the retina of MAIDS-10 mice Day 10 post-infection with MCMV or media control.
Whole eyes from MAIDS-10 mice were harvested at 10 dpi, fixed in 10% formalin, and assessed by immunofluorescent staining of GSDMD protein (red) and counterstained with DAPI (blue). Viewed at 40x magnification.
1st row: MCMV infected retina with primary antibody. 2nd row: MCMV negative isotype control (shared for 10 dpi IL-1β and IL-18). 3rd row: non-infected control (media injected) with primary antibody
4th row: media negative isotype control (shared for 10 dpi IL-1β and IL-18)
Key: star labels the photoreceptor side of the retina for orientation where still intact.
6 DISCUSSION AND CONCLUSIONS

Aim 1 tested the central hypothesis that explored the role necroptosis has on CMV retinitis in the murine model and in human patients, while Aim 2 tested the role of pyroptosis in the same regard. Specific aim 1 hypothesis: Necroptosis molecules RIPK1, RIPK3 and MLKL will be upregulated during MCMV retinitis in MAIDS model mice. Specific aim 2 hypothesis: Pyroptosis molecules caspase 1, caspase11 and GSDMD will be upregulated during MCMV retinitis in MAIDS model mice. This study investigated the relationship between the potential upregulation of programmed necrototic and pyroptotic cell death and retinal degeneration in the experimental murine model. Although a combination of previous mRNA and total protein findings for necroptosis and pyroptosis-associated molecules were found to be stimulated during MAIDS-related MCMV retinitis, the full extent of these cell death pathways in MCMV retinitis had yet to be elucidated. Both aims were pursued when needed by assessment of mRNA and total protein via real-time RT-PCR quantitative analysis and western blot respectively and protein localization via IHC staining in the murine model eyes. The findings herein confirm that necroptosis-associated RIP1, RIP3, MLKL and pyroptosis-associated caspase 1, caspase 11, GSDMD, IL-1b and IL-18 were indeed stimulated during the progression of MAIDS-related MCMV retinitis and that they precede retinal necrosis development. These findings and related studies are essential in the accurate quantification of cell death players for anti-molecule targeted therapies [17].
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