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STIMULATION OF RIPK3 IN ARPE-19 CELLS BY HUMAN CYTOMEGALOVIRUS UNDER H_2O_2-INDUCED OXIDATIVE STRESS

by

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Under the Direction of Richard D. Dix, PhD

ABSTRACT

Approximately 83% of the world's population is seropositive for human cytomegalovirus (HCMV) and will endure lifelong latency. An immunocompromising disease such as HIV/AIDS increases susceptibility to reactivation and HCMV diseases. If an AIDS patient's CD4+ T cell count falls below 50 cells/μL of peripheral blood, HCMV is reactivated which causes a sight-threatening retinitis. The disease affects up to 42% of AIDS patients and causes retinal necrosis. To further investigate the mechanisms of AIDS-related HCMV retinitis we studied RIPK3 stimulation in HCMV-infected ARPE-19 cells under oxidative stress, through pursuit of two experimental hypotheses: (1) HCMV-infected ARPE-19 cells will upregulate RIPK3 in the presence of oxidative stress induced by H_2O_2 and (2) HCMV-infected MRC-5 cells will not express RIPK3 stimulation in the presence of oxidative stress induced by H_2O_2. Our data suggests that RIPK3 stimulation presents a cell-specific dose-dependent response to H_2O_2 concentrations in ARPE-19 cells during HCMV infection.

INDEX WORDS: Human cytomegalovirus (HCMV), Oxidative stress, RIPK3, AIDS-related cytomegalovirus retinitis, ARPE-19 (arising retinal pigmented epithelial), Fibroblasts, MRC-5 (Medical Research Council cell strain 5), Redox stress, Reactive oxygen species (ROS), Free radicals

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DEDICATION

This manuscript is dedicated to my amazing and wonderful family: to my angelic mother who prayed for me and encouraged me regardless of her arduous days; to my loving father who kept me grounded despite his health battles; to my church family who constantly uplifted me and reminded me of my strength in God; to my amazing lab mates who motivated me through my toughest times; to my genius P.I. whose guidance and patience allowed me to complete this work better than I imagined; to my aunts, uncles, and my cousin Jaimie whose sacrifices made way to give me peace during my most difficult times; and to my additional mentors, friends, and former coworkers all of whom bear witness to me and encouraged me to remain steadfast in fulfilling my purpose along my journey in life. Thank you all.

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LIST OF ABBREVIATIONS

RIPK1	Receptor-interacting sering/threonine-protein kinase 1
RIPK3	Receptor-interacting sering/threonine-protein kinase
MLKL	Mixed lineage kinase domain like psuedokinase
HCMV	Human Cytomegalovirus
GCLC	glutamate-cysteine ligase
SOD3	superoxide dismutase 3
Nrf2	nuclear factor erythroid 2-related factor 2
GPX	Glutathione peroxidase
AIDS	Acquired immunodeficiency syndrome
ROS	Reactive Oxygen Species
RPE	Retinal Pigmented Epithelial
ATP	Adenosine triphosphate
ADP	Adenosine Diphosphate
ILM	inner limiting membrane
NFL	nerve fiber layer
GCL	ganglion cell layer
IPL	inner plexiform layer
INL	inner nuclear layer
OPL	outer plexiform layer
ONL	outer nuclear layer
OLM	outer limiting membrane
PL	photoreceptor layer
ART	antiretroviral therapy
ER	endoplasmic reticulum
TR1	triplex monomer 1
TR2	triplex dimer 2
MCP	major capsid protein
PORT	portal protein
pp	Phosphorylated
SCP	smallest capsid protein
ERGIC	ER-Golgi intermediate compartment
DAMPs	Damage-associated molecular patterns
CYLD	cyclindromatosis
RHIM	RIP homotypic interaction motif
dpi	Days post infection
hpi	Hours post infection
ARPE-19	Arising retinal pigmented epithelia
MRC-5	Medical Research Council Cell Strain 5

1 INTRODUCTION

"Do not allow your mind to be imprisoned by majority thinking. Remember that the limits of science are not the limits of imagination"- Patricia Bath, MD

Human cytomegalovirus (HCMV) is a ubiquitous, species-specific virus that establishes lifelong latency in bone marrow and monocytes [47]. As an opportunistic pathogen, HCMV affects various organs causing a variety of diseases including encephalitis, colitis, hepatitis, pneumonitis, and retinitis [13]. HCMV is a double-stranded DNA (dsDNA) virus within the Herpesviridae family under the subfamily of Betaherpesvirinae [2]. The viral dsDNA lies within an icosahedral capsid, composed of 162 capsomeres, adjacent to a viral tegument and surrounded by a viral envelope [58]. Structurally, the capsid consists of four virus-encoded proteins critical for viral replication, triplex monomer (TR1), triplex dimer (TR2), major capsid protein (MCP), and the small capsid protein (SCP) [5, 58, 61]. Along with a specialized pore on the single side of the icosahedron, known as a portal protein (PORT), to act as a channel for viral genome release [5]. The PORT is essential to package viral DNA and utilizes two gene products, UL89 and UL56, to help release viral genome [58,61]. The viral genome is comprised of over 25 membrane glycoproteins and over 230 genes [6-10]. The five essential glycoproteins for HCMV replication, are gB, gM:gN, gH:gL, while gB and gH:gL allow for viral entry [58].

The viral DNA enters through the nuclear pore of a host cell. Following viral DNA entry, the viral genome replicates transcribing its DNA sequentially through a regulated cascade of immediate-early (IE) genes, early (E) genes, and late genes (L). Within minutes of viral infection, the IE genes are first transcribed to regulate the expression for early enzymes [58]. The IE gene is essential for viral DNA replication and encoded by gene product IE1 [3]. The IE genes

regulate the expression of early enzymes for DNA replication through early genes. New virions enclosed in vesicles are transported to the plasma membrane, where the vesicles fuse with the host's membrane and release viral progeny. In fibroblasts cells, such as MRC-5 cells (human lung fibroblasts) in vitro studies, this process lasts longer compared to RPE cells with an elapsed time of up to two days [19,55].

HCMV has shown to infect many cell types and is capable of replicating in epithelial cells, macrophages, endothelial cells, hepatocytes, smooth muscle, stromal cells, progenitor cells, and fibroblasts [17,18]. Among the cell types, HCMV expresses viral tropism for fibroblasts, seen in MRC-5 cells [13], and epithelial cells, seen in ARPE-19 cells [58,59,19,55]. HCMV enters epithelial cells and endothelial cells through endocytosis, while it enters fibroblasts through direct fusion of the viral envelope to the host's cell membrane**.** After viral entry HCMV begins to recruit host cell machinery through the host cell's endosomes, endoplasmic reticulum (ER), and the ER-Golgi intermediate compartment (ERGIC) after binding to the host cell surface receptor [4]. This proceeds down the cascade regulation to initiate IE, E, and L as mentioned earlier to develop virions [6].

Virions are infectious virus particles and dense bodies enclosed in a lipid bilayer envelope derived from ERGIC [12]. Within a virion are 23 viral glycoproteins. Presently, three viral glycoproteins are essential for attachment to allow for viral entry, gB and gH:gL, while gM:gN allow for maturation of the virus [12]. Additional help may also come from pUS16 if gH:gL is silent or deleted [12]. The viral tegument (or matrix) of HCMV packs in-between the nucleocapsid and the envelope containing a minimum of 32 virally-encoded tegument glycoproteins, including the host's protein RNA [3, 58]. Numerous tegument proteins are phosphorylated (pp), such as viral protein pp65, and can provoke an immune response reactivity

in a host [4]. Pp65 targets major histocompatibility class (MHC) class I CD8 T cells and MHC class II CD4 T cells [58]. Immediately upon viral entry, pp65 localizes to the nucleus of the infected cell. This localization allows pp65 to modulate the immune system by reducing the interferon response at the site of infection and enter its latent state. Once HCMV enters a host cell, it will establish latency for the remainder of the host's life [58]. Latency entails long-term maintenance of viral genomes in an immunocompetent host with sustained viral genomes in hematopoietic cells and in CD4+ T cells.

An overview of HCMV replication and life cycle in **Figure 1**.

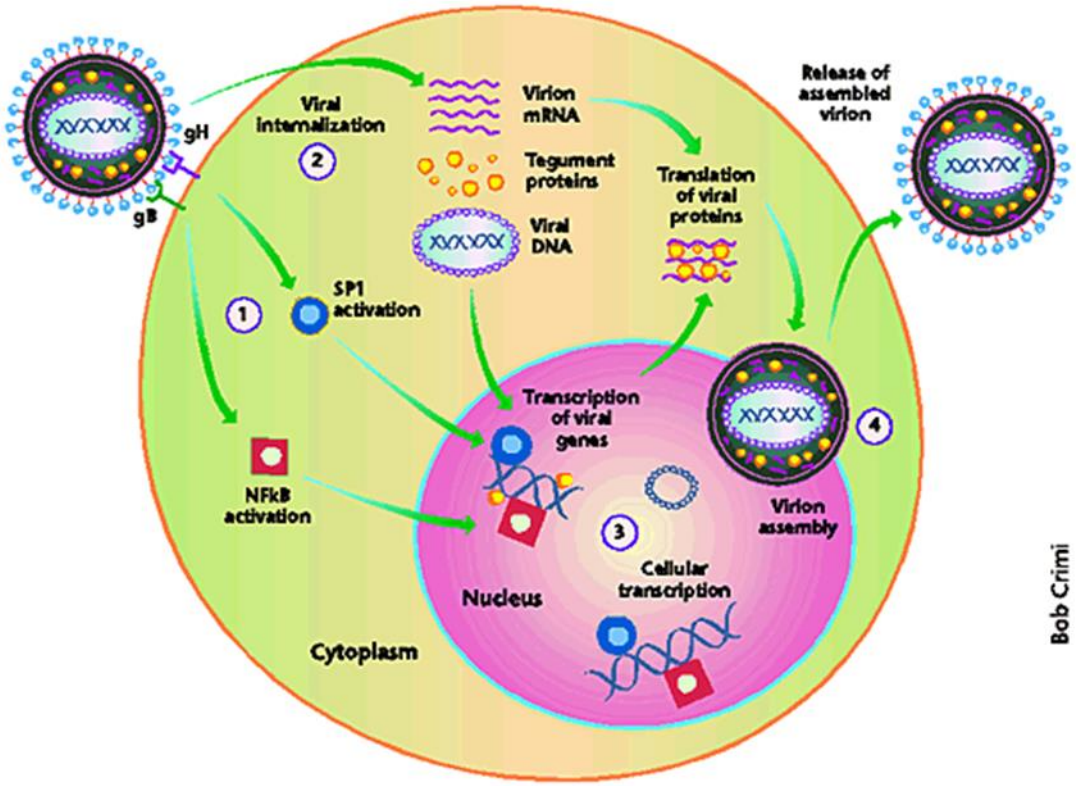

Figure 1. HCMV replication cycle within a host cell. HCMV utilizes its cellular receptors gB [and the understood gH:gL protein complex] to activate transcription factors NF-κB and Sp1 for entry into a host cell. Upon entry, the virus releases viral DNA into the cytoplasm of the host cell, which will be transported to the host nucleus. Within the nucleus, HCMV utilizes the host's transcription factors to code for its own DNA polymerase and replicate its viral DNA. The viral

components and DNA are packaged in the virion envelope, and the infectious viral particles are released from the host cell [11].

HCMV can cause congenital infections, which may lead to the death of a fetus or developmental abnormalities in approximately 0.2% to 2% of the United States infant population each year [58, 59]. The percentage of women in America of childbearing age at risk for infection is 86% and the percentage of the global population is 86%. Primary HCMV-infection is often acquired during childhood through saliva, breast milk, or urine. Moreover, other ways to contract HCMV is through genital secretions, usually during adolescence or adulthood, through sexual intercourse or in cases of childhood rape [14]. Upon primary infection, there is viremia. A leukocyte-associated viremia that allows active HCMV to remain in the blood. Infectious viral particles shed through urine, saliva, or semen for months or years, even in a functional immune system [51]. However, primary infection generally causes asymptomatic symptoms in healthy immunologically normal children and adults [15]. Their primary infection is under control by innate and adaptive immune responses. If primary infection of HCMV is acquired as an adult, a nonspecific illness will transpire [16]. The illness will produce a fever lasting 1–3 weeks associated with transient lymphocytosis and a developing abnormal liver [16].

In addition to primary infection, HCMV travels to the bone marrow to establish latency and remains undetected by the immune system for the duration of the host's life. HCMV infects CD34+ myeloid progenitor cells in the bone marrow but is blocked to transcribe into more virions when bound to the IE promotor gene. This viral block puts HCMV in the latency stage within the bone marrow expressing only a limited viral load within the nucleus.

During chronic infection, low viral loads are shed continuously from the host for an extended period of time. The length of time the virus sheds depends on the health of the host. Chronic virus shedding may branch off from the primary infection or may occur after

reactivation of the virus in a latent stage. Latent infection in cells allows HCMV to travel throughout the body and is the main contributor of systemic infection if the virus reactivates [58]. Reactivation of a latent virus is due to a weakened immune system [15]. This mechanism favors the viral life span of HCMV within the host by exiting latent virus reservoirs within the host to promote viral spread amongst new hosts. If an individual is no longer an immunologically normal person the virus will leave the latency stage and proliferate. AIDS patients are an example of individuals who have acquired an impaired immune system and are no longer immunologically normal.

1.1 AIDS-Related Human Cytomegalovirus Retinitis

When the immune system in a host becomes weakened due to HIV infection, it puts a host in danger of acquiring an opportunistic infection such as HCMV. HCMV is an opportunistic infection that travels to the retina, liver, fibroblasts, and GI tract [47]. The global percentage of people currently infected with HCMV has increased from approximately 80% to 83% [20]. Activated HCMV will cause a cytokine driven inflammatory response that will continue to drive the cell to differentiate and reactivate the virus locally or systemically. If left untreated, HCMV will remain active and cause varied diseases such as encephalitis, colitis, hepatitis, pneumonitis, and retinitis [59]. In AIDS patients, HCMV-infection can develop into a sight-threatening disease known as AIDS- related cytomegalovirus retinitis. The progression of AIDS, is distinguished after the host's CD4+ T cell count, falls below 200 cells/μL of peripheral blood. If the host does not respond to antiretroviral therapy (ART) or doesn't have access to ART, their CD4+T cells will continue to drop. When the host's CD4+ T cell count falls below 50 cells/μL of

peripheral blood HCMV will reactivate in a seropositive individual and cause AIDS-related human cytomegalovirus retinitis [15,21].

Due to AIDS-related HCMV retinitis affecting up to 42% of AIDS patients [14], our work focuses on the effects HCMV has on the retina. HCMV travels to the retina in infected macrophages and monocytes through the retinal blood vessels to proliferate in the retinal pigmented epithelium (RPE), illicit an inflammatory response, and cause retinal destruction [15,21]. The damaged RPE is caused by enlarging lesions in the retina of individual foci that lead to vision loss in an average of six months to a year [15,21]. Failure to treat this disease through antivirals such as ganciclovir, valganciclovir, cidofovir, and foscarnet to inhibit HCMV replication is typically followed by vision loss in the contralateral eye within one year [15,21].

Vision loss occurs due to HCMV infection in dendritic cells and macrophages in the bone marrow exiting the latency stage to reactivate within the nucleus, through HCMV detaching from the IE promotor gene. Production of new infectious viral particles can now take place. The advantage the virus has to sustain virulence is due to the challenge of the immune system to detect limited viral genes within the cells. Therefore, the infected host myeloid progenitors cells divide as usual and differentiate into monocytes spreading the virus further [15].

During HCMV retinitis, the virus enters into the RPE through endocytosis resulting in full-thickness retinal necrosis and retinal hemorrhage [22]. The shrewdness of this virus utilizes viral protein products to prevent antigen presentation from traveling in the blood undetected by cytotoxic T cells and avoid attack [22]. HCMV also created a HLA class 1 homolog to remain unrecognized by natural killer cells (NKC), since NKC adapt a way to detect downregulation of HLA class 1 [22]. Furthermore, HCMV will bind a created cytokine homolog to the host's receptor, such as IL-10, to downregulate the immune response and prevent signaling of CD4+ T

cells. The mechanisms of blindness caused by HCMV retinitis involves the destruction of the retina, retinal detachment, uveitis, and damage to the fovea, observed in **Figure 2.**

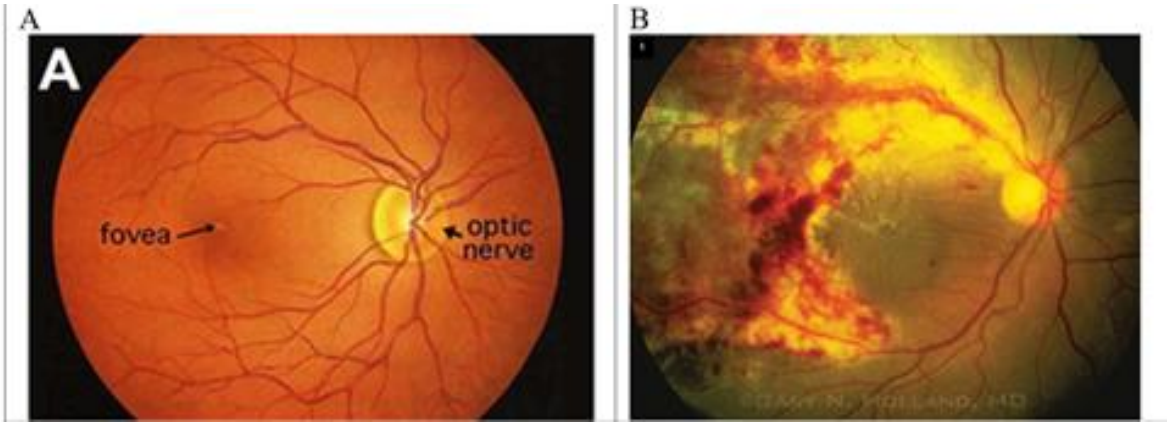

Figure 2. Photograph of a Posterior View of a Human Eye through an Ophthalmoscope. (A) Healthy patient. Optic nerve and fovea are intact with no retinal destruction observed. (B) Fundus of an eye in an AIDS- related HCMV retinitis patient. Morphology shows the continuation of retinal destruction, retinal hemorrhages, white granular foci, vascular sheathing, and panuveitis.

AIDS-related HCMV retinitis progresses after an immunocompromised host succumbs to a significant decrease in their T lymphocytes. The expansion of ART within the United States to stabilize the HIV/AIDS pandemic as well as increase T lymphocytes has lowered a substantial amount of HCMV retinitis cases within the last decade [22]. Nevertheless, ART is not a drug that is consistently available worldwide, which condemns HIV patients outside of the United States. Moreover, individuals within the United States suffer from ineffective responses to ART. The inconvenient access to ART medication, quality of life, and cost that is associated with immunotherapy will increase the burden on AIDS-patients as well as the health care system. Finding enhanced methods to prevent the disease is both essential for improving economic burdens as well as the health of the population. The mechanisms as to how HCMV retinitis progresses in AIDS patients is vital for treatment improvement and vision loss prevention.

1.2 The Human Retina

The human retina is an immune-privileged site acting as an extension of the brain through the optic nerve located in the posterior segment of the globe of the eye [23]. The retina is composed of 10 layers listed in order from the layer touching the vitreous humor to the Bruch's membrane: the inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), the outer limiting membrane (OLM), the photoreceptor layer (PL), and lastly the RPE. A visual representation of the position of the retinal layers in the eye is displayed in **Figure 3.**

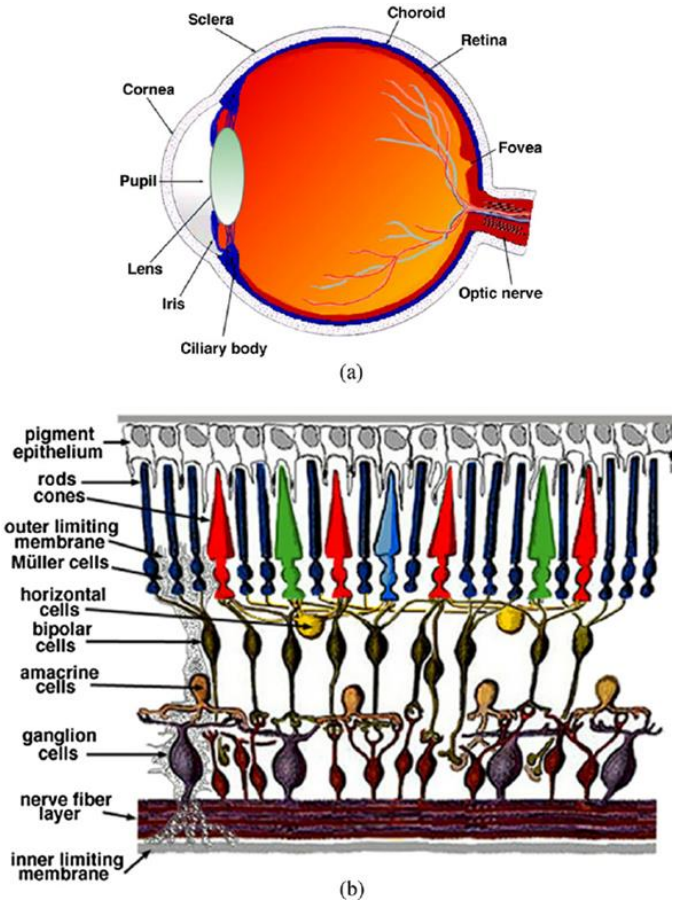

Figure 3. Human Eye Globe and Cell Layers of the Huma Retina. (a) A sagittal view of the brief anatomy of a human eye and (b) the Diagram of its respective retinal layers [24].

There are no resident inflammatory cells within the retina; therefore, the retina does not elicit a typical inflammatory immune response. The blood supply of a human neurosensory retina comes from the retinal artery that enters the eye through the optic nerve. The retinal artery branches off into a capillary network to form a blood-ocular barrier. The blood-ocular barrier allows the movement of macrophages and leukocytes through the Bruch's membrane to perfuse between the RPE. However, the RPE blood flow is supplied by the choroidal vessels and can assist in the transfer of leukocytes into the remaining layers of the retina [58].

The RPE functions as the main defense layer of the retina. A physiological advantage of the RPE is how it contains tight junctions at the apex of its cells to regulate molecules coming into the retina. Another purpose of the RPE is its role as a blood-retinal barrier between the photoreceptor cells and the Bruch's membrane. In the event of an inflammatory response, tight junctions uncouple to allow access of macromolecules to diffuse between the RPE and the blood-retinal barrier.

The RPE is a pigmented cell monolayer layer that has several functions to help provide nourishment, support, and protection to the photoreceptor cells to maintain visual acuity in the host. Photoreceptors detect light and transmit signals through several neurons into the ganglion cell, which exits through the optic nerve for visual processing in the brain. The photoreceptors are located on the apical side of the RPE as it sheds its outer segment daily. Part of the RPE function is to phagocytose the daily shed of the segments and regulate the flow of ions to the photoreceptors for phototransduction.

The RPE is essential for retinal homeostasis, transporting nutrients [25], and removing waste from the photoreceptor cells [26]. In the retina, the RPE is of main concern compared to the other retinal layers due to its high concentration of mitochondria to supply energy to

continually carry out these cell protective functions [27]. In addition to its vital properties, cell dysfunction or cell death of the RPE is fatal for vision because the choroidal blood supply to the photoreceptors will be lost as well as.

1.3 Reactive Oxygen Species

One factor suspected to accelerate the progression of HCMV retinitis is the increase in oxidative stress within the RPE caused by the accumulation of reactive oxygen species (ROS). ROS are natural byproducts of the mitochondria [28]. ROS molecules, also labeled as free radicals, are derived from molecular oxygen in the electron transport chain (ETC)/ oxidative phosphorylation metabolic pathway during aerobic respiration [30]. In the case of cellular stress in the RPE, the cell will increase its ATP output by its need to increase its cellular defense mechanism [31]. Thus a faster production of ATP establishes a higher chance of error that increases the output of ROS, which is why ROS serves as a signal molecule for cellular stress [29].

Oxidative phosphorylation is the key generator of the potential detriment to the retina due to an over-accumulation of ROS molecules [31,32]. The generation of a superoxide ion, is within the mitochondrial matrix and dependent on the proton motive force within the inner mitochondrial membrane. Many free radicals are formed from the imbalance of the redox reaction of superoxide [28]. CoEnzyme Q, the NADH/NAD+ complex, and ratios of molecular oxygen already present surrounding the ATPase in the inner mitochondrial membrane are involved in free radical formation [28]. A diagram that depicts free radical formation is shown in **Figure 4.**

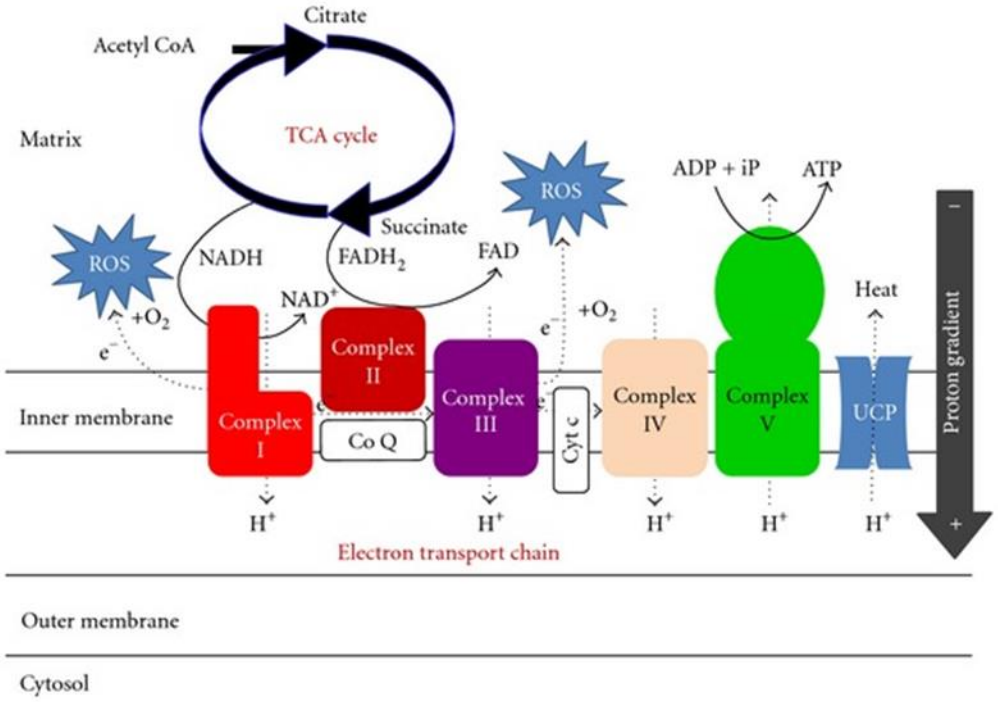

Figure 4. ROS production from the electron transport chain in the Inner Mitochondrial Membrane. Tricarboxylic acid (TCA), nicotinamide adenine dinucleotide (NADH and NAD+), flavin adenine dinucleotide (FADH2 and FAD), (adenosine diphosphate) ADP, adenosine triphosphate (ATP), coenzyme Q (Co Q), cytochrome c (cyt c), uncoupling protein (UCP) [29, 33].

The most prevalent form of ROS is hydrogen peroxide serving as a precursor molecule to many of the free radical species. ROS aggressively increases when the cell is put under highly stressful conditions [31]. When the RPE undergoes stress from incidences such as viral infection, excessive light exposure, or complications due to natural aging, the RPE accelerates its mitochondrial function for more ATP to restore homeostasis in the retina. The ATPase rotates more frequently to join more ADP and phosphate groups together, which leads to a higher byproduct amount of unreduced free radicals producing more ROS. Epithelial cells possess a natural defense against naturally occurring ROS molecules known as antioxidants. Antioxidants are present to reduce free radicals through redox reactions, converting free radicals to water.

There are many antioxidants present, but common molecules studied in the cell are glutathione peroxidase (GPx), glutamate-cysteine ligase (GCLC), superoxide dismutase 3 (SOD3), and nuclear factor erythroid 2-related factor 2 (NRF2). GPX is an antioxidant that converts hydrogen peroxide to water [34]. SOD3 detoxifies the environment by decreasing the number of free radicals in the retinal environment [35]. Nrf2 is a transcription that further lowers toxicity by reducing the reactivity of ROS molecules [35]. When the number of free radicals outnumber the available antioxidants in the cellular environment, it increases toxicity within the cell. Under high concentrations of ROS, RPE is at an increased risk for cell death due. ROS is capable of degrading cell membranes, reducing the formation of ATP, reacting with phospholipids inducing inflammation by creating a permeable membrane, and decrease metabolic function [30],[36].

Mitochondrial degeneration within the RPE diminishes RPE function and, in return, causes a breakdown of photoreceptor cells due to loss of defensive aid [26, 36, 37]. Increased ROS species leads to decreased membrane density within the RPE and cell death, such as necroptosis within the cell [28, 36].

1.4 RIPK3 Influence

RIPK3 is an essential molecule for tissue repair and inflammation brought on from injury in the cell [47]. RIPK3 kinase activity can mediate necroptosis activity and cytokine expression [47]. The scaffolding function of RIPK3 has added speculation in previous literature for its involvement in non-necrotic RIPK3 signaling mediated by oxidative stress [47].

Necroptosis is an alternative programmed pathway induced when caspase-8 activity is blocked. RIPK3- dependent necroptosis is mediated by RIPK3 (receptor-interacting serine/threonine-protein kinase) and will cause swelling, membrane lysis, and ATP depletion in

the cell. RIPK1 recruits RIPK3 through its RIP homotypic interaction motif (RHIM) to activate RIPK3. Activated RIPK3 phosphorylates mixed lineage kinase domain-like (MLKL) molecule to form an oligomer complex with other phosphorylated MLKL molecules. The MLKL oligomer complexes migrate to the plasma membrane to rupture the cell membrane through pore formation and cause cell lysis [38, 39]. The release of cellular contents such as damage-associated molecular patterns (DAMPs) recruits a highly pro-inflammatory response, which further progresses the cell death cycle [38]. Previous data have shown that necroptosis markers were upregulated in RPE cells under high oxidative stress [36].

Furthermore, *in vitro* studies had revealed a significant increase in RPE viability when necroptosis was inhibited, confirming it is the leading cell death pathway occurring in oxidative stressed RPE cells [36, 40]. An increase of oxidative stress concentration in the RPE upregulates necroptosis as a cellular defense response to protect the health of the retina [36].

ROS can play a role in the signaling functions of non-necrotic RIPK3 [4]. More work is necessary to identify the different role ROS plays in necrosis-independent RIPK3 signaling, including the different antioxidant effects [4].

Table 1. Molecules Mediated by RIPK3 Opposed to RIPK3-Dependent Necroptosis

Molecules Mediated/ Effected by RIPK3	**Overview of Regulatory Factors and Mechanism Involved**
TNF	RIPK3 may serve as a control for TNF-induced necrosis by switching TNF mediated cell death from apoptosis to necroptosis [47].
IKK α/β	IKK α/β phosphorylate RIPK1 to block the formation of RIPK1-RIPK3 complex
TRAF-2	TRAF-2 inhibits necroptosis by binding to MLKL to block RIPK3 and MLKL interaction
NF-κB	NF-kappaβ is a latent transcription factor that effects RIPK3 in cell injury and during innate inflammatory responses. NF-κB can negatively control RIPK3-dependent necroptosis. For example, in response to TLR stimulation, NF-κB brings about inflammatory cytokine expression [47].
p38- MAPK	p38-MAPK pathway suppresses RIPK3 activation [48]
DC (dendritic cells)	RIPK3-independent mechanisms can regulate DC and cause inflammation [47].
IL-23 and IL-1β	RIPK3 controls IL-22 expression through stimulation of IL-23 and IL-1β. RIPK3 allows IL-1β processing in dendritic cells and macrophages. RIPK3 dependent necroptosis and IL-1βare induced by the ablation of caspase 8 [49].
K48-linked Polyubiquitination	K48-linked to the kinase domain within RIPK3 through polyubiquitination inhibiting necroptosis causing RIPK3 to be rapidly degraded by proteasomes
DAI (DNA-dependent activator of IFN-regulatory factors)	RIPK3 may trigger necroptosis through the influence of DAI and RIPK1
C-terminal RHIM	C-terminal RHIM-containing cleavage product of RIPK3 was shown to induce non-necrotic cell death, while the N-terminal kinase domain-containing product was unable to trigger any form of cell death

FADD	RIPK3 can function as a pro-apoptotic converter to recruit RIPK1 and FADD. The interaction will lead to the activation of caspase-8 and subsequently, apoptosis

Table 1. Molecules Mediated by RIPK3 Opposed to RIPK3-Dependent Necroptosis.

Taking everything into account, RIPK3 has demonstrated the ability to be activated independent of necroptosis, as well as mediate necroptosis. Therefore, with the RPE primary function as the caretaker of the retina to protect against cellular stress, this gives a plausible reason why we should continue the investigation of RIPK3 influence through ARPE-19 cell cells [54].

ARPE-19 cells are a spontaneously arising pigmented epithelial human cell line which were created by selective trypsinization of a primary RPE from a 19-year old male. The cell culture shows normal structure and function of the cell nuclei similar to human RPE (hRPE). The cell line exhibits stable cell replication and resulted in a uniform replication similar to hRPE [16]. The ARPE-19 monolayer cells were proven to be a reliable cell line by evaluation of tight junction formation, morphology, and structural properties, for *in vitro* studies [62, 64]. Regardless of the success of these studies [16,18], we cannot ignore that ARPE-19 cells are a transformed cell line and leave the opportunity not to portray a 100% accurate depiction of what will occur in hRPE [63].

HCMV replication has proved to cause an imbalance of the oxidant/antioxidant ratio, increasing the production of ROS [6], and in the RPE, oxidative stress can cause necroptosis. Although HCMV inhibits RIPK3-mediated necroptosis, the upregulation of RIPK3 in the RPE led us to pursue our hypothesis, **(1) HCMV-infected ARPE-19 cells will upregulate RIPK3 in the presence of oxidative stress induced by H_2O_2.** HCMV exhibits cell-specific pathogenesis. Hence, to determine if stimulation of RIPK3 is cell-specific to ARPE-19 cells, we decided to

compare our future findings to another cell type. HCMV establishes viral tropism for fibroblasts and endothelial cells [58]. Since, fibroblast cells are not found in the eye we decided to use a fibroblast cell line, MRC-5 cells as a comparison. Therefore, our second hypothesis tests **(2) HCMV-infected MRC-5 cells will not express RIPK3 stimulation in the presence of oxidative stress induced by H_2O_2.**

2 MATERIALS AND METHODS

2.1 Cell Culture

ARPE-19 cell line was purchased from ATCC (Manassas, VA, No. SCRC-1002) and was passaged (TC-MCMV) in 75-cm2 flasks in growth media Dulbecco's modified eagle media F-12 (DMEM, Corning Life Sciences, Manassas, VA, #10-013) supplemented with 10 % fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, #FR-0500-A), 0.5 mM sodium pyruvate, 50 U/mL penicillin/50 mg/mL streptomycin, and 4 mM L-glutamine. MRC-5 cells were acquired from ATCC and grown in a base media of DMEM in 10% fetal bovine serum (FBS) media supplemented with gentamicin, sodium bicarbonate, and penicillin. T-75 flasks were replaced with new media every 2 days to sustain cellular health. Cells were maintained at 37°C and 10% CO2. Single layer confluent cells were grown in 12-packs for *in vitro* viral infection of HCMV treated with hydrogen peroxide concentrations 100mM, 200mM, and 300mM in the appropriate ARPE-19 and MRC-5 media stated above.

2.2 Viral Stock

Human cytomegalovirus, Towne strain, was disseminated and quantified in MRC-5 cells stored at -80° C. MRC-5 cells were infected from a clarified viral stock; frozen, thawed, and propagated until the cytopathic effect increased to a MOI of 5. All experiments for viral infection

in ARPE-19 cells were performed using ARPE-19 cell media maintained in a 1:1 mixture of Dulbecco's modified eagle media F-12 (DMEM), with 10 % fetal bovine serum (FBS), 0.5 mM sodium pyruvate, 50 U/mL penicillin/50 mg/mL streptomycin, and 4 mM L-glutamine. All experiments for viral infection in MRC-5 cells were performed using the appropriate MRC-5 cell media maintained in a 1:1 mixture of DMEM in 10% fetal bovine serum (FBS) media supplemented with gentamicin, sodium bicarbonate, and penicillin.

2.3 RNA Extraction from Cell Monolayers

Cell monolayers experimentally treated as specified were harvested at indicated time points in TRIzol® reagent (Ambion/ThermoFisher). Total RNA was isolated by chloroform extraction, purified over PureLink® RNA Mini Kit spin cartridge filters according to the manufacturer's instructions (Ambion/ThermoFisher), and stored frozen at -80°C until use in downstream applications.

2.4 Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

ARPE-19 cells and MRC5 cells were harvested in 0.5 mL of Trizol reagent (Invitrogen Life Technologies) at 1, 2, and 4 hour-post-infection (hpi). Continued harvest in 0.5 trizol at 1, 2, 3, 4, 5, and 6 day-post-infection (dpi). Complete RNA was isolated by chloroform and purified using reagents in the PureLink® RNA Mini Kit spin cartridge filters acquired from Ambion/Thermo-Fisher Scientific. RNA was reverse- transcribed into cDNA using SuperScript III first-strand synthesis system. Real-time RT-PCR procedure was done using Applied Biosystems 7500 Fast Real-Time PCR System and using a Power SYBR Green Master mix. Human specific primers for RIPK1, RIPK3, MLKL, and Nrf2 were obtained from QIAgen. Housekeeping gene GAPDH was used to normalize analysis and mRNA fold change was determined using the 2-ΔΔCt method.

2.5 Statistical Analysis

All quantitative data for Real-time RT-PCR assays were performed from at least two independent experiments compared to the control with +/- standard deviations. Statistical analyses were performed using GraphPad Prism and analysis of variance were performed through two-way ANOVA with p values; *$p < 0.05$; **$p < 0.001$ considered significant.

3 RESULTS

RIPK3 STIMULATION IN HCMV-INFECTED OXIDATIVE STRESSED ARPE-19 CELLS

Hypothesis **(1)** tested that **HCMV-infected ARPE-19 cells will upregulate RIPK3 in the presence of oxidative stress induced by H_2O_2.** Commonly, when RIPK3 activation is observed, necroptosis is the leading cell death pathway to account for this cellular degradation. HCMV has shown to inhibition of necroptosis [6], but also requiring fatty acid synthesis and histone methylation for replication. An increased amount of ROS induces oxidative stress and depletes both fatty acid synthesis and histone methylation in a cell [68]. An abundant amount of ROS found in ARPE-19 has shown an increased level of RIPK3 in ARPE-19 cells. RIPK3 is an essential molecule for tissue repair and inflammation brought on by cell injury [47]. RIPK3 can mediate necroptosis activity and non-necrotic signaling induced by oxidative stress [47]. Therefore, in this study we performed qPCR to test mRNA expression of RIPK1, RIPK3, MLKL, Nrf2, SOD3, GCLC, and GPX-1. To investigate if HCMV-infection and oxidative stress in ARPE-19 cells stimulates RIPK3 as synergistic or additive effect.

3.1 mRNA expression of RIPK3 in HCMV-infected ARPE-19 cells.

To begin, in determining if RIPK3-mediated necroptosis is the cell death pathway at work during early HCMV-infection in ARPE-19 cells, we tested mRNA levels of key necroptosis markers: RIPK1, RIPK3, and MLKL.

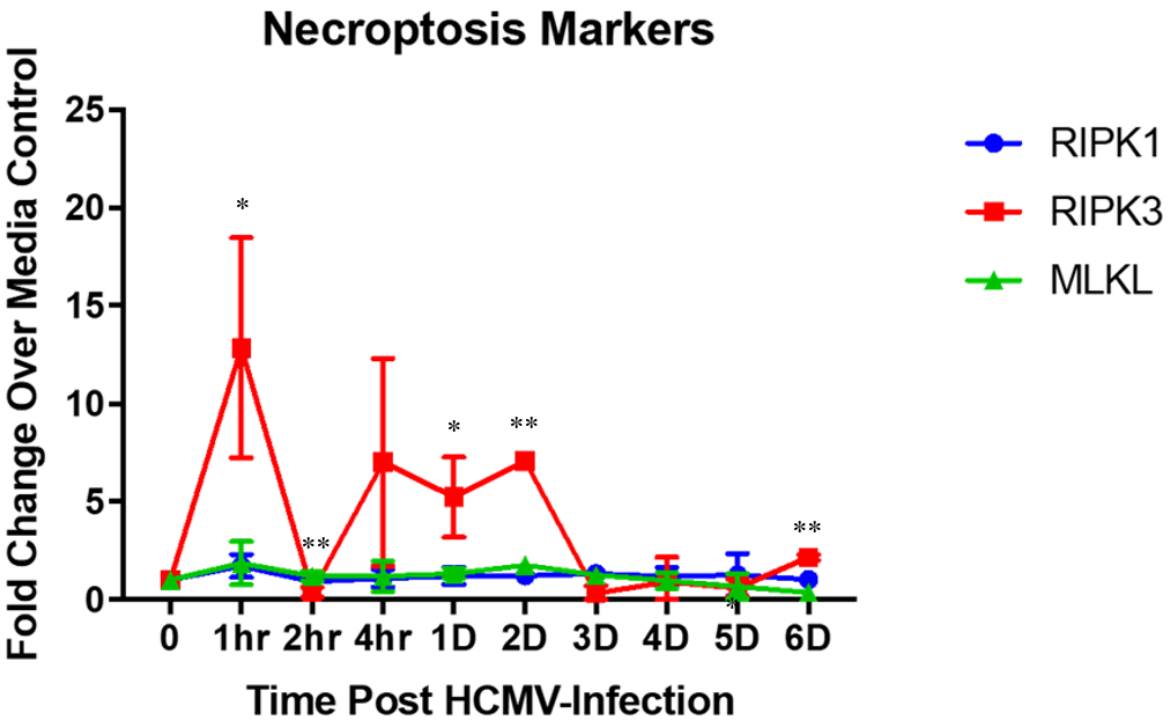

Figure 5. mRNA expression of RIPK3 in HCMV infected ARPE-19 cells. ARPE-19 cells were harvested at 0 hpi, 1 hpi, 2 hpi, 4hpi, 1 dpi, 2 dpi, 3 dpi, 4 dpi, 5 dpi, and 6 dpi. HCMV (Towne strain) at a MOI=5. These samples were accessed through mRNA expression of RIPK3-mediated necroptosis markers RIPK1, RIPK3, and MLKL through RT PCR and compared back to the media- treated control. All three experimental repeats were performed on different days with the same time points. * $p<0.05$ and ** $p<0.01$ all samples

There is a prominent fold change of increased RIPK3 mRNA expression seen at 1 hpi and 4 hpi. RIPK3 stimulation continues over 2 days post-infection, then quickly decreases for the reminder of the tested time points. There is a decline of RIPK3 shown at 2 hpi, while mRNA expression of RIPK1 and MLKL show minimal increased stimulation in response to HCMV-infection in ARPE-19 cells. To confirm RIPK3-mediated necroptosis as the leading cell death

pathway all three markers are expected to be upregulated. Therefore, based off Fig. 5. we cannot assume RIPK3-mediated necroptosis is an active mechanism at work in RPE HCMV- infection over 6 days, but it may be a mechanism that is initiated by the cell during early-onset infection.

3.2 mRNA expression of Antioxidant Markers in HCMV infected ARPE-19 cells.

In addition to our findings, we tested four antioxidant molecules that may be involved in RIPK3- independent mechanisms and/or oxidative stress. If there is an increase or decrease of these markers in HCMV-infection without hydrogen peroxide treatment, it may lead us closer to answering why there is an pronounced increase of RIPK3 [10].

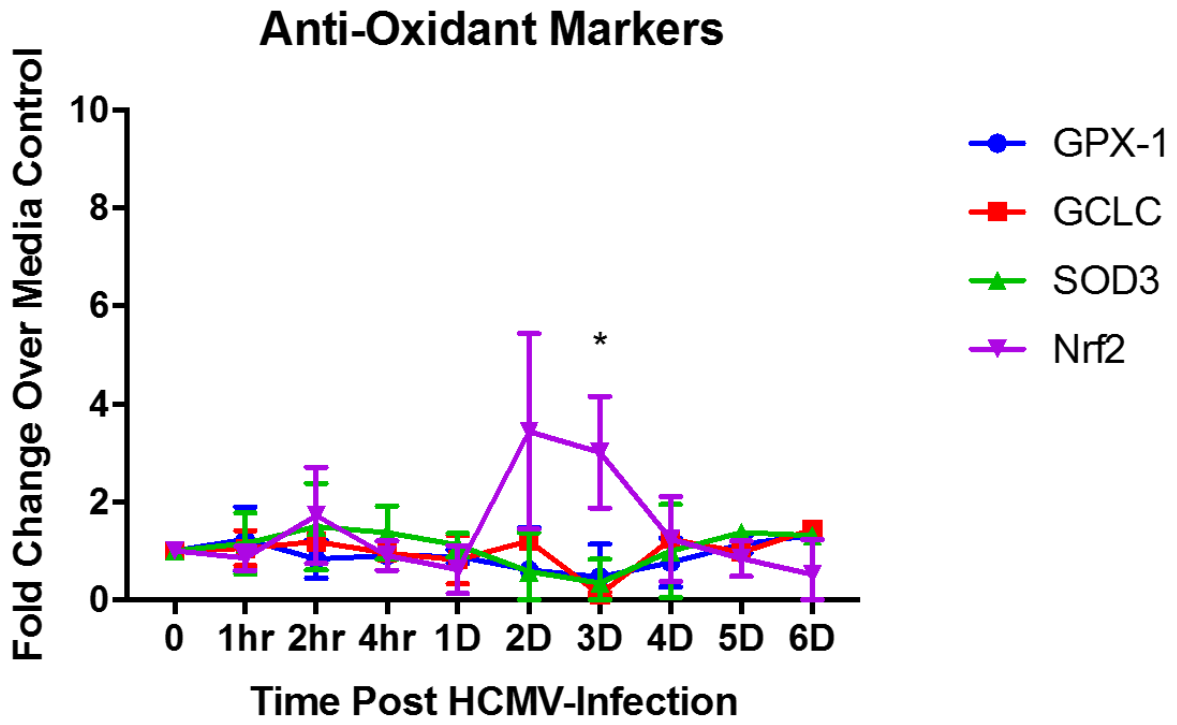

Figure 6. mRNA expression of Antioxidant Markers in HCMV-infected ARPE-19 cells. HCMV (Towne strain) MOI=5 infected ARPE-19 cells. All three experimental repeats were infected and harvested at the same time points. mRNA expression of GPX-1, GCLC, SOD3, and Nrf2 were all compared back to media- treated control cells. * $p<0.05$ and ** $p<0.01$ all samples.

There were little to no stimulation of GPX-1, GCLC, or SOD3 at these specific time points tested. However, there is an increased activity of Nrf2 mRNA expression shown over 2-dpi then declined for the remainder of the time course.

3.3 mRNA Expression of Necroptosis Markers and Nrf2 in HCMV-infected ARPE-19 Cells from 1 to 4 hours.

RIPK3 presents a high fold change at 1-hpi, 4-hpi, but immediately declines at 2-hpi throughout our experimental repeats. Our next step was to investigate the stimulation of these markers over a shorter time frame (4 hours), where we saw the most engaging activity of RIPK3.

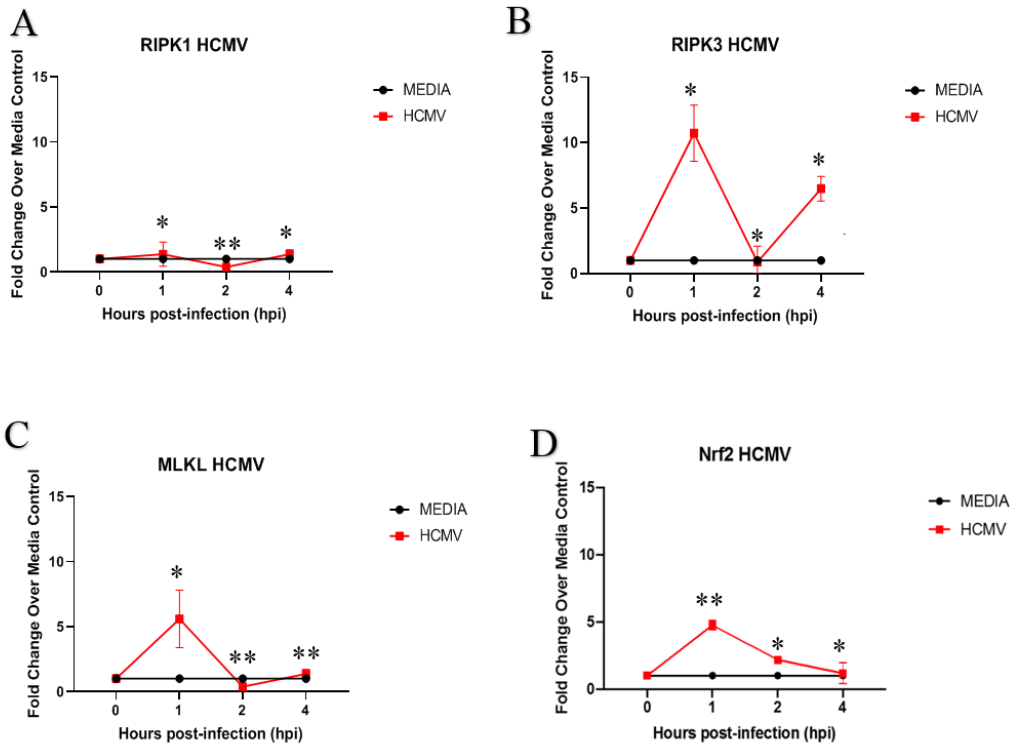

Figure 7. mRNA Expression of Necroptosis Markers and Nrf2 in HCMV-infected ARPE-19 cells from 1 to 4 Hours. HCMV (Towne strain) MOI=5 infected ARPE-19 cells all infected at the same time and harvested at the same time points. * $p<0.05$ and ** $p<0.01$, HCMV groups were compared back to the media-treated control.

RIPK1 shows no stimulation during the first four hours of HCMV-infection in ARPE-19 cells. While RIPK3 shows repeated results compared to, **Fig. 5.** through a consistent pattern of upregulation at 1 hpi, a decline at 2 hpi, and an increase at 4 hpi. MLKL and Nrf2 show a moderate increase at 1 hpi, but proceeds to decrease for the remaining 4 hour time period. We know based on our repeated results in **Fig 6.** that there will be another increase of Nrf2 in 2 days. Nrf2 is usually a molecule in cellular senescence; this peak at 1 hpi suggests it serves as either an aid or a hindrance to HCMV survival upon early infection.

Furthermore, since RIPK3 has shown consistent upregulation in mRNA expression upon early infection, we needed to compare these findings to cells without HCMV-infection. In addition to testing the stimulation or suppression of RIPK1, RIPK3, MLKL, and Nrf2 in an oxidative stressed cellular environment. Hydrogen peroxide is the main byproduct of a redox imbalance, causing oxidative stress in the cell. Therefore, we simulate an oxidative stressed environment in a cell through a building concentration of hydrogen peroxide. Therefore, we performed qPCR on mock-infected ARPE-19 cells to test RIPK1, RIPK3, MLKL, and Nrf2 in different concentrations of hydrogen peroxide.

3.4 Mock-Infected ARPE-19 cells Treated with Different H_2O_2 Concentrations

We treated ARPE-19 cells with 100mM, 200mM, and 300mM concentrations of hydrogen peroxide to investigate whether stimulation of RIPK3 is also dose-dependent. Since cell death mechanisms are dependent on molecular influence, cell type, and cellular environment [11, 12], we considered it essential to continue testing for necroptosis markers RIPK1 and MLKL, in addition to Nrf2.

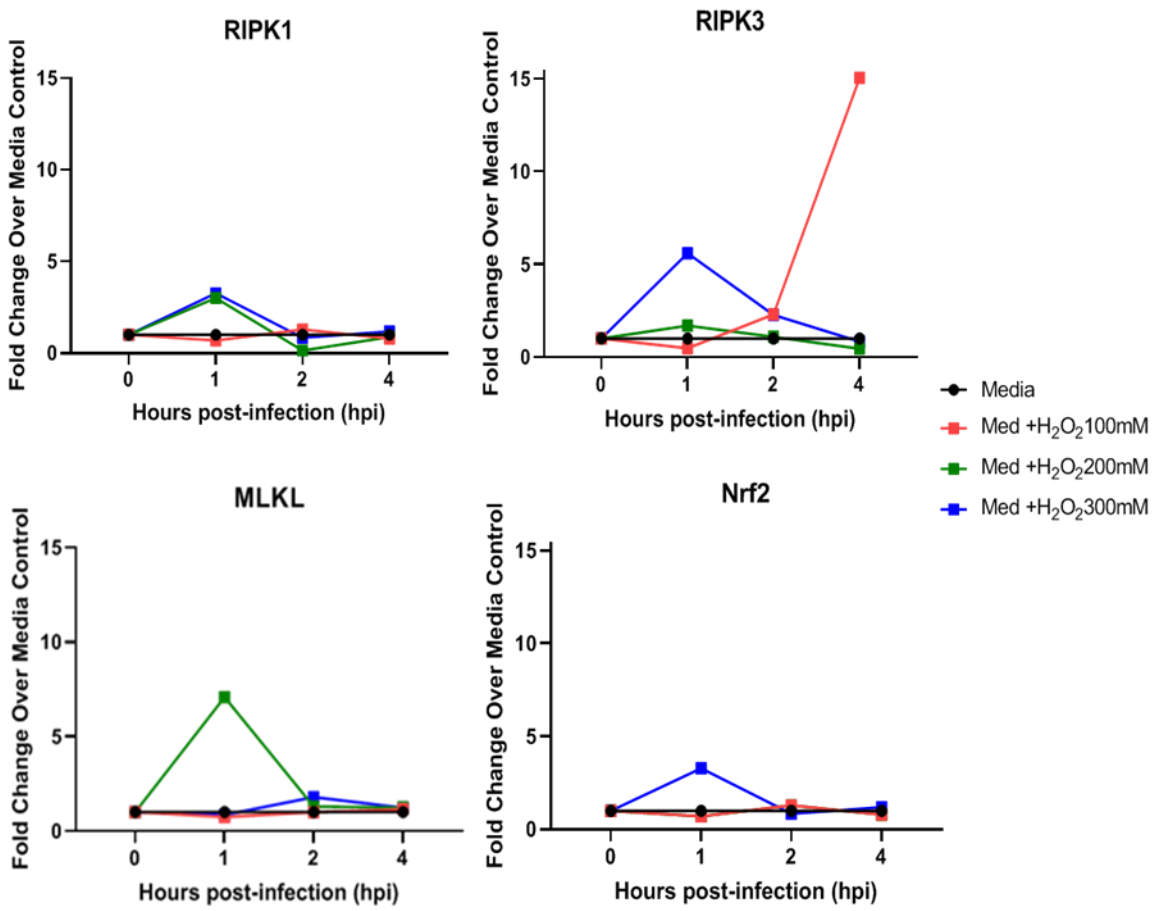

Figure 8. Mock-Infected ARPE-19 cells Treated with Different H_2O_2 Concentrations. APRE-19 samples treated with 100mM, 200mM, and 300mM H_2O_2 solutions were all harvested at the same time for all respective time points. All experiments were compared back to the media control.

RIPK1 mRNA expression presented a moderate increase at 1 hpi in treated 200mM and 300mM hydrogen peroxide concentration, but declined for the remainder time period. MLKL mRNA expression was upregulated at 1 hpi in 200mM hydrogen peroxide treatment and showed little to no stimulation under the other concentrations over time. Nrf2 mRNA expression showed a moderate increase in 300mM hydrogen peroxide treatment at 1 hpi, with no stimulation in the other concentrations over time. The only increase of mRNA RIPK3 expression at 1 hpi was in 300mM hydrogen peroxide treatment, with a repeated downregulation at 2 hpi.

In mock-infected ARPE-19 cells, RIPK1, MLKL, and Nrf2 mRNA expression do not show a pattern of increase or decrease treated with varying hydrogen peroxide concentrations. However, RIPK3 mRNA expression does present an increase dependent on the concentration of hydrogen peroxide. In cells treated with a lower concentration, 100mM, RIPK3 increase occurs at a later time point, while treated with a stronger concentration, 300mM, RIPK3 increases sooner at 1 hpi.

Furthermore, since RIPK3 has shown a dose dependent relationship treated with hydrogen peroxide our next consideration was to test the same markers in the prescence of HCMV. We treated HCMV-infected ARPE-19 cells with hydrogen peroxide concentrations of 100mM, 200mM, and 300mM to test if RIPK3 stimulation is dose-dependent. Studies have found HCMV to have stimulated replication due to treatment of exogenous hydrogen peroxide in another cell type [49]. Since cell death mechanisms are dependent on molecular influence, cell type, and cellular environment [53, 54], we considered it essential to continue testing for necroptosis markers RIPK1 and MLKL, in addition to RIPK3. Subsequently, while seeing an increase of Nrf2 in **Fig 7.** we continued qPCR testing of this marker to observe it's stimulation under oxidative stress.

3.5 HCMV- Infected ARPE-19 Cells Treated with Different Concentrations of H_2O_2.

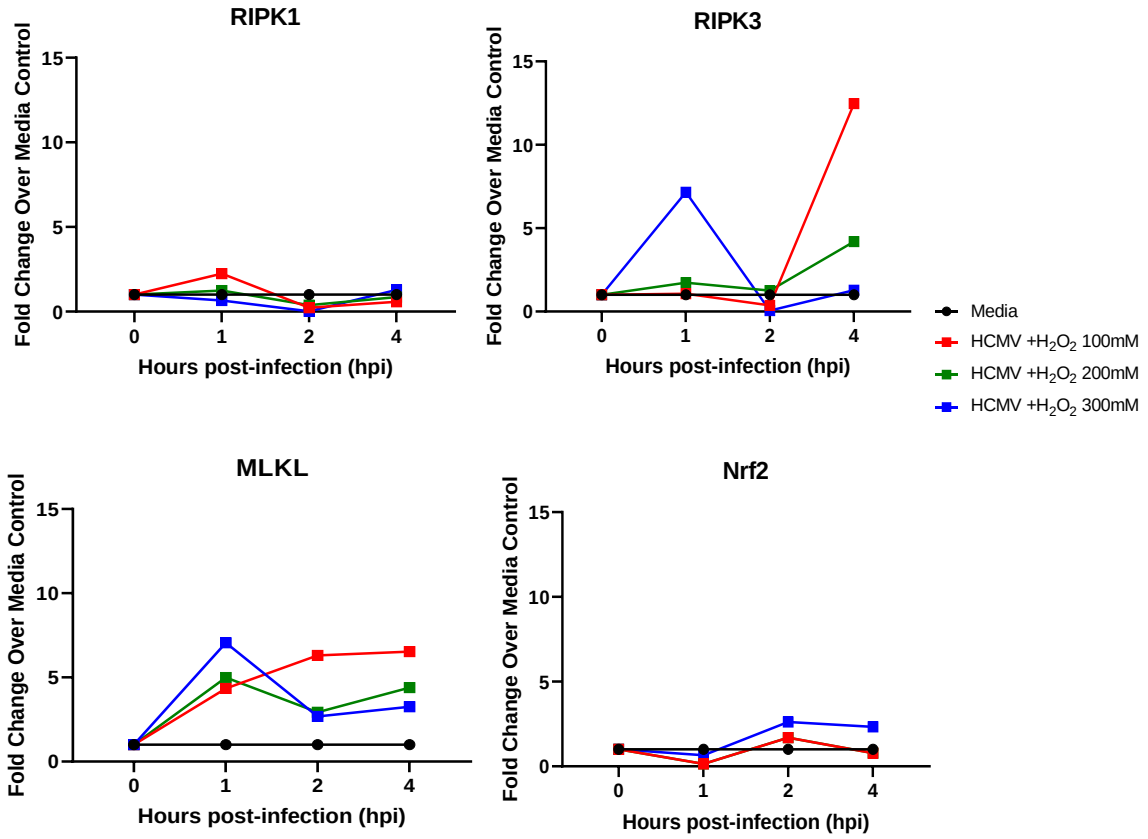

Figure 9. HCMV-infected ARPE-19 cells Treated with Different Concentrations of H_2O_2. HCMV (Towne strain), MOI=5, infected ARPE-19 cells all infected at the same time and harvested at the same time points. ARPE-19 samples with 100mM, 200mM, and 300mM H2O2 solutions were treated and collected at the same time for all time points. All experiments were compared back to the media control.

RIPK1 and Nrf2 mRNA expression show little to no stimulation in hydrogen peroxide treatment. Similar to the results in mock-infected cells, in **Fig. 8**, at 4 hpi in 300mM hydrogen peroxide treatment, there is a decline in RIPK3 expression and an increase in 100mM concentration. Exhibiting a dose dependent relationship between RIPK3 during HCMV infection. In cells treated with 100mM and 200mM hydrogen peroxide, there is a dampening effect of RIPK3 mRNA expression in HCMV-infection. Suppression of RIPK3 favors HCMV survival. In 300mM hydrogen peroxide treatment RIPK3 is decreased at 1 hpi compared to mock-infected cells in **Fig. 8.** This decrease of RIPK3 would no longer be in favor of host cell

survival and will aid in prolonging AIDS-related HCMV retinitis. At 2 hpi there is no increase of RIPK3 in all hydrogen peroxide concentrations. MLKL mRNA shows moderate stimulation at 1 hpi in a stimulation pattern dependent on the dose of hydrogen peroxide. The higher the concentration of hydrogen peroxide, the more robust MLKL mRNA expression present at 1 hpi becomes. As time progresses, the inverse reaction occurs where the lowest dose of hydrogen peroxide causes a higher stimulation of MLKL compared to the highest concentration of hydrogen peroxide.

3.6 RIPK3 Stimulation in HCMV-infected and Mock-infected ARPE-19 cells with H_2O_2 treatment.

Through our studies, we have determined RIPK3 stimulation is dose-dependent on hydrogen peroxide concentration in HCMV-infection over time. To show this comparison, we plotted our repeat experiments of only RIPK3 stimulation in HCMV-infected ARPE-19 cells to mock-infected ARPE-19 cells within each graph. Our graphs below create a better presentation of how the upregulation of RIPK3 is either sustained or dampened at specific hydrogen peroxide concentrations. We tested mRNA expression through qPCR of RIPK3 at 100mM, 200mM, and 300mM H_2O_2 at 1 hpi, 2 hpi, and 4 hpi.

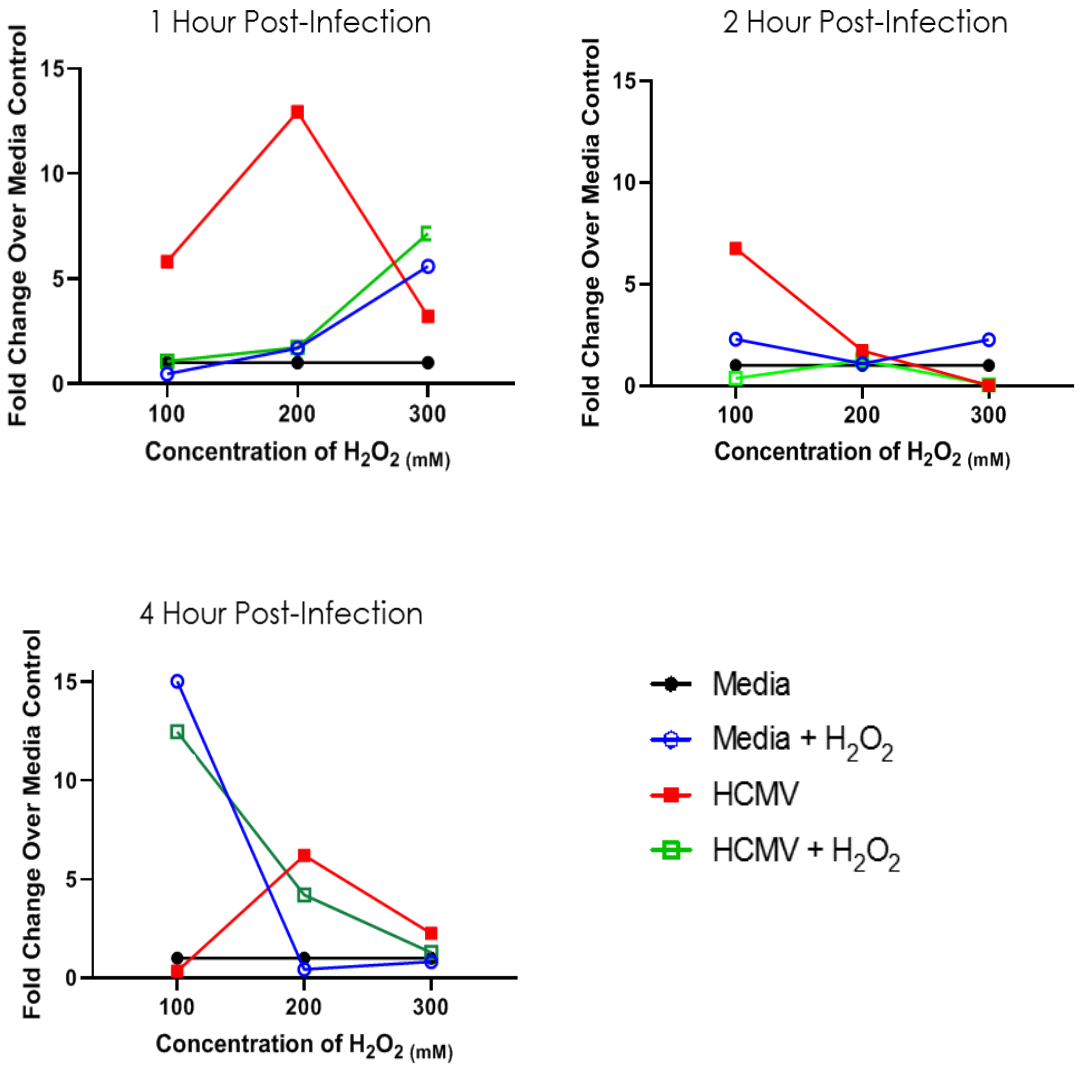

Figure 10. RIPK3 Stimulation in HCMV-infected and Mock-infected ARPE-19 cells with H_2O_2 treatment. HCMV (Towne strain) MOI=5 infected ARPE-19 cells all infected at the same time and harvested at the same time points. APRE-19 samples with 100mM, 200mM, and 300mM H2O2 solutions were treated and harvested at the same time for all time points. All experiments were compared back to the media control.

We notice a repeated pattern at 1 hpi, where RIPK3 mRNA expression is upregulated during HCMV-infection. When low concentrations of hydrogen peroxide (100mM and 200mM) is present in the cell RIPK3 shows a prominent decrease. While, in ARPE-19 cells

treated with 300mM hydrogen peroxide RIPK3 no longer shows a higher expression compared to HCMV alone.

At 2 hpi there is again no prominent activity of RIPK3. Although the addition of 100mM H_2O_2 does present a lesser expression of RIPK3 compared to the expression in HCMV alone without H_2O_2, there is not a prominent difference.

At 4 hpi RIPK3 upregulation shows a fascinating presentation. As the same in **Fig. 5.** and **Fig. 7.** upon HCMV-infection alone RIPK mRNA increases, but with the addition of 100mM H_2O_2 there is a higher fold change in RIPK3. A higher fold change in mock-infected cells agrees with the ARPE-19 cell's natural response to activate a mechanism to repair cellular damage. Although for cells infected with HCMV and H_2O_2 no longer seems to be an aid to the virus in suppressing RIPK3- mediated mechanisms to sustain virulence. While at 200mM and 300mM H_2O_2 (simulating oxidative stress building within the cell), hydrogen peroxide appears to work back in HCMV favor and suppressing RIPK3. **Therefore, RIPK3 mRNA expression exhibits a dose-dependent response over time in HCMV-infected ARPE-19 cells in 100mM, 200mM, and 300mM H_2O_2 concentrations.**

3.7 Nrf2 Stimulation in HCMV- infected and Mock-Infected ARPE-19 cells treated with H_2O_2.

Next, was to determine if there is a pattern shown in Nrf2 mRNA expression by observing each fold change over 100mM, 200mM, and 300mM hydrogen peroxide concentrations as we tested for RIPK3 mRNA expression.

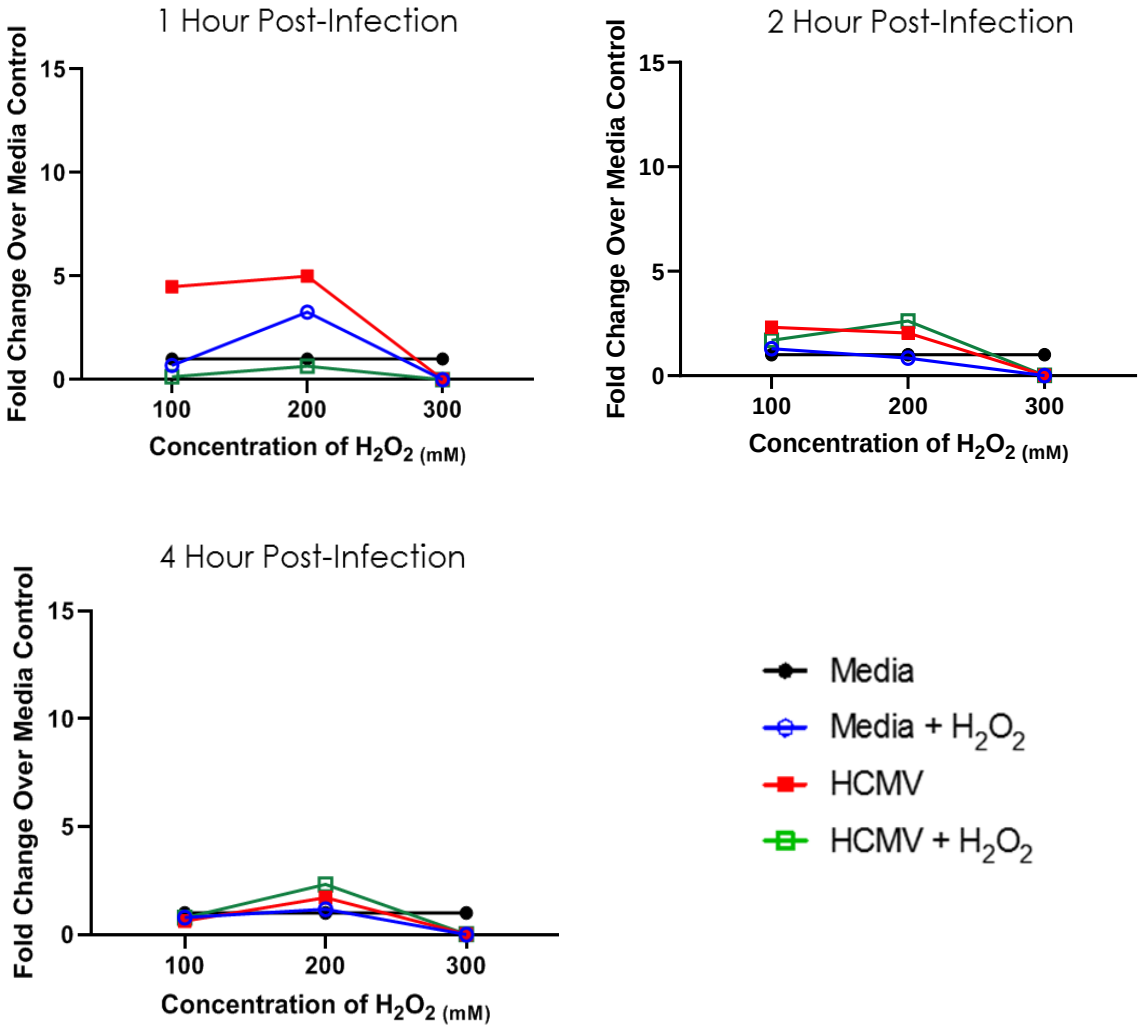

Figure 11. Nrf2 Stimulation in HCMV-infected and Mock-infected ARPE-19 cells. HCMV (Towne strain) MOI=5 infected ARPE-19 cells were all infected and harvested at the same time. ARPE-19 samples treated with 100mM, 200mM, and 300mM H_2O_2 solutions were treated and harvested at the same time for all time points. All experiments were compared back to the media control.

At early onset, HCMV-infection of Nrf2 mRNA expression is decreased when hydrogen peroxide is added to HCMV-infected ARPE-19 cells. The Nrf2 marker that would typically be stimulated indirectly upon HCMV-infection is now suppressed in the presence of high oxidative stress in the cell. After 1 hpi expression Nrf2 declines, but based on **Fig. 5.** we know that Nrf2 will be peak again at 2 dpi.

RIPK3 STIMULATION IN OXIDATIVE STRESSED HCMV-INFECTED MRC-5 CELLS

Human fibroblasts are a target of HCMV replication [13, 14]. From our previous results in **Fig. 5.- Fig.10.**, RIPK3 stimulation in ARPE-19 cells were evident, so to determine if RIPK3 stimulation is cell-specific to APRE-19 cells, we tested the same variables on MRC-5 cells. To determine if stimulation of RIPK3 is cell-specific to ARPE-19 cells, we decided to test identical variables in another cell type. We took into account the other pathologies HCMV has in different cell types, such as fibroblasts and endothelial cells. Therefore, we decided to use a fibroblast cell, MRC-5, that has tropism for HCMV as well as not found in the eye to confirm our results. Leading our second hypothesis to test that **(2) HCMV-infected MRC-5 cells will not express RIPK3 stimulation in the presence of oxidative stress induced by H_2O_2.**

We began by performing qPCR to observe if there would be a baseline upregulation of RIPK3 in early hours of HCMV-infection in MRC-5 cells. We continued testing RIPK1 and MLKL markers to discern if RIPK3-mediated necroptosis was the mechanism at work and if not, would Nrf2 play a role in RIPK3 rising out of a quiescence state.

3.8 mRNA Expression of Necroptosis Markers and Nrf2 in HCMV-infected MRC-5 cells.

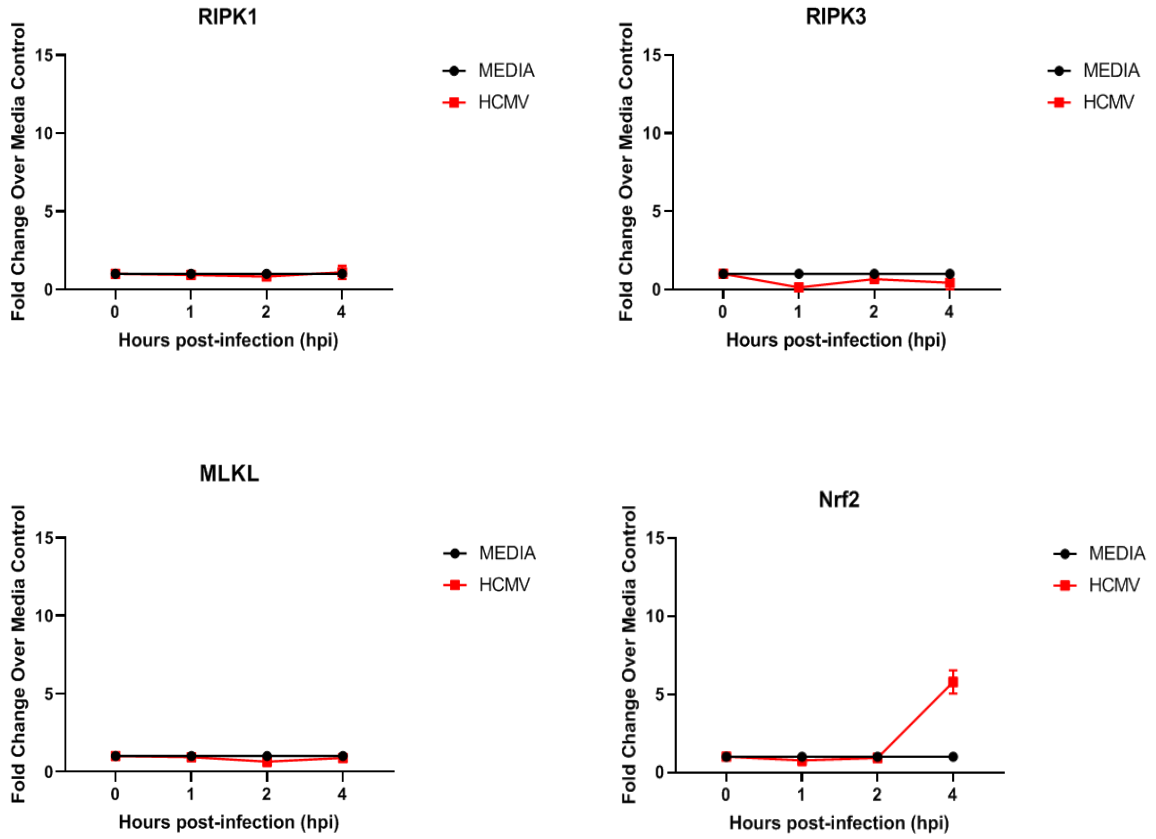

Figure 12. mRNA Expression of Necroptosis Markers and HCMV-infected MRC-5 cells. HCMV (Towne strain) MOI=5 infected MRC-5 cells were all infected and harvested at the same time for respective time points post-infection. * $p<0.05$ and ** $p<0.01$, HCMV groups were compared back to the media-treated control. All samples in all 4 graphs were **$p<0.01$.

There was no stimulation in MRC-5 cells of RIPK3, RIPK1, or MLKL at these time points tested in early HCMV-infection. Nrf2 was the only molecule tested that showed a stimulation at 4 hpi.

3.9 mRNA Expression of Necroptosis Markers and Nrf2 in Mock-Infected MRC-5 Cells

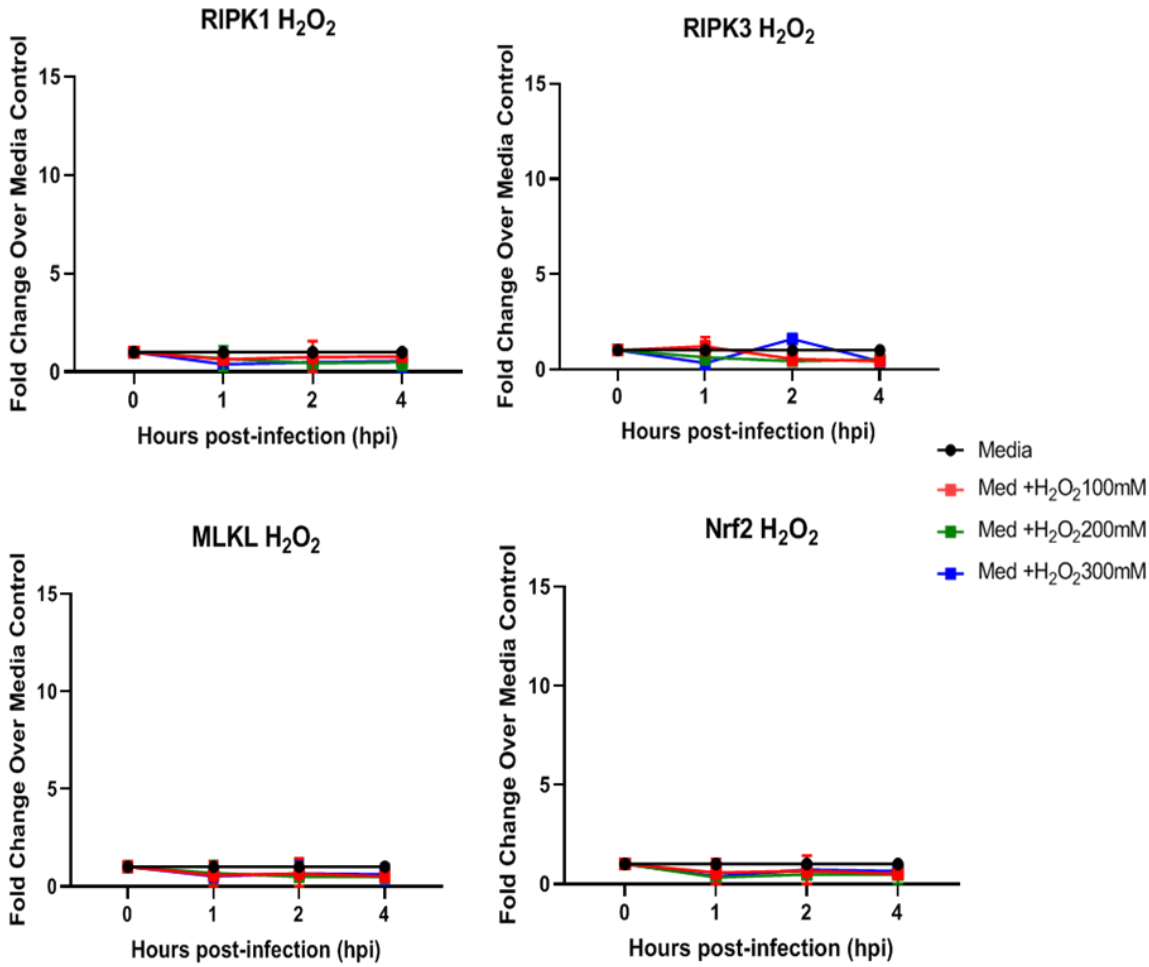

Figure 13. mRNA Expression of Necroptosis Markers and Nrf2 in Mock-Infected MRC-5 Cells. All MRC-5 samples treated with 100mM, 200mM, and 300mM hydrogen peroxide solutions were harvested at the same time for all respective time points. All experiments were compared back to the media control and **p<.01 significant.

Furthermore, even under the tested hydrogen peroxide concentrations in mock-infected MRC-5 cells, RIPK1, RIPK3, MLKL, and Nrf2 did not display a significant stimulation of either markers.

3.10 Necroptosis and Nrf2 mRNA Expression in HCMV-Infected MRC-5 Cells Treated with Different Concentrations of H_2O_2

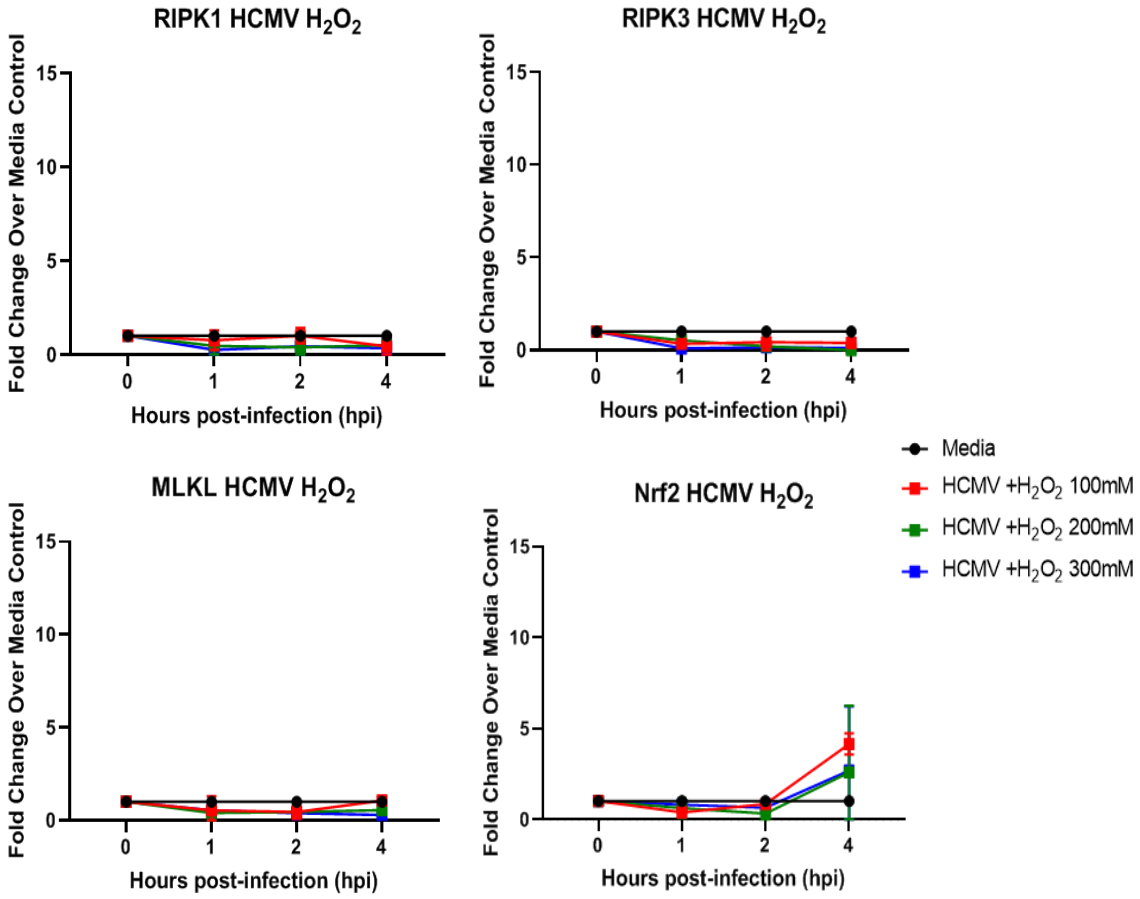

Figure 14. Necroptosis and Nrf2 mRNA expression in HCMV-infected MRC-5 Cells treated with Different Concentrations of H_2O_2. HCMV (Towne strain), MOI=5, infected MRC-5 cells were all infected and harvested at the same time points. MRC-5 samples treated with 100mM, 200mM, and 300mM H2O2 solutions were treated and harvested at the same time for all time points. All experiments were compared back to the media control and **p<.01 significant.

RIPK1, RIPK3, and MLKL did not show an increase in any tested hydrogen peroxide concentrations in HCMV-infected MRC-5 cells. This is an important support for the stimulation of RIPK3 found in APRE-19 cells proving the RIPK3 stimulation is cell-specific.

3.11 RIPK3 mRNA Expression in HCMV-Infected MRC-5 Cells Treated with Different Concentrations of H_2O_2.

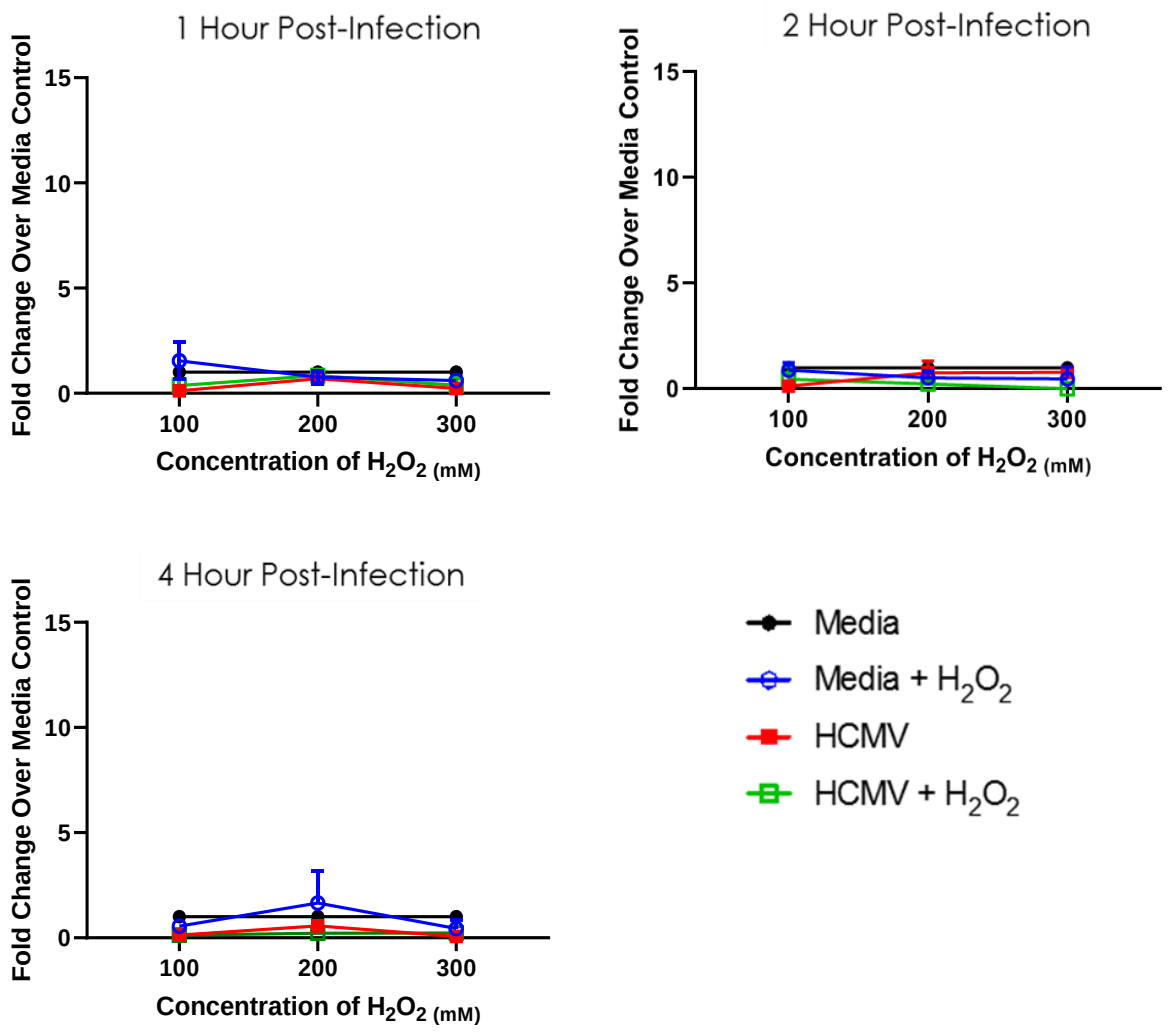

Figure 15. RIPK3 mRNA Expression in HCMV-Infected MRC-5 Cells Treated with Different Concentrations of H_2O_2. HCMV (Towne strain) MOI=5 infected MRC-5 cells treated with 100mM, 200mM, 300mM hydrogen peroxide solutions were all infected and harvested at the same time. * $p<0.05$ and ** $p<0.01$, all groups were compared back to the media-treated control and **$p<0.01$ significant.

RIPK3 was not upregulated in MRC-5 cells during HCMV-infection, HCMV-infection treated with hydrogen peroxide of the three tested concentrations, or in mock-infected MRC-5

cells. To continue to see if this activity is cell specific to ARPE-19 cells we tested the mRNA expression of Nrf2 under the same conditions.

3.12 Nrf2 mRNA Expression in HCMV-infected and Mock-infected MRC-5 cells under Induced Hydrogen Peroxide at Several Concentrations.

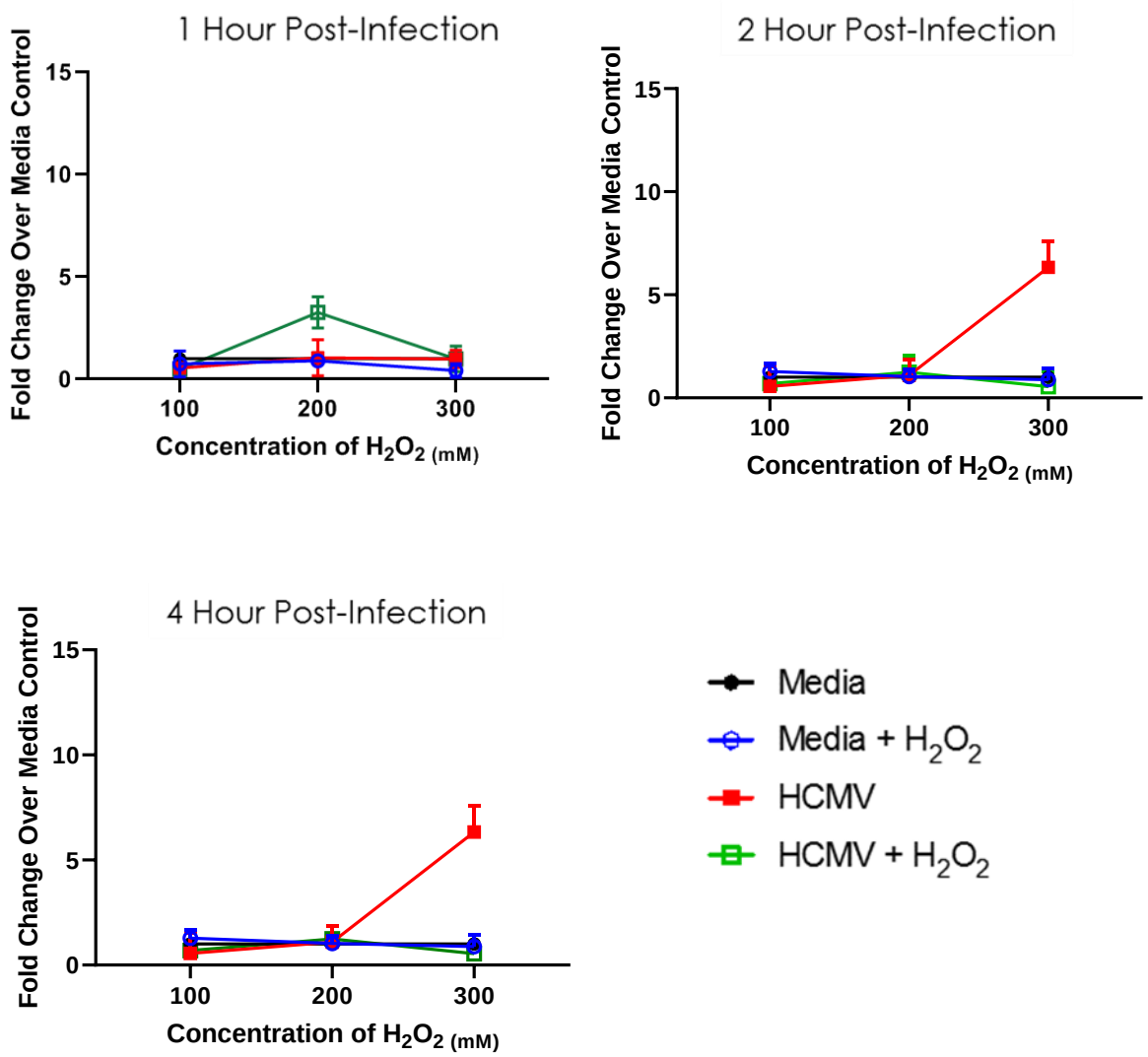

Figure 16. Nrf2 mRNA Expressoin in HCMV-infected and Mock-infected MRC-5 Cells under Induced Hydrogen Peroxide at Several Concentrations. HCMV (Towne strain), MOI=5. MRC-5 cells were treated with 100mM, 200mM, 300mM hydrogen peroxide solutions and were all infected and harvested at the same time. * $p<0.05$ and ** $p<0.01$, all groups were compared back to the media-treated control. All sample groups were **$p<0.01$ significant.

Nrf2 only showed a moderate upregulation at 1 hpi under 200mM hydrogen peroxide concentration. Aside from the slight stimulation at 1 hpi, increasing hydrogen peroxide concentration displayed no upregulation when compared back to media during the first 4 hours of acute infection.

4 DISCUSSION

Two hypotheses were tested to broaden our understanding of RIPK3 activity during HCMV-infection amongst oxidative stress present in the RPE. Hypothesis (1) tested that HCMV- infected ARPE-19 cells will upregulate RIPK3 in the presence of oxidative stress induced by H_2O_2. To validate cell specificity based on previous results, our second Hypothesis tested that (2) HCMV-infected MRC-5 cells will not express RIPK3 stimulation in the presence of oxidative stress induced by H_2O_2. As stated in the introduction, one of RPE functions is to prevent or alleviate cell injury. Accumulation of hydrogen peroxide left untreated can result in cell death. Despite HCMV showing inhibitory effects of RIPK3-mediated necroptosis, there is an increase in RIPK3 activity during early infection, **Fig. 5.** It then became essential to determine whether the two variables, HCMV and oxidative stress, influence on RIPK3 upregulation, were additive or synergistic.

Without the addition of hydrogen peroxide in HCMV-infected ARPE-19 cells, RIPK3 increases during early infection, as shown in **Fig. 5.** and **Fig. 7.** at 1 hpi and at 4 hpi. When hydrogen peroxide is added to HCMV-infected RPE cells, RIPK3 is decreased at 1 hpi. There is also a noticeable difference in the sustained downregulation of RIPK3 in the 200 mM hydrogen peroxide solution in **Fig. 9.** Therefore, **we see that amongst an excessive accumulation of hydrogen peroxide, there is a dampening effect of RIPK3 stimulation during early HCMV-**

infection. All things considered, this can lead to the reduction of RIPK3- mediated necroptosis and RIPK3-independent mechanisms, which ultimately encourages HCMV replication and sustain retinal destruction.

During the progression of AIDS-related HCMV retinitis, oxidative stress may be a factor to influence host cell death by lessening a defense mechanism of the RPE. These findings and related studies are essential in the accurate detection of which cell-defense markers are necessary to be included as targets in antiviral therapies.

While it does not escape our attention that other antioxidants are present. The commonly tested antioxidants such as SOD3, GCLC, and GPX-1, initially investigated in this study, displayed inconsistent stimulation in mRNA expression to continue analysis during early infection. We conclude that SOD3, GCLC, and GPX-1, markers are not adequate targets for future investigation to improve antiviral therapies in cytomegalovirus retinitis patients.

Due to the pathogenesis of HCMV still under investigation, understanding the development of this disease is imperative to develop safe, innovative, and effective treatments for the prevention and management of AIDS-related HCMV retinitis. All HIV infected patients do not elicit a positive response to ART. In addition to the ineffective response to the current drug therapies for HIV/AIDS, antiviral drug-resistant of HCMV strains have emerged throughout the study of this disease. Antivirals created such as ganciclovir, valganciclovir, cidofovir, and foscarnet suppress HCMV through targeting its virus-encoded DNA and showed positive suppression in HCMV-infected patients. Despite the hopeful responses during the early years of antiviral treatment, viral mutations circumvent ganciclovir and valganciclovir attack through the pUL97 kinase producing ganciclovir and valganciclovir- resistant HCMV strains. Therefore, it

would behoove us to look into other target molecules that may indirectly initiate or maintain viral replication.

For instance, another way activated HCMV indirectly sustains its viral replication may occur as follows. As viral replication transpires in the cell, it induces cellular stress, including stress on the endoplasmic reticulum (ER) [41]. Viral stress on the cell activates a host cell survival response known as the unfolded protein response (UPR) [41]. UPR is enabled to remove misfolded proteins, upregulate chaperones to reestablish homeostasis during oxidative stress, and sustain cellular metabolic state [41]. A branch of the UPR system that becomes activated is known as PERK (Protein R-like ER kinase). PERK functions as an adaptive response to modify protein synthesis and induce KEAP1 (Kelch-like ECH-associated protein 1)[43, 55]. KEAP1 is a protein that acts as an intracellular detector of free radicals/ ROS by releasing antioxidant Nrf2 into the cytoplasm. Nrf2 acts as an antioxidant to eradicate free radicals. As it begins its attempt to diminish the oxidative stress on the cell, it in turn removes the molecules that are initially activating the UPR system. HCMV may take advantage of this negative feedback loop and continue replication.

Furthermore, within this mechanism, activated PERK phosphorylates eIF2α (eukaryotic initiation factor 2). Phosphorylated eIF2α works in favor of viral replication. It enables the Translation Attenuation mechanism, which safeguards proper transcription and translation to continue viral replication [44]. This potential mechanism is demonstrated in the self-illustrated pathway in **Fig. 17**. In summary, UPR's primary responsibility to aid in cell survival may result in support of HCMV survival through indirectly continuing viral replication and ultimately progressing HCMV retinitis.

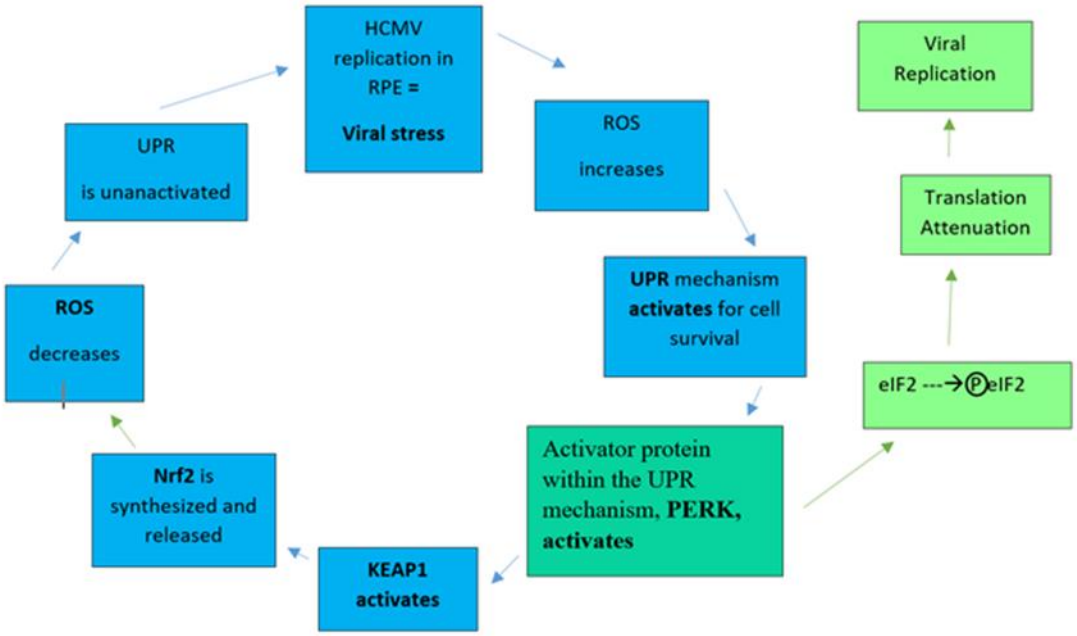

Figure 17. The Indirect Sustained HCMV Replication through two Pathways. Through a cell survival mechanism of UPR, HCMV has acquired yet another way to sustain its virulence. Blue colored boxes represent one pathway, and the green represents the second pathway. The connectivity point of these two pathways are illustrated through the blue-green box, which describes PERK activation, insinuating the UPR mechanism can indirectly activate eIF2 through phosphorylation and initiate HCMV replication.

Considering there are mechanisms indirectly enhancing viral replication, antiviral therapies should improve its content to reduce the accumulation of free radicals causing oxidative stress in the RPE and lessen the concentration of Nrf2 within the cell. Nrf2 is one of many antioxidants synthesized to combat increased levels of ROS [45]. Thus, targeting Nrf2 levels would potentially remove another favorable molecule for HCMV replication and lessen the progression of vision loss in AIDS- related cytomegalovirus retinitis.

FUTURE DIRECTION

Although the SOD3, GCLC, and GPX-1antioxidants did not yield a successful result in mRNA expression, we should not reject the notion that there are several other antioxidants present that may be involved in enhancing HCMV replication in the RPE. However, due to the

upregulation of Nrf2 it may be a new potential target molecule for further investigation in allowing continued replication of HCMV in RPE cells. Furthermore, subsequent protein assays are necessary to assess protein levels of the markers tested as added support of mRNA expression [37][38].

Also, to continue comparison studies of RIPK3 stimulation in ARPE-19 cells and MRC-5 cells, future experiments should be performed together on the same day for all time points. Also, in vivo studies can be done through using knockout RIPK3 -/- mice to test for any markers provided in **Table 1.** The new markers would be tested to know which independent RIPK3-mediated mechanism is at work during early HCMV-infection.

CLINICAL RELEVANCE

HCMV cytopathic effect in AIDS-related HCMV retinitis, reigns a persistent problem for AIDS patients who do not have access or show a negative response to antiviral treatment. One way AIDS-related HCMV retinitis can be inhibited is to stop replication of the virus through effective HCMV antiviral treatment. Due to the pathogenesis of HCMV still under investigation, antiviral drug-resistant strains of HCMV have emerged throughout the study of this disease. Therefore, it is essential to continue investigation of target molecules that may indirectly initiate or maintain viral replication.

Our findings suggest that hydrogen peroxide is a factor in limiting cell survival by lessening a host cell defense mechanism mediated by RIPK3. HCMV initially, as seen in **Fig. 7.,** showed elevated RIPK3 mRNA expression at 1 hpi and 4 hpi. However, when HCMV infected ARPE-19 cells were treated with hydrogen peroxide, a molecule seen to increase RIPK3-mediated cell death, RIPK3 expression was decreased at 1 hpi and 4 hpi. Due to this suppression, hydrogen peroxide that was once an aid to host cell survival is now an aid to HCMV survival; essentially

progressing retinitis. Future investigations should continue experiments related to RIPK3 stimulation during HCMV retinitis *in vitro* and *in vivo.* In addition to RIPK3, separate studies can be done to see the effects of PERK in the UPR system and Nrf2 to see confirm if they play a role in sustaining HCMV replication. These future findings will be essential to further our knowledge of which cell-defense markers is HCMV manipulating to sustain life, so there can be improvement in HCMV antiviral therapies.

5 CONCLUSION

Herein we tested our hypothesis, **(1) HCMV-infected ARPE-19 cells will upregulate RIPK3 in the presence of oxidative stress induced by H_2O_2.** The results of this study suggest the following conclusions:

(i) *In vitro* infection of HCMV upregulated RIPK3 mRNA expression and may facilitate independent RIPK3- mediated mechanism upon early infection,

(ii) RIPK3 stimulation presents a dose-dependent response given the tested hydrogen peroxide concentrations,

(iii) Within the first-hour post-infection, the more hydrogen peroxide present in the cell inducing oxidative stress favors the host cell cellular defense and increase RIPK3 expression, and

(iv) As time progresses within 4 hours post-infection, and oxidative stress increases, RIPK3 is downregulated favoring HCMV sustained replication, leading to the reduction of RIPK3- mediated necroptosis and RIPK3-independent mechanisms, which ultimately encourages HCMV replication and sustain retinal destruction.

Our second hypothesis pursued **(2) HCMV-infected MRC-5 cells will not express RIPK3 stimulation in the presence of oxidative stress induced by H_2O_2.**

(i) *In vitro* infection of HCMV did not stimulate RIPK3 and will not facilitate independent RIPK3- mediated mechanism upon early infection, and

(ii) Due to lack of stimulation of RIPK3, RIPK1, MLK, and Nrf2 in MRC-5 cells prove that our findings in ARPE-19 are cell-specific

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