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Virus Production and Cell Viability of HSV-1-infected Murine Keratinocytes (HEL-30) Co-cultured with Murine Macrophages (RAW 264.7)

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Virus Production and Cell Viability of HSV-1-infected Murine Keratinocytes (HEL-30) Co-cultured with Murine Macrophages (RAW 264.7)

A thesis submitted in partial fulfillment

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Master of Science.

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Barry Graffagna ENTITLED Virus Production and Cell Viability of HSV-1-infected Murine Keratinocytes (HEL-30) Co-cultured with Murine Macrophages (RAW 264.7) BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Graffagna, Barry. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2018. Virus Production and Cell Viability of HSV-1-infected Murine Keratinocytes (HEL-30) Co-cultured with Murine Macrophages (RAW 264.7).

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Keratinocytes are the most abundant type of cell in the outer layer of skin, the epidermis, and provide barrier against pathogens from invading. However, Herpes Simplex Virus Type 1 (HSV-1) targets these keratinocytes for infection, and later infects neurons to establish lifelong latency. The keratinocytes stimulate the innate immune system to engage and to destroy the virus. Among the cells of the innate immune system to respond to the viral invasion is the macrophage. In this study, RAW 264.7 macrophage and HEL-30 keratinocyte monolayers were challenged in vitro with HSV-1 at a multiplicity of infection (MOI) of 0.1 to investigate the individual responses. A co-culture was established using a ratio of 1:5 with the macrophages and keratinocytes, respectively after the keratinocytes were challenged with HSV-1 with an MOI of 0.1. The viabilities and the viral titers of both monolayers and the co-culture were recorded after 24, 48, and 72 hours from the initial infection with HSV-1. The viabilities of both monolayers decreased over time to confirm HSV-1 infection in both cell lines. Further confirmation was made with the results of the viral titers increasing over time for both monolayers.

Interestingly, the co-culture resulted in a different outcome than of the individual cultures. The infected co-culture viabilities remained high and were comparable to the uninfected co-culture over the 72 hours. The viral titer of the co-culture was significantly lower than that of the two monolayer titers at 48 and 72 hours. However, there was a significant increase in viral production from 48 to 72 hours. This suggests the RAW 264.7 macrophage in a co-culture with HEL-30 keratinocytes were able to slow the HSV-1 infection and demonstrate its protective role within the innate immune system.

HYPOTHESIS

HSV-1 virus replication in epithelial cells, e.g. Vero cells, leads to maximum virus yield within three to five days. Keratinocytes are epithelial cells and the cell type initially infected in humans by HSV-1. To mimic initial infection of keratinocytes by HSV-1, a co-culture system comprised of macrophages and HSV-1-infected keratinocytes was developed. In this static system it was hypothesized that RAW 264.7 murine macrophages can inhibit HSV-1 replication in cultured murine HEL-30 keratinocytes over a 72 hour observation period.

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LIST OF ABBREVIATIONS

HSV-1 = Herpes Simplex Virus Type 1

HVEM = Herpes Virus Entry Mediator

TLR = Toll-like Receptor

PRR = Pattern Recognition Receptor

HSPG = Heparan Sulfate Proteoglycan

3-O-HS = 3-O-Heparan Sulfate

IE = Immediate-Early

TNF-α = Tumor Necrosis Factor – α

IFN- α/β = Interferon – α/β

IL-12 = IL-12

LAT = Latency Associated Transcript

ICP27 = Infected Cell Protein – 27

IRF3 = Interferon Response Factor 3

MHC = Major Histocompatability Complex

MyD88 = Myleoid Differentiation Primary Response 88

MOI = Multiplicity of Infection

PFU = Plaque Forming Unit

HEL-30 = Keratinocyte cell line

RAW (264.7) = Macrophage cell line

M_0 = Unpolarized Macrophage Phenotype

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is part of the *Herpesviridae* family along with HSV-2, Cytomegalovirus, and Varicella-Zoster virus. The structure of HSV-1 includes the tegument and the capsid which holds the double stranded DNA (dsDNA), enclosed by a lipid envelope that has several glycoproteins which aid it to enter and infect the cell (Figure 1) (Rogalin & Heldwin, 2016). HSV-1 is believed to have infected approximately 60-95% of the global adult population (Marchi, Trombetta, Gasparini, Temperton, & Montomoli, 2017). The most common symptom of an HSV-1 infection is the painful lesion that forms in the mouth and face; however, HSV-1 capable of infecting the eyes and genitals (Marchi, Trombetta, Gasparini, Temperton, & Montomoli, 2017). HSV-1 has a primary infection which occurs in the epithelium cells, where it undergoes lytic replication and infects nearby neurons to establish lifelong latency (Szpara et al., 2013). Severe symptoms of HSV-1 would include blindness and in individuals who are immunocompromised, HSV-1 can cause encephalitis (Marchi, Trombetta, Gasparini, Temperton, & Montomoli, 2017).

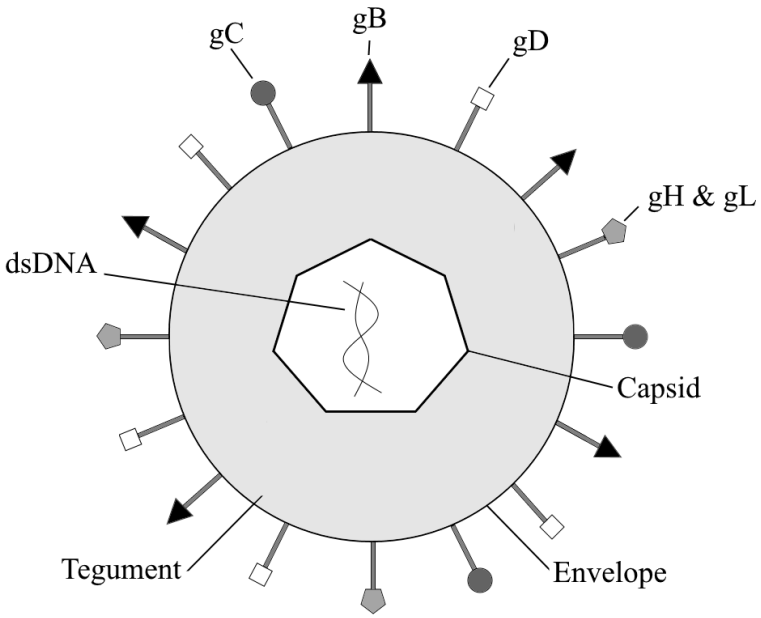

Figure 1: The structure of Herpes Simplex Virus Type 1.

Keratinocytes are an important cell which make up most of the epidermal layer of skin, the outermost layer, and serves as the primary defense against pathogens (Wu et al., 2011). Keratinocytes also make up the outermost layer of oral mucosa to serve the same purpose in creating a barrier to prevent pathogens from entering the tissue (Wu et al., 2011). However, this barrier can be compromised by a break, which can allow the pathogens to enter into the tissue. In the event there is any damage sustained in the barrier, the keratinocytes secrete growth factors to help repair the barrier (Hobbs & Watt, 2003). HSV-1 is able to enter the tissue through the cracks in the keratinocyte barriers and begin infecting cells. The primary methods of entry into the cell are through endocytosis and fusion with the outer membrane of the cell utilizing its glycoproteins to bind to specific receptors, such as glycoprotein-D (gD) and the cell's nectin-1 receptor (Figure 2) (Montgomery, Warner, Lum, & Spear, 1996). Herpes virus entry mediator (HVEM) is another major protein utilized by HSV-1 for entry and is found on the surface of the host cell (Montgomery, Warner, Lum, & Spear, 1996).

Once a keratinocyte has been infected by HSV-1, it can begin its antiviral response by producing cytokines and chemokines to alert neighboring keratinocytes and nearby immune cells, such as macrophages and dendritic cells, that there is an invader (Frey et al., 2009). However, HSV-1 has shown to inhibit anti-viral responses and even inhibit apoptosis pathways (Jerome et al., 1999). Should HSV-1 succeed in replicating in the keratinocytes, it can migrate and infect nearby cells until it reaches a neuron where it will infect and lie dormant in a latent state until reactivation (Szpara et al., 2013).

Macrophages are a vital cell within innate immune system that is one of the first to respond to infection or tissue damage. These cells are monocytes that either migrate through the blood or are tissue specific resident macrophages. Upon detection of a pathogen, macrophages

can enter the tissue from the bloodstream or resident macrophages can migrate to the site of infection (Lee & Ghiasi, 2017). Macrophages are capable of secreting cytokines to aid in an immune response against pathogens (Fraternale et al., 2014). To maintain homeostasis, macrophages can also secrete growth factors to aid in tissue repair, and phagocytize any infected, dead, or dying cells so that it may be degraded along with any intracellular pathogen in those cells; it can also phagocytize the pathogen itself if it is found extracellularly (Arandjelovic & Ravichandran, 2015). During an HSV-1 infection, macrophages can be alerted through the Toll-like receptors (TLRs), pattern recognition receptors (PRRs), and several different cytokine receptors (Gregory & Devit, 2004). HSV-1 can activate TLRs which will trigger the MyD88 pathway in macrophages to begin the production of interferons and cytokines that are important for an antiviral response (Leoni, Gianni, Salvioli, & Campadelli-Fiume, 2012).

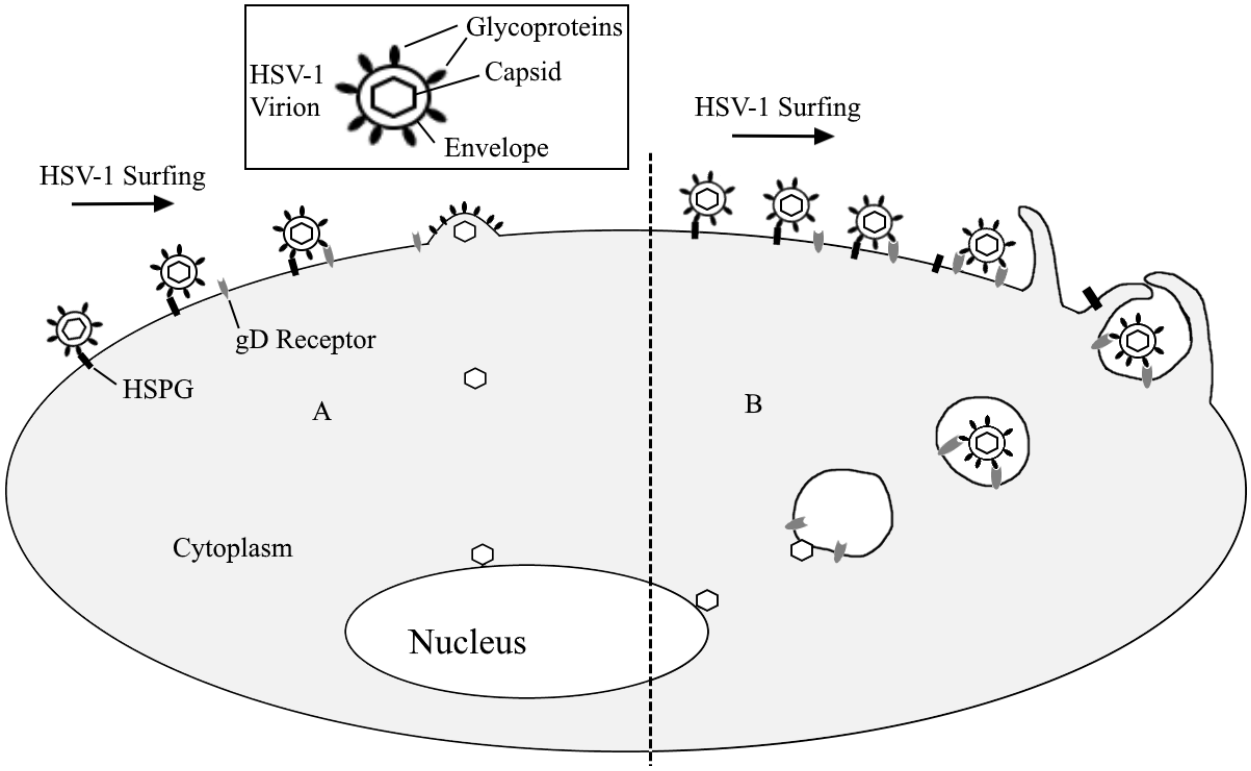

Figure 2: HSV-1 Entry Mechanisms: Herpes Simplex Virus Type 1 can utilize two different mechanism to enter a cell. HSV-1 can undergo a pH-independent fusion by binding glycoprotein B (gB) and gC to heparan sulftate proteoglycan (HSPG) so that the virion may surf on the surface of the cell until gD binds to one of the three gD receptors: nectin-1, herpes virus entry mediator, or 3-O-heparan sulfate; then the virion can fuse its envelope with the cell and allow the capsid and tegument to migrate to the cell's nucleus where it can begin replication (**A**). The process of endocytosis is a pH-dependent method of entry into the cell utilized by HSV-1. The virion first needs to bind to HSPG with gB and gC so that it may eventually bind to a gD receptor. Multiple bindings between the virion's gDs and the cell's gD receptors can take place while the cell begins the process of endocytosis. Once in an endosome, the virion eventually undergoes fusion to release its capsid and tegument, where they will migrate to the nucleus (**B**). Adapted from Akhtar & Shukla, 2009.

LITERATURE REVIEW

Herpes Simplex Virus Type 1

HSV-1 is a prevalent pathogen that infects humans and is largely contagious. HSV-1 has five major components to its structure, all of which are important for its ability to infect and replicate: dsDNA, capsid, envelope, glycoproteins, and tegument (Figure 1) (Döhner et al., 2002). The genome of HSV-1 is made up of dsDNA (152 kb) with approximately ~80 genes that are conserved and protected by the capsid (Smith, Reuven, Mohni, Schumacher, & Weller, 2014; Szpara et al., 2013). The lipid envelope around the virus contains many glycoproteins on the surface that allow it to enter a host cell (Heldwein & Krummenacher, 2008). HSV-1 has a tegument contains proteins that aid in viral replication once inside of the host cell (Döhner et al., 2002). Upon replication and release from the cell, HSV-1 will attempt to migrate to nearby nerve endings where enter and remain in a latent state of infection (Agelidis & Shulka, 2015). HSV-1 can be reactivated and migrate out of the nerve endings back into the tissue to re-infect nearby keratinocytes (Halford, Gebhardt, & Carr, 1996). In cases where the immune system is completely compromised, HSV-1 has been known to migrate to the brain and cause encephalitis (Maertzdorf, Lelij, Baarsma, Osterhaus, & Jerjans, 2001).

HSV-1 Infection and Replication Mechanisms

The process by which HSV-1 infects cells is through endocytosis or fusion through the cellular membrane (Figure 2) (Clement et al. 2006). Endocytosis can be done through a pH-dependent mechanism (Nicola, McEvoy, & Straus, 2004); however, fusion can be done through a pH-independent mechanism (Gianni et al., 2010). HSV-1 utilizes the glycoproteins along the outside of its envelope to bind to specific receptors and gain entry into the cell (Nicola & Straus, 2003). Currently, there are three well studied receptors used for entry by HSV-1 involving the nectin-1 receptor, HVEM, and 3-O-heparan sulfate (3-O-HS) (Thier et al., 2017). These receptors are found on keratinocytes, which make them prime targets for infection (Spear, 2004). However, many other types of cells express these receptors making susceptible to HSV-1 infection (Montgomery, Warner, Lum, & Spear, 1996).

The first interaction between HSV-1 and the host cell is the binding of gB and gC to heparin sulfate proteoglycans (HSPG), where it will allow the virus to remain attached to the surface of the cell, but will not give it entry (Herold, WuDunn, Soltys, & Spear, 1991). The second interaction requires gD to bind to the host cell's nectin-1 receptor. However, HVEM has also been recognized as a target for binding for gD as a separate way to enter the host cell (Petermann et al., 2015). There is another method for HSV-1 to enter the cell by the binding of gD with 3-O-HS (Shukla et al., 1999). Glycoproteins gH and gL have been reported to aid in the fusion of the virus envelope with the host cell's membrane (Gianni et al., 2010).

Once HSV-1 has succeeded in entering the host cell, the tegument proteins are utilized for migrating the viral dsDNA to the nucleus, which then aid in the transcription and translation of the immediate-early (IE) genes (Loret, Guay, & Lippé, 2008). The early genes require the

products of the IE transcription and translation, and in the continuation of the cascade, the late genes require the products of the IE and early genes to be transcribed and translated in order to be expressed (Harkness, Kader, & DeLuca, 2014). Once the necessary components have been produced, assembly of HSV-1 begins until the cell has been lysed, where the virions will go onto infect other nearby cells (Tal-Singer et al., 1997).

The Role of the Macrophage during HSV-1 Infection

The innate immune system is an incredibly important line of defense against pathogens that aim to infect cells or tissue. One of the cell types that make up the innate immune system is the macrophage. These cells are derived from monocytes and originate from the bone marrow where they flow through the bloodstream until they sense a danger signal (Ma & He, 2014). However, macrophages also develop tissue specific residency during the embryonic stage where they renew without ever leaving (Gautier et al., 2012). The job of the macrophage in a state without infection is primarily focused on phagocytizing and degrading dead and dying cells (Rossi et al., 1998). In addition to being able to clear dead cells, they can secrete growth factors to repair any tissue and help maintain homeostasis (Bhat, Wooten, & Jayasuriya, 2013).

Macrophages have TLRs and PRRs which helps it recognize the presence of a pathogen (Zhou, Cao, Fang, Li, & Fan, 2015). HSV-1 has been reported to bind the gH-gL dimer with its TLR2 and begin the MyD88 pathway which will lead to the production of cytokines (Figure 3) (Leoni, Gianni, Salvioli, & Campadelli-Fiume, 2012). Both of these are important during an HSV-1 infection because keratinocytes infected with HSV-1 can produce IL-1α to stimulate an immune response (Parpaleix et al., 2016). Once the macrophage has been activated, it can begin secreting pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), interferon α /β (IFN-

α /β), and interleukin-12 (IL-12) to begin an antiviral response (Figure 3) (Fraternale et al., 2014).

HSV-1 Immune Evasion

HSV-1 has evolved multiple methods of evading the immune system as well as inhibiting cellular functions all in an attempt to allow itself to replicate. The inhibition of apoptosis gives a strong advantage in forcing the cell to continue HSV-1 replication (Inman et al., 2001). One of the known methods of apoptosis inhibition is by utilizing the latent associated transcript (LAT) which targets the cleavage of caspase 3 (Carpenter et al., 2007). A second method reported HSV-1 inhibiting apoptosis by targeting the Fas receptor involved in the Fas-induced apoptosis pathway (Jerome et al., 1999). Infected cell protein 27 (ICP27) is an HSV-1 protein that will target IFN response factor 3 (IRF3), which is important in the production of IFN-β, a cytokine that stimulates the innate immune system (Johnson, Song, & Knipe, 2008). ICP0 has also shown to have a role in inhibiting IRF3 as well (Melroe, Silva, Schaffer, & Knipe, 2007). HSV-1 can cause the host cell to begin production and cleavage of IL-1β. However, the cell is rendered unable to release it (Johnson, Chikoti, & Chandran, 2013). Major histocompatibility complexes (MHC) are key to alerting the immune system of invaders. However, HSV-1 has the ability to target MHC II by blocking it with gB (Neumann, Eis-Hübinger, & Koch, 2003); MHC I is targeted for inhibition as well (Orr et al., 2005).

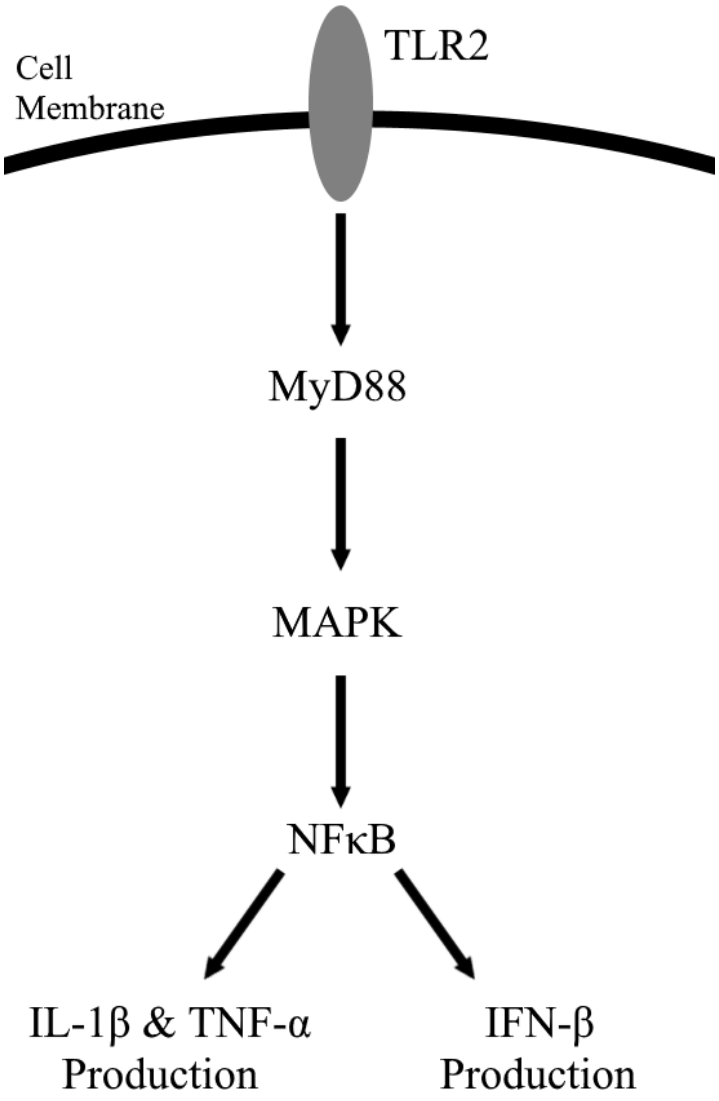

Figure 3: Signaling pathway for TLR2, which leads to the production of pro-inflammatory cytokines IL-1β, TNF-α, and IFN- β in macrophages. Adapted from Zhou & Amar, 2007.

MATERIALS AND METHODS

Cell Cultures and Virus

HEL-30 keratinocytes derived from C3H mice (initially provided by Dr. D Germolec of the NIEHS in Durham, NC). RAW 264.7 macrophages were derived from BALB/c mice which were induced into a tumor using Abelson murine leukemia virus, and were purchased from the American Type Culture Collection (ATCC). Vero cell line originated from African green monkey kidney epithelial cells (CCL-81 from ATCC). The cells were stored in liquid nitrogen and thawed, then seeded into T-25 flasks (25 cm^2). Cells were passaged, split, and transferred into T-75 flasks (75 cm^2). Cells were grown using Dulbecco's Modified Eagle Medium (DMEM; HyClone) with 10% fetal bovine serum (FBS). Cell cultures were incubated in a humidified incubator at 37^oC and 5% CO_2.

Herpes simplex virus - 1 (HSV-1; syn 17+) was used for infection (initially provided by Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH). The virus stock concentration was 1.90 x 10^7 viruses per mL.

Infection of HEL-30 Keratinocytes

HEL-30 keratinocytes were seeded at a density of 1.00 x 10^5 in 12 well plates with 1 mL of 10% FBS media and allowed to incubate for 24 hours. The old media was removed and the cells were washed with phosphate buffer solution (PBS) before 0.50 mL of DMEM was added to each well. Wells selected for virus treatment had 0.52 µL of HSV-1 stock added to have a 0.1 multiplicity of infection (MOI). The 12 well plates were then incubated at 37^oC with 5% CO_2 for 2 hours. The wells were washed with PBS and 2 mL of 10% FBS was added to each well. Separate plates were allowed to incubate at 37^oC with 5% CO_2 for 24, 48, and 72 hours after the

infection. At the 24, 48, and 72-hour time points the media was placed into a 1.5 mL tube and centrifuged at 487 xg for 5 minutes, then placed into a 4^{o}C refrigerator. The cells were washed with PBS after the media was removed and later trypsinized. The old media was removed from the 4^{o}C refrigerator and transferred to fresh 1.5 mL tubes, then placed into a -80^{o}C freezer. Once the cells had finished trypsinization, the cells were placed into the respective tubes initially used to centrifuge the old media, then the cells were centrifuged at 487 xg for 5 minutes. This procedure was done to ensure that any floating cells were accounted for during viability. The supernatant was aspirated and 1 mL of fresh 10% FBS was used to re-suspend the cells. The viability of each well was calculated afterwards.

Infection of M_0 RAW 264.7 Macrophage

Unpolarized (M_0) RAW 264.7 macrophages were seeded at a density of 1.00 x 10^5 in 12 well plates with 1 mL of 10% FBS media and allowed to incubate for 24 hours. The old media was removed and the cells were washed with phosphate buffer solution (PBS) before 0.50 mL of DMEM was added to each well. Wells selected for virus treatment had 0.52 µL of HSV-1 stock added to have a 0.1 multiplicity of infection (MOI). The 12 well plates were then incubated at 37^{o}C with 5% CO_2 for 2 hours. The wells were washed with PBS and 2 mL of 10% FBS was added to each well. Separate plates were allowed to incubate at 37^{o}C with 5% CO_2 for 24, 48, and 72 hours after the infection. At the 24, 48, and 72-hour time points, a cell scraper was used to detach the cells. The media was placed into a 1.5 mL tube and centrifuged at 487 xg for 5 minutes, then the supernatant was placed into a fresh 1.5 mL tube and stored in a -80^{o}C freezer. One milliliter of fresh 10% FBS was used to re-suspend the cells. The viability of each well was calculated afterwards.

Infection of Co-culture (HEL-30 & RAW 264.7)

HEL-30 keratinocytes were seeded at a density of 8.00 x 10^4 in 12 well plates with 1 mL of 10% FBS media and allowed to incubate for 24 hours. The old media was removed and the cells were washed with PBS before 0.50 mL of DMEM was added to each well. Wells selected for virus treatment had 0.52 µL of HSV-1 stock added to have a 0.1 MOI. The 12 well plates were then incubated at 37ºC with 5% CO_2 for 2 hours. The wells were washed with PBS and 2 mL of 10% FBS was added to each well. M_0 RAW cells were added to each well at count of 1.6 x 10^4 to obtain a 1:5 ratio of macrophages to keratinocytes. Separate plates were allowed to incubate at 37ºC with 5% CO_2 for 24, 48, and 72 hours after the infection. At the 24, 48, and 72-hour time points the media was placed into a 1.5 mL tube and centrifuged at 487 xg for 5 minutes, then placed into a 4ºC refrigerator. The cells were washed with PBS after the media was removed and later trypsinized. The old media was removed from the 4ºC refrigerator and transferred to fresh 1.5 mL tubes, then placed into a -80ºC freezer. Once the cells had finished trypsinization, the cells were placed into the respective tubes initially used to centrifuge the old media. The cells were centrifuged at 487 xg for 5 minutes. Reusing the first 1.5 mL centrifuge tube was done to ensure that any floating cells were accounted for during viability. The supernatant was aspirated and 1 mL of fresh 10% FBS was used to re-suspend the cells. The viability of each well was calculated afterwards.

Cell Viability

Viability of the cells was determined using trypan blue staining, as the stain will only be absorbed by dead cells, leaving living cells a clear color. Staining was done in a 1:2 ratio of cells to stain, 50 µL of suspended cells and 100 µL of trypan blue. Ten microliters of the stained cells were added to a hemocytometer and was used to count the live and dead cells using a light microscope with 10x magnification. The viability was calculated by dividing the live cells from the total cell count.

$$Viability\ \% = \frac{Live}{\substack{Total\ Cell\ Count \\ (Live + Dead)}} \times 100$$

Plaque Assay

A 12 well plate was seeded with Vero cells and allowed to reach 100% confluency. HSV-1 stock was obtained and using serial dilution of taking 120 µL of the stock and adding it to 1080 µL of DMEM, a dilution factor of 1.0×10^{-1} was obtained. This procedure was repeated until the dilution factor 1.0×10^{-8} was reached. Supernatant containing virus was diluted in the same method. The 12 well plate was marked with two controls where no virus was added and the chosen dilutions were added to each well following the controls in increasing dilution factors. Before the virus is added to the wells, they are washed with PBS, then 0.5 mL of the virus dilution was added to a well; the control was given 0.5 mL of virus-free DMEM. The virus was allowed to incubate 37ºC with 5% CO_2 for 2 hours, which was then removed and the wells were washed with PBS. One milliliter of 2% methylcellulose gel was added to each well. The plates were allowed to incubate at 37ºC with 5% CO_2. Plaques began to form after 3-5 days after infection. Once the plaques were formed, 4% paraformaldehyde solution was added to the wells and allowed to incubate at room temperature for 3 hours. Afterwards, tap water was used to

remove the gel and paraformaldehyde, and 0.5 mL of 1% crystal violet was added to each well for 5-10 minutes. The crystal violet was removed using tap water. Clear circles formed by the virus lysing the Vero cells are plaques. These circles are counted to obtain the plaque forming unit (PFU), which is divided by the dilution factor and the mL of inoculum. The result is the PFU/mL.

$$\frac{PFU}{mL} = \frac{number\ of\ plaques}{(dilution\ factor) \times (mL\ of\ inoculum\ per\ well)}$$

Statistical Analysis

All experiments were done in triplicate. SigmaPlot 12 software was used to run one-way ANOVA on all data to calculate significance. P-values ≤ 0.05 are considered statistically significant. Varying degrees of significance are annotated with * as $P \leq 0.05$, ** as $P \leq 0.01$, *** as $P \leq 0.001$, and no significance as $P > 0.05$.

RESULTS

Cell Viability

Cell Viability of HEL-30 Keratinocytes Infected with HSV-1 after 24, 48, and 72 Hours.

The HEL-30 keratinocytes were designated to be left uninfected as control and infected with herpes simplex virus – 1 (HSV-1) with a multiplicity of infection (MOI) of 0.1. HEL-30 keratinocytes showed no significant difference in viability after 24 hours from infection ($P = 0.458$) (Figure 4). However, at the 48-hour time point the viability of the infected keratinocytes was significantly decreased from the uninfected control ($P = 0.002$). After 72 hours from infection, the viability of the infected keratinocytes was greatly decreased compared to the control ($P = < 0.001$). When comparing the infected HEL-30 keratinocytes' time points, the 72-hour time point showed the greatest significant decrease in viability than the 24 and 48-hour time points ($P = < 0.001$; $P = < 0.001$), respectively. There was moderately significant decrease in viability difference between the 24 and 48 hour groups ($P = 0.003$).

Cell Viability of RAW 264.7 M_0 Macrophages Infected with HSV-1 after 24, 48, and 72 Hours.

RAW 264.7 macrophages were initially seeded and inoculated with HSV-1 in an unpolarized (M_0) state at 0.1 MOI. At 24 hours, the RAW macrophages showed no significant difference in viability from the control ($P = 0.190$) (Figure 5). The 48-hour time point had no significant change in viability compared to the control ($P = < 0.670$). After 72 hours, the viability of RAW macrophages had the greatest decrease when compared to the control ($P = < 0.001$). The comparison of infected RAW macrophages at 72 hours had a large significant decrease in viability compared to the 24 hour and 48 hour groups ($P = < 0.001$; $P = < 0.001$),

respectively. The infected RAW macrophages at 48 hours no significant difference in viability compared to the 24 hour infected group (P = < 0.657).

Cell Viability of HEL-30 Keratinocyte and RAW 264.7 M_0 Macrophages Co-culture Infected with HSV-1 after 24, 48, and 72 Hours.

HEL-30 keratinocytes were initially infected with HSV-1 with an MOI of 0.1 or left uninfected as control. RAW macrophages were added at a ratio of 1:5 macrophages to keratinocytes after 2 hour of infection. When comparing the infected groups to the respective uninfected controls, all three time points: 24, 48, and 72 hours showed no significant difference in viability (P = 0.055, P = 0.060, P = 0.103), respectively (Figure 6). The 24 and 48 hour infected co-culture groups had no significant difference in viabilities (P = 0.355). The 72 hour infected co-culture group had a slight significant decrease in viability when compared to the 24 and 48 hour infected groups (P = 0.039; P = 0.014), respectively.

Cell Viability Comparison between Infected HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Model after 24 Hours.

The RAW macrophages have a decreased viability compared to the HEL-30 keratinocytes, as well as, the co-culture after 24 hours since infection (P = 0.007, P = 0.012), respectively (Figure 7). The viabilities of the HEL-30 keratinocytes and co-culture had no significant difference (P = 0.579) (Figure 7).

Cell Viability Comparison between Infected HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Model after 48 Hours.

The HEL-30 keratinocytes began to show a significant decrease in viability after 48 hours when compared to the co-culture viability (P = 0.024) (Figure 8). The RAW macrophages had no significant difference in viability compared to the HEL-30 keratinocytes (P = 0.627). The

difference between RAW macrophages and co-culture viabilities was significantly decreased in comparison ($P = < 0.001$).

Cell Viability Comparison between Infected HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Model after 72 Hours.

After 72 hours since infection, the RAW macrophages had no significant difference in viability compared to the HEL-30 keratinocytes ($P = 0.536$) (Figure 9). The viabilities of HEL-30 keratinocytes and RAW macrophages had greatly decreased compared to the co-culture ($P = < 0.001$; $P = < 0.001$), respectively.

Plaque Assay

Plaque Assay of HEL-30 Keratinocytes Infected with HSV-1 after 24, 48, and 72 Hours.

The HEL-30 keratinocytes after 24 hours post-infection had the lowest viral titer, however, when compared to 48 hours and 72 hours post-infection, a significant increasing of viral titer was seen ($P = < 0.001$; $P = < 0.001$), respectively (Figure 10). Even the 48 and 72 hours viral titers had a significant increase over time ($P = < 0.001$).

Plaque Assay of RAW 264.7 M_0 Macrophage Infected with HSV-1 after 24, 48, and 72 Hours.

The viral titer at 24 hours compared to the 48 and 72 hour titers had a significant increase with both times ($P = < 0.001$; $P = < 0.001$) (Figure 11). The difference in viral titers between 48 and 72 hours had no significance ($P = 0.739$).

Plaque Assay of HEL-30 Keratinocyte and RAW 264.7 M_0 Macrophage Co-culture Infected with HSV-1 after 24, 48, and 72 Hours.

The viral titer at 24 hours and 48 hours showed no significant difference (P = 0.230) (Figure 12). However, after 72 hours, the viral titer had significantly increased compared to the 24 and 48 hour titers (P = < 0.001; P = < 0.001).

Comparison of HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Plaque Assays 24 Hour after Infection with HSV-1.

HEL-30 keratinocytes and RAW macrophages had no significant differences in viral titers when compared to the co-culture titer after 24 hours from the initial HSV-1 infection (P = 0.404, P = 0.404), respectively (Figure 13). The RAW macrophages and HEL-30 keratinocytes also had no significant difference in viral titers (P = 0.101).

Comparison of HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Plaque Assays 48 Hour after Infection with HSV-1.

HEL-30 keratinocyte viral titer was significantly higher than the co-culture titer after 48 hours since the initial HSV-1 infection (P = < 0.001) (Figure 14). The RAW macrophage viral titer was significantly higher when compared to the HEL-30 keratinocyte and co-culture titers (P = < 0.001; P < 0.001), respectively.

Comparison of HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture Plaque Assays 72 Hour after Infection with HSV-1.

The co-culture viral titer was significantly lower compared to the RAW macrophage and HEL-30 keratinocyte viral titers after 72 hours since the initial HSV-1 infection (P = < 0.001; P = < 0.001), respectively (Figure 15). The RAW macrophage viral titer was slightly significantly higher than the HEL-30 keratinocyte titer (P = 0.011).

DISCUSSION

This study aimed to use an in vitro co-culture model between keratinocytes (HEL-30) and macrophages (RAW 264.7) during a herpes simplex virus – 1 (HSV-1) infection. Most importantly the model was made to show the specific role that the macrophage has during a viral challenge while interacting with a tissue. Cells were inoculated with a multiplicity of infection (MOI) of 0.1 to simulate the initial infection of HSV-1 in a tissue.

A decreasing viability over 24, 48, and 72 hours when infected with HSV-1 was seen in keratinocytes after infection. The viral titers increased over 24, 48, and 72 hours which correlated with the decrease in cell viability. HEL-30 keratinocytes behave similarly to another mouse keratinocyte: PAM212 infected with HSV-1. PAM212 produced far more virus after 48 and 72 hour compared to HEL-30 (Alradi, 2018). Overall, HEL-30 cells demonstrated it is susceptible to HSV-1 infection and replication.

For the first two days of the infection, the viability of the macrophage culture looked as if the infection was under control. Then after 72 hours the amount of viable macrophages diminished as the virus overran the system. The macrophage were able to control the virus infection for the first 24 hours, which after there was a massive increase in viral titer at 48 hours. Interestingly, RAW 264.7 macrophages has been reported to undergo an immune response and lower the viral titer from 24 to 48 hours, but this was not seen in this study (Alradi, 2018). However, the peculiarity of having high viability and viral titer was not exclusive to this study, as another study reported unpolarized RAW 264.7 macrophage having a high viability and viral titer after 72 hours (Alanazi, 2018). It is possible that HSV-1 was able to inhibit the macrophages' ability to undergo apoptosis (Jerome et al., 1999).

Interestingly, the co-culture of HEL-30 keratinocytes and RAW 264.7 macrophages showed strong viabilities at each of the time points. At 24, 48, and 72 hours there were no significant decreases in viability. However, while not significant, the viability of the infected co-culture at 72 hours began to show some difference compared to the uninfected co-culture. It could suggest that at 96 hours, the viability of the infected co-culture would rapidly decrease like that of the monolayers. However, after 96 hours the media might be depleted of nutrients and prove difficult to assess whether any decrease in viability is due to the virus or the lack of nutrients available. The viabilities of a PAM212 keratinocyte and RAW 264.7 macrophage co-culture decreased significantly over 72 hours, while the HEL-30 and RAW 264.7 co-culture did not have a significant decrease in viability (Alradi, 2018).

Viral titers of the co-culture's plaque assay increased over the 72 hours observation time. However, the only significant change was seen from 48 to 72 hours. There was no significant increase in viral titer from 24 to 48 hours, which was different from what was observed in both of the monolayer viral titers. Even when comparing the co-culture with PAM212 keratinocytes and RAW 264.7 macrophages, the viral titers are extremely low (Alradi, 2018). Ultimately, the different cell viabilities and viral titers associated with HEL-30 and PAM212 keratinocytes suggests that HSV-1 proliferates more in PAM212 cells than HEL-30 cells. The co-culture's viral load 72 hours after infection is similar the HEL-30 cells after 48 hours.

The lag seen between 24 and 48 hours where the co-culture viability and viral titer deviates from the monolayer may represent the macrophage playing a protective role against the HSV-1 infection. Also the infected keratinocytes could be producing cytokines that help activate the macrophages. One of the cytokines that could be produced from the keratinocytes infected with HSV-1 is interferon (IFN) – α/β (Malmgaard, Melchjorsen, Bowie, Mogensen, & Paludan,

2004). However, it seems after 72 hours the macrophages are not capable of controlling the infection any longer in a closed system and the virus replicated rapidly. In this system, fresh macrophages could not enter the site of infection. In addition to the limit of macrophages within the system, HSV-1 has the capability to inhibit the production of IFN-β by targeting the interferon inducible protein (IDI) 16 for ubiquitous degradation (Eidson, Hobbs, Manning, Carlson, & DeLuca, 2002). IFN- α has been shown to play a key role in controlling HSV-1 (Malmgaard, Melchjorsen, Bowie, Mogensen, & Paludan, 2004; Rong, Alexander, Koski, & Rosenthal, 2003). TNF- α is another cytokine that can play a role in slowing the HSV-1 infection and has even shown to have a synergetic effect with IFN- α/β in activating macrophages sooner than both cytokines alone when there is a viral challenge (Ellerman-Eriksen, 1993).

Another possibility behind the lag in viral production could be partly due to how the co-culture system was prepared. The macrophages were added after the keratinocytes were infected with HSV-1. It is possible that the macrophages were able to detect the infection before HSV-1 was able to inhibit any antiviral response through danger signals, which would allow them to release cytokines, such as TNF-α and IFN-β (Paluden, Bowie, Horan, & Fitzgerald, 2011). This would have given the macrophages enough time to react to the infection.

FUTURE STUDIES

This study focused solely on the role of macrophages during an HSV-1 infection in keratinocytes, however, macrophages are only one type of immune cell that would be localized in tissue. There are other immune cells like dendritic cells, neutrophils, and natural killer cells would be present in the tissue or be capable of responding to an infection. Seeing the outcome of HSV-1 infection after substituting the macrophage with another innate immune cell could potentially have different results entirely.

In this study, only the keratinocytes were initially infected in the co-culture. A second set of experiments could be prepared by infecting the keratinocytes and macrophages at the same time in a co-culture system. By doing this, there is a possibility that the macrophages might not be able to handle the HSV-1 infection. Performing this setup is important because in vivo, there are macrophage present around the keratinocytes naturally.

A point of interest would be in altering the system and initial infection of the cells. In this study, the keratinocytes were infected with HSV-1, then the macrophages were added and the system remain closed. Instead, the macrophages could be added in a ratio of 1:5 macrophages to keratinocytes at several times during the first 24 hours of infection while keeping an MOI of 0.1 of the initial quantity of cells. Adding fresh macrophages to the system would mimic the migration of immune cells to a site of infection.

It would be highly beneficial to understand the kind of cytokine signaling occurring during the three days of infection. A cytokine analysis was not performed in this study, however, identifying what cytokines were produced and at what concentration could further explain the higher viabilities seen in the co-culture. Specifically, the cytokines of interest are IFN-α/β, IL-1β, TNF- α, and IL-10. In addition to cytokine analysis, characterization of the

extracellular surfaces both the HEL-30 keratinocytes and the RAW 264.7 macrophages would help identify any receptors that was responsible synergy.

FIGURES

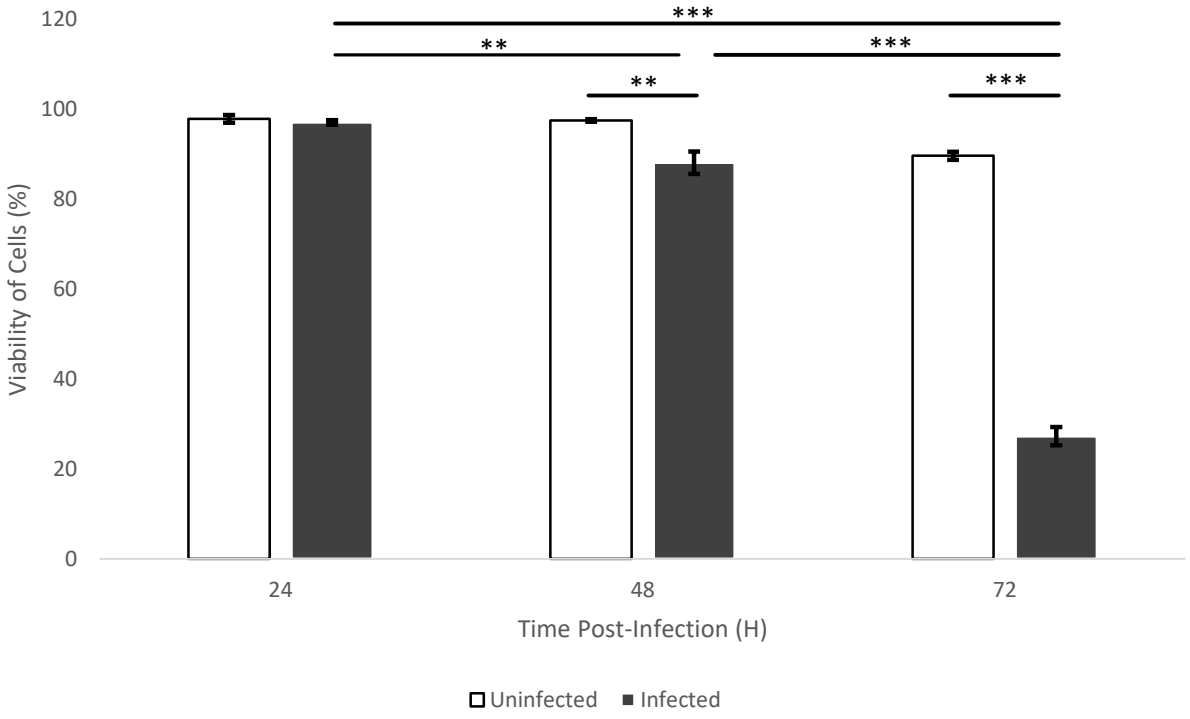

Figure 4: Cell viability of HEL-30 keratinocytes at 24, 48, and 72 hours after infection with HSV-1. HEL-30 keratinocytes showed no significant difference after 24 hours since infection with HSV-1 (P = 0.458). However, at the 48-hour time point, HEL-30 keratinocytes show a decrease in viable cells (P value = 0.002). The 72-hour time point shows the greatest decrease in viable HEL-30 keratinocytes (P value = < 0.001). When comparing the viabilities of the infected HEL-30 keratinocytes, the 24 and 48 time point shows significance in difference (P = 0.003). The 72 hour viability for the infected HEL-30 keratinocytes in comparison to the 24 and 48 hour viabilities is still greatly lower (P = < 0.001, P = < 0.001), respectively. SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

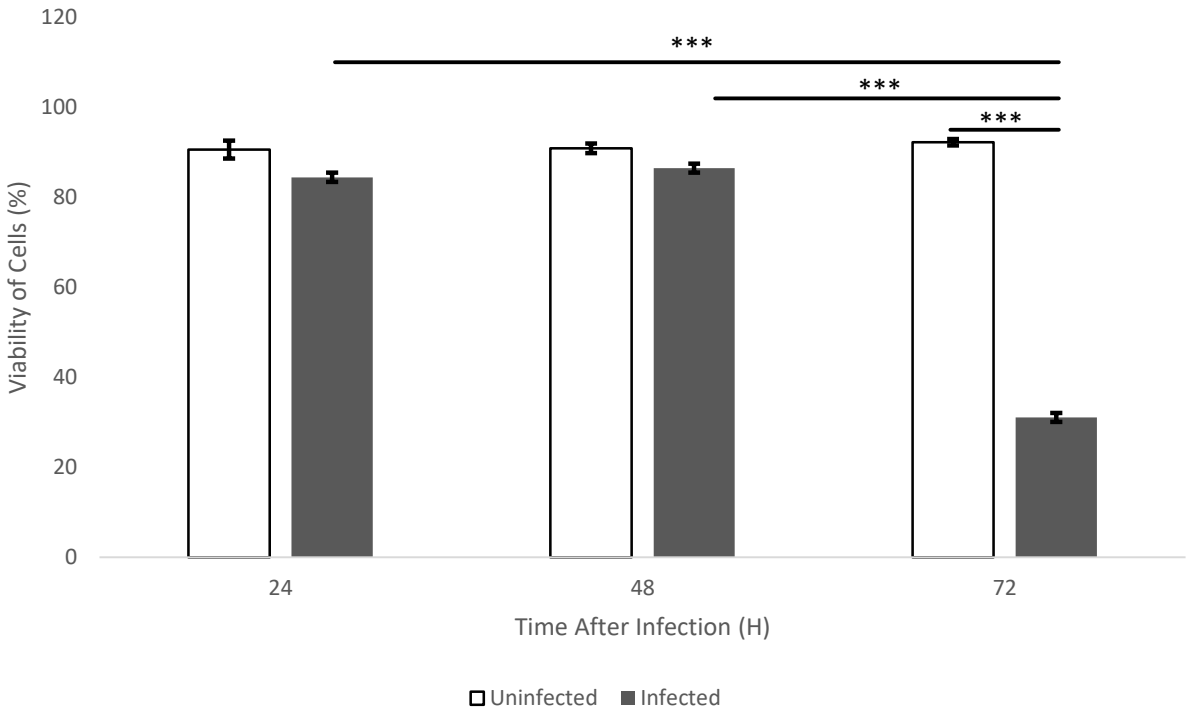

Figure 5: Cell viability of RAW 264.7 M_0 macrophages at 24, 48, and 72 hours after infection with HSV-1. At 24 and 48 hours, the RAW macrophages showed no significant differences in viability when compared to the uninfected controls (P = 0.190, P = 0.670), respectively. However, after 72 hours there was a large decrease in the infected RAW macrophage cultures compared to the uninfected RAW macrophages (P = < 0.001). There was no significant difference between 24 and 48 hour viabilities of the infected RAW macrophages (P = 0.657). Upon comparing the 24 and 48 hour infected RAW macrophages with the 72 hour RAW macrophages, the decrease was significant (P = < 0.001, P = < 0.001), respectively. SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

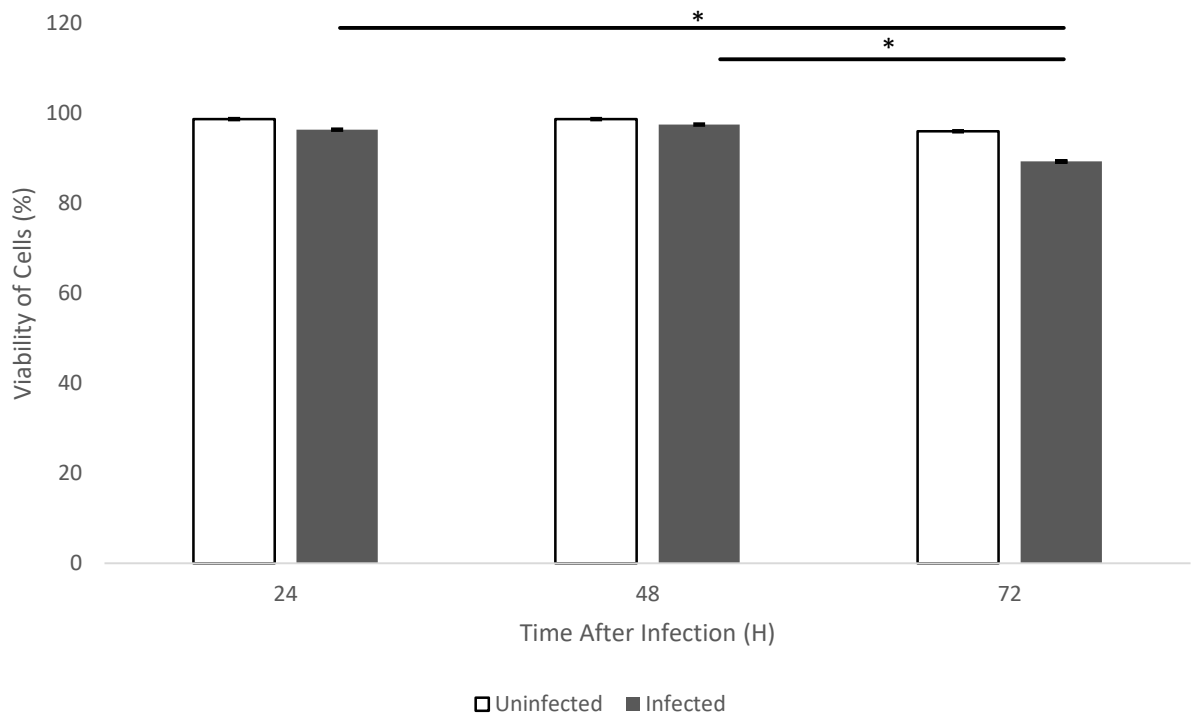

Figure 6: Cell viability of HEL-30 keratinocytes and RAW 264.7 macrophages co-culture at 24, 48, and 72 hours after infection with HSV-1. The viability of the co-culture after 24 hours since infection showed no significant decrease (P = 0.055). The 48 hour viability also showed no significant decrease between the infected and uninfected cells (P = 0.060). After 72 the viability of the infected co-culture showed no significant difference from the uninfected co-culture (P = 0.103). The comparison of infected co-cultures at 24 and 48 hours showed no significant decrease in viability (P = 0.355). The 72-hour time point showed slight significant differences between the 24 and 48-hour time points (P = 0.014, P = 0.039), respectively. SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

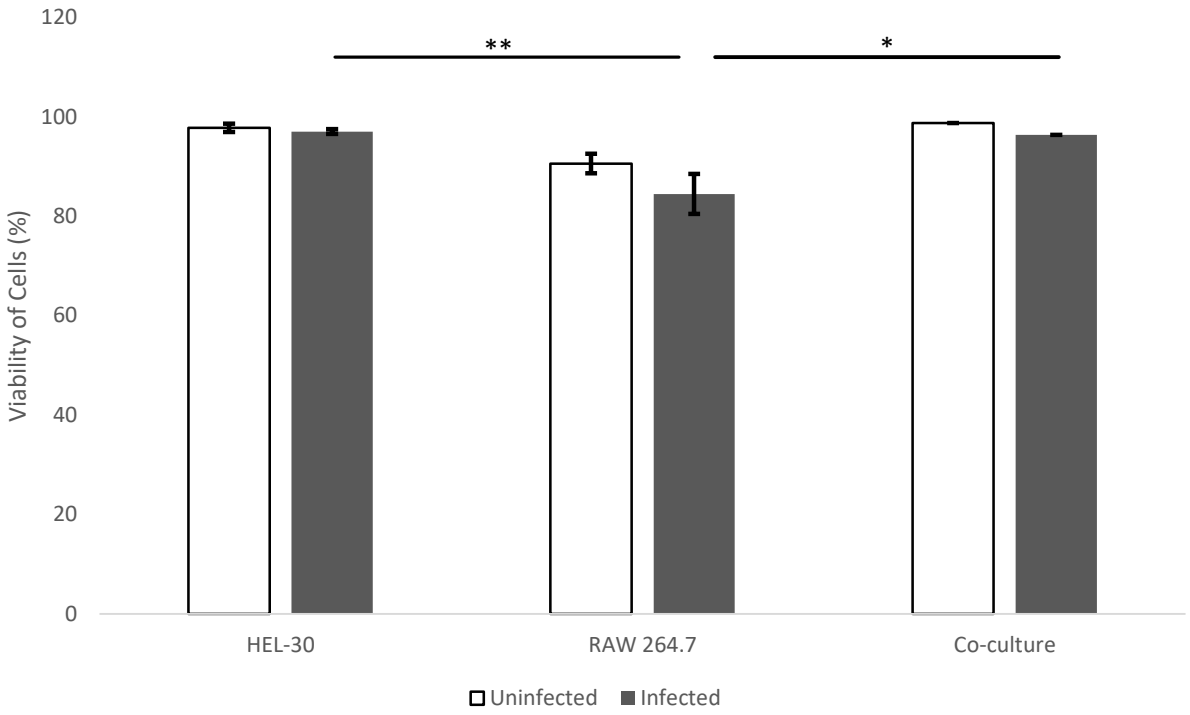

Figure 7: Cell viability Comparison between HEL-30 Keratinocytes, RAW 264.7 M_0 Macrophages, and Co-culture at 24 hours after HSV-1 Infection. The HEL-30 keratinocytes, RAW macrophages, and the co-culture showed no significant difference in viability when compared to the uninfected controls (P = 0.458, P = 0.19, P = 0.055), respectively. The infected RAW macrophages had significantly lower viability compared to the HEL-30 keratinocytes and the co-culture (P = 0.007, P = 0.012), respectively. HEL-30 keratinocytes and the co-culture had no significant difference (P = 0.579). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

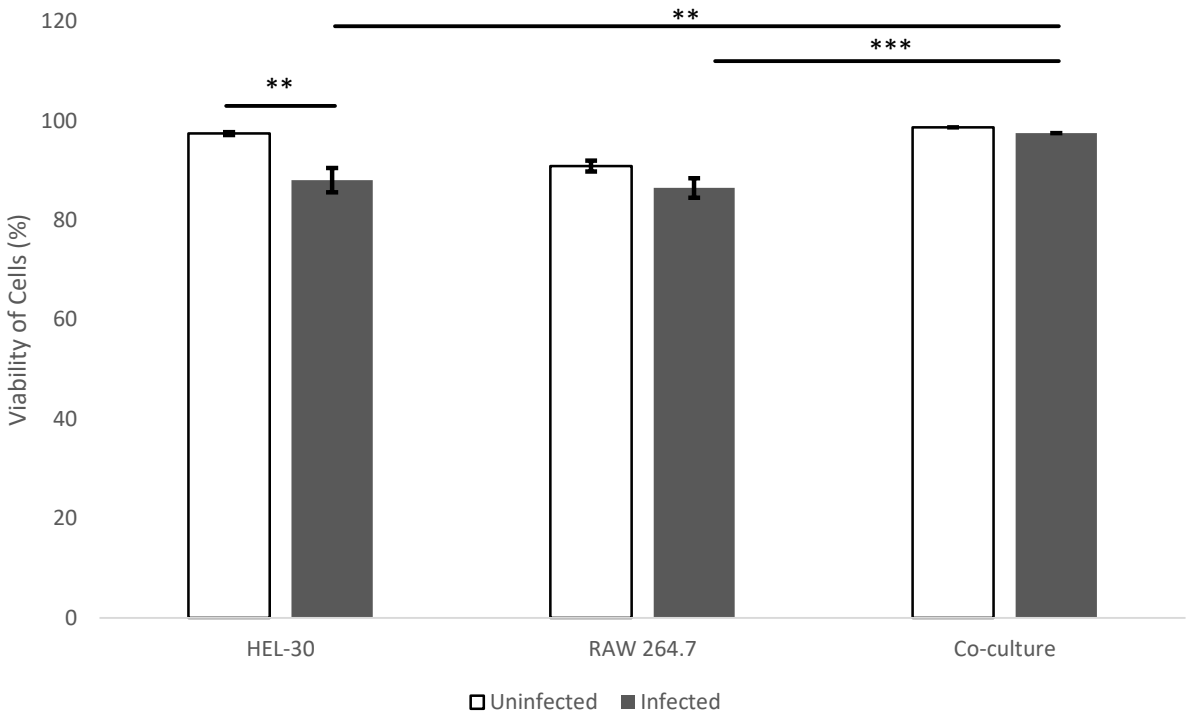

Figure 8: Cell viability comparison between HEL-30, RAW 264.7, and co-culture at 48 hours after HSV-1 infection. After 48 hours post-infection the HEL-30 keratinocytes had a significant decrease in viability compared to the control (P = 0.024). The RAW macrophages had no significant difference in viability when compared to its respective control (P = < 0.670). The co-culture had no significant difference in viability with its control (P = 0.060). When the infected HEL-30 keratinocyte and RAW macrophage viabilities were compared to the infected co-culture cell viability, both had significantly lower viabilities (P = < 0.002, P = < 0.001), respectively. The viabilities between the infected HEL-30 keratinocytes and RAW macrophages were not significant (P = 0.627). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

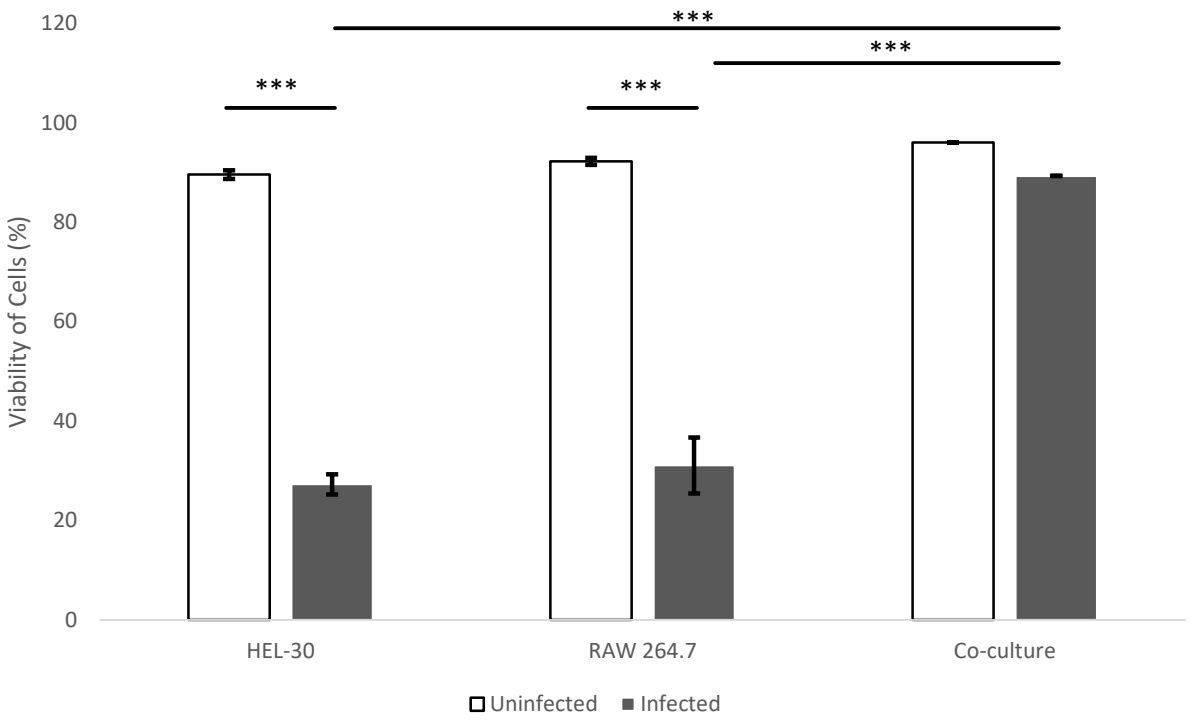

Figure 9: Cell viability comparison between HEL-30, RAW 264.7, and co-culture at 72 hours after HSV-1 infection. The HEL-30 keratinocytes and the RAW macrophages had a greatly decreased viability than their respective controls after 72 hours since infection (P = < 0.001, P = < 0.001), respectively. However, the co-culture had no significant difference in viability with its uninfected control (P = 0.103). The viability of infected HEL-30 keratinocytes and RAW macrophages had no significant differences (P = 0.536). The co-culture had significantly higher viability compared to the HEL-30 and RAW viabilities (P = < 0.001, P = < 0.001), respectively. SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

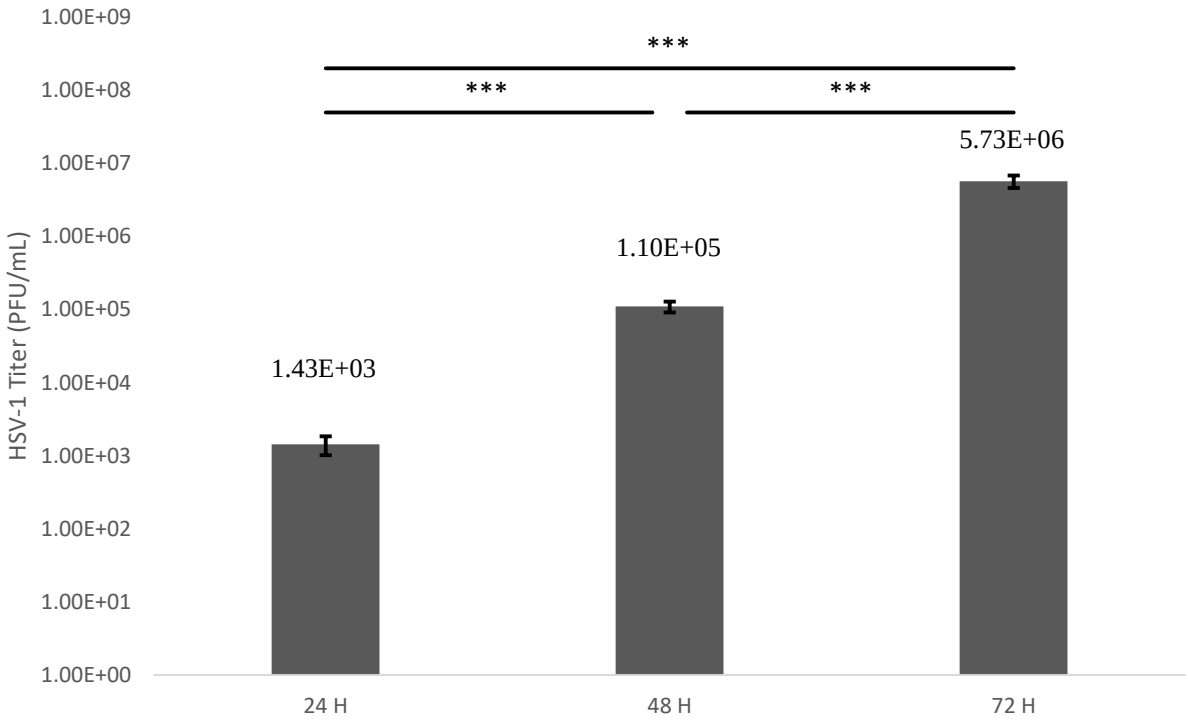

Figure 10: Plaque assay of HEL-30 keratinocytes infected with HSV-1 for 24, 48, and 72 hours. The HEL-30 keratinocytes showed signs of viral replication after 24 hours since the initial infection of HSV-1. At 48 hours, there was a large increased of viral titer from the 24-hour time point (P = < 0.001). The 72-hour time point also had a significant increase from the 48-hour time point (P = < 0.001). When comparing the 24 and 72-hour time points, there is a great significance between the two points (P = < 0.001). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

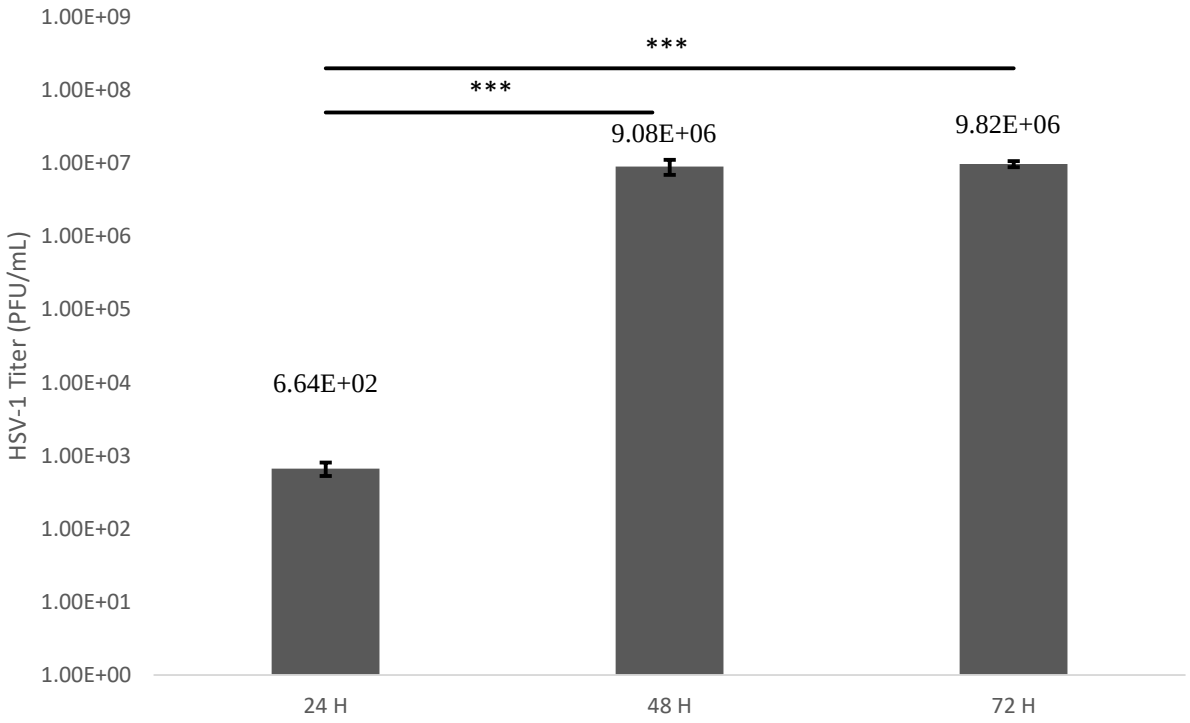

Figure 11: Plaque assay of RAW 264.7 M_0 macrophage infected with HSV-1 for 24, 48, and 72 hours. The RAW macrophages showed a significant increase in HSV-1 titer from 24 hours to the 48 hours after infection (P = < 0.001). From 48 to 72 hours there was no significant change in viral titer (P = 0.739). The 24 and 72-hour time points have a large significant difference (P = < 0.001). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

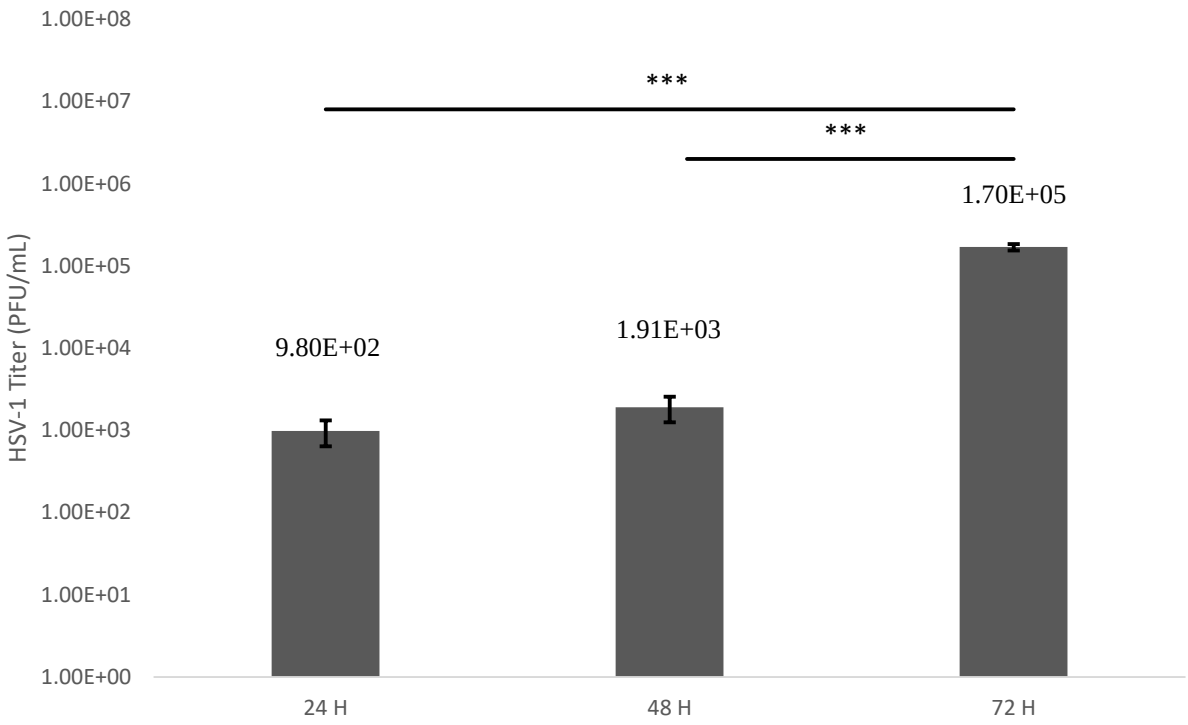

Figure 12: Plaque assay of HEL-30 keratinocyte and RAW 264.7 M_0 macrophage co-culture infected with HSV-1 for 24, 48, and 72 hours. The co-culture of HEL-30 keratinocytes and RAW 264.7 macrophages showed no significant difference in HSV-1 titer between 24 hours and 48 hours after infection (P = 0.230). However, from 48 hours to 72 hours there was a significant increase in the viral titer (P = < 0.001). The comparison of 24 to 72 hour viral titer shows a significant increase (P = < 0.001). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

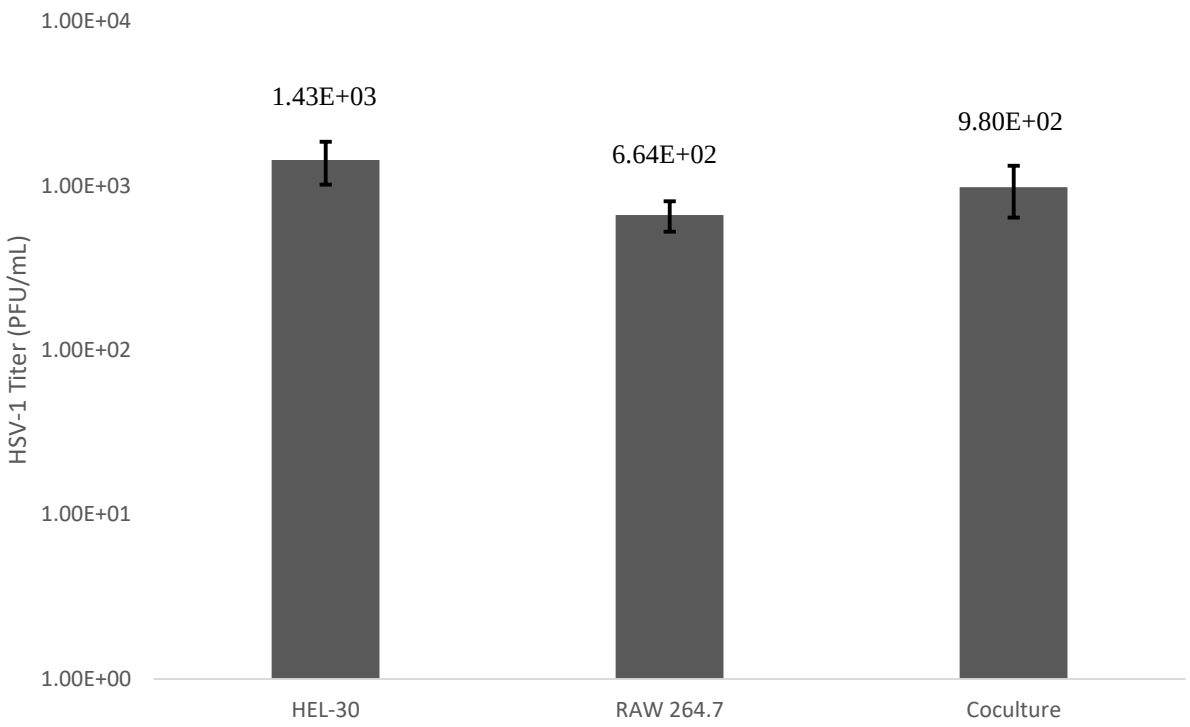

Figure 13: Comparison of HEL-30 keratinocytes, RAW 264.7 M_0 macrophages, co-culture 24 hours after HSV-1 infection. After the first 24 hours since the HSV-1 infection, the HEL-30 keratinocytes and RAW macrophages had no significant differences (P = 0.101). Comparisons of the co-culture viral titer to both HEL-30 keratinocytes and RAW macrophages viral titers were also not significantly different (P = 0.404, P = 0.404), respectively. SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

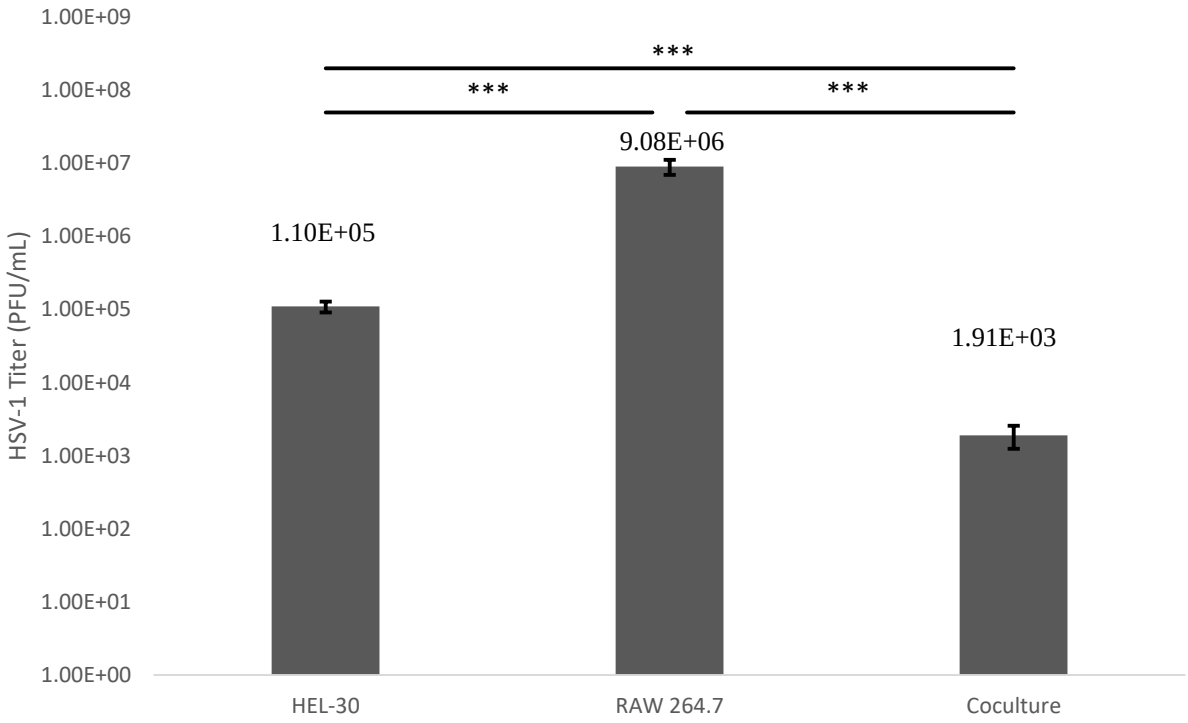

Figure 14: Comparison of HEL-30 keratinocytes, RAW 264.7 M_0 macrophages, co-culture 48 hours after HSV-1 infection. After 48 hours since HSV-1 infection, RAW macrophages had the highest viral titer in comparison to the HEL-30 keratinocytes and the co-culture (P = < 0.001; P = < 0.001), respectively. The HEL-30 viral titer was significantly higher than the co-culture viral titer (P = < 0.001). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

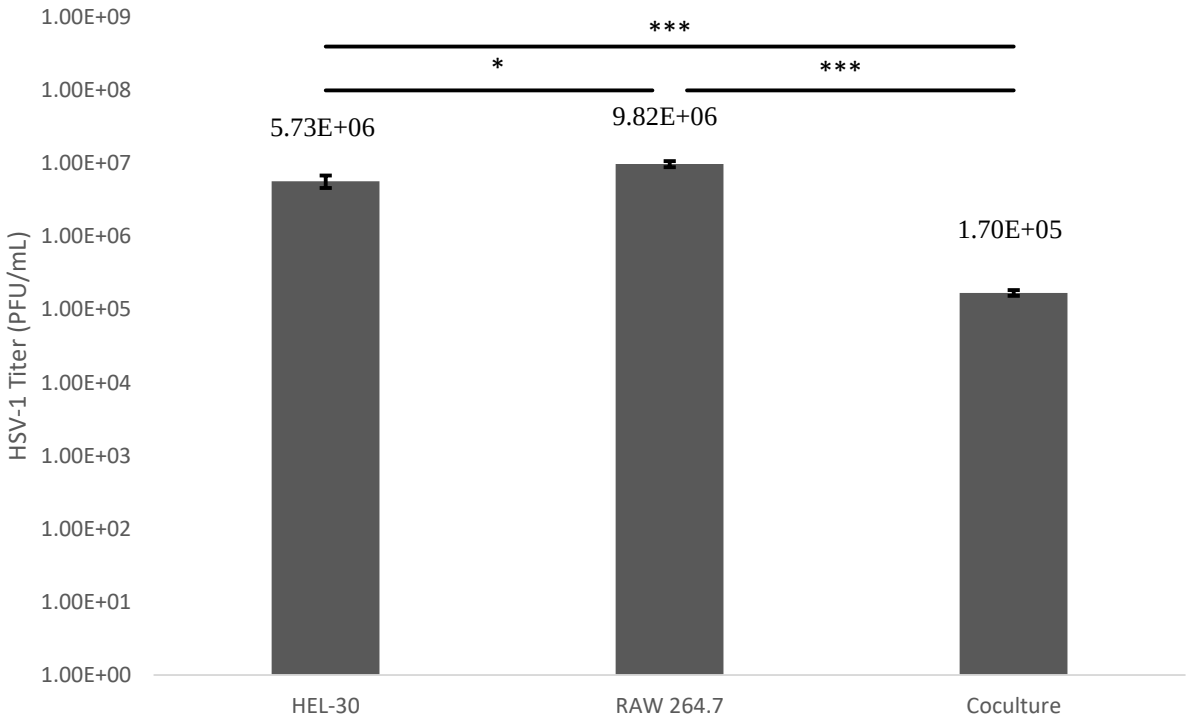

Figure 15: Comparison of HEL-30 keratinocytes, RAW 264.7 M_0 macrophages, co-culture 72 hours after HSV-1 infection. The RAW macrophage HSV-1 titer was slightly higher than the HEL-30 keratinocyte viral titer after 72 since the initial infection (P = 0.011). However, when compared to the co-culture viral titer, the RAW viral titer was significantly higher (P = < 0.001). HEL-30 viral titer also was significantly higher than the co-culture viral titer (P = < 0.001). SigmaPlot 12 was used to calculate one-way ANOVAs between the uninfected and infected group. Error bars represent the standard error ±. Stars (*) indicate levels of significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. (n = 3)

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