HSV-2 Δ*gD*-Induced Cellular Anti-Viral Response and Death of Human Keratinocytes

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Abstract

Introduction: Herpes Simplex Virus has become a growing problem worldwide and in the United States. A vaccine using an attenuated strain of HSV-2 that is genetically deleted in the gene for gD, HSV-2 Δ*gD*, has been shown to invoke an immune response in the murine disease model. Another strain, HSV-2 *dl5-29*, is defective for replication in human cells and has been shown to provide partial protection. The objective of this study was to determine why protective immunity is induced by HSV-2 Δ*gD* but not by wild type HSV-2 or HSV*dl5-29*.

Methods: The cellular anti-inflammatory response and apoptosis of human keratinocytes in vitro, a primary target of virus replication were quantified, following multiple time points post-infection. Secretion levels of inflammatory proteins were quantified by multiplex bead array, and virus-induced cellular death was quantified by the release of lactate dehydrogenase (LDH) following HSV-2 Δ*gD* infection as compared to wild type and *dl5-29* virus strains.

Results: The data demonstrated that HSV-2 Δ*gD* induces increased late production of monocyte chemoattractants and far less cellular death. HSV-2 *dl5-29* proved to induce increased levels of proinflammatory cytokines and a higher level of cytotoxicity than even the wild strain of the virus.

Conclusions: HSV-2 Δ*gD* and HSV-2 *dl5-29* induce different pathways. The pathway induced by HSV-2 Δ*gD* may promote the priming of protective immunity, due to the increased monocyte chemoattractants that are produced and likely resulting in a different innate immune response to this vaccine strain. The data also suggest that HSV-2 *dl5-29* has a greater capacity to infect and kill noncomplementing human cells than HSV-2 Δ*gD*, making HSV-2 *dl5-29* not as safe to use as a vaccine. Future research could include analyzing the pathways in vivo following vaccination and quantifying the different innate immune cell populations activated by vaccination.

Determining the different mechanism of immune activation following HSV-2 Δ*gD* vaccination will elucidate how to most effectively induces protective immunity against HSV infection, yielding more therapeutic targets for this infectious disease.

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1.0 Review of Literature

Herpes simplex virus, or HSV, has two serotypes: herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). HSV infection is most common in the skin and mucosal membranes. Specifically, HSV-1 typically infects the oral mucocutaneous area, and HSV-2 typically infects the genital tract. HSV-1 tends to infect children, whereas HSV-2 is usually transmitted sexually and therefore tends to infect adolescents and adults (Whitley et al., 2001). Both HSV-1 and HSV-2 can only infect a person after coming into contact with mucosal surfaces or cut skin, causing a primary infection. In addition, HSV-1 can cause corneal blindness as wells as oral mucocutaneous diseases. In the United States, there are over 50,000 new eye infections caused by HSV-1 each year (Lairson et al., 2003). Given HSV-2 typically infects the genitals causing recurrent virus shedding and genital ulcers, HSV-2 is also the cause of neonatal herpes and increases the risk of contracting human immunodeficiency virus (HIV) 3-fold (Freeman et al., 2006). Neonatal herpes occurs in 54 out of every 100,000 live births in the United States (Brown et al., 2003). These data highlight that severe morbidities are associated with HSV infection and due to the high prevalence of infection further clinical treatments and a prophylactic vaccine need to be developed.

HSV-2 has infected over 500 million people worldwide and continues to infect an estimated 23 million people every year (Looker et al., 2008). In the United States alone, 58% of people ages 14-49 are seropositive for HSV-1 and 16% percent are seropositive for HSV-2 (Xu et al., 2006). This means that about 1 in 6 people in the United States have genital herpes ("Genital Herpes- CDC Fact Sheet," 2015). The majority of individuals who are infected become asymptomatic and are unaware that they are infected, but still have frequent episodes of viral shedding and are capable of transmitting virus (Johnston et al., 2016). It's estimated that 87.4% of people who are infected with HSV have never been diagnosed and are unaware of their

infection status ("Genital Herpes- CDC Fact Sheet," 2015). In symptomatic individuals, genital herpes usually causes painful sores to appear around the genitals, rectum, or mouth. Sometimes these symptoms can be very mild, especially during later periods of reactivation. Given the majority of infected individuals are unaware of their HSV reactivation and production of infectious virus, HSV transmission rates have not been inhibited and the virus continues to be a public health concern.

In a primary HSV infection, the virus initially replicates in the mucosal epithelial cells and subsequently travels to the sensory nerve cells down to the dorsal root ganglia to establish latency (Cunningham et al., 2006). The virus is neurovirulent, meaning it is capable of replicating in neuronal cells (Fields et al. 2001), but because the virus doesn't cause apparent symptoms when establishing latency in the nerve cells people often don't realize they are infected (Cunningham et al., 2006) (Whitley et al., 2001). During latency periods, those who are infected are asymptomatic (Auslander et al., 2005). Reactivation of the virus, during which HSV resumes a high level of replication in the neurons, traveling back to the mucosal surface, causing viral shedding at the primary infection site, and ending the period of latency (Fields et al. 2001). During these episodes of reactivation symptoms can reappear (Johnston et al., 2011).

There have been many attempts to develop drugs to combat HSV. Although the use of acyclovir is commonly used as therapy for HSV infections, valacyclovir and famciclovir have also been licensed (Whitley et al., 2001). Acyclovir, given intravenously, has been shown to greatly help control symptoms and is more effective than topical therapy (Whitley et al., 2001). Other benefits of acyclovir treatment include a shorter recovery time, reduced virus-induced disease severity, and the prevention of complications (Szenborn et al., 2016). However, the virus can develop resistance to acyclovir, resulting in circulating strains where the frontline medical

treatment is ineffective. In addition acyclovir treatment does not decrease the transmission rate of HSV (Szenborn et al., 2016). Therefore additional treatment options or an efficacious vaccine to protect against HSV are necessary.

There have also been many attempts to create a vaccine that provides full protection against HSV infection by preventing disease or transmission without any success. Vaccines can be either therapeutically or prophylactically administered. The first type, a therapeutic vaccine, functions like a disease treatment and is administered after infection has already occurred (Johnston et al., 2011). It seeks to either eradicate the disease from the body, or more realistically, to reduce transmission by viral shedding and reactivation. On the other hand, a prophylactic vaccine aims to limit pathogen replication and prevent the induction of disease, much like a Polio or Tdap vaccine currently administered by medical doctors. An effective prophylactic vaccine for HSV would need to limit HSV replication at the infection site, inhibit disease symptoms, and prevent the establishment of latency in nerve cells (Johnston et al. 2011). One potential way to accomplish this is by generating a live-attenuated HSV vaccine strain that has an impaired ability to replicate and induce disease but still capable of inducing a protective immune response. By genetically altering HSV-2, the *dl5-29* mutant strain, deleted in the replication genes U_L5 and U_L29, is defective for replication in human cells making it a potential vaccine (Costa et al., 2000). This vaccine candidate is currently in phase I clinical trials and has provided partial protection against HSV-1 induced ocular disease and HSV-2 in the murine disease model (Rezka et al., 2010) (Lint et al., 2007). However, latent HSV virus is still established in approximately 15 percent of animal with no significant difference in reactivation episodes between immunized and nonimmunized guinea pigs following HSV challenge (Bernard et al., 2015).

Many researchers have also been investigating the use of a prophylactic glycoprotein subunit vaccine since the viral envelope glycoproteins are the immunodominant viral antigens and essential for the virus to enter cells (Connolly et al., 2011). HSV enters the cell through membrane fusion, which involves multiple glycoproteins on the viral envelope. The viral glycoprotein D (gD) binds to one of its cellular receptors, herpes virus entry mediator (HVEM) or Nectin-1 to induce a conformational change and permit viral glycoproteins gH/gL and gB to mediate fusion with the cellular membrane (Fields et al. 2001). Many immune cells actually express HVEM, so it is very often used by HSV to enter immune cells (Kwon et al., 1999). Therefore, when activated $CD4^+$ T cells arrive to the infection site, the virus can easily infect them and induce apoptosis (Han et al., 2007). Although HSV induces apoptosis in immune cells, it also has mechanisms for preventing apoptosis in infected epithelial cells in order to evade the immune system (Aubert et al., 2001). HSV glycoprotein subunit vaccines generally consist of injecting the immunodominant surface glycoproteins gB and/or gD into the body to induce an antibody response (Roth et al., 2013), with the rationale that when the virulent virus infects the body, the surface proteins would be recognized by the immune response and entry into target cells would be prevented. Numerous vaccines have specifically targeted glycoproteins because of their vitality for viral entry into cells and natural immunodominance, without success in generating protective HSV immunity in humans (Bright et al., 2012). Corey et al. found that a recombinant subunit vaccine containing gB and gD induced levels of HSV-2 specific neutralizing antibodies, but the vaccine was not efficacious in providing protection against HSV-2 infection (1999).

Researchers in the Jacobs and Herold laboratories at Albert Einstein College of Medicine took a different route and created an attenuated vaccine strain of HSV-2 that is genetically

deleted in the gene for gD (Petro et al., 2015). Since gD is needed for HSV to enter cells, the attenuated virus deficient in *gD* (HSV-2 Δ*gD)* was grown in Vero cells that express HSV-1 gD (VD60 cells). As a result, this progeny strain only contains the gD protein and therefore can only enter non-gD complementing cells once because the virus does not contain the *gD* gene to produce the protein. The researchers found that prime-boost vaccination with HSV-2 Δ*gD* complemented with the gD protein limited virus replication, prevented the establishment of latency, and protected against vaginal, skin, and neural disease in the murine disease model (Petro et al., 2015). Furthermore, they demonstrated that protection can be passively transferred by administering HSV-2 Δ*gD*-elicited antibodies capable of mediating antibody-dependent cell-mediated cytotoxicity (Petro et al., 2015). Contrarily, HSV-2 *dl5-29* elicits neutralizing antibodies (Hoshino et al., 2005). Some of the antibodies that are elicited by HSV-2 *dl5-29* target against ICP8, which is a protein required for HSV-1 replication, and are not sufficient to provide protection (Diaz et al., 2016). The difference in the antibody response may be the reason why HSV-2 Δ*gD* is protective.

2.0 Research Questions and Hypothesis

This research focused on elucidating why protective immunity is induced by HSV-2 Δ*gD* but not by wild type HSV-2 or HSV*dl5-29*. The hypothesis was that HSV-2 Δ*gD* would induce the least cellular death and the largest anti-viral inflammatory response in order to elicit the protective immunity observed in the animal model. To test this hypothesis the cellular anti-inflammatory response and apoptosis of human keratinocytes in vitro, a primary target of virus replication were quantified, following multiple time points post-infection. Secretion levels of inflammatory proteins were quantified by multiplex bead array, and virus-induced cellular death was quantified by the release of lactate dehydrogenase (LDH) following HSV-2 Δ*gD* infection as compared to wild type and *dl5-29* virus strains. The data demonstrated that HSV-2 Δ*gD* induces increased late production of monocyte chemoattractants and far less cellular death. HSV-2 *dl5-29* proved to induce increased levels of proinflammatory cytokines and a higher level of cytotoxicity than even the wild type strain of the virus.

3.0 Methods

3.1 Cell lines and virus strains

In this experiment, three different strains of HSV-2 were used: HSV-2 wild type (WT) strain G, HSV-2 Δ*gD*, and HSV-2 *dl5-29*. HSV-2 WT was grown and harvested from Vero cells, which originate from the kidney epithelial cells of an African Green Monkey. HSV-2 Δ*gD* and HSV-2 *dl5-29* are both attenuated strains and are potential HSV vaccine candidates. HSV-2 Δ*gD* is the HSV-2 virus genetically deleted for U_S6, which encodes the gene for glycoprotein D in HSV-2 (Petro et al., 2015). It was grown and harvested from VD60 cells, which are Vero cells that have been genetically altered to express gD from HSV-1 (gD-1) to complement the viral envelope with gD protein and permit entry of progeny viruses. HSV-2 *dl5-29* is an attenuated strain genetically deleted for viral replication genes U_L5 and U_L29 (Dias et al., 2016). Its complementing cell line is called V529, which are Vero cells that have been altered to express HSV-2 U_L5 and U_L29. Human keratinocyte (HaCaT) cells were also used during these experiments in order to compare the cellular response between the HSV-2 strains.

3.2 Infections

Infections were done in 24-well plates by plating 2 x 10^5 HaCaT cells/well the previous day. Prior to infection, cells were washed once with PBS. Infections were performed at a multiplicity of infection (MOI) of 1 by diluting the concentrated virus stock in serum free DMEM media. Diluted virus in serum-free media was placed on the cells and incubated at 37°C for 1.5 hours with manual rocking every 15 minutes. Equal volumes of 10% FCS DMEM media was subsequently added to wells. At 0, 16, 24, and 40 hours post infection (hpi), the supernatant was removed from each well and flash frozen in liquid nitrogen for later analysis. PBS was then added to the wells with Trypan Blue dye and bright field images were captured with Nikon Ti

microscope. Each experimental condition was performed in technical triplicates and independently repeated three to five times.

3.3 Cytokine Bead Assay

Proteins secreted into the supernatants were analyzed using a LEGENDplex bead based immunoassay kit (BioLegend, Inc.) and a CANTO flow cytometer (BD Biosciences, Inc). This kit allowed for the simultaneous analysis of thirteen proteins from one sample. The raw data from this assay was analyzed using the manufacturer's software and subsequently transferred to Prism for graphical display and statistical analysis.

3.4 LDH Assay

An LDH Cytotoxicity Detection Kit (CloneTech,) was used to quantify cellular death. The kit measures the level of LDH, a cytoplasmic enzyme that is released when the cytoplasmic membrane integrity is compromised, relative to the media alone and 100% lysis controls as calculated following manufacturer's protocol. Calculations of the raw O.D. values obtained on a Victor, were performed in Excel (Microsoft) and entered into Prism for graphical display and statistical analysis.

3.5 Statistical Analysis

All the experiments were performed in triplicate and independently repeated three to five times. The technical triplicate mean from each independent experiment is represented in the figures. For statistical analysis a two way ANOVA with a Dunnet's post test was performed. Each vaccine strain was compared to the mock group.

4.0 Results

4.1 HSV-2 Δ*gD* induces sustained increased production of monocyte chemoattractants

To determine the cellular anti-viral response of human keratinocytes following infection with HSV-2 strains, protein secretion was quantified by performing a multiplex bead assay at various time points. At 16, 24, and 40 hpi, HSV-2 *dl5-29*-induced increased production of the highly pro-inflammatory cytokines TNF-α and IL-6 (Figure 1A-B). However the secretion of MCP-1 and IL-8, both monocyte chemoattractants, were increased following HSV-2 Δ*gD* infection of HaCaT cells through 40 hpi (Figure 1C-D). Together, the data suggests that the HSV-2 Δ*gD* and *dl5-29* vaccine strains are activating the immune response by alternative pathways inducing the production of different inflammatory cytokines and chemokines.

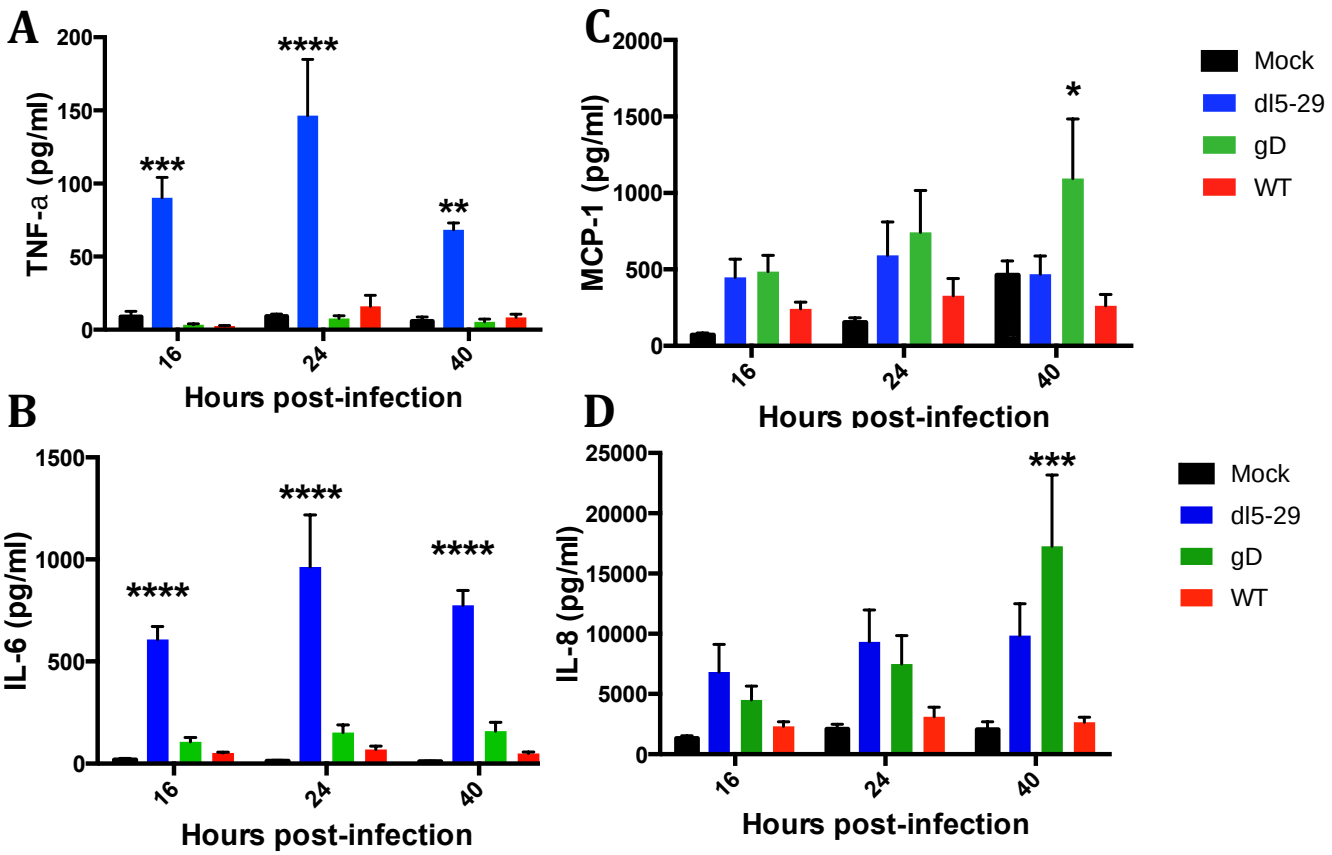

Figure 1. Increased late-production of monocyte chemoattractants following HSV-2 ΔgD infection. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. Secreted concentrations of TNF-α (A), IL-6 (B), MCP-1 (C), and IL-8 (D) was quantified by a multiplex bead array assay in the supernatant at 16, 24, and 40 hpi. 13 proteins, including IL-1β, IFN-α, IFB-γ, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33, were analyzed but are not displayed because they were either not detected or not statistically different as compared to the mock control. This experiment was repeated independently three times in triplicate, displaying the technical average from each independent experiment (*n*=3). For statistical analysis a two way *ANOVA* with a Dunnet's post test was calculated. *,p<0.05, **, p<0.01, ***, p<.001, ****, p<0.0001.

4.2 HSV-2 Δ*gD* induces less cellular death than HSV-2 *dl5-29* and HSV-2 WT

To determine the ability of each of the strains to induce cellular death in human cells, images were taken of the cells at various time points and the levels of LDH secreted by the HaCaT cells were quantified using an LDH assay. The microscope images suggest that at the time of infection the cellular monolayers were similar between the different virus strains (Figure 2). However the image data also demonstrate that HSV-2 WT induced the most cellular death, with the least amount of viable cells within the first sixteen hours after infection (Figure 3). The data also suggest that HSV-2 *dl5-29* induces almost as much cellular death as the wild type virus by 24 hpi, though the kinetics of viral-induced cellular death may be delayed since at 16 hpi there appears to be increased death in the wild type infection (Figures 3-4). HSV-2 Δ*gD* also appeared to induce some cellular death, but not nearly as much as either HSV-2 wild type or *dl5-29* (Figures 2-5). The only cellular death in the mock group was due to the overpopulation of cells.

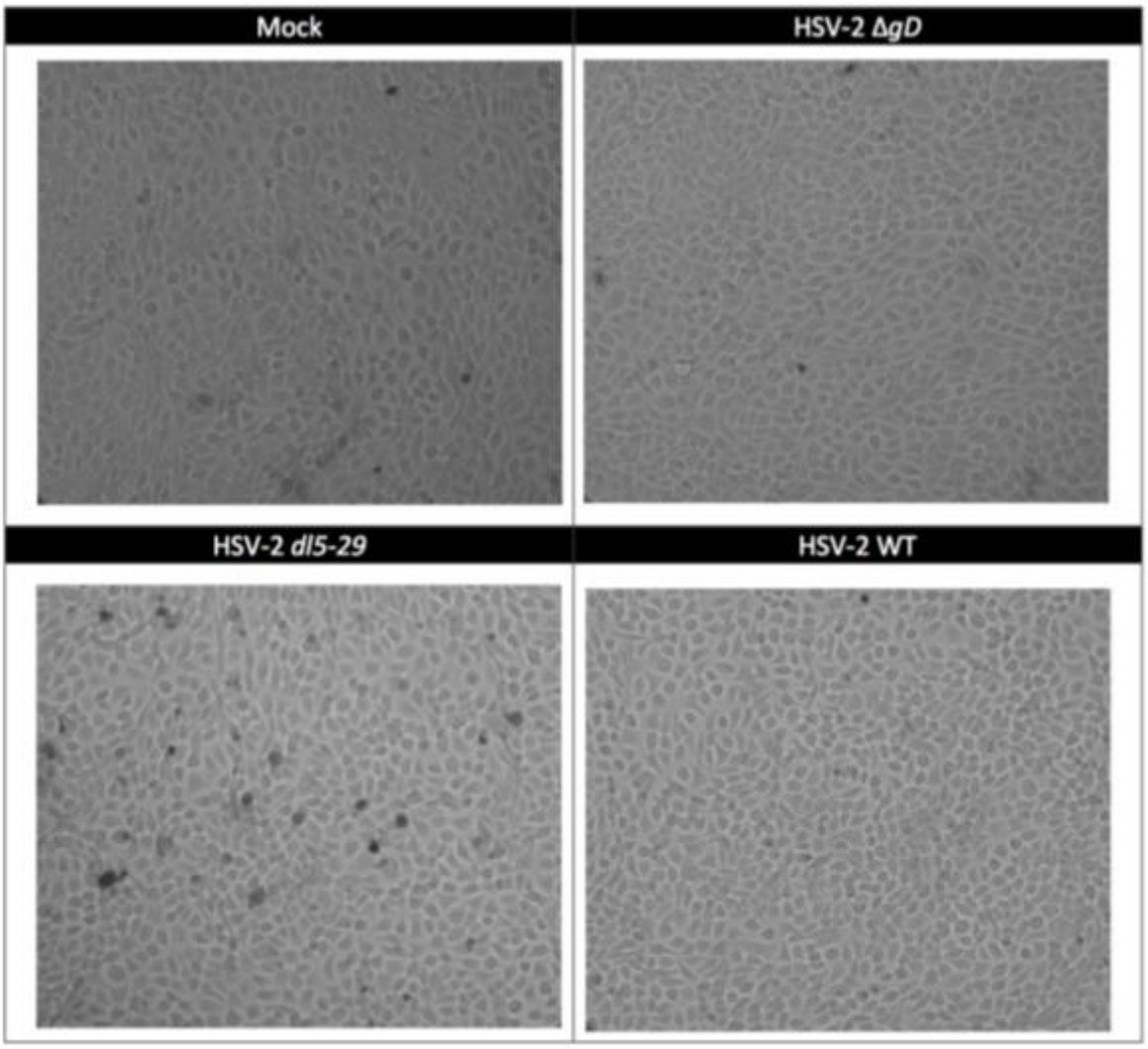

Figure 2: Consistent cell confluency at 0 hpi. All cells started out 100% confluent. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. The cells were stained with Trypan Blue diluted in PBS. Images of the stained cells were taken by a Nikon Ti Microscope at 0 hpi. The experiment was performed in triplicate displaying representative images from one of three independently repeated experiments.

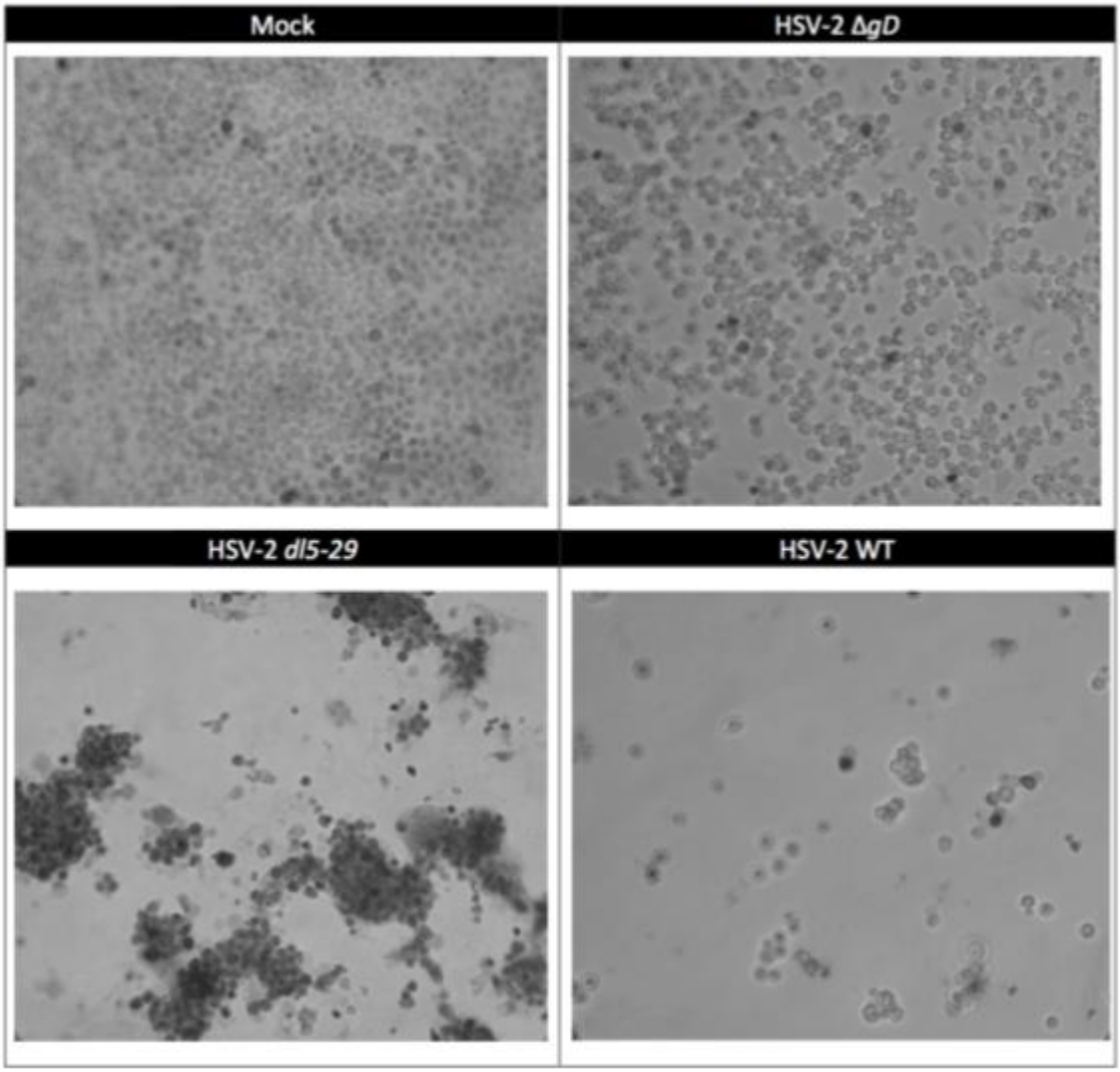

Figure 3: Increased cell death of HSV-2 *dl5-29* and HSV-2 WT at 16 hpi. All cells started out 100% confluent. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. The cells were stained with Trypan Blue diluted in PBS. Images were taken of the stained cells were taken by a Nikon Ti Microscope at 16hpi. This experiment was performed in triplicate displaying representative images from one of three independently repeated experiments.

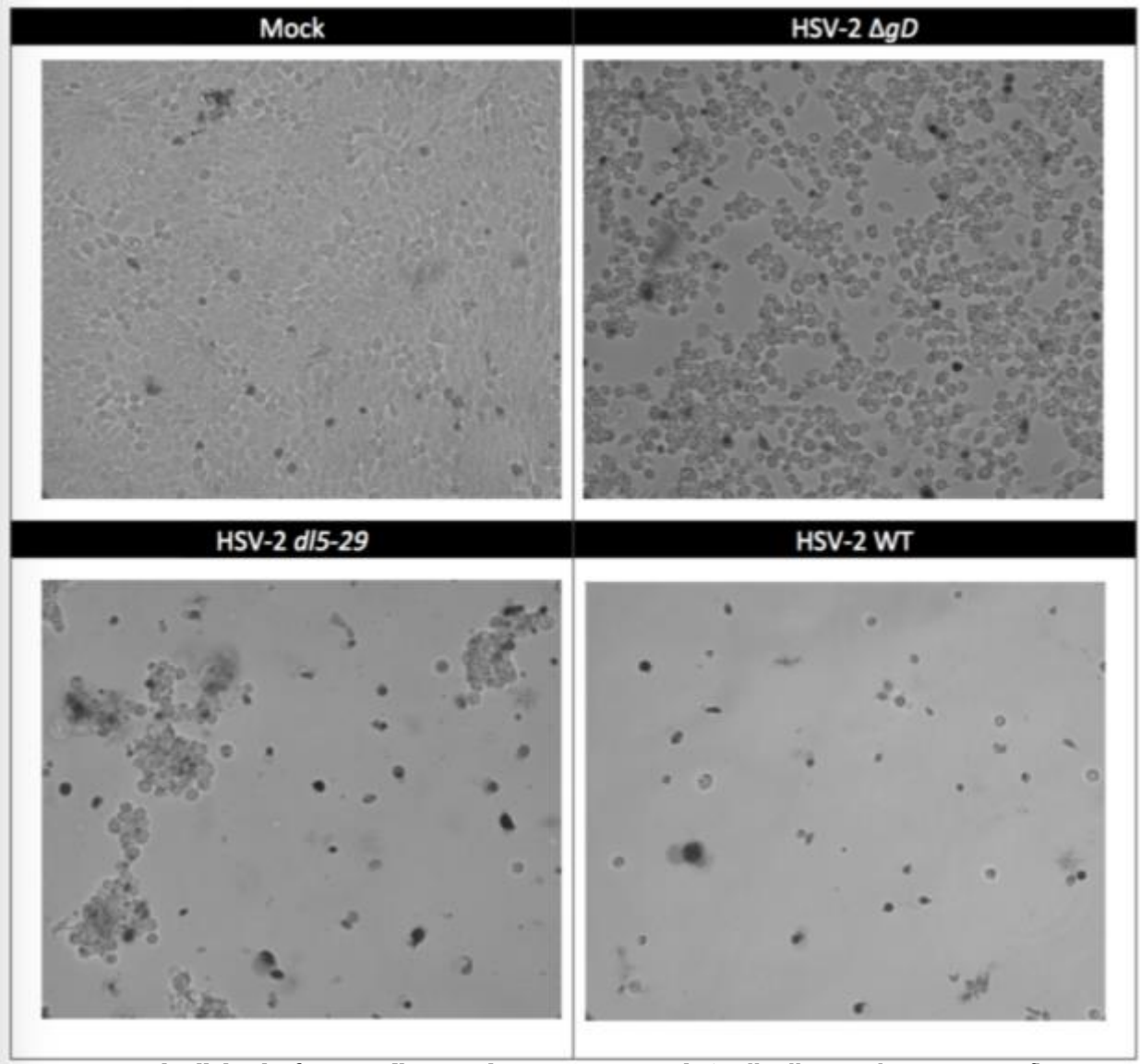

Figure 4: Increased cell death of HSV-2 *dl5-29* and HSV-2 WT at 24 hpi. All cells started out 100% confluent. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. The cells were stained with Trypan Blue diluted in PBS. Images were taken of the stained cells were taken by a Nikon Ti Microscope at 24 hpi. This experiment was performed in triplicate displaying representative images from one of three independently repeated experiments.

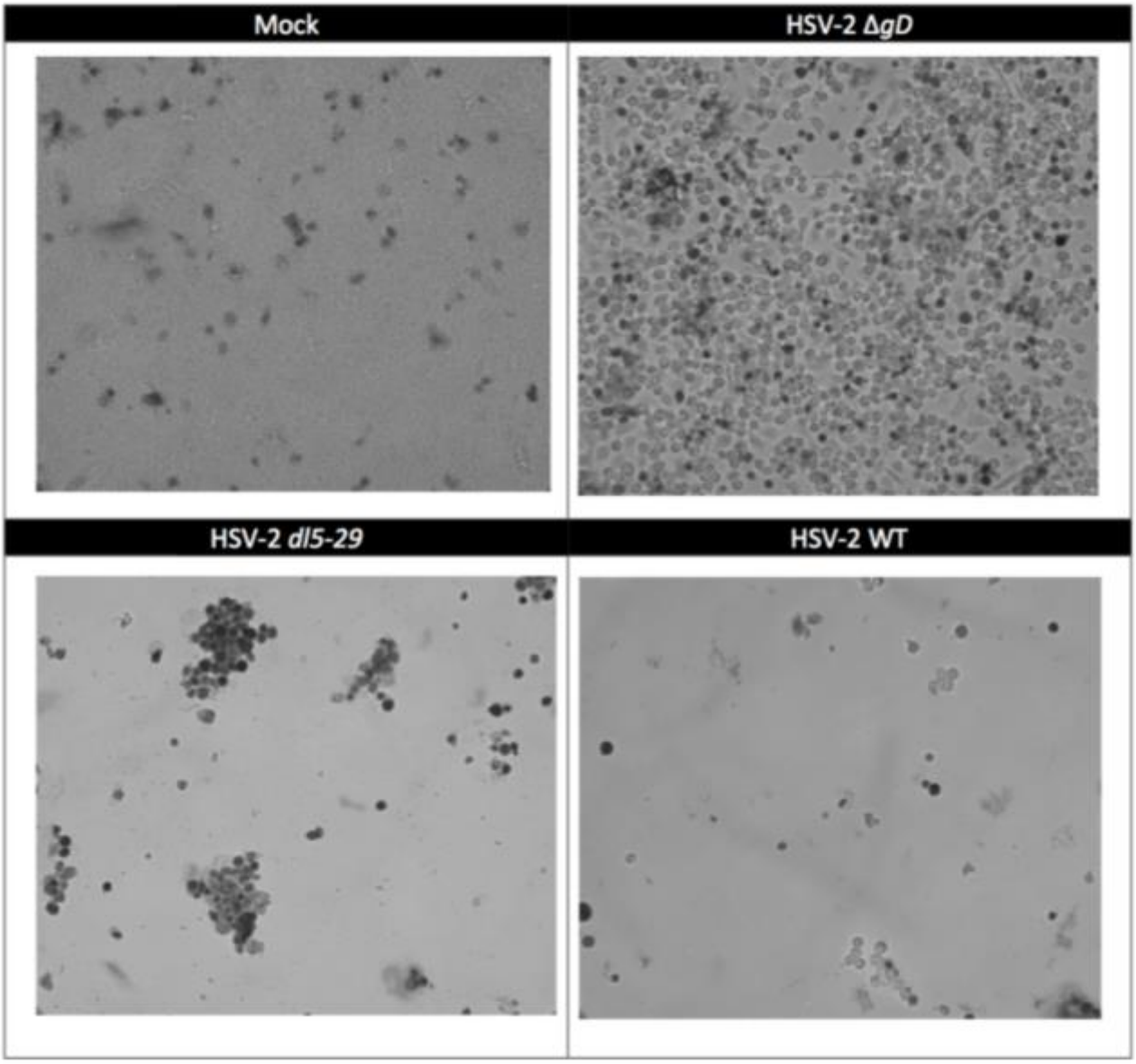

Figure 5: Increased cell death of HSV-2 *dl5-29* and HSV-2 WT at 40 hpi. All cells started out 100% confluent. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. The cells were stained with Trypan Blue diluted in PBS. Images were taken of the stained cells were taken by a Nikon Ti Microscope at 40 hpi. This experiment was performed in triplicate displaying representative images from one of three independently repeated experiments.

To support the image data following tryphan blue staining of HSV-infected HaCaT cells, the cellular release of LDH was also quantified. Although the images suggest similar virus-induced cellular death following either HSV-2 WT of *dl5-29* infection, the LDH assay indicated that HSV-2 *dl5-29* infected cells had the highest percent toxicity (Figure 6). The data demonstrates a significant increase in the level of cellular death, as determined by the LDH level in the supernatant of the HSV-2 *dl5-29* and HSV-2 WT infected cells between 24 hpi and 40 hpi (Figure 6). Although the HSV-2 Δ*gD* infected cells had a higher percent cytotoxicity than the mock infected cells, it was decreased as compared to the HSV-2 *dl5-29* and HSV-2 WT infected cells. These data suggest that HSV-2 *dl5-29* has a greater capacity to infect and kill noncomplementing human cells than HSV-2 Δ*gD*, and is therefore not as safe to use as a vaccine.

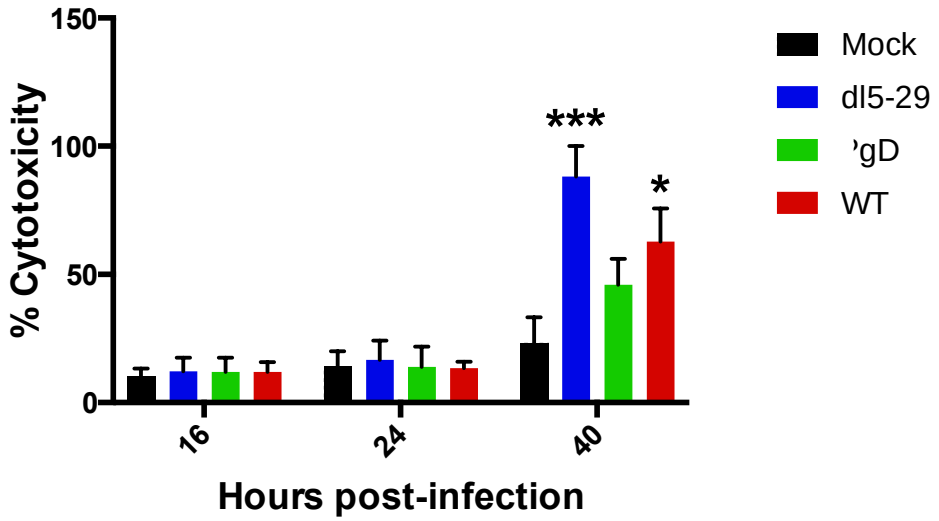

Figure 6: Increased cytotoxicity of HSV-2 *dl5-29* and HSV-2 WT infected cells. HaCaT cells were infected at an MOI 1 by either HSV-2 *dl5-29*, HSV-2 Δ*gD*, or HSV-2 WT virus. At 16, 24, and 40 hpi, the supernatant was removed and flash frozen in liquid nitrogen for later analysis. The secreted concentrations of LDH were measured and the percent cytotoxicity was determined as compared to uninfected and 100% lysis controls. This experiment was repeated independently three times in triplicate, displaying the technical average from each independent experiment (*n*=3). For statistical analysis a two way *ANOVA* with a Dunnet's post test was calculated. *,p<0.05, **, p<0.01, ***, p<.001, ****, p<0.0001.

5.0 Discussion

This study suggests that increased protective immunity elicited by HSV-2 Δ*gD* may be because of an alternative inflammatory pathway induced resulting in a different cytokine and chemokine profile and induction of cellular death as compared to other HSV strains. The cytokine bead assay showed that there was increased production of TNF-α and IL- 6 in HSV-2 *dl5-29* infected cells, and very little production of these two proteins in HSV-2 Δ*gD* infected cells (Figure 1). TNF-α and IL- 6 are both proinflammatory genes and immune cell mediators that studies have shown may be produced by $CD4^{+}$T cells and neurons in response to HSV infection in the nervous system (Fields et al., 2001). This assay also revealed HSV-2 Δ*gD* infection results in increased late production of MCP-1 and IL-8 following infection. MCP-1 regulates migration of monocytes and macrophages, and has an important role in immunological surveillance (Deshmane et al., 2009). IL-8 has been shown to induce changes in neutrophils, such as the migration of cells (Bickel et al., 1993). This suggests that different pathways are induced by HSV-2 *dl5-29* and HSV-2 Δ*gD*. The pathway induced by HSV-2 Δ*gD* may promote the priming of protective immunity, due to the increased monocyte chemoattractants that are produced and likely resulting in a different innate immune response to this vaccine strain.

Furthermore, the HSV-2 *dl5-29* is a "leaky vaccine" in that it can sometimes induce disease and establish latent infection in the animal model (Bernard et al., 2015). This may question the safety of this vaccine for clinical use. Our in vitro data demonstrates that infection with this strain caused a higher percentage of cell cytotoxicity similar to wild type HSV-2, which also suggests that the attenuation may not be sufficient (Figures 2-6). Given HSV-2 Δ*gD* does not cause latency to develop and induced decreased cell death as compared to HSV-2 *dl5-29* or HSV-2 WT, it may be a safer strain to use as a live-attenuated vaccine.

The results from this study are important moving forward because it demonstrates that the HSV strains induce different cell-intrinsic responses following infection. Furthermore, suggesting that to elicit protective HSV-2 immunity the increased production of monocyte chemoattactants may need to be induced following vaccination. Future research could include analyzing the pathways in vivo following vaccination and quantifying the different innate immune cell populations activated by vaccination. Determining the different mechanism of immune activation following HSV-2 Δ*gD* vaccination will elucidate how to most effectively induces protective immunity against HSV infection, yielding more therapeutic targets for this infectious disease.

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