AN INQUIRY INTO THE EFFECTS OF THE PRODUCER CELL TYPE ON HERPES SIMPLEX VIRUS TYPE-1 INFECTIVITY AND THE VIRION-ASSOCIATED PROTEOME

by

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DEDICATION

This dissertation is dedicated to my wife, Celia, who is incredibly supportive of everything that I do. She made friends in Bozeman, but moving here was difficult for her. Her family is incredibly important to her. Moving here meant that she would be further apart from her family than she had ever been, but she did it anyway to support my pursuit of becoming a scientist. She has been very understanding when I've had to dedicate a great amount of time to school, lab work, and writing. I ultimately experienced far more in Montana because of her than I ever would have experienced on my own. Some of my favorite memories will always be of the trails that we hiked together with our dogs.

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I learned a great deal from Matt, but discussions and feedback from other scientists that have different perspectives have also been important for my growth as a scientist. My initial interest when coming to MSU was on the topic of host-pathogen interactions, particularly with a slant towards immunology. My committee consisting of Dr. Jovanka Voyich, Dr. Agnieszka Rydna-Apple, and Dr. Mark Quinn helped me to design a research project that centered around uncovering the interactions that take place between HSV-1 and neutrophils. This is a topic that is still somewhat of a mystery in the field of HSV-1 virology. Even though this project did not result in a publication, I learned a tremendous amount about virology and immunology. My committee continued to offer valuable insights and perspectives as I progressed through new topics of HSV-1 research.

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ABSTRACT

Viruses inherently rely upon the host cell to produce infectious virions, and many viruses can infect several cell types. The type of cell used to produce a virus, i.e. the producer cell type, has been shown to alter the macromolecular composition of the virions that are produced. These alterations can change the capacity of virions to infect cells, spread within the host, and interact with the immune system. Herpes Simplex Virus Type-1 (HSV-1) is a ubiquitous human pathogen that can elicit disease outcomes ranging from the development of orofacial lesions to blindness or encephalitis. This range of disease is possible because HSV-1 replicates in multiple cell types. To study HSV-1 replication and disease, researchers must propagate virus that is subsequently used to establish infection in cell culture or animal models. Currently, there is no standard cell line used for the propagation of HSV-1 in research, although the vero cell is among the most used cell types for this purpose. We hypothesize that the producer cell type of HSV-1 alters the macromolecular composition and infectivity of the virions that are produced. To test this hypothesis, we propagated HSV-1 from African green monkey kidney cells (vero), immortalized human keratinocytes (HaCaT), and primary human foreskin fibroblasts (HFF-1). The capacity for virions to replicate was measured by quantifying viral gene expression, protein production, and the output of infectious virions from infected target cells. We also used liquid chromatography-mass spectrometry (LC-MS/MS) to quantify proteins that are incorporated into virions derived from each cell type. We found that HSV-1 derived from HaCaTs produce more viral protein and infectious virions from infected cells relative to HSV-1 derived from vero or HFF-1 cells. We also identified many viral and cellular proteins that are differentially incorporated into HSV-1 virions in a producer cell-dependent manner. This data suggests that the producer cell type of HSV-1 alters virion infectivity and protein composition, which may have implications for how the virus spreads in humans. In addition, these results allude to the possibility that the producer cell type may influence experimental outcomes in studies that investigate HSV-1 replication and pathogenesis.

INTRODUCTION

A brief history of herpes simplex virus type 1 (HSV-1) infection in humans

HSV-1 has a long history of infecting human beings. The timing of the emergence of HSV-1 is currently under debate. Some estimates of its origin stem from the initial human migration out of Africa, whereas others estimate that HSV-1 originated approximately 7000 years ago (1,2). Regardless of the exact timing of its origins, humans and HSV-1 have long been bedfellows. Recently, HSV-1 DNA was extracted and sequenced from the teeth of deceased individuals from the UK, Russia, and the Netherlands. The sequences were obtained from individuals that had died as early as 253 CE and as recently as 1700 CE, illustrating the prevalence of the virus throughout human history (3). The virus infects ~67% of humans worldwide in the modern day (4). HSV-1 has long been recognized as a causal factor in severe disease and is among the first viruses of humans to be isolated and studied by science (5–7).

HSV-1 Disease

HSV-1 has a broad cell tropism, meaning the virus can infect many different cell types *in vivo*. In the majority of cases, HSV-1 infection is either asymptomatic or causes manifestation of recurrent, self-resolving, lesions of the orofacial or genital epidermis and mucosa. However, broad tropism of HSV-1 can cause a wide range of disease outcomes in patients (Fig. 1) (8–10). Severe disease is more common in immune compromised adults and neonates (11–18). HSV-1 disease is most severe when the virus replicates in the eye, central nervous system (CNS), or disseminates to multiple organs. Infection of the eye can result in Herpes stromal keratitis

(HSK). Although HSK typically resolves, reactivation of HSV-1 can cause recurrent infection of the corneal stroma. In developed nations HSV-1 causes blindness in more people than any other pathogen each year (8). HSV-1 is also the leading diagnosed cause of sporadic viral encephalitis, referred to as herpes simplex encephalitis (HSE). HSE has a high mortality rate in patients, even when treatment is administered. HSE is caused from the virus spreading from either the olfactory bulb to the central nervous system (CNS), resulting in forebrain HSE, or from neurons of the trigeminal sensory ganglia resulting in brainstem HSE (13). The broad tropism of HSV-1 is perhaps best observed, tragically, in neonates, where infection is often lethal (12,15,16). Mothers with HSV-1 infection within the genital tract can transmit the virus to the infant upon delivery. In neonates that become infected, the virus can disseminate within the skin, mucosa, eyes, brain, and multiple other organs. HSV-1 that has disseminated systemically or to the brain in neonates is typically lethal without treatment, and survival of HSE can result in the development of lifelong neurological disorders.

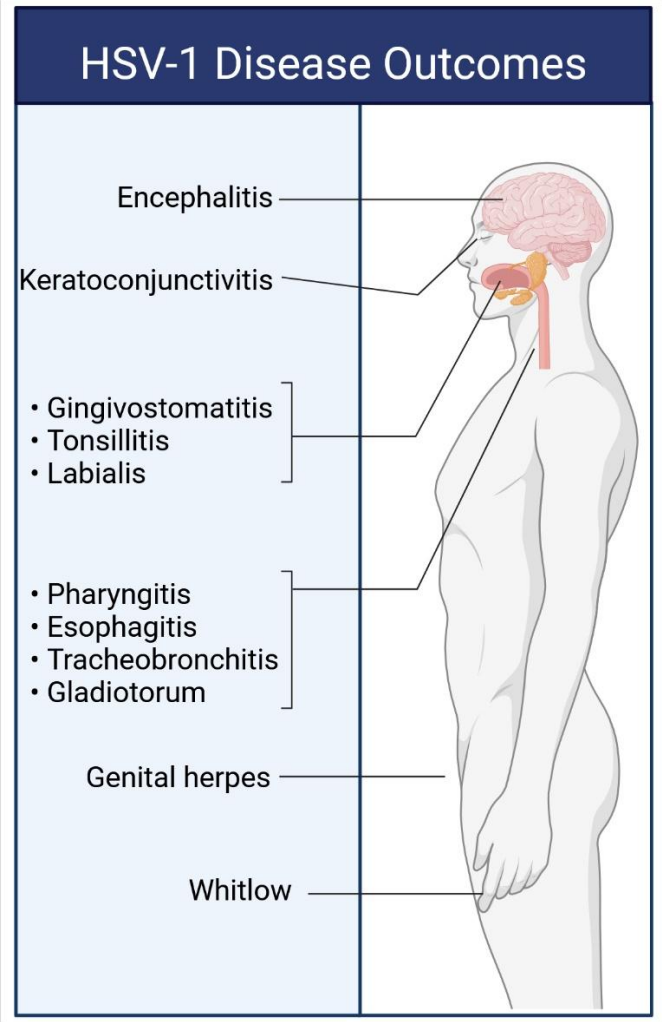


Figure 1: HSV-1 disease outcomes. HSV-1 can infect several tissue types, including those associated with the skin, peripheral nervous system, eyes, and central nervous system among others. Viral replication within these tissues can lead to mild symptoms or severe disease.

HSV-1 replication and structure

HSV-1 is a double-stranded DNA virus with a 152 kb genome. The genome is encapsulated by a proteinaceous icosahedral capsid. This capsid is surrounded by tegument proteins composed of a combination of mostly viral and some cellular proteins. The tegument layer, capsid, and genome are contained within the viral envelope, which is composed of a lipid

bilayer decorated with viral glycoproteins (Fig. 2). Despite its capacity to infect many cell types, the virus primarily infects keratinocytes associated with the skin or oral mucosa prior to spreading to neurons associated with the peripheral nervous system. HSV-1 infection of non-neuronal cells results in lytic replication in which viral protein coding genes are expressed, and mature infectious virions are formed. These mature virions egress from the infected cell to establish infection in new cells (19).

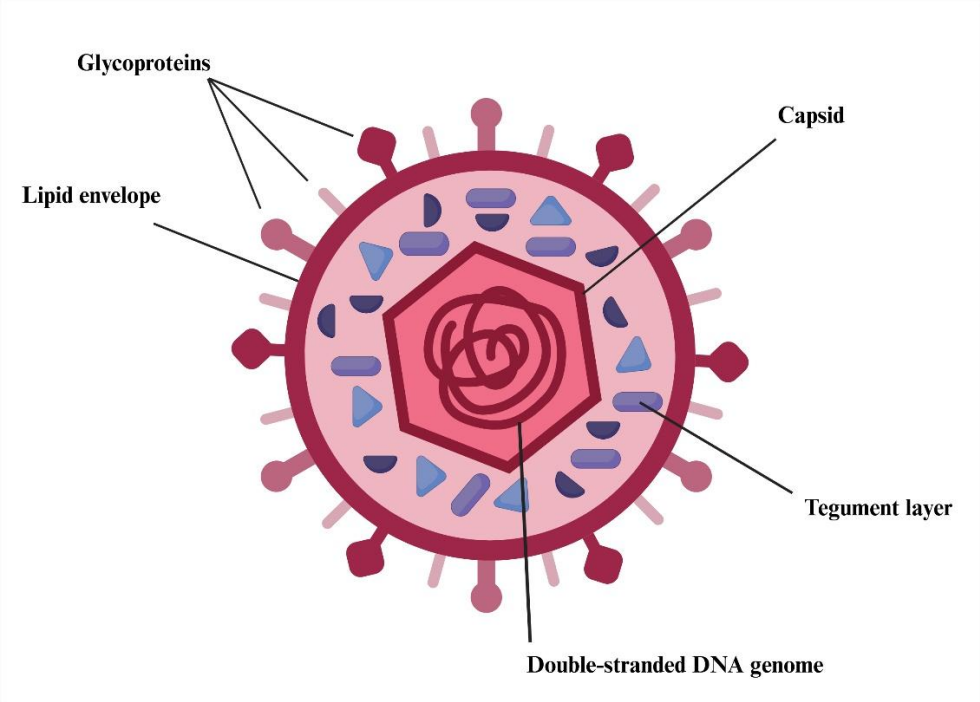


Figure 2: HSV-1 virion structure. HSV-1 is composed of a protein capsid that protects the 152 kb dsDNA genome. The capsid is surrounded by a tegument layer composed of multiple structural and non-structural viral proteins. The outer layer of the virion is made up of a lipid bilayer decorated with glycoproteins that are required for the binding of cellular receptors and entry into cells.

HSV-1 can lytically replicate in neuronal cells, but the virus often instead establishes a latent infection in peripheral neurons (Fig. 3). Generally, latent HSV-1 infection of neuronal cells is described as the persistence of the viral genome within the nucleus of the neuronal cell in the

absence of viral protein synthesis. Few viral genes are expressed during latency including the latency associated transcript (LAT) and a subset of viral microRNAs (20–22). The function of LAT during viral latency is not well understood, but it likely plays a role in the suppression of viral gene expression and reactivation in neurons (23). Infectious virions are not produced during latent infection. The trigeminal ganglion, which consists of sensory neurons, serves as a reservoir for HSV-1 latency in humans. Neurons that are latently infected allow the virus to persist within the human host, and viral spread can occur upon reactivation of HSV-1 in neurons. The mechanisms of reactivation are currently under investigation, but it is generally understood that conditions of stress can result in reactivation of the virus. Reactivation results in lytic replication of HSV-1 within the latently infected neuron. Mature virions are produced that can then spread to other neurons, non-neuronal cells and other hosts (19).

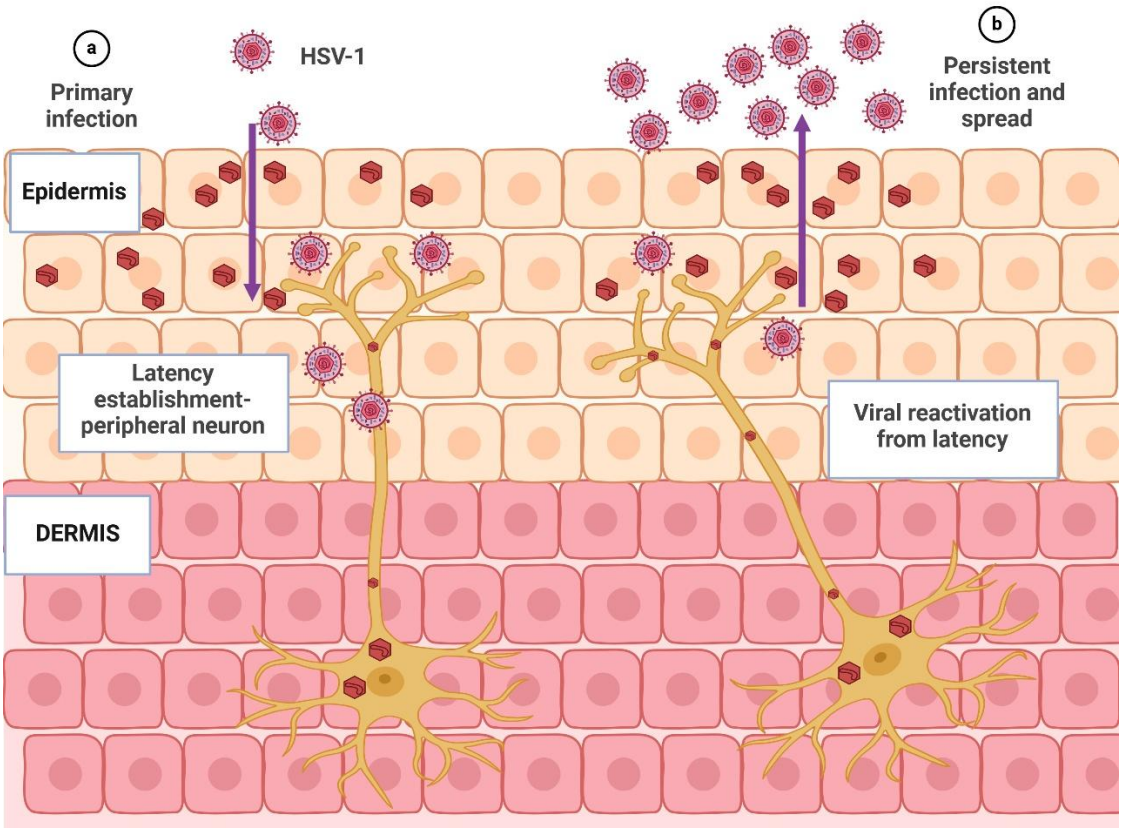


Figure 3: HSV-1 primary infection and reactivation. HSV-1 infects keratinocytes associated with the skin or oral mucosa during primary infection. The infectious virions that are produced eventually encounter neurons of the peripheral nervous system. Capsids that have entered neurons undergo retrograde transport from the neuronal axon to the nucleus. The dsDNA genome is injected into the neuronal nucleus, where the genome is maintained through latency in the absence of viral protein production. Reactivation of the virus causes lytic replication of HSV-1, which results in anterograde transport of capsids from the nucleus to the axon, where mature infectious virions egress. Virions derived from reactivated neurons spread to keratinocytes and other hosts.

To establish infection within a cell, an HSV-1 virion must first bind to a viable cellular receptor expressed on the outer surface of the cellular plasma membrane (Fig. 4). These cellular receptors include nectin-1 and the herpesvirus entry mediator (HVEM). Binding of these cellular receptors is required for entry of the virion into the host cell to initiate replication. The viral envelope protein glycoprotein D (gD) is responsible for binding these cellular receptors, but

additional viral glycoproteins are required for entry of the virus into cells post-attachment. Ultimately, the virion enters the cell via endocytosis or plasma membrane fusion in a cell-type dependent manner (24). Upon viral entry, the virion is shed of the viral envelope and the outer tegument proteins dissociate from the capsid. Several tegument proteins including ICP0 and virion host shutoff (VHS) serve to manipulate the infected cell into a state that is permissive to viral replication. ICP0 will be discussed further in the context of HSV-1 gene expression. VHS is a viral RNase that degrades cellular transcripts, prevents the synthesis of host proteins, and inhibits antiviral responses. Other tegument proteins, such as ICP4, are directly involved with the beginning stages of HSV-1 lytic replication post-entry. ICP4 is a transactivator of viral gene expression and is required for the regulation of viral transcription. The inner tegument proteins remain attached to the capsid (25). The capsid utilizes the microtubule network for transport to the nucleus. After docking with the nuclear pore, the capsid injects the HSV-1 genome into the nucleus to initiate viral gene expression (26).

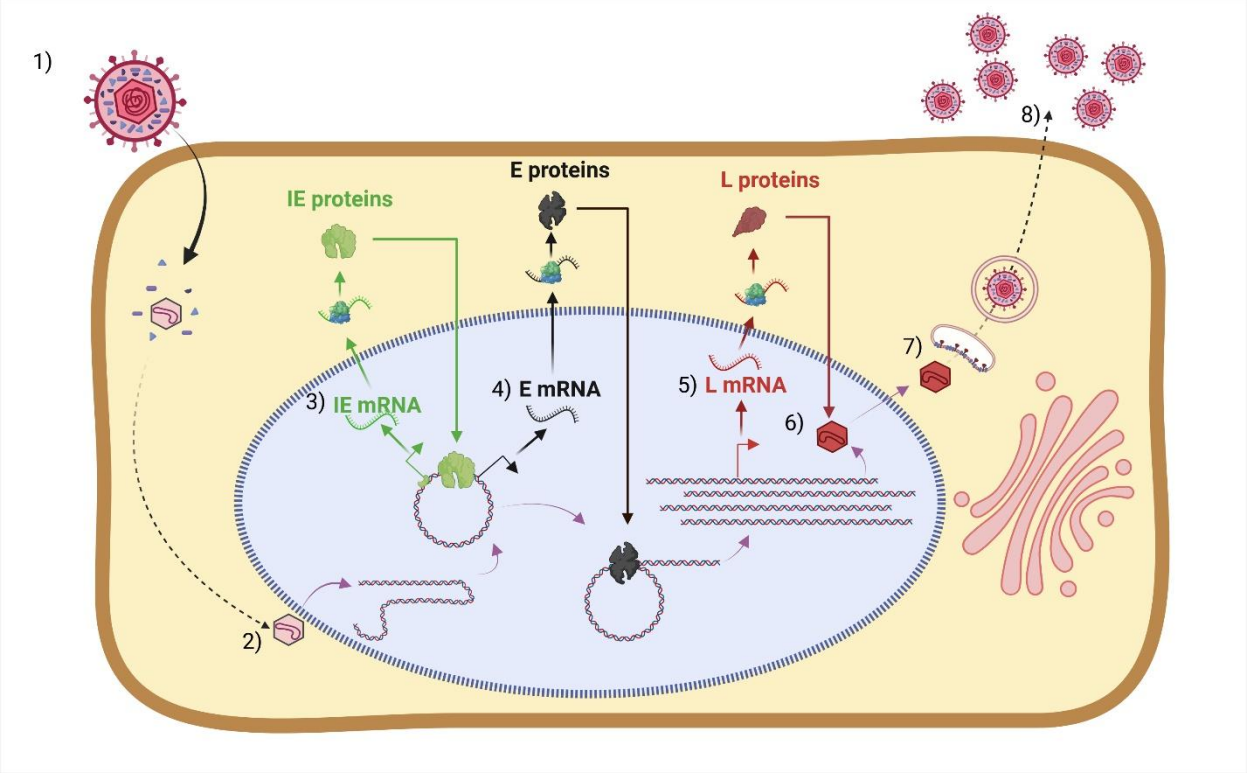


Figure 4: HSV-1 replication cycle. 1) HSV-1 binds an appropriate cellular receptor and enters the cell using plasma membrane fusion or endocytosis. The tegument proteins dissociate from the capsid and the virion uses motor proteins to traffic along the microtubule network to the nucleus. 2) Upon docking with the nuclear pore, the dsDNA genome is injected from the capsid into the nucleus. 3) Immediate-early genes are expressed, and the proteins regulate the expression of downstream early and late genes. 4) Expression of early genes produces viral proteins that are required for the replication of the HSV-1 genome. 5) Late gene expression produces the structural capsid proteins that. 6) The viral genome copies are packaged into the capsid. The capsid egresses from the nucleus into the cytosol in a process known as primary envelopment, and acquires proteins associated with the inner tegument layer. 7) The capsid is enveloped by membranes that contain proteins of the outer tegument layer as well as the glycoproteins in a process known as secondary envelopment. These membranes are likely derived from the trans Golgi network. 8) The virus egresses from the cell as a mature infectious virion that can infect new cells.

HSV-1 lytic gene expression takes place as a regulated cascade of immediate-early, early, and late expression throughout infection (27). Immediate-early genes encode transcriptional activators and repressors that are required for the expression of early genes and regulation of HSV-1 transcription. In addition, the immediate-early gene ICP0 functions to inhibit antiviral

responses by the cell and also operates as a ubiquitin ligase to degrade cellular proteins (28–33). Expression of early genes are required for the transition to late gene expression, and these genes encode proteins that are responsible for the replication of the viral genome including UL30/UL42, which make up the viral DNA polymerase (34). Late genes encode viral structural proteins, such as the major capsid protein VP5, that are required to form the capsid that protects and delivers the viral genome (35). The late genes also encode the viral glycoproteins that, among other functions, are required for binding and entry of the virus into cells. Capsids that contain a viral genome egress from the nucleus and enter the cytoplasm. Inner tegument proteins are assembled onto the capsid either upon or directly after nuclear egress. Cytoplasmic capsids are then enveloped within a lipid bilayer that contains the outer tegument proteins and viral envelope proteins prior to egressing from the cell as a mature, infectious virion through the plasma membrane (25,36)

Propagation of HSV-1 for research

HSV-1 was first isolated in 1919, and it was first established that the virus was a transmissible agent by infecting rabbits (5). Like all viruses, HSV-1 requires living cells to replicate. This made the ability to study the virus difficult early on because cell culture was still in its infancy. The scientific and medical interest in HSV-1 meant that models were needed to study viral infection, replication, and disease. Much of our modern understanding of HSV-1 replication is derived from work in cell culture models. A cell line used to propagate and study viruses needs to be relatively cheap and easy to maintain. Immortalized cancer cell lines were and still are widely used for the study of viruses because of their propensity to continually divide. Interestingly, many of the cell lines that are used to study viruses that infect humans are

derived from other species and tissue types that are not necessarily relevant to disease (37). A primary example of this would be the widely used vero cell line for the propagation and study of HSV-1 in laboratories. The vero cell line is arguably the most common cell line employed for the growth of HSV-1 and other viruses that are used in downstream experiments (38,39). This cell line was initially isolated from the kidney of the African green monkey in 1962 (40). This relatively early isolation in combination with the ease of growth, its general susceptibility to viral infection and ability to produce high viral titers have all made the vero cell a staple of virology (39). It was even used by several labs to propagate SARS CoV-2 when it first emerged in late 2019, although this practice generally fell out of favor because propagation of SARS CoV-2 from vero cells select for mutations that lead to a loss in viral infectivity *in vivo* (41). Despite the popularity of the vero cell, several other cell lines have been used for the propagation and study of HSV-1. These cell lines include BHK-21 and HeLa cells. BHK-21 is an immortalized kidney fibroblast cell line first isolated from Syrian gold hamsters in 1961 (42). HeLa is a cervical cancer cell line isolated in 1951 and served as the first human cell line ever isolated (37). HSV-1 can infect many cell types both *in vitro* and *in vivo*. Interestingly, the type of cell used to produce a virus, i.e. the producer cell type, has been shown to change the infectious properties and molecular composition of the virions that are produced for several viruses (43). These factors have led our group to question whether the producer cell type of HSV-1 alters virion properties, specifically comparing virions derived from the historically relevant vero cell to those derived from cell lines that are medically relevant to HSV-1 disease.

Infectious virion composition

Mature HSV-1 virions consist of both viral and cellular proteins. Several studies have used mass spectrometry to determine the proteomic contents of HSV-1 virions by purifying virions away from cellular material. These analyses have identified at least 44 viral proteins and 49 cellular proteins that are incorporated into mature virions, although the list of identified virion-associated proteins varies greatly depending on the study (44–46).

The viral proteins that make up a virion play an important role in establishing successful infection within a cell. There are structural proteins that are used to form the viral capsid that houses the double-stranded DNA (dsDNA) genome which are absolutely required for capsid formation. In addition, as previously discussed, a subset of glycoproteins are attached to the viral envelope that are critical to viral entry into cells (47). However, the tegument layer of mature virions features the most diverse array of proteins in the virion as far as function (25,48). Several of these functions were described earlier in the chapter regarding the tegument proteins ICP0, ICP4, and VHS. The function of the cellular proteins that are incorporated into the virions are largely unknown in the context of viral infection, although a previous study demonstrated that preventing the incorporation of a subset of these cellular proteins can dampen the viral replication capacity of progeny virions (49).

There may be several reasons as to why the results from proteomics studies of HSV-1 virions feature inconsistencies in the specific viral and cellular proteins that are detected. First, several purification schemes have been used to isolate virions from cellular material. Most purification schemes utilized by HSV-1 virologists rely upon ultracentrifugation on a density gradient or cushion formed by various substrates. Virions are separated from cellular material on

this gradient based on their unique density relative to other viral assemblies or cellular components. The substrates that are chosen for this process often differ between studies, such as sucrose or Ficoll, and the choice of substrate may affect virion purity (44–46). Second, the identification of viral proteins often relies upon mass spectrometry analysis. Mass spectrometry has greatly increased in sensitivity over time (50). This increase in sensitivity has likely led to detection of virion-associated proteins or non-virion associated contaminating proteins that were previously not detected (51). Third, each set of published data has relied upon identification of proteins of a different strain of HSV-1. Strain variability is not well studied regarding HSV-1. However, strains differ in their infectious properties and genomic sequences (52). HSV-1 strains can have varying replication capacities in both neuronal and non-neuronal cell types and elicit different disease severity. For example, the HSV-1 strain McKrae replicates at higher levels in the nervous system and causes more severe disease in animal models relative to other viral strains (53–58). Replication differences between strains may alter molecular interactions that are happening within infected cells to the point where protein incorporation into virions becomes distinct for each strain. Fourth, different types of cells have been utilized to generate HSV-1 virions for mass spectrometry in each data set. HSV-1 incorporates both viral and cellular proteins into virions. Because different cell types express unique proteomes, the molecular interactions that occur between viral and cellular proteins within different cell types likely change in the context of HSV-1 infection. These differences in molecular interactions may change the pool of both viral and cellular proteins that are available for incorporation into virions.

The problems highlighted in reference to the identification of proteins in HSV-1 virions, specifically regarding strain and producer cell type-dependent differences that alter virion composition, extend to the HSV-1 literature as a whole. Scientific papers in the field often produce contradictory findings because of the use of different experimental systems. Some of these experimental systems rely upon different HSV-1 strains for infection and/or different cell types to produce HSV-1 for use in subsequent experimentation. While strain-dependent differences in viral replication have been experimentally documented when studying HSV-1, the effect that the producer cell type has on HSV-1 is often overlooked. Very few studies have tested whether the producer cell type can alter the macromolecular composition of virions. Furthermore, a dearth of knowledge exists as to how the producer cell type of HSV-1 virions impacts the infectious properties of the virus. We hypothesize that the producer cell type alters the macromolecular composition and infectious properties of mature HSV-1 virions.

Research aims and hypotheses

HSV-1 virology has historically relied upon the use of several cell lines to propagate HSV-1 for research. Previous studies have found that the producer cell type of many viruses can alter virion properties (43). This phenomenon has received little attention in the context of HSV-1, but a previous study using the HSV-1 strain KOS previously showed that the viral proteins ICP0 and ICP4 are incorporated into virions in a producer cell-dependent manner (59). The impact of this producer cell-dependent incorporation on infectivity was never determined. The study also relied on western blot for protein identification and quantification, which is a targeted analysis with undefined sensitivity for virion protein content. We hypothesize that the producer cell type of HSV-1 alters the capacity of the virions that are produced to lytically replicate in

target cells. This includes the ability of virions to express viral genes, viral proteins, and produce infectious virions. We also hypothesize that the incorporation of both viral and cellular proteins into virions is producer cell type dependent. It is important to determine whether the producer cell type of HSV-1 alters virion quality for several reasons. First, several cell lines are used for HSV-1 propagation in the laboratory. This lack of consistency may alter experimental results and interpretations of HSV-1 research and lead to a lack of reproducibility of scientific results. Second, this question has relevance to HSV-1 disease in humans because the virus infects many cell types *in vivo*. If the producer cell type of HSV-1 alters the proteomic composition and infectious capacity of virions, this could have implications for how the virus causes disease and spreads within and between hosts.

Chapter 2 is a manuscript published in Future Virology that reviews previous studies of producer cell-dependent effects on viruses. These viruses are associated with the *Coronaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Retroviridae*, and *Togaviridae*. The review discusses producer cell-dependent alterations to the macromolecular composition of virions including the genome, lipidome, glycome, and proteome. The review also covers producer cell-dependent changes to virion infectivity, spread, and immune recognition. The summary of literature in this review offers guidance into how the producer cell type of HSV-1 may change virion quality and which experiments we should perform to test our hypothesis.

Chapter 3 is a manuscript that has been submitted for peer review in the Journal of Virology. The experiments in this chapter directly test the hypothesis that the producer cell type of HSV-1 alters the infectious capacity of virions and their proteomic composition. The capacity for HSV-1 to replicate was tested by producing the virus from vero, immortalized human

keratinocyte (HaCaT) cells, and primary human foreskin fibroblast (HFF-1) cells. Vero cells were chosen due to their current and historical relevance in HSV-1 research as a propagation cell line for experimentation. HaCaT and HFF-1 cells were selected as a comparison because they more closely resemble a cell type that HSV-1 would encounter in humans. HSV-1 replication was measured by quantifying viral protein production and the output of infectious virions from infected target cells. Our data clearly demonstrates that the producer cell type of HSV-1 alters the infectious properties of virions. In addition, an LC-MS/MS based quantitative analysis of viral and cellular proteins associated with HSV-1 virions purified from each cell line demonstrated that the producer cell type alters virion protein content.

Chapter 4 provides further data supporting the hypothesis that the producer cell type alters HSV-1 infectivity. This chapter uses two different strains of HSV-1 to test our hypothesis. Two separate assays were used to monitor producer cell-dependent changes to viral gene expression. First, viral transcript abundance of HSV-1 McKrae was measured from infected cells using quantitative reverse-transcription PCR (qRT-PCR). Second, a fluorescent reporter HSV-1 strain 17 was employed to visualize the timing of gene expression using live imaging fluorescent microscopy. The data not only reaffirms that the producer cell alters virion infectivity but also shows that producer cell-dependent effects apply to multiple HSV-1 strains.

Chapter 5 provides an overall conclusion to the data presented in this dissertation and what it might mean in the context of HSV-1 infection. It also offers future directions to further test how the producer cell type may alter HSV-1 virion infectious properties and macromolecular composition. Some of the future experiments proposed include the use of neurons as a model to

study producer cell-dependent effects on HSV-1, as neurons are the reservoir for latent HSV-1 infection.

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CHAPTER TWO

THE HIDDEN IMPACT OF PRODUCER CELLS ON VIRION COMPOSITION AND INFECTIVITY

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Contributions: Literature summary, writing, table design, editing, and preparation of manuscript

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Contributions: Figure design, writing, editing, preparation of manuscript

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REVIEW

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The hidden impact of producer cells on virion composition and infectivity

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ABSTRACT

The cells infected by a virus *in vivo* are critical determinants of infection and disease. These same susceptible cells can also provide a wide range of options for viral propagation. The type of cell used to produce a virus, i.e. the producer cell type, can change the macromolecular composition of viruses and other factors associated with viral inoculum independent of genetic selection. Changes in the post-translational modifications of viral proteins, virion protein and lipid composition, and the types of viral structures released from different producer cells have been observed for several virus families. These producer cell-dependent changes can have wide ranging consequences on subsequent infection by altering viral tropism, antigenicity, and overall infectious capacity. The changes imparted by the producer cell impact experimental outcomes and influence viral spread and disease *in vivo*. In this review, we discuss the literature documenting the effects that producer cell type has on the macromolecular composition and infectious properties of virions and viral inoculum. We discuss the evidence of producer cell-dependent changes on the outcome of infection and antigenicity from diverse viral families. These observations highlight the need to better understand the impact producer cell type has on viral infections and disease.

PLAIN LANGUAGE SUMMARY

Viruses need cells to make copies of themselves and to make people sick. The kind of cell used to make a virus, called the producer cell, can change what the virus is made of. This review presents information about how producer cells have been seen to change with six different virus families. These changes are seen in the different proteins, fats, and sugars that go into the virus. The producer cell can also change the make-up of material used to infect other cells. All these changes can alter how easily the virus infects cells, how our bodies fight the virus, and how sick we get. We need to study more about how producer cells change viruses. This will help us understand how viruses spread and cause sickness. Knowing this could help us make better vaccines and drugs to stop viral infection.

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Tropism; virion; proteome; post-translational modification; antigenicity; glycosylation

1. Introduction

The infectious particle produced over the course of a viral infection, the virion, is necessary for the propagation of infection from cell-to-cell, within tissues, and between hosts. Virions can be simple in composition, consisting solely of a nucleic acid genome surrounded by a proteinaceous capsid shell. They can also be extraordinarily complex, containing multiple layers of viral and cellular proteins enclosed within lipid bilayers that are studded with viral proteins. Despite differences in virion complexity, all viruses ultimately rely upon the machinery of a host cell to replicate virions and propagate infection. The ability to infect and produce infectious virions in specific cell types is a defining feature of the tropism of a virus. Viruses can exhibit narrow or broad cellular tropisms both *in vivo*, where disease manifests, and *in vitro*, in the use of experimental models [1]. In this review, we will refer to the cell type used to produce virions as the producer cell type.

Experimentation with viruses requires propagation of infectious material, i.e., viral inoculum, which can be used to establish infection in subsequent experiments. Propagation of viral inoculum typically involves inoculation and harvest of infectious virus from cultured cells. In addition to infectious virions, viral inoculum can consist of noninfectious viral materials and cellular components depending on the method of harvest. Infectious virions are sometimes purified away from noninfectious viral materials and cellular components to refine interpretations of experimental infection. Some viruses are limited to a few selected cell types that can be used for propagation *in vitro*. However, other viruses can be reliably grown on a wide variety of cell types, many of which may not closely resemble the natural host cells that the virus would typically encounter and infect. Ultimately, the choice of cell line used to generate viral inoculum can depend on several factors. First and foremost, the cell line must be capable of producing high quantities of infectious virus. Second, the cell line should be relatively easy and cost-effective to maintain in the laboratory. Third, the cell line must be readily available. This may seem straightforward, but early virology research faced limitations in cell culture techniques, which restricted the types of cells available for viral propagation and infection studies [2]. These three factors are commonly considered when selecting cell lines for viral inoculum production.

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Article highlights

- The type of cell used to produce viruses can alter the macromolecular composition of the virions that are produced. Producer cell-dependent alterations to virion macromolecular composition include changes in glycosylation patterns of viral envelope proteins and differential incorporation of proteins and lipids into viruses.
- The producer cell type can alter the composition of viral inoculum that is used to infect cells. This does not necessarily involve alterations to virions, but instead retention of factors within the producer cell or changes to non-virion secreted factors.
- Producer cell-dependent changes to virions or virion inoculum can alter the outcome of immunogenic responses, viral tropism, and overall viral infectivity and spread within both *in vitro* and *in vivo* models.
- Evidence of producer cell-dependent effects on viruses affiliated with the *Coronaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Retroviridae,* and *Togaviridae* are discussed in this review.

However, other factors may be considered for various purposes including generation of attenuated virus for vaccine production or reproduction of scientific results from previous studies [3–5]. While these factors are important in choosing a producer cell type, an often-overlooked aspect is how the cell line used for viral propagation can influence the qualities of the viral inoculum that is produced.

Cell types that have been used for viral propagation in the literature have been derived from an array of different species and tissue types. These cell lines often become immortalized through spontaneous mutations or deliberate genetic manipulation [6]. Cells derived from different species will retain the genome from the host organism and therefore possess a transcriptome and proteome unique to that host. Furthermore, transcriptomic and proteomic profiles differ between cell types derived from the same species based on the differentiation of those cells to perform specialized functions, such as neurons or immune cells [7]. These dissimilarities are variables that the virus must contend with to successfully replicate and spread. The physiological differences between cell types can lead to producer cell-dependent changes to virion morphology [8] and the macromolecular composition of progeny virions including the glycome [9,10], lipidome [11–13], proteome [14–16], and genome [17–21] (Figure 1). Moreover, the overall content of the viral inoculum, including factors beyond just the virions, can be influenced by the producer cell type [22–24]. Ultimately, these producer cell-dependent alterations can result in shifts in viral infectivity and immune recognition.

Beyond *in vitro* experiments, understanding how the producer cell type alters infectious properties of virions helps us to understand how viruses spread between different tissues and hosts to ultimately cause disease. A summary of known producer cell-dependent alterations to virion macromolecular composition, antigenicity, and infectivity can be found in Table 1. A description of the producer cell types discussed in this review, including their species and tissue of origin, can be found in Table 2. This review will specifically examine how the producer cell type alters virion envelope protein glycosylation, virion proteomic content, lipidomic changes, and the overall composition of the viral inoculum, extending beyond just the progeny virion. It will also discuss the ramifications of these producer cell-dependent variations, highlighting their impact on viral infection.

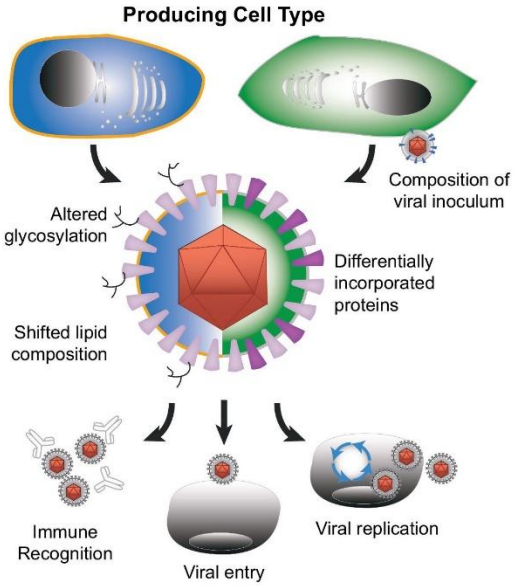


Figure 1. Producer cell-dependent effects on viruses. The type of cell used to produce the virus can result in changes to virion composition and subsequent attributes of infection. Virions produced from different cell types can exhibit alterations to protein glycosylation, lipid composition, and the viral and cellular proteins that are incorporated. These producer cell dependent changes can subsequently impact recognition by immune products such as antibodies and complement, restrict virion entry into some cell types, result in reduced capacity of viral replication, and limit pathogenesis in *in vivo* infection models.

2. Producer cell-dependent changes to virion protein glycosylation

Enveloped viruses depend upon virally encoded glycoproteins to both evade host immune responses and bind to cellular receptors to establish infection [1,25]. Some cell types can be selected for genetic changes in viral glycoproteins that alter the antigenicity, cellular tropism, and overall infectivity of a virus. An example of this phenomenon has been covered with an in-depth review of rapid mutation in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein when propagated in Vero cells. Briefly, the propagation of SARS-CoV-2 in Vero cells selects for mutations within the spike protein furin-cleavage site that results in a loss of infectivity in several animal models [17]. However, the function of viral glycoproteins is not completely dependent upon the genetic sequence of the protein. Virally encoded glycoproteins rely upon host cell glycosylation pathways for glycan modification [26]. Eukaryotic glycosylation pathways are very diverse. This diversity is evident when comparing protein glycosylation in insect and mammalian cells, and even human cell lines to those derived from other primates. Insect cells typically produce glycoproteins that are made up of short mannose

Table 1. Summary of producer cell effects on virions/viral inoculum and viral infection.

Virus	Producer cell types used in study	Description of virion or viral inoculum alteration	Observed effects from producer cell-dependent alterations
Coronaviridae			
Severe acute respiratory syndrome cornavirus (SARS-CoV 2)[13,17,53]	Vero, Vero E6, Vero h-Slam, Calu-1, Calu-3	Genomic mutation-Deletions in Furin-cleavage site of Spike protein in Vero and Vero E6-derived SARS-CoV 2	Loss of infectivity in hamster and primate animal models by Vero and Vero E6-derived SARS-CoV 2
	A549, Vero	Lipid composition of viral envelope-differential incorporation of lipids	n.d.
	A549, Calu-3	Virion proteome-differential incorporation of viral and host cellular proteins	n.d.
Flaviviridae			
Dengue virus (DENV)[12,30]	C6/36, LLC-MK2	Lipid composition of viral envelope	n.d.
	C6/36, Vero, DC	Envelope protein glycosylation	Loss of DC tropism by DC-derived Denv
Japanese encephalitis virus[21]	C6/36, BHK	Differential selection of genetic variants	n.d.
West Nile virus (WNV)[31]	C6/36, Vero, BHK-21	Envelope protein glycosylation	Loss of immune recognition by lectin complement system of Vero or BHK-derived WNV
Herpesviridae			
Epstein–Barr virus (EBV)[60]	AGS gastric carcinoma, Akata-Burkitt lymphoma	Glycoprotin incorporation-reduced incorporation of gH-gL-gp42 into virion envelopes of B cell-derived EBV	Cell tropism switch-B cell-derived EBV has higher affinity for epithelial cell infection and loss of infectious capacity for B cells
Herpes simplex virus-1 (HSV-1)[22,23]	Vero, HEp-2, BHK-21	Virion proteome content-loss of ICP0 and ICP4 when produced from BHK-21 High ratio of L-particles:mature virion in BHK-21-derived inoculum compared to HEp-2	n.d.
	DC, BHK-21	Change to proteomic content of DC-L-particles compared to BHK-21-L-particles	n.d.
Herpes simplex virus-2 (HSV-2)[18]	HaCaT, Vero	Selection for greater quantity of minor variants when produced on HaCaT	n.d.
Human cytomegalovirus (HCMV)[24]	HUVEC, Human foreskin fibroblast (HFF)	Cellular retention of endothelial cell-tropic virion population when propagated in HUVEC but not HFF	HUVEC-derived HCMV inoculum unable to infect endothelial cells
Orthomyxoviridae			
Influenza A virus (IAV)[8,33–35,39,61]	Vero 76, Vero C1008, BHK-21, HEC-1	Virion morphology-production of filamentous virions by Vero C1008 and HEC-1 but production of mostly spherical virions by BHK-21	n.d.
	Egg, MDCK, Vero, AGE1.CR, A549, HepG2	Changes to Hemagglutinin through glycosylation	Altered infectivity and antibody recognition
	Egg, MDBK	Virion proteome-differential incorporation of host cellular proteins into virions	n.d.
Influenza B virus (IBV)[32,38]	Egg, MDCK	Changes to Hemagglutinin through either mutation or glycosylation	Altered antibody recognition
Retroviridae			
Human immunodeficiency virus (HIV-1)[11,14,20]	Sup-T1, Thp1	Virion proteome-differential incorporation of viral and host cellular proteins into virions	n.d.
	MT4, HeLa	Lipid composition of viral envelope-differential incorporation of lipids	n.d.
	T-cells, monocyte-derived macrophages	Different recombination and mutation rates of HIV-1 genome	n.d.
Togaviridae			
Barmah Forest virus[36]	C6/36, BHK-21	Envelope protein glycosylation	Increased type I interferon secretion and decreased infectivity when infecting DC's with BHK-21-derived virus compared to C6/36-derived virus
Ross River virus (RRV)[36,37]	C6/36, BHK-21	Envelope protein glycosylation	Increased type I interferon secretion and decreased infectivity when infecting DC's with BHK-21-derived virus compared to C6/36-derived virus
	C6/36, Vero	Envelope E2 protein glycosylation	Results come from infected mice. Differences in viral dissemination within multiple tissues. More abundant antiviral signaling and immune cell infiltration of infected tissues when infected with Vero-RRV. Exacerbated disease outcome when infecting with C6/36-RRV.
Venezuelan equine encephalitis virus[36]	C6/36, BHK-21	Envelope protein glycosylation	Increased type I interferon secretion and decreased infectivity when infecting DC's with BHK-21-derived virus compared to C6/36-derived virus

List of viruses where an observed phenotype is associated with the type of cell producing the infectious material. Viruses are organized alphabetically by family. n.d. means no data provided.

Table 2. Summary of producer cells used for propagation of viral inoculum covered in review.

Cell Line/Type	Species	Tissue/Organ	Cell type or morphology	Virus propagated
A549	*Homo sapien* (human)	lung	epithelial	IAV, SARS CoV-2
AGE1.CR	*Cairina moschata* (Muscovy duck)	retina		IAV
AGS (gastric adenocarcinoma)	*Homo sapien* (human)	stomach	epithelial	EBV
Akata-Burkitt lymphoma	*Homo sapien* (human)		B cell	EBV
BHK-21	*Mesocricetus auratus* (Syrian golden hamster)	Kidney	fibroblast	Barmah Forest virus, HSV-1, Japanese encephalitis virus RRV, Venezuelan equine encephalitis virus, WNV
C6/36	*Aedes albopictus* (Asian tiger mosquito)	larva		Barmah Forest virus, DENV, Japanese encephalitis virus, RRV, Venezuelan equine encephalitis virus, WNV
Calu-3	*Homo sapien* (human)	lung	epithelial	SARS CoV-2
Dendritic cell	*Homo sapien* (human)		Dendritic cell	DENV, HSV-1
HaCaT	*Homo sapien* (human)	epidermis	keratinocyte	HSV-2
HEK 293T	*Homo sapien* (human)	kidney	epithelial	DENV
HeLa	*Homo sapien* (human)	cervix	epithelial	HIV-1
HEp-2	*Homo sapien* (human)	HeLa cell line contaminant	epithelial	HSV-1
HepG2	*Homo sapien* (human)	liver	epithelial	IAV
Human foreskin fibroblast	*Homo sapien* (human)	foreskin	fibroblast	HCMV
HUVEC	*Homo sapien* (human)	umbilical vein	endothelial	HCMV
Macrophage	*Homo sapien* (human)	peripheral blood	macrophage	HIV-1
MDBK	*Bos taurus* (cow)	kidney	epithelial	IAV
MDCK	*Canis familiaris* (dog)	kidney	epithelial	IAV, IBV
MT-4	*Homo sapien* (human)	blood	T cell	HIV-1
NIH/3T3	*Mus musculus* (mouse)	embryo	fibroblast	DENV
Sup-T1	*Homo sapien* (human)		T lymphoblast	HIV-1
THP-1	*Homo sapien* (human)	peripheral blood	monocyte	HIV-1
Vero	*Chlorocebus aethiops* (African green monkey)	kidney	epithelial	DENV, HSV-1, RRV, SARS CoV-2, WNV

Information regarding cell types was obtained from the American Type Culture Collection (ATCC) and Cellosaurus. The list of cell lines used for viral propagation is not comprehensive and reflects the cell lines used for virus propagation in the studies covered in this review.

glycans, called paucimannosidic glycans, and oligomannose-type glycans. These are considered less complex than the N-glycans produced by mammalian cells that often terminate in N-acetylneuraminic acid (NeuAc) [27,28]. Further evidence of divergence in glycosylation pathways can be found in primates. The enzyme responsible for N-glycolylneuraminic acid (Neu5Gc) formation, a type of sialic acid added to proteins post-translationally, is expressed in most mammals including old world primates but not new world primates or humans [27,29]. As evidenced by these examples, host species and cell type are important factors in determining viral glycoprotein composition. Viruses that exhibit changes in envelope protein glycosylation based on the producer cell type include viruses of *Flaviviridae* [30,31], *Orthomyxoviridae* [32–35], and *Togaviridae* [36,37]. Changes in envelope protein glycosylation within these viruses have a range of outcomes including changes to host cell tropism [30], antigenicity [31,32,38,39], and infectivity [33,36,37].

Influenza A virus (IAV) exhibits a wide host range and can circulate between populations of birds and mammals. This host range is mediated by the viral receptor-binding protein hemagglutinin (HA) which binds cellular sialic acids for viral entry [40]. Previous studies have revealed that the glycosylation pattern of HA can change in a producer cell-dependent manner [34,35]. Schwarzer *et al.* 2009 observed that HA of both IAV subtypes H1N1 (H1) and H3N2 (H3) is variable in molecular weight when grown on MDCK (M-H1 or M-H3), AGE1.CR (C-H1), Vero (V-H1 or V-H3), A549 (A-H1), and HepG2 (H-H1) cells. The changes in glycosylation of IAV HA protein produced from these cells were observed by capillary gel electrophoresis-laser induced fluorescence [35,41,42]. H1N1 and H3N2 hA glycans, both undigested and digested, showed differences in N-glycan fingerprints based on migration time and peak number when derived from the cells presented earlier. Further work utilizing similar assays to Schwarzer *et al.* 2009 verified that the H1N1 hA glycosylation pattern is indeed changed when propagated from different cell types, specifically when comparing HA of M-H1 and V-H1 [34]. The impact of these producer cell-dependent changes to HA glycosylation on the overall infectivity and antigenicity of the virus remains to be demonstrated. However, several past studies relied on simpler assays to determine that the producer cell type elicits changes to IAV [33,39] and Influenza B virus [32,38] antigenicity and infectivity, likely due to alterations of HA.

Flaviviruses are a type of arbovirus transmitted from mosquitoes to a vertebrate host as part of the sylvatic cycle or as a zoonotic infection of people [43]. At least one flavivirus, West Nile virus (WNV), has been shown to have its envelope glycan composition altered by host cells in a species-dependent manner [31]. This change to glycoprotein composition was detected when comparing WNV derived from mammalian cells to WNV derived from mosquito cells. The producer cell-dependent change to protein glycosylation caused alterations to immune recognition by the lectin complement pathway. Proteins of the lectin complement pathway recognize pathogen-associated carbohydrates and can prevent cellular entry of several viruses [44–46]. A cell line isolated from *Aedes albopictus* mosquitoes, C6/36, is frequently used as a producer cell for flaviviruses, including WNV. WNV derived from C6/36 cells (C-WNV) was bound by

naïve C57BL/6 [31] mouse serum complement proteins C3 and mannose-binding lectin (MBL). WNV derived from mammalian cells, including BHK21 (B-WNV) and Vero (V-WNV), was not bound by MBL. B-WNV and V-WNV were capable of plaque formation after incubation with mouse serum, but not C-WNV. C-WNV regained the ability to form viral plaques when incubated with serum from MBL-A/C KO mice, which lack a functional lectin-dependent complement pathway. A membrane fusion assay was used to show that loss of plaque formation by C-WNV was most likely due to inhibition of viral entry into cells after serum treatment. It was determined that this loss of infectivity by C-WNV may be due to a difference in glycosylation of viral envelope proteins. A follow-up experiment demonstrated that the inhibition of N-glycosylation in BHK cells with deoxymannojirimycin [47] (DMJ) rendered B-WNV susceptible to MBL binding. Exposure of B-WNV derived from DMJ treated cells to mouse serum resulted in a significant loss to infectivity as demonstrated by plaque assay.

Changes to glycoprotein content induced by the producer cell have also been characterized in the flavivirus dengue virus (DENV), which results in a change of cell tropism [30]. DENV produced in mammalian cells including Vero (V-DENV) and HEK 293T (H-DENV) was able to infect dendritic cells (DCs). Interestingly, DENV produced in DCs (DC-DENV) retained a tropism for Vero cells but was incapable of producing infectious virus or viral proteins in DCs. DENV is known to utilize DC-SIGN and L-SIGN as viral receptors for entry [48]. Infection of NIH/3T3 cells transfected with L-SIGN or DC-SIGN revealed that DENV produced in C6/36 (C-DENV) was able to bind and infect both transfected cell lines. DC-DENV was only capable of binding and infecting 3T3 cells expressing L-SIGN but not DC-SIGN. Researchers relied on a lectin blot analysis to distinguish differences in glycosylation of C-DENV, V-DENV, and DC-DENV envelope (E) protein. This analysis employed a panel of plant lectins that each has binding affinities for unique glycosylation patterns. From this analysis, researchers observed that each cell type produced a unique pattern of glycosylated E protein. More specifically, DC-DENV was found to lack high- and paucimannose-type N-glycans that are present in C-DENV and V-DENV. Digestion of DENV E protein with endoglycosidase H or peptide-N-glycosidase F further revealed differences in the molecular weight of glycosylated DENV E protein on western blot when comparing E protein of each producer cell type. These producer cell-dependent changes to glycosylation of DENV E protein likely give rise to the differences observed in binding and infectivity of DCs by DENV. Producer cell-dependent effects on glycosylation have also been described for other arboviruses including togaviruses. Shabman *et al.* found that three different togaviruses including Ross River virus (RRV), Venezuelan equine encephalitis virus, and Barmah Forest virus infect DCs less efficiently when the virus is produced from mammalian cells as opposed to mosquito-derived cell lines [36]. This reduction in infectivity of mammalian-derived togavirus (MA-T) was due to significantly greater amounts of type I interferon secretion by DC's when infected with MA-T as opposed to mosquito-derived togavirus. Like DENV, these differences were attributed to producer cell-dependent changes to envelope protein glycosylation.

The previous studies are important for understanding producer cell-dependent changes to viral envelope protein glycosylation and how it might impact viral tropism and immunity. However, few studies have tested whether these glycosylation changes influence viral spread and disease outcome *in vivo*. Tharmarajah *et al.* used glycan mass spectrometry to identify changes to glycosylation of the E2 protein of RRV when produced from Vero cells (V-RRV) or C6/36 cells (C-RRV) [37]. Both C-RRV and V-RRV E2 protein contained high-mannose glycans. However, V-RRV E2 protein also exhibited more complex glycosylation that featured galactosylation, fucosylation, and sialylation. Importantly, the producer cell type of RRV had a significant impact on viral dissemination and disease outcome in mice. Disease severity, duration of infection, and the distribution of virus in different organs were all affected by the producer cell type of the viral inoculum. Gene expression profiles of the skin and skin-draining lymph nodes showed that V-RRV stimulated earlier and more robust antiviral responses and greater immune cell infiltration of infected tissues than C-RRV. While these studies are compelling, more research is required to connect producer cell-dependent changes to protein glycosylation and the altered capacity to replicate and spread *in vivo*.

3. Producer cell-dependent changes to the virion proteome

Viruses encode multiple structural and non-structural proteins that are vital to re-programming host cells for viral replication, antagonizing immune responses, and producing mature infectious virions. Virions incorporate genomically encoded viral proteins and several viruses have been shown to incorporate cellular proteins into progeny virions [14,49–55]. The function of these cellular proteins in the context of virion incorporation and subsequent virion infection remains largely unclear. However, a subset of studies have shown host cell proteins that associate with progeny virions influence viral replication [56–58]. The producer cell type has been shown to impact the incorporation of both viral and cellular proteins into virions for some viruses including those of the *Coronaviridae* [53], *Herpesviridae* [23,59], *Orthomyxoviridae* [60], and *Retroviridae* [14].

Herpes Simplex virus-1 (HSV-1) is an alphaherpesvirus known to infect several cell types *in vivo* and *in vitro*. *In vivo*, HSV-1 primarily infects keratinocytes of the epidermis and oral mucosa prior to establishing latency in neurons of the peripheral nervous system [61]. *In vitro*, many cell types are used to propagate HSV-1 for downstream experiments. HSV-1 encodes 84 known viral proteins and incorporates at least 44 virally encoded and 49 cellular proteins into virions [1,22,52,62]. Yang & Courtney purified HSV-1 from Vero (V-HSV), BHK-21 (B-HSV), and HEp-2 (H-HSV) cells and found that HSV-1 virions derived from these cell types differed in the composition of virally encoded proteins [23]. Specifically, western blot analysis showed that the immediate-early tegument proteins Infected Cell Protein 0 (ICP0) and ICP4 were present in V-HSV and H-HSV but not B-HSV. Both ICP0 and ICP4 exhibit multiple functions, and their gene expression is required for successful viral replication. These functions include regulation of viral gene expression, evasion of cellular antiviral responses, and ubiquitination of host cell proteins [63,64].

Furthermore, this producer cell-dependent incorporation of ICP0 and ICP4 may be a strain-dependent phenomenon. Yang & Courtney did not detect ICP0 and ICP4 in purified B-HSV using the KOS strain. However, other studies detected both proteins in purified B-HSV when using strain 17 [22,65] or strain F [52]. Although it seems likely that a lack of ICP0 and ICP4 incorporation into virions would reduce the infectious capacity of HSV-1 virions, the consequences of these missing proteins have yet to be demonstrated.

As mentioned previously, SARS-CoV-2 can undergo selection for mutation in the furin cleavage site of the spike protein based on the producer cell type [17]. There is evidence suggesting that producer cell-dependent changes to SARS-CoV-2 virions can happen independently from viral genome mutation. Murigneux *et al.* identified and quantified proteins associated with SARS-CoV-2 virions derived from A549-ACE2 (A-SARS) and Calu-3 (C-SARS) using LC-MS [53]. The abundance of viral and cellular proteins in purified virions changed based on the producer cell type. All known SARS-CoV-2 structural proteins were detected in virions derived from each cell type. However, one viral non-structural protein was detected in A-SARS, ORF7a, but was not detected in C-SARS. ORF7a antagonizes antiviral responses and may be involved in regulation of autophagic flux [66–68]. A total of 284 and 120 cellular proteins were identified in A-SARS and C-SARS, respectively, only 48 of which were incorporated into virions derived from both cell types [53].

The detection of incorporated cellular proteins can depend not only on cell type but also on the method of virion purification. Within this study, subsequent LC-MS analysis of A-SARS utilized an alternative method to purify virions away from cellular material. The subsequent LC-MS analysis of purified A-SARS detected only 70 cellular proteins in comparison to the 284 that were originally detected, 57 of which were identified in both analyses. This same analysis was not performed with C-SARS, but it is indicative that differences in purification schemes may provide varying levels of virion purity that can elicit divergent results when analyzing the proteomic contents of virions. Nevertheless, producer cell-dependent differences within the virion proteome have also been detected by mass spectrometry in Human immunodeficiency virus-1 (HIV-1) and IAV. Similar to SARS-CoV2, propagation of HIV-1 in different human cells including Sup-T1 T lymphocytes and the monocyte model cell line THP-1 result in differential protein incorporation [14]. Furthermore, IAV contains cellular proteins that are unique or differentially incorporated into virions depending on whether they are produced from mammalian or avian cell lines [60]. Like HSV-1 and SARS-CoV-2, the consequences of differential protein incorporation into IAV and HIV-1 virions are unknown. Collectively, these results may indicate that producer cell-dependent changes to virion proteomic composition is a common feature of viruses. This topic requires further study to understand its full implications to viral spread and disease outcomes.

The consequences of producer cell-dependent alterations to virion proteomes have remained elusive for most viruses. This is not the case for the gammaherpesvirus Epstein–Barr virus (EBV). EBV undergoes lytic replication in epithelial cells and establishes a latent infection in B cells. EBV latency, and subsequent reactivation from a latent to lytic state, is a feature that allows EBV to persist and spread to new hosts [69]. EBV cell tropism is determined by producer cell-dependent alterations to the virion proteome. More specifically, lytic replication in B cells results in a tropism switch from B cells to epithelial cells [59]. The viral envelope requires a three-part complex of glycoprotein H (gH), gL, and gp42 for successful entry into B cells [70,71], but gp42 is inhibitory to EBV entry of epithelial cells [71]. Borza & Fletcher found that EBV produced from gastric adenocarcinoma (AGS) epithelial cells (E-EBV) produced greater quantities of EBV in B cells than EBV produced from the B cell-like Akata-Burkitt lymphoma cell line (B-EBV) [59]. They also found the inverse to be true, in which B-EBV was more infectious toward epithelial cells when compared to E-EBV. Antibody staining of EBV gp42 and gH in combination with flow cytometry revealed that B-EBV possessed less gp42 but equivalent quantities of gH in comparison to E-EBV. The reduced gp42 in B-EBV virions is related to cell type-specific expression of proteins. Human leukocyte antigen class II (HLA II) is primarily expressed in immune cells including B cells, but the majority of epithelial cells lack HLA II expression [72]. The authors hypothesized that the degradation of gp42 in HLA II expressing cells influences the loss of B cell infectivity by B-EBV. An experiment that relied on HLA II expressing AGS cells for EBV production resulted in a similar loss of virion B cell infectivity as B-EBV from the produced virions. This data shows that producer cell-dependent changes to the virion proteome can alter the tropism of viruses.

Proteomic analysis of virions is challenging, and analysis of virions have yielded different results for protein composition within and across studies of several viruses including HSV-1 [22,52,62], human cytomegalovirus [51,73] (HCMV), SARS-CoV -2 [53], and HIV-1 [14,15]. The differences in virion protein content of the same virus across studies could be due to a multitude of factors. As already mentioned, different cell lines are often utilized to propagate virions for analysis which likely drive changes in virion protein content. Differences between viral strains may also cause deviation in virion protein incorporation. In addition, several methodologies exist to purify virions away from cellular material. Common virion purification techniques often rely upon density ultracentrifugation, filtration, or affinity purification [74]. As discussed previously regarding SARS-CoV 2 virion purification, these different purification methodologies may not result in equivalent virion purity or yield. Although the identification of virion proteins is most reliably performed using mass spectrometry, various other assays have been employed for protein detection in virions including western blot, flow virometry [75], or immunoelectron microscopy [16]. It is also important to highlight that sensitivity of mass spectrometry analysis has greatly improved over time [76], making it difficult to compare results between older and newer studies. This increase in sensitivity may also allow detection of contaminating proteins that were not previously detected in purified virions, and it has become increasingly difficult to determine whether a protein is incorporated into virions or whether said protein is a contaminant co-purifying with virions. Follow-up experiments are often warranted to delineate candidate proteins of interest as either contaminants or virion incorporated and assess their function.

4. Producer cell-dependent changes to the lipidome

Enveloped viruses acquire at least one lipid bilayer that engulfs the protein capsid of the virion prior to egress from an infected cell. The source of the lipid envelope is dependent on the virus and can include the plasma membrane or internal membranous structures such as the Golgi apparatus or the endoplasmic reticulum. A viral envelope can be composed of several classes of lipids. In addition to protective effects, such as shielding virion components from antibody binding, the envelope plays an important role in virion stability and entry into cells. After the virus has bound a viable cellular receptor and with the aid of fusion proteins, the lipid envelope facilitates entry into the cell by fusing with the plasma membrane or the membrane of an endocytic vesicle. The role of lipids within the producer cell in the context of viral infection and their function post virion incorporation has been reviewed extensively [77–81]. The lipid composition of viral envelopes is critical to viral infectivity. For example, depletion of cholesterol can lead to membrane permeability and virion inactivation [82,83]. Cholesterol also directly interacts with some viral envelope proteins and can be a critical mediator of envelope protein distribution and viral entry [84–87]. In addition, the lipid phosphatidylserine is exposed on the surface of several viral envelopes, and this lipid is implicated in inhibition of antiviral responses in DCs [88]. Importantly, the lipid composition of the viral envelope can be altered in a producer cell-dependent manner in some viruses including those of the *Coronaviridae* [13], *Flaviviridae* [12], and *Retroviridae* [11].

The retrovirus HIV-1 acquires its lipid envelope from the plasma membrane of the infected cell upon virion egress. The lipid composition of the HIV-1 envelope has been determined by mass spectrometry for HIV-1 isolated from HeLa (H-HIV) and MT-4 (M-HIV) cells [11]. H-HIV and M-HIV envelopes featured lipid contents that were distinct from the overall lipid composition of the plasma membrane derived from their producer cell. Similarities were observed in the HIV-1 envelopes derived from both cell lines, including enrichment of cholesterol, sphingomyelin, phosphatidylserine, and plasmalogen-phosphatidylethanolamine. Despite these similarities, many of the lipids that were identified within the envelopes of HIV-1 in this study were found to be incorporated at different abundances including sphingolipids and glycerophospholipids. In addition, dihydrosphingomyelin (DHSM) was uniquely incorporated into M-HIV. Interestingly, DHSM is known to inhibit HIV-1 entry into cells by causing rigidity within the plasma membrane [89]. Whether DHSM incorporation in the M-HIV virions impacts viral envelope rigidity and subsequent entry remains to be determined. Similar reports of differential lipid incorporation into viral envelopes have been published that compare the lipidome of SARS-CoV-2 virions derived from A549 and Vero cells [13] as well as DENV derived from C6/36 and LLC-MK_2 cells [12]. The functional impact that producer cell-dependent alterations to virion lipid content has on overall viral infectivity requires further study. Future experiments are warranted to test whether these differences in lipid composition can alter processes such as viral entry, virion stability, and susceptibility to disinfectants and detergents. Furthermore, it is important to note that the lipid content of the viral envelope often does not match the lipid content of the producer cell plasma membrane or the producer cell as a whole. Indeed, the aforementioned lipidomic studies determined that each virus was enriched for and diminished of specific lipid types compared to the host cell. This would indicate that producer cell-dependent effects on the lipid content of the viral envelope may not be predictable purely based on the lipid content of the producer cell or the membranes that are associated with the viral egress pathway.

5. Producer cell-dependent changes to viral inoculum

The effects of the producer cell type described in this review thus far have been focused on the direct alteration of virions. However, producer cell-dependent differences have also been identified in the viral inoculum itself, independent of changes to the virion's macromolecular composition. These producer cell-dependent changes in the viral inoculum can manifest in different ways depending upon the virus. These changes can result in altered infectivity of viral inoculum. Additionally, these changes may lead to enhanced or reduced immune cell activation. This review will focus on producer cell-dependent changes to viral inoculum that have been observed for members of the *Herpesviridae* family [22–24].

HSV-1 produces extracellular virions to facilitate the spread of infection to new cells, but these virions are not the only product of HSV-1 infection. Cells infected by HSV-1 produce virus-like particles called Light particles (L-particles). L-particles consist of a viral envelope and tegument proteins but lack a viral capsid or genome [90]. The role of L-particles during HSV-1 infection is not well understood. Although they lack the capacity to establish infection, L-particles can serve as a vector for delivery of viral proteins into cells. There is evidence that L-particles may play a role in immune modulation. L-particles have been shown to downregulate CD83 and MHC II in DCs, and DCs exposed to L-particles have a significantly reduced propensity for T-cell activation [22,65]. Interestingly, the quantity of L-particles produced as well as their proteomic content can be producer cell type-dependent.

L-particles can be purified away from cellular materials using similar methodologies as previously described for virion isolation in proteomics studies. Yang & Courtney were able to separate HSV-1 virions from L-particles by density gradient ultracentrifugation [23]. This separation revealed that BHK-21 and HEp-2 cells produce a ratio of L-particles to mature viral particles of 1:1 and 1:1300, respectively. Western blot analysis also showed that the ratio of ICP0 to ICP4 that was incorporated into BHK-21 derived L-particles (B-LP) appeared to be far lower than what was incorporated into Hep-2 derived L-particles. A more extensive characterization of L-particles purified from human monocyte-derived mature DCs (DC-LP) and BHK-21 cells was performed by Birzer *et al.* [22]. Mass spectrometry identified 63 viral proteins associated with B-LPs compared to 41 viral proteins in DC-LPs. Several viral proteins that were incorporated into each were present within L-particles at different ratios. Of note, a subset of proteins that are known to be present only in mature HSV-1 virions were detected in both DC-LPs and B-LPs in relatively low abundance during analysis, likely indicating a small amount of mature virion contamination in the Ficoll density band that contained L-particles. DC-LPs also contained a greater number of host cellular proteins compared to B-LPs. Because L-particles associated with viral inoculum can enter

cells and deliver HSV-1 viral proteins, it seems likely that producer cell-dependent differences observed in L-particle quantity and protein composition could alter various aspects of viral infection, including immune cell modulation.

The betaherpesvirus HCMV possesses a broad cellular tropism both *in vivo* and *in vitro*. Despite this, propagation of HCMV *in vitro* can be challenging. Human fibroblasts have become a standard for reliable propagation of HCMV for laboratory experiments [91]. Much like EBV, HCMV virions can possess different glycoprotein complexes that impact cell tropism. HCMV can incorporate a gH/gL/gO complex or a gH/gL/pUL(128-131A) complex. gH/gL/gO limits HCMV infection to a small subset of cells that include neurons and fibroblasts [92,93]. HCMV can gain a tropism toward leukocytes, DCs, epithelial, and endothelial cells after incorporating a gH/gL/pUL(128-131A) complex [92–95]. The tropism of HCMV inoculum can change based on the producer cell type. This change is not due to differential incorporation of glycoprotein complexes, as previously described for EBV. Instead, it has been shown that in at least some cell types, HCMV produces two populations of virions, each of which possess a unique composition of glycoprotein complexes [24]. Distinct populations of virions can be retained in some cell types, resulting in a change to cell tropism of the viral inoculum.

HCMV viral inoculum produced from human foreskin fibroblasts (F-HCMV) can infect both fibroblasts and endothelial cells. However, HCMV viral inoculum produced from HUVEC cells (E-HCMV), a primary endothelial cell line, has a greatly reduced infectious capacity toward endothelial cells but retains fibroblast infectivity. This change to cellular tropism was attributed to a lack of gH/gL/pUL(128-131A)-containing virions in the E-HCMV inoculum. To mimic this effect, F-HCMV was deprived of its endothelial-tropic viral population using an antibody-based depletion assay targeting pUL131A or pre-incubation of F-HCMV with endothelial cells. These treatments revealed that viral inoculum derived from fibroblasts could be depleted of endothelial-tropic HCMV without losing fibroblast infectivity. Like fibroblasts, endothelial cells produced HCMV that has the capacity to infect other endothelial cells, but this endothelial-tropic subset of HCMV virions was retained by the producer endothelial cells through an unknown mechanism. As a result, the retained viral population of endothelial-tropic HCMV could not spread infection to endothelial cells, whereas the released viral population of fibroblast-tropic HCMV was able to establish infection in fibroblasts. This data supports that two subsets of HCMV are produced in both fibroblasts and endothelial cells, each of which exhibit a tropism toward either fibroblasts or endothelial cells. However, the viral inoculum obtained from endothelial cells loses endothelial cell infectivity. This loss of infectivity is due to retention of endothelial-tropic HCMV virions within the producer endothelial cell. This data demonstrates that the cell type used to propagate viral inoculum can influence its infectivity and spread, independent of changes to the virion's macromolecular composition, by retaining specific virion populations within the producer cells.

6. Conclusion

This review has discussed findings from multiple studies that indicate the producer cell type of viruses can alter the macromolecular composition of virions as well as the overall content of viral inoculum. These studies focused on viruses affiliated with the *Coronaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Retroviridae*, and *Togaviridae*. These changes to virions include alterations to glycan modifications of virion envelope proteins, differential incorporation of proteins, and changes to lipid envelope composition. Although the consequences of such changes remain speculative for several viruses, various outcomes have been attributed to these alterations across studies. These consequences include changes in immunogenicity and infectivity of viruses both *in vitro* and *in vivo*.

7. Future perspective

Producer cell-dependent effects on viruses remain an understudied aspect of many viral infections. It is likely that we do not understand the full range of ramifications associated with producer cell effects on progeny virions, either *in vitro* or *in vivo*. Although this review centers on producer cell-dependent alterations to the virion glycome, lipidome, and proteome, producer cell-dependent alterations to virion morphology [8] and genetic changes to the viral genome [17,18,20,21] have also been documented in other viruses. Producer cell-dependent changes to virions or viral inoculum are often left unrecognized without dramatic shifts in cell tropism or loss of infectivity in animal models. Indeed, producer cell-dependent effects may alter aspects of viral infection in ways that can change experimental outcomes, interpretation of results, and the reproducibility of scientific findings. Though understudied, interest in cell-based modifications and producer cell-specific effects on viruses have gained interest in some fields and will likely become a point of emphasis in the future. Further research is needed to determine whether producer cell-dependent changes can alter viral spread within and between hosts and whether these effects can be exploited for prevention and treatment of disease.

Beyond the effects on viral infection and replication, the producer cell can alter immune recognition and antiviral responses to viruses. This review has discussed previously documented changes to antibody and complement recognition, yet there are likely further impacts to antiviral immunity that need to be investigated. The macromolecular composition of virions likely affects the recognition of viral components by cellular pathogen-recognition receptors and subsequent activation of antiviral responses in both immune and nonimmune cells. Uptake and processing of producer cell-altered viral antigens may also influence peptide recognition and subsequent activation of T cells. The sum of these potential differences not only implicates broader immune recognition and clearance but can also guide vaccine design strategies. Similarly, the efficacy and design of antiviral drugs is also likely to be shaped by the producer cell. Many antivirals are specifically engineered against conserved viral proteins, which limits their utility across different families of viruses [96,97]. The targeting of conserved viral proteins can also render these antiviral drugs more susceptible to the development of drug resistance by viruses [98]. Continuing research efforts for broad-spectrum antivirals have looked to cellular pathways to inhibit the replication of multiple viruses [99]. A better understanding of the ways in which host cells alter the macromolecular composition of virions, subsequent

immunogenicity, and infectivity can lead to the development of therapeutics. For example, small molecules inhibitors could be developed that prevent the incorporation of a specific cellular protein or several cellular proteins into virions that are needed for infectivity. Viruses that replicate in similar tissue and organ systems may possess common sets of alterations by host cells that enhance or are required for efficient replication and dissemination. Viruses that have broad tropism with potential for systemic spread *in vivo* may have an initial entry point for replication in which dissemination could be inhibited by targeting specific cellular modifications of virions specific to the initial point of entry. Further study of producer cell-dependent modifications to viruses will continue to advance our understanding of viral replication and spread. Ultimately, it may become a major factor in the development of antiviral drugs and preventative vaccines.

The influence of the producer cell type on viruses has profound implications for our understanding of viral infection and experimental approaches for research. The development and accessibility of relevant cell culture models will become increasingly critical. A more nuanced analysis of how producer cell type affects virion composition and viral inoculum is essential for resolving discrepancies across experimental studies and properly modeling disease outcome. As evidenced by the studies discussed in this review, the impact that the producer cell type has on viral macromolecular composition, infectivity, and immunogenicity is widespread across various viral families. Continued research will be key to identifying producer cell-dependent changes to viruses, linking them to outcomes in viral infectivity and pathogenesis, and exploiting those changes for the development of therapeutics.

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CHAPTER THREE

THE PRODUCER CELL TYPE OF HSV-1 ALTERS THE PROTEOMIC CONTENTS AND INFECTIOUS CAPACITY OF VIRIONS

Contribution of Authors and Co-Authors

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Abstract

The cell that a virus replicates in i.e., the producer cell, can alter the macromolecular composition and infectious capacity of the virions that are produced. HSV-1 primarily infects keratinocytes of the epidermis or oral mucosa prior to establishing latency in neurons of the peripheral nervous system, allowing the virus to persist for the lifetime of the host. Many cell lines that are used to amplify Herpes Simplex virus type 1 (HSV-1) are derived from species and tissue types that are less physiologically relevant to HSV-1 disease. To understand if the producer cell type influences HSV-1 infection, we tested the infectivity of HSV-1 derived from immortalized African green monkey kidney cells (vero), immortalized human keratinocytes (HaCaT), and primary human foreskin fibroblasts (HFF-1). We observed that the producer cell type alters the capacity of HSV-1 to produce viral proteins and infectious virions from infected cells and susceptibility to inhibition of replication by interferon treatment. HaCaT-derived HSV-1 consistently exhibited enhanced replication over HFF-1 or vero-derived virus. To determine if the producer cell type changes the protein composition of virions, we performed an untargeted LC/MS-MS analysis of virions purified from each cell line. Comparison of virion associated proteins revealed quantitative differences in composition of both cellular and viral proteins including ICP0, pUL24 and pUL42. These results highlight the influence that the producer cell-type has on HSV-1 infection outcomes and suggest that cell type specific factors can alter HSV-1 and potentially support or hinder HSV-1 replication.

Importance

Approximately 67% of the human population harbors HSV-1 infection. To study HSV-1 infection, laboratories utilize several different cell lines to propagate HSV-1 for downstream experiments. The type of cell used to produce a virus, i.e. the producer cell type, can alter the macromolecular composition, immunogenicity, and infectivity of the virions that are produced across several virus families. We found that the producer cell type of HSV-1 alters virion infectivity and virion protein composition. Therefore, the producer cell type may have implications in the spread of HSV-1 and subsequent disease outcomes in humans. Our results also raise concerns about how the use of different ceil types to propagate HSV-1may alter the outcome, interpretation, and reproducibility of experimental results.

Introduction

Herpes Simples Virus type 1 (HSV-1) inherently relies upon the resources of a host cell to replicate and spread. HSV-1 possesses a broad tropism both *in vitro* and *in vivo*, meaning it can successfully replicate and produce infectious virions from multiple cell types. Different cell types express distinct transcriptomes and proteomes depending on multiple factors that include the function of the cell, the extracellular environment, and cell-cell signaling. Because HSV-1 infects multiple cell types, the virus must contend with these dissimilar host environments to successfully replicate. The state of the cellular environment can change fundamental aspects of viral infection and influence the macromolecular composition and infectious properties of the virions that are produced (1–27). An example of this phenomenon has been described for the gammaherpesvirus Epstein-Barr virus (EBV) (5). The incorporation of viral glycoprotein

complexes into the viral envelope of EBV changes based on whether the virus was produced from B cells or epithelial cells, likely due to the expression of MHC II in B cells. The change in glycoprotein incorporation results in altered cellular tropism of EBV. Beyond members of the *Herpesviridae*, producer cell-dependent effects have been described for members of at least 5 other viral families, eliciting changes in cellular tropism, immune responses, and replicative capacity (27). It is likely that producer cell-dependent effects remain to be described for a wider range of viruses.

There are many cell lines used to propagate HSV-1 for laboratory experiments that study infection and replication. Commonly used cell lines include vero, BHK-21, and HeLa among others (28). In this paper, we will refer to the type of cell used to propagate HSV-1 as the producer cell type. The producer cell type of HSV-1 has previously been described to alter virion protein composition. Evaluation of HSV-1 virions produced from vero and HEp-2 cells contained ICP0 and ICP4, but these proteins were not detected in BHK21-derived virions (11). Although ICP0 and ICP4 expression are critical to the establishment of successful HSV-1 infection, it is currently unknown whether their absence in virions alters virion infectivity. Indeed, the effects that the producer cell type has on HSV-1 infectivity remains understudied. To test our hypothesis that the producer cell type of HSV-1 alters virion infectivity, we propagated HSV-1 from immortalized human keratinocytes (HaCaT), African green monkey kidney cells (vero), and human foreskin fibroblasts (HFF-1). We then compared the capacity of HSV-1 from each cell line to replicate in non-neuronal cells. Our results show that the producer cell type impacts the subsequent expression of viral proteins, production of infectious virions, and the capacity to replicate in cells stimulated by interferon in all infected cell types that were tested. To begin

characterizing the molecular features that lead to these producer cell dependent differences, we performed an untargeted analysis of the proteomic composition of HSV-1 virions. We observed that proteomic composition is dependent not just on the producer cell type observing changes in the content of both viral and cellular proteins present in purified virions.

Results

Producer cell-dependent effects on genome:PFU of HSV-1

Replication often produces non-infectious virions. Despite their lack of infectivity, non-infectious virions can still influence the replication of infectious virions. Whether non-infectious virions enhance or hinder viral replication is dependent on several factors including the type of virus and the viral strain (29–32). We hypothesized that the producer cell type of HSV-1 alters the ratio between the number of genomes and the number of plaque forming units (PFU) (genome:PFU). To test this hypothesis, we propagated HSV-1 McKrae viral stocks from HaCaT (HaCaT-HSV), vero (Vero-HSV), and HFF-1 (HFF-HSV) cells. Each viral stock was propagated in triplicate. We used a plaque assay to determine the viral titer of HaCaT-HSV, Vero-HSV, and HFF-HSV and quantitative PCR (qPCR) to determine the absolute genome content of HSV-1 derived from each producer cell type. The genome:PFU of each producer cell type was quantified specifically for the viral stocks that were used to infect target cells for the experiments described in this paper. HaCaT-HSV had the lowest genome:PFU ratio in comparison to Vero-HSV and HFF-HSV (Table 3). This data supports the conclusion that the producer cell type alters the PFU:genome of viral stocks. The subsequent experiments discussed in this paper rely upon infection of target cells using an equivalent multiplicity of infection (MOI) based on the PFU applied per cell. It is important to note that normalizing MOI to the genome content of the viral

stocks rather than the PFU/cell would result in a lower PFU/cell applied to target cells that were infected with Vero-HSV or HFF-HSV relative to cells infected with HaCaT-HSV. Previous work has shown that higher MOI infections result in enhanced replication of HSV-1 (33).

Table 3. Producer cell-dependent effects on the HSV-1 genome to PFU ratio.

HSV-1 producer cell	Average genome:PFU ratio	HSV-1 infectious MOI when normalized to HaCaT-HSV genome content
HaCaT-HSV	4.6 ± 0.8	10.0
Vero-HSV	14.4 ± 1.8 **	3.0
HFF-HSV	17.0 ± 3.4 **	3.0

The genome to PFU ratio of HSV-1 derived from HaCaT, vero, or HFF-1 cells. Genome and PFU content were determined using qPCR to quantify genome content and plaque assay to quantify PFUs, respectively, with values being represented with ± the SEM. Analysis of the statistical significance was performed relative to HaCaT-HSV using unpaired t-test. ** indicates $P<0.01$.

Producer cell-dependent effects
on HSV-1 protein expression

To determine whether the producer cell type alters the capacity of virions to express viral proteins, HaCaT, vero, and HFF-1 cells were infected with HSV-1 propagated from the aforementioned cell types at an MOI of 1 and 10. Infected cells were fixed at 3 hours post infection (hpi) for MOI 10 or 6 hpi for MOI 1 and 10. Detection of viral proteins was achieved by staining with a polyclonal HSV-1 antiserum (Fig. 1a). Except for 6 hpi in HaCaTs, HaCaT-HSV consistently produced more detectable viral proteins in cells when infected at an MOI of 10 (Fig. 1b). At an MOI of 1, HaCaT-HSV produced a greater quantity of viral proteins in HaCaTs but produced equivalent amounts of protein compared to Vero-HSV in vero and HFF-1 cells (Fig. 1c). HFF-HSV consistently produced the lowest quantity of detectable viral proteins in all treatment conditions and did not produce detectable amounts of protein in HFF-1 cells at MOI 1

at either time point. In the cell types that were tested, these results show that HSV-1 produced from HaCaT cells express greater amounts of detectable viral proteins compared to HSV-1 derived from vero or HFF-1 cells.

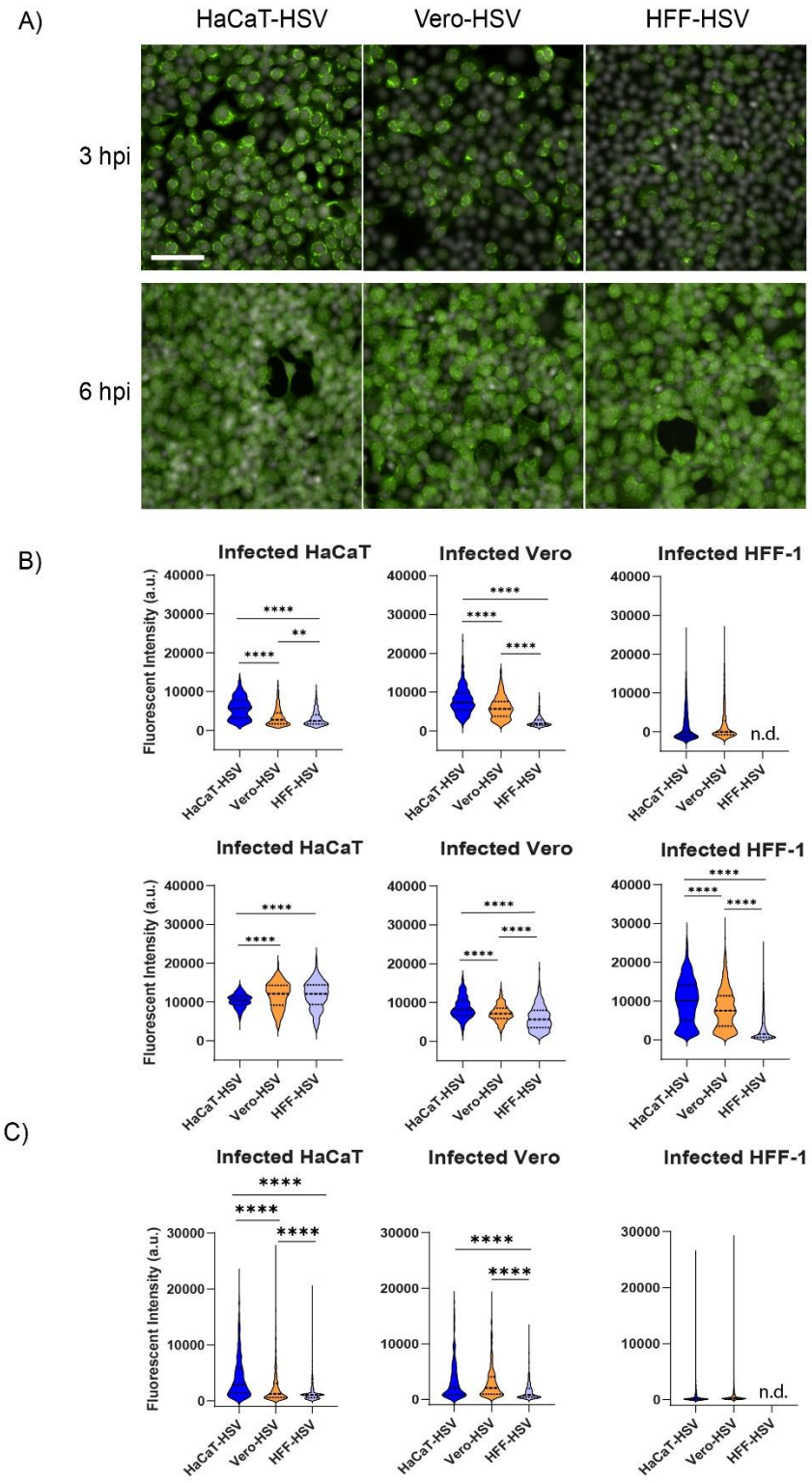

A)
HaCaT-HSV
Vero-HSV
HFF-HSV
3 hpi
6 hpi
B)
Infected HaCaT
Infected Vero
Infected HFF-1
Fluorescent Intensity (a.u.)
40000
30000
20000
10000
0
****
**
n.d.
HaCaT-HSV
Vero-HSV
HFF-HSV
C)
Infected HaCaT
Infected Vero
Infected HFF-1
Fluorescent Intensity (a.u.)
30000
20000
10000
0
****
n.d.
HaCaT-HSV
Vero-HSV
HFF-HSV

Figure 1. HaCaT-HSV produces more viral proteins early in infection. (A) Images of HaCaT cells infected with HSV-1 McKrae derived from HaCaT, vero, or HFF-1 cells at an MOI of 10. Infected HaCaTs were fixed at 3 and 6 hpi and stained with rabbit polyclonal HSV-1 anti-serum (green) and Hoescht (blue). Equivalent exposure presentation values are applied for each channel across all presented images. Scale bar = 200 μm. (B) Quantification of fluorescent intensity of viral proteins in HaCaT, vero, or HFF-1 cells infected with HSV-1 McKrae derived from HaCat, vero, or HFF-1 at an MOI of 10 or (C) MOI 1. Infected cells were fixed and stained as described in A at 3 hpi (top row-B and C) and 6 hpi (bottom row-B). Quantification of fluorescent intensity was performed using Image J. n.d. = no detection. Statical comparison was performed using one-way ANOVA with Tukey's multiple comparisons test. ** indicates $p < 0.01$, **** indicates $p < 0.0001$.

Producer cell-dependent effects on the production of infectious HSV-1

To determine whether the producer cell type of HSV-1 alters the quantity of infectious virions produced in subsequently infected cells, HSV-1 propagated from the aforementioned cell types was inoculated onto HaCaT, vero, and HFF-1 cells at an MOI of 10. The amount of infectious virus that was produced at the indicated time points from infected cells was determined by plaque assay (Fig. 2). HaCaT-HSV consistently produced higher quantities of infectious HSV-1 in HaCaT, vero, and HFF-1 cells at 8 and 12 hpi in comparison to Vero-HSV and HFF-HSV. The output of infectious virions was roughly equivalent in all tested conditions by 24 hpi. This data shows that HaCaT-HSV has a propensity for producing higher quantities of infectious virions in infected cells.

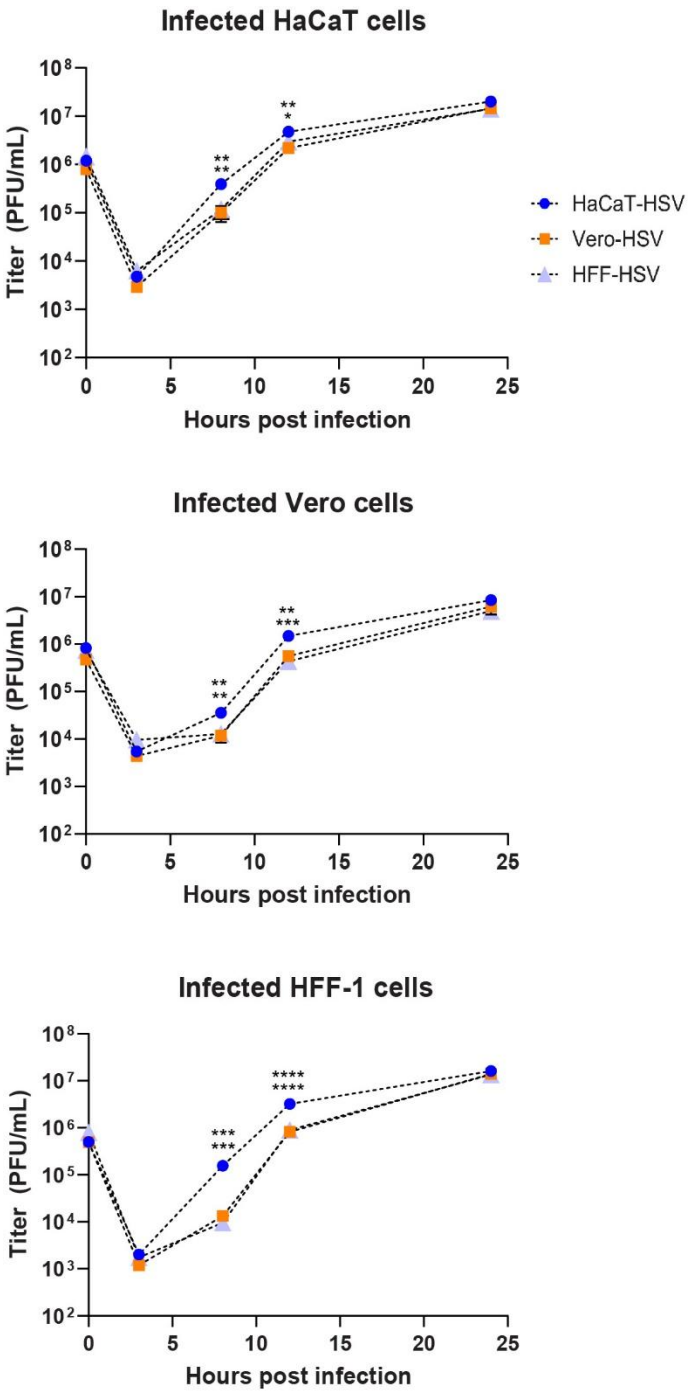


Figure 2. HaCaT-HSV produces higher viral titers early in infection. HaCaT, vero, and HFF-1 cells were infected with HSV-1 McKrae derived from HaCaT, vero, or HFF-1 cells at an MOI of 10. Virally infected cells and supernatant were harvested at corresponding time points after infection and titered using plaque assay on vero cells. The values represent means from three biological replicates per treatment. Error bars indicate the SEM. * indicates $p < 0.05$, **

indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p<0.0001$ using one-way ANOVA with Tukey's multiple comparisons test. Statistical comparison shows HaCaT-HSV relative to Vero-HSV (top) and HaCaT-HSV relative to HFF-HSV (bottom).

Producer cell-dependent effects on the sensitivity
of HSV-1 to Type I and Type II interferon

Cells express pathogen recognition receptors (PRRs) that are capable of recognizing pathogen associated molecular patterns (PAMPs), including those associated with HSV-1 virions or replication products such as double-stranded (ds) DNA, dsRNA, or glycoproteins (34–38). PRR recognition of viral PAMPs elicits secretion of interferons (IFN) (39). HSV-1 expresses several viral proteins that inhibit recognition of PAMPs and downstream signaling post-PAMP recognition, but HSV-1 PAMPs eventually trigger IFN secretion from infected cells (40–43). Over the course of viral spread, HSV-1 inevitably encounters cells that have entered an antiviral state through uptake of IFNs, including IFN-γ and IFN-β (44–47). IFN secretion limits viral replication and spread within hosts, and HSV-1 replication is severely dampened in cells pre-treated with type I and type II IFN (45,48–60). To test if the producer cell type influences the sensitivity of HSV-1 to IFN responses, we evaluated HSV-1 replication following pre-treatment of cells with IFN-β or IFN-γ. Target cells were pre-treated for 16 hours with 100 U/mL (HaCaT) or 1000 U/mL (HFF-1) of IFN-β or IFN- γ (Fig. 3) prior to infection with HSV-1 McKrae propagated from the previously identified cell types. In both target cell types, IFN-β had a greater inhibitory effect on viral replication based on output viral titer in comparison to IFN- γ. Interestingly, HaCaT-HSV maintained the ability to produce higher quantities of infectious virions in comparison to Vero-HSV and HFF-HSV when infecting HaCaT and HFF-1 cells pre-treated with each interferon. Vero-HSV and HFF-HSV did not produce a significantly different quantity of infectious virions from IFN-treated cells relative to one another. These results show

that HSV-1 produced from HaCaT cells is less sensitive to the antiviral effects of type I and II IFN in the cell types that were infected.

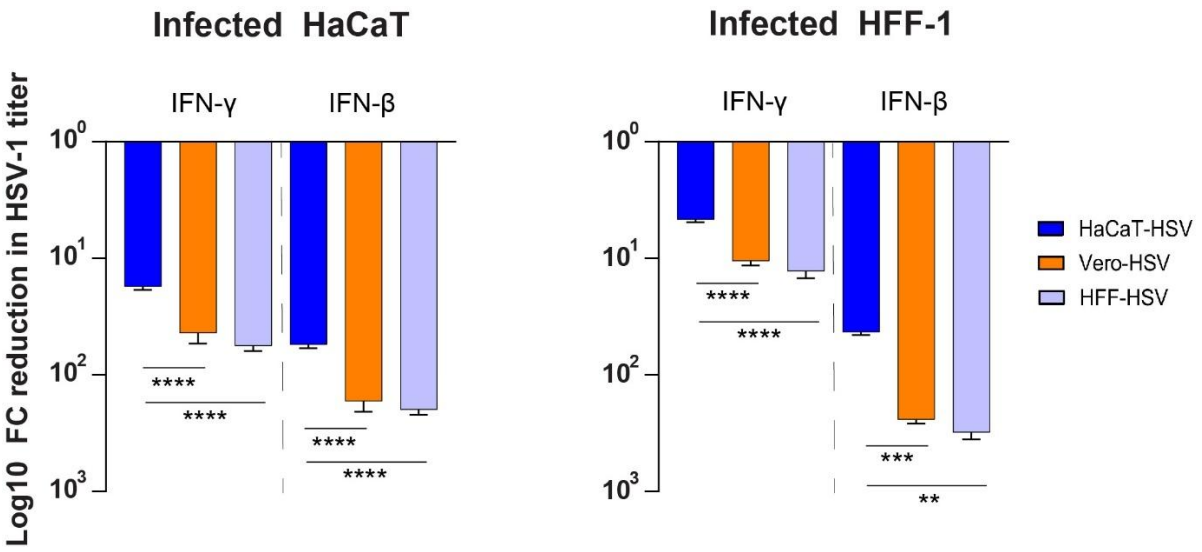


Figure 3. HaCaT-HSV replication is less sensitive to pre-treatment with type I and type II interferon. Cells were pre-treated with 100 U/mL (HaCaT) or 1000 U/mL (HFF-1) for 16 hours prior to infection with HSV-1 McKrae at an MOI of 10. Virally infected cells and supernatant were harvested 12 hpi and titered on vero cells. The values represent the mean reduction in viral titers across two experiments from three biological replicates relative to infected cells that did not receive IFN pre-treatment. Error bars indicate the SEM. Statistical comparison of the producer cell-dependent PFU/cell output between conditions used one-way ANOVA with Tukey's multiple comparisons test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p<0.0001$.

Producer cell-dependent effects
on the HSV-1 virion proteome

Our results have shown that HSV-1 replication changes based on the producer cell type of HSV-1 virions. HSV-1 expresses 84 viral proteins during infection that perform a wide array of functions critical to viral infectivity and replication (61). At least 44 of these proteins have been previously identified to be incorporated into mature HSV-1 virions (62–64). We hypothesized that the phenotypes we have observed are due to producer cell-dependent changes to the

proteome associated with HSV-1 virions. To test this hypothesis, HSV-1 McKrae was propagated from HaCaT, vero, and HFF-1 cells and extracellular virus was subjected to a modified version of a previously described purification protocol (64). Briefly, HSV-1 virions were separated away from cellular components using a combination of centrifugation, filtration, and DNase digestion prior to further purification of virions by ultracentrifugation on a 10% Ficoll cushion. Purified virions were then processed for liquid chromatography and tandem mass spectrometry (LC-MS/MS) of tryptic peptides using a Data Independent Analysis (DIA) workflow. Proteins associated with purified HSV-1 virions were identified and quantified to determine which viral and host cellular proteins are differentially detected between the different samples (Sup. Table 1). To determine if proteins were either virion-associated or contaminating proteins that co-purify with virions, mock infected cells were scraped and frozen at -80 °C. After thawing, the mock infected cell supernatants were subjected to the same purification scheme, and proteins that were detected from mock-infected cells were identified as potential contaminants and removed from further analysis (Sup. Table 3a-b). As an additional control, separate replicates of HaCaT-HSV virions were concentrated and digested with Proteinase K prior to Ficoll separation (65). Proteins that are not contained within lipid bilayers are accessible to Proteinase K digestion, resulting in a reduction in the detection of peptides. We considered many of these proteins, other than those known to be virion envelope proteins, to be either a likely contaminant or partial contaminant, meaning that fractions of the protein were both virion and non-virion associated (Sup. Table 2d).

LC-MS/MS identified several viral and cellular proteins that were differentially incorporated into HSV-1 virions based on the producer cell type. 56 total viral proteins were detected in HSV-1 McKrae. Three proteins that are not incorporated into the viral envelope,

thymidine kinase, pUL7, and pUL51 exhibited reduced detection following Proteinase K digestion (Sup. Table 2d) (64). These proteins were previously identified as tegument proteins, meaning that a subset of the protein was likely inside the virion whereas another portion was external to the virion envelope in our digested samples. A fourth non-membrane associated protein, pUL50 deoxyuridine triphosphatase, was also reduced by Proteinase K digestion, but has not been previously characterized as a tegument protein. All other Proteinase K accessible viral proteins were envelope proteins, with patterns of reduced detection in line with prior reports of viral membrane proteins (65). A quantitative comparison of viral proteins showed that 25 and 34 proteins were differentially incorporated into HFF-HSV and Vero-HSV, respectively, relative to HaCaT-HSV (Fig. 4, Sup. Table 2a-c). When applying a stricter threshold for defining differential protein incorporation (greater than 1 $\log_2$ fold-change with a $p<0.01$) 39 out of 55 proteins were found to be equivalently detected between HaCaT-HSV and HFF-HSV compared to 26 out of 56 proteins when comparing HaCaT-HSV to Vero-HSV (Fig. 5A). A total of seven viral proteins were detected in HaCaT-HSV and HFF-HSV that were not detected in Vero-HSV. Some of this lack of detection is likely due to the limits of mass spectrometry, including the limited detection of low molecular weight proteins (66). For example, neither VP26 nor pUs9 were detected in Vero-HSV, and each of these proteins exhibited relatively low or variable detectability between replicate samples in both the HaCaT-HSV and HFF-HSV samples. ICP8 was uniquely detected in Vero-HSV. To address the hypothesis that the virion proteome is contributing to the enhanced infectivity of HaCaT-HSV, we compiled a list of viral proteins that were incorporated into HaCaT-HSV at greater than 1 $\log_2$ fold-change with a $p<0.01$ in comparison to both Vero-HSV and HFF-HSV. These proteins included gG, gK, ICP0, and ICP10.

We were also interested in viral proteins that may inhibit viral replication. Viral proteins that were incorporated into Vero-HSV and HFF-HSV at greater than 1 $\log_2$ fold-change with a $p<0.01$ in comparison to HaCaT-HSV include pUL24 and pUL42.

A quantitative analysis of human cellular protein incorporation into virions was performed comparing incorporation into HaCaT-HSV relative to HFF-HSV (Sup. Table 2b). Comparison of cellular protein incorporation between HaCaT-HSV and Vero-HSV was determined to be unreliable due to protein heterogeneity between species (Sup. Table 2c). Across all samples and controls, 3,225 proteins were identified. A total of 1061 human proteins were detected in both mock infected and HSV-1 infected samples. 185 of these 1061 proteins were detected in greater quantities in viral samples compared to mock. Although some of the cellular proteins identified in mock samples were enriched in viral samples, we decided to treat these as contaminating proteins in the context of our data analysis. Removal of control detected proteins and low-quality detections left 1,880 cellular proteins uniquely detected in HSV-1 infected samples (Sup. Table 3a-c). Additionally, 344 human cellular proteins were accessible to proteinase k digestion in HaCaT-HSV, indicative of either their membrane topology or presence as an extra-virion contaminant (Sup. Table 2d). There were 620 cell proteins that were equivalently detected between HaCaT-HSV and HFF-HSV (Sup. Table 3h). HaCaT derived HSV-1 contained 163 unique proteins, in comparison to 127 proteins unique to HFF-HSV (Sup. Table 3d & 3f). An additional 365 proteins were enriched in HaCaT-HSV relative to HFF-HSV, compared to 283 proteins enriched in HFF-HSV relative to HaCaT-HSV (Sup. Table 3e & 3g). It is difficult to determine what the role of these proteins are in the context of their association with the virion. However, many of the virion-associated cellular proteins are affiliated with vesicular

transport, exocytosis, and metabolic processes among others. A summary of the differential detection of viral and cellular proteins based on the producer cell type of HSV-1 can be found in Fig. 5a and 5b.

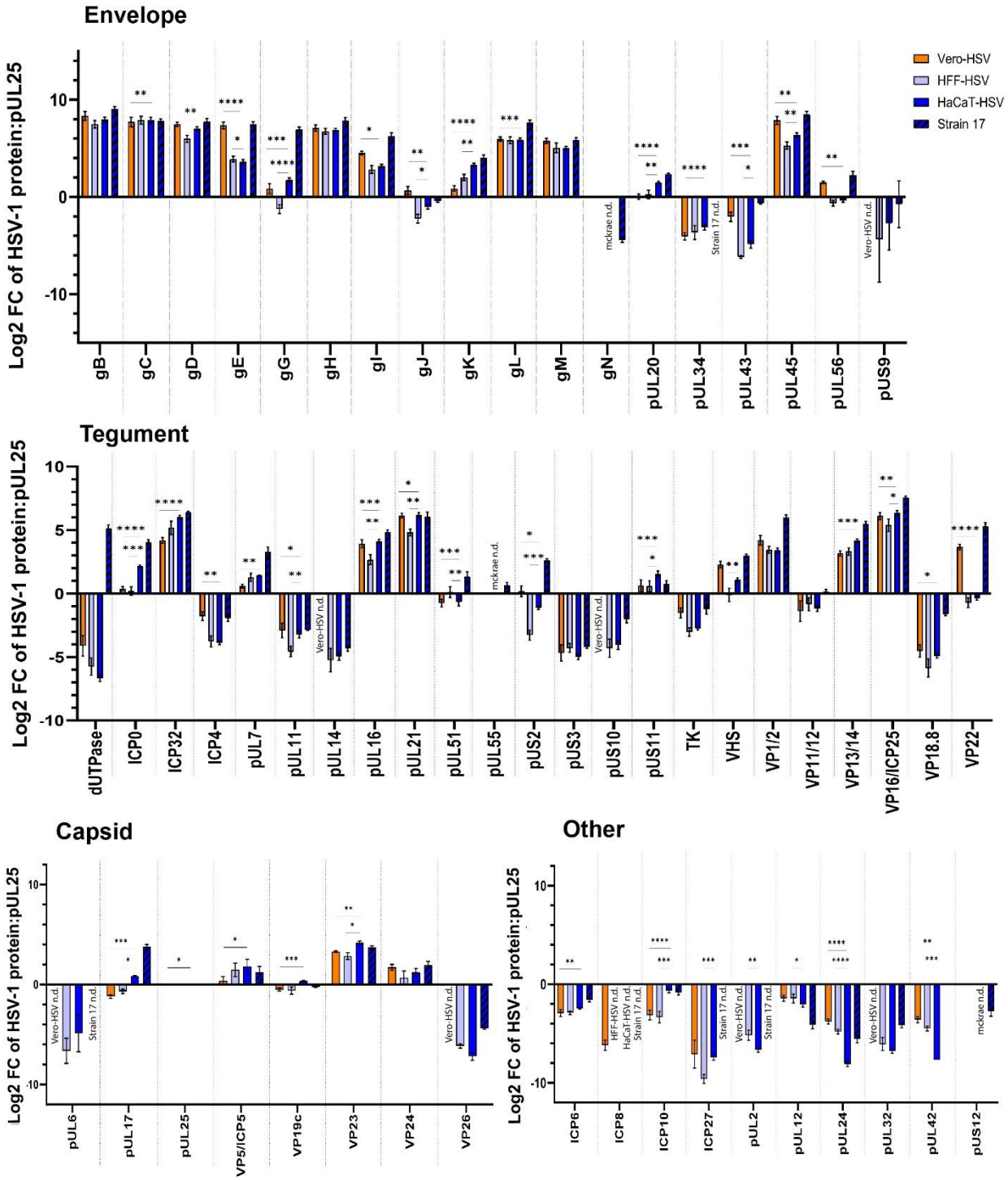
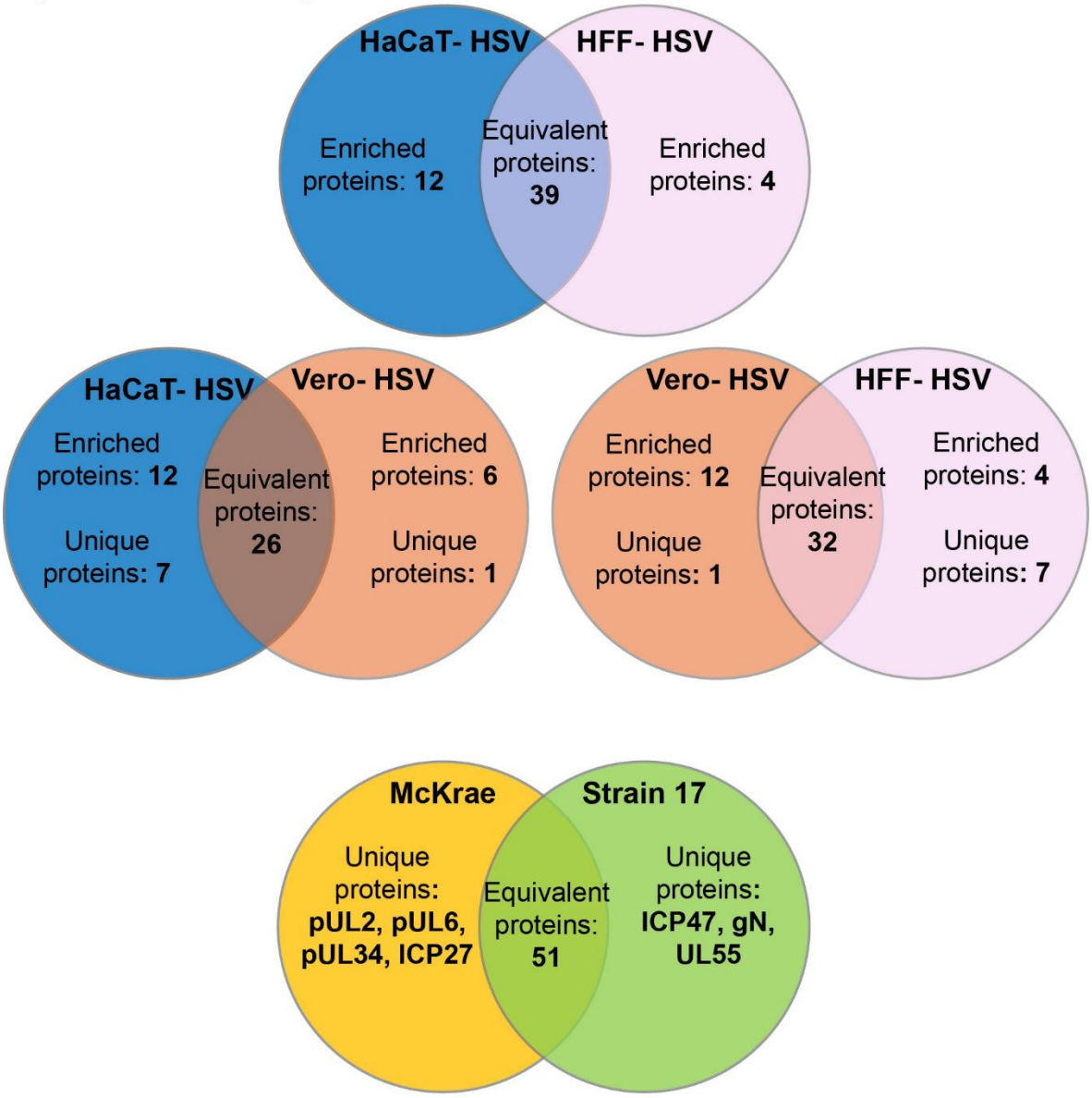


Figure 4. HSV-1 virions incorporate viral proteins in a producer cell and strain-dependent manner. HSV-1 virions were purified from vero and HFF-1 cells (McKrae) or HaCaT cells (McKrae and strain 17). LC-MS/MS was performed on purified virions using an Orbitrap 480

mass spectrometer and protein identification and quantification was based on data-independent acquisition. Relative protein abundance of each viral protein is expressed as the Log2 fold change (FC) relative to pUL25 detected in each treatment and organized based on the protein localization within the virion. Error bars indicate the SEM. n.d.: no detection. * Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p<0.0001$ using limma and eBayes.

A) HSV-1 viral proteins



B) Human cellular proteins

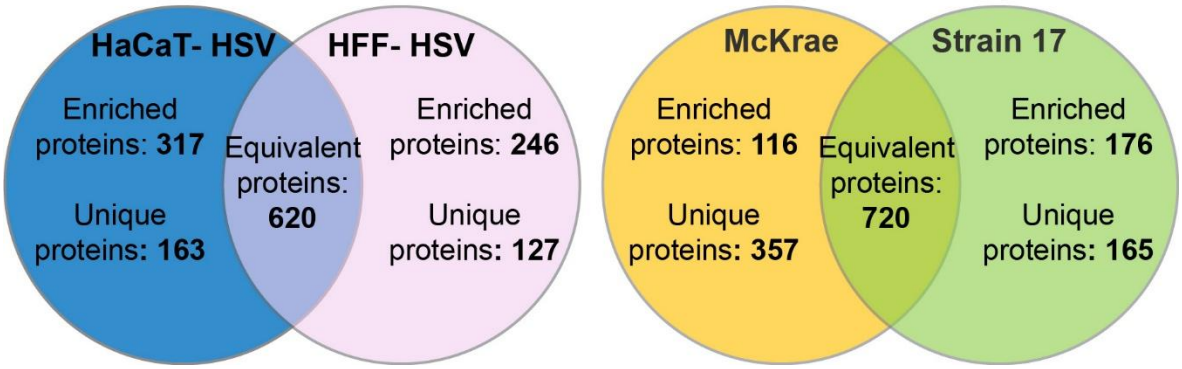


Figure 5. Summary of HSV-1 protein incorporation into virions. Venn diagrams show commonalities and differences in HSV-1 viral protein (A) and cellular protein (B) incorporation based on the producer cell type and strain.

Strain dependent effects on the HSV-1 virion proteome

Several previous studies have used LC-MS/MS to identify HSV-1 virion incorporated proteins. These studies have used different viral strains and producer cell types for virion production and analysis (Table 4) (62–64). Because the incorporation of viral and cellular proteins into HSV-1 virions often varies between studies, we isolated HSV-1 strain 17 from HaCaT cells to compare the proteome of HaCaT-derived McKrae and strain 17 virions.

Table 4. Detection of HSV-1 proteins in purified virions across studies.

		HSV-1 protein detection in this study		HSV-1 protein detection from previous studies		
HSV-1 Gene	**HSV-1 Protein**	**Strain McKrae (HaCaT, Vero, HFF-1)**	**Strain 17 (HaCaT)**	**Strain F (HeLa) (64)**	**Strain Sc16 (HaCaT) (62)**	**Strain 17+ (BHK-21) (63)**
Envelope						
UL1	gL	+[a]	+	+	+	+
UL10	gM	+	+	+	+	+
UL20		+[a]	+	WB	+	+
UL22	gH	+	+	+	+	+
UL27	gB	+	+	+	+	+
UL34		+[a]	-[b]	-	+	+
UL43		+[a]	+	-	-	x
UL44	gC	+[a]	+	+	+	+
UL45		+[a]	+	+	+	+
UL49.5	gN	-	+[b]	-	-	+
UL53	gK	+[a]	+	-	+	+
UL56		+[a, c]	+	+	+	+
US4	gG	+[a, c]	+	+	+	+
US5	gJ	+[a]	+	-	-	-
US6	gD	+[a,c]	+	+	+	+
US7	gI	+[a, c]	+	+	+	+
US8	gE	+[a, c]	+	+	+	+
US9		+[a, c]	+	+	-	+

Table 4 Continued

Capsid						
UL6		+[a]	-[b]	+	+	+
UL17		+[a]	+	+	+	+
UL18	VP23	+[a]	+	+	+	+
UL19	VP5/ICP5	+[a]	+	+	+	+
UL25		+[a]	+	+	+	+
UL26	VP24	+	+	+	+	+
UL35	VP26	+[a]	+	+	+	+
UL38	VP19c	+[a]	+	+	+	+
Tegument						
Rs1	ICP4	+[a]	+	+	+	+
RL1	ICP34.5	-	-	+	-	-
RL2	ICP0	+[a]	+	+	+	+
UL7		+[a]	+	+	+	+
UL11		+[a]	+	+	+	+
UL13	VP18.8	+[a]	+	+	+	+
UL14		+[a]	+	+	+	+
UL16		+[a]	+	+	+	+
UL21		+[a]	+	+	+	+
UL23	TK	+[c]	+	+	+	+
UL31		-	-	-	+	+
UL36	VP1/2	+	+	+	+	+
UL37	ICP32	+[a]	+	+	+	+
UL41	VHS	+[a]	+	+	+	+

Table 4 Continued

UL46	VP11/12	+	+	+	+	+
UL47	VP13/14	+[a]	+	+	+	+
UL48	VP16/ICP25	+[a, c]	+	+	+	+
UL49	VP22	+[a, c]	+	+	+	+
UL50	dUTPase	+	+	+	+	+
UL51		+[a]	+	+	+	+
UL55		-	+[b]	+	+	+
US1	ICP22	-	-	-	+	+
US2		+[a]	+	+	+	+
US3		+	+	+	+	+
US10		+[a]	+	+	+	+
US11		+[a]	+	-	+	+
Unknown						
UL2		+[a]	-[b]	-	+	+
UL3		-	-	-	-	+
UL4		-	-	-	-	-
UL5		-	-	-	-	+
UL8		-	-	-	-	-
UL9		-	-	-	-	-
UL12		+	+	-	-	+
UL15		-	-	-	-	-
UL24		+[a]	+	-	-	+
UL28	ICP18.5	-	-	-	-	-
UL29	ICP8	+[a]	-[b]	-	-	+
UL30		-	-	-	-	+

Table 4 Continued

UL32		+[a]	+	-	-	+
UL33		-	-	-	-	-
UL39	ICP6	+[a]	+	-	+	+
UL40	ICP10	+[a]	+	-	+	+
UL42		+[a]	-	-	-	+
UL52		-	-	-	-	+
UL54	ICP27	+[a]	-[b]	-	+	+
US12	ICP47	-	+[b]	-	-	+

Comparison of HSV-1 protein detection by mass spectrometry between our study and previous studies. +: Detected in purified HSV-1 virions, -: not detected in purified HSV-1 virions, a: producer cell-dependent incorporation of the HSV-1 McKrae viral protein incorporated into purified virions within the current study, b: strain-dependent effect on viral protein incorporated into purified virions within the current study, c: the viral protein was accessible to proteinase k digestion within the current study, WB: the viral protein was detected by Western blot but not by mass spectrometry, x: the viral gene was deleted from the HSV-1 genome in the study.

A quantitative comparison of HSV-1 McKrae and strain 17 viral protein incorporation was complicated by protein sequence heterogeneity between the two strains. However, LC-MS/MS analysis did reveal a subset of viral proteins that are uniquely incorporated into virions in a strain-dependent manner (Sup. Table 2e). In total, 55 and 54 viral proteins were detected in HSV-1 McKrae and strain 17 derived from HaCaT cells, respectively (Fig. 4). ICP47, gN, and pUL55 were each detected in strain 17 but were undetected in McKrae. Conversely, pUL2, pUL6, pUL34 and ICP27 were all detected in McKrae but not strain 17. Human cellular proteins were abundant in both strains. Strain 17 virions contained 1166 total cellular proteins whereas McKrae incorporated 1361 cellular proteins (Sup. Table 2e). 720 cellular proteins were equivalently detected between each strain, with 181 and 119 of those proteins enriched (greater than 1 $\log_2$ fold-change with a $p<0.05$) in strain 17 and McKrae, respectively. Even though each strain was derived from HaCaT cells, 165 unique proteins were detected in strain 17 compared to 357 unique proteins detected in McKrae. The results from our LC-MS/MS analysis show that both the producer cell-type and strain of HSV-1 influences the incorporation of both viral and cellular proteins into virions. A summary of the differential detection of viral and cellular proteins based on the strain can be found in Fig. 5a and 5b.

Discussion

The producer cell type can alter the infectivity, antigenicity, and cellular tropism of several viruses. Some of these phenotypes have been attributed to producer cell type-dependent changes to the macromolecular contents of the virions themselves. These changes in virion

content include altered protein glycosylation patterns, differential protein incorporation, distinct lipid compositions, and selection for genomic mutations. The goal of the work presented here was to determine whether cell type alters the capacity of the produced HSV-1 virions to initiate infection and replicate. The cell types that were selected for these experiments were relevant to HSV-1, including HaCaT and HFF-1 cells. In addition, vero cells were chosen due to their common use as a producer and experimental cell line for HSV-1. The replication capacity of virions from each producer cell type was assessed on multiple cell lines, as measured by viral protein production and the output of infectious virions. When comparing HaCaT-HSV, Vero-HSV, and HFF-HSV infectivity of cells, HSV-1 produced in HaCaT cells exhibited the most robust replication in target cells overall, including cells that had been pre-treated with type I and type II interferon. We hypothesized that the observed differences in HSV-1 replication in target cells are due to producer cell-dependent alterations to the proteome of HSV-1 virions. LC-MS/MS analysis revealed that virions from different producer cells contain unique and differing quantities of both viral and cellular proteins. In addition, HSV-1 McKrae and HSV-1 strain 17 derived from HaCaTs produce unique virion associated proteomes relative to one another. From the data provided by the presented experiments, we can conclude that the producer cell has a direct impact on both the complement of proteins associated with HSV-1 virions and the capacity of virions to replicate. Connecting the changes in the proteome to the producer cell-dependent effects on viral replication will require greater analysis of the individual factors that differ between the samples tested.

Critical to evaluating the effects of a producer cell on HSV-1 is the evaluation of viral protein incorporation. Several viral proteins were differentially detected in both a producer cell-

type and strain-dependent manner. 58 viral proteins were identified in purified virions of HSV-1, although one of these proteins, ICP8, was unique to Vero-HSV samples. Viral proteins that were detected at greater quantities (greater than 1 $\log_2$ fold-change with a $p<0.01$) in HaCaT-HSV included gG, gK, ICP10, and ICP0. The increased incorporation of ICP0 could explain some of the observed differences in viral replication. ICP0 has multiple activities that include activation of viral gene expression, protein degradation, inhibition of antiviral responses, cell cycle arrest, and inhibition of the DNA damage response (67–73). Knockout or mutations to ICP0 result in significantly less viral replication in cells that have been pre-treated with type I or type II interferon (73). In our experiments, HSV-1 derived from each cell type was sensitive to type I and type II interferon in both HaCaT and HFF-1 cells. However, the greater abundance of ICP0 in HaCaT-HSV may have contributed to the reduced sensitivity of HaCaT-HSV infections to interferon pre-treatment. Interestingly, deletion of ICP0 from HSV-1 results in either severely dampened or lack of replication in the cornea and trigeminal ganglia of mice. However, this replication is either partially or fully restored in IFN-α/βR$^{-/-}$ or IFN-α/β/γR$^{-/-}$ mice (72). Although differential incorporation of viral proteins into virions is not similar to the complete knockout of a viral gene, it does demonstrate the importance of these proteins *in vivo*, and it is possible that producer cell-dependent changes to protein incorporation impacts viral replication and spread *in vivo*. Further testing of the changes to viral protein incorporation will be required to understand if the altered capacity of HSV-1 infection that we have observed is related to the differential incorporation of any of these identified viral proteins.

An alternative interpretation of the cell type differences is that replication is hindered by inclusion of certain viral proteins by Vero-HSV and HFF-HSV. Viral proteins that were

incorporated into Vero-HSV and HFF-HSV at greater than 1 $\log_2$ fold-change with a $p<0.01$ in comparison to HaCaT-HSV include pUL24 and pUL42. pUL24 and pUL42 each serve many pro-viral roles within infected cells including nuclear remodeling, DNA replication, and the inhibition of antiviral responses (74–79). Interestingly, both proteins can bind the NF-κB p50 and p65 subunits and prevent the nuclear translocation of NF-κB (80–83). Although several antiviral roles have been attributed to NF-κB activation, HSV-1 subverts NF-κB function and instead uses it as a pro-viral factor in several cell types including keratinocytes (84–90). Regions of the HSV-1 genes that encode ICP0 and VP16 each contain κB binding sites, and NF-κB activation is needed for optimal viral gene expression early in infection (87,89,91). It is possible that the reduced capacity for productive replication by Vero-HSV and HFF-HSV relative to HaCaT-HSV is due to enhanced cytoplasmic sequestration of NF-κB by the pUL24 and pUL42 proteins that are incorporated into virions at greater abundance.

Evaluation of our LC-MS/MS results needs consideration of prior studies that have evaluated HSV-1 virion associated proteomes and the factors that complicate direct comparison. The differential incorporation of proteins by HSV-1 virions based on both the strain and producer cell type may explain why the proteins identified in purified HSV-1 virions often vary between studies (62–64) (Table 4). As previously mentioned, Yang & Courtney found that HSV-1 strain KOS purified from vero and HEp-2 cells contained ICP0 and ICP4, but not virions derived from BHK21 cells (11). However, subsequent studies that purified HSV-1 strain 17 or HSV-1 strain F from BHK21 cells were able to detect ICP0 and ICP4 associated with virions (63,64,92). These results allude to the possibility that producer cell-dependent effects on HSV-1 virions can change in a strain-dependent manner. Our analysis detected ICP0 and ICP4 in all treatments, although

both proteins were incorporated into virions at different quantities in a producer cell-dependent manner. However, several studies have relied on different methodologies for virion purification and mass spectrometry analysis which may result in changes to protein detection. In addition, mass spectrometers have become far more sensitive and therefore are more likely to detect peptides that may have been undetected in previous studies (93). Furthermore, it is possible that several of the proteins that have been identified within our LC-MS/MS analysis, as well as other analyses, are not actually associated with HSV-1 virions. Although our purification scheme utilizes several steps to isolate virions away from extra-virion material, our proteinase k-treated sample revealed that several proteins identified by LC-MS/MS were susceptible to digestion. While a subset of these proteins is likely associated with the virion membrane, it is unlikely that this is the case for all proteins that were digested. In addition, we detected several markers that are known to be associated with extracellular microvesicles such as exosomes.

Interpretation of the producer cell-dependent phenotypes must address changes in the complement of cellular proteins that are detected. Due to the large quantity of cellular proteins that were detected in purified virions, it is unclear as to whether these proteins are specifically incorporated into virions or other structures. Stegen et. al previously used siRNA knockdown experiments to determine that a subset of cellular proteins that are incorporated into HSV-1 are important for replication (94). However, several of these proteins were either not detected in our LC-MS/MS analysis or were incorporated at similar quantities between cell types. Several classes of proteins that were identified have been detected in several viruses, such as translation initiation factors, RNA binding proteins, and cytoskeletal proteins (9,25,62–64,94–103). Proteins that are known to be associated with exosomes such as tetraspanins, heat shock proteins, and

ESCRT proteins are commonly identified in purified virions by LC-MS/MS analysis of several viruses including HSV-1 (9,25,62–64,97,100,102,104). It is possible that extracellular vesicles commonly co-purify with HSV-1 virions, perhaps through aggregation, making it difficult to determine which proteins are truly virion-associated. Previous studies that explicitly isolate exosomes using density ultracentrifugation have not detected HSV-1 DNA within these bands (105,106). Despite the potential for contamination of our purified virions with extracellular vesicles, it is interesting that the contents of these vesicles may differ based on the producer cell type that is infected. If this is the case, extracellular vesicles excreted during HSV-1 infection may be contributing to the observed differences in HSV-1 replication in infected target cells. Alternatively, it is possible that these proteins that are commonly identified in both exosome and virion purifications are incorporated into HSV-1 virions. A previous study that relied on electron microscopy identified several exosome associated proteins within mature HSV-1 virions (107). These proteins were specific to exosomes secreted by neurons, none of which have been identified in any previous LC-MS/MS analysis of HSV-1 virions that are isolated from non-neuronal cells. An additional study using a human oligodendroglia cell line identified a fraction of infectious HSV-1 that was secreted in microvesicles (108). Other studies have determined that HSV-1 directly interacts with exocytosis machinery in non-neuronal cells, providing further evidence that HSV-1 utilizes the exocytosis pathway for egress and as a result, may be incorporating exosome proteins into virions (109). Indeed, further experiments are needed to validate incorporation of these proteins into virions or to determine whether virions are associated with extracellular vesicles.

An alternative, or perhaps complementary hypothesis as to why the producer cell type of HSV-1 alters the replication capacity of virions is that non-infectious particles produced from each cell line are altering replication. These non-infectious particles can include light particles (L-particles) and defective virions. L-particles produced by HSV-1 infected cells contain viral tegument proteins and an envelope but lack a viral capsid and genome (110). Previous work has determined that L-particles can function to antagonize immune responses and enhance viral replication of naked HSV-1 DNA (92,111,112). Inquiries into the nature of L-particles have found that the producer cell type can alter the proteomic contents of L-particles, as well as the ratio of L-particles to mature virions that are produced (11,112). The role of defective virions in HSV-1 infection requires further research, and a wide array of phenotypes have been observed when studying non-infectious virions in the context of infection for several viruses (29–32). These roles can include activating or antagonizing antiviral immunity, enhancement or inhibition of viral replication, and induction of cell death depending on the virus or even viral strain that was observed. Our analysis demonstrated that the HSV-1 genome:PFU ratio was lowest in HaCat-derived viral inoculum and that HFF-derived inoculum had the highest genome:PFU ratio. Further testing is needed to determine the role of non-infectious virions in the context of HSV-1 infection. However, inhibitory effects by non-infectious virions could contribute to the differences in HSV-1 replication when the inoculum is derived from different producer cells.

A critical question for these and other studies of HSV-1 virions is the relevance to disease. Studies that have looked at virion associated proteomes of HSV-1 have relied on many cell types, including vero, BHK-21, and HeLa among others, to propagate material for experiments. The use of different producer cell lines for the propagation of viruses can influence

experimental outcomes *in vitro* and *in vivo*. For example, the propagation of SARS-CoV-2 and Ross River virus from vero cells results in different outcomes of infection in animal models relative to virus that was propagated from more physiologically relevant cell lines (2,23). These differential outcomes of infection include altered immune responses, viral dissemination, and a reduction in replication. Future experiments are needed to determine whether the producer cell type of HSV-1 influences infection in animal models. Additionally, HSV-1 can infect many cell types in humans. The spread of HSV-1 primarily begins with replication in keratinocytes associated with the skin or oral mucosa prior to establishing latency in neurons of the peripheral nervous system. In healthy individuals, HSV-1 spread is generally restricted and is either asymptomatic or manifests as cold sores. However, HSV-1 exhibits a broad tropism in humans resulting in viral replication within tissues of the eye, liver, and central nervous system among others, and replication can even result in systemic spread in some patients (113–116). Understanding producer cell-dependent effects on HSV-1 may help us to understand viral spread within and between individuals as well as disease outcomes in patients with severe disease.

Methods

Cell culture and viruses

HaCaT, vero, and HFF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) (get actual units) at 37 °C in 5% CO_2. HSV-1 McKrae was propagated on HaCaT, vero, and HFF-1 cells in DMEM supplemented with 2% FBS and 1% pen/strep. All HSV-1 stocks were tittered by plaque assay on vero cells.

Fluorescence microscopy

Epifluorescence imaging was performed on a Nikon Ti-Eclipse inverted microscope (Nikon Instruments) equipped with a Spectra X LED excitation module (Lumencor) and fast-switching emission filter wheels (Prior Scientific). Fluorescence imaging used paired excitation/emission filters and dichroic mirrors for DAPI, green fluorescent protein (GFP) and tetramethylrhodamine (TRITC) filters (Chroma corp.). All brightfield images were acquired using phase-contrast configuration at 4x or 20x optical magnification. Imaging immunofluorescent stained samples were set to the same exposure and power when comparing treatments within the same sampling group as determined by cell type and staining time post infection.

Quantification of HSV-1 antigen

Cells were infected with HSV-1 McKrae using an MOI of 1 or 10. Cells were washed with DPBS 1hpi and replaced with DMEM supplemented with 2% FBS and 1% pen/strep. Cells were fixed at 3 or 6 hpi as described above. Immunofluorescent stains of HSV-1 antigen in infected cells were quantified for fluorescent intensity using Fiji ImageJ. One image from each biological replicate was selected for analysis at each time point. All of the cells within the image were selected as an independent region of interest (ROI). The mean gray value was measured for each ROI and background noise subtracted based on the mean gray value of mock infected cells.

Purification of HSV-1 virions

HSV-1 McKrae was purified from HaCaT, vero, and HFF-1 cells using a modified purification scheme that was previously described (Lippe). Cells were infected with HSV-1 McKrae at an MOI of 0.01. Inoculum was removed 1 hpi. Cells were washed with DPBS and replaced with serum-free DMEM. HSV-1 was propagated for 48 hrs (HaCaT) or 72 hrs (vero and HFF-1). Viral supernatants were harvested and centrifuged at 300 xg for 10 min. The supernatant was then filtered through a 0.45 μm filter. Virions were pelleted by centrifugation at 20,000 xg for 30 min at 4 °C. Virions were resuspended in PBS, sonicated, and incubated with 100 U/mL of DNAse I at 37 °C for 1 hr. Virions that received proteinase k treatment were incubated with 0.1 mg/mL of Proteinase K for 45 min at room temperature and subsequently treated with 2 mM of phenylmethylsulfonyl fluoride (PMSF). Samples were further diluted in DPBS, loaded onto a 10% ficoll cushion, and centrifuged at 26000 x RPM for 2 hrs at 4 °C on an SW41 Ti rotor. The pellets were resuspended in 100 uL of 0.75% NP-40 for 1 hr on ice. Purified HSV-1 was stored at -80 °C. This same protocol was used for HSV-1 strain 17 purified from HaCaT cells and mock-infected HaCaT, vero, and HFF-1 cells that were scraped and frozen at -80 °C prior to purification.

HSV-1 genome quantification using qPCR

HSV-1 genome was concentrated and isolated to form a standard curve for qPCR quantification of HSV-1 viral genomes from viral stocks. To do this, HSV-1 supernatants were harvested from HaCaT cells infected with HSV-1 McKrae at an MOI of 10 for 20 hrs. Supernatants were centrifuged at 300 xg for 10 min and filtered through a 0.45 μm filter. Virions were pelleted by centrifugation at 20,000 xg for 30 min at 4 °C. Virions were resuspended in PBS, sonicated, and incubated with 100 U/mL of DNAse I at 37 °C for 1 hr to remove

contaminating cellular DNA. HSV-1 genome was then isolated from digested virions using the Zymo Quick-DNA miniprep kit (cat# D3024). The HSV-1 viral genome concentration was calculated using nanodrop prior to building the standard curve for qPCR analysis.

HSV-1 virion-associated genome quantitation was calculated by comparing HSV-1 genome content to known standard curve values of HSV-1 genomic DNA. Prior to qPCR analysis, HSV-1 viral stock was treated with DNAse I and processed through the Zymo Quick-DNA miniprep kit as previously described. qPCR analysis was performed using PowerUp SYBR Green kit (Applied Biosystems, cat no. 4367659), targeting HSV-1 ICP27. 28S rRNA was targeted as quality control to ensure no cellular DNA was present in standard curve or sample DNA.

qPCR primers:

28S fwd 5'- GGG CCG AAA CGA TCT CAA CC −3'

28S rev 5'- GCC GGG CTT CTT ACC CAT T −3'

ICP27 fwd 5'- AGA CGC CTC GTC CGA CGG −3'

ICP27 rev 5'- AGG CGC GAC CAC ACA CTG T −3'

Fixation and immunofluorescent staining

Cells were washed with DPBS and fixed with ice-cold 50:50 methanol:acetone for 20 minutes at RT. Methanol:acetone was removed, dried, and cells were blocked with 2% bovine serum albumin (BSA) in DPBS for 10 min at RT. Cells were incubated with rabbit polyclonal HSV-1 anti-serum (1:500, Dako) diluted in antibody solution (1% BSA in DPBS) for 1 hr at RT. Cells were washed 3 x with DPBS and incubated with Donkey anti-Rabbit IgG Alexa Fluor 488

(2 drops/mL) diluted in antibody solution for 1 hr at RT. Cells were washed with DPBS and incubated with 1:5000 hoescht in DPBS for 10 min. Cells were washed 2 x with DPBS prior to fluorescent imaging.

Plaque assay

Cells were infected with HSV-1 McKrae at an MOI of 10. The inoculum was removed 1 hpi and cells were washed with DPBS and incubated in cell media consisting of DMEM supplemented with 2% FBS and 1% pen/strep at 37 °C in 5% CO_2. Cells and supernatants were harvested by scraping at corresponding time points. Samples were stored at -80 °C and sonicated after thawing for measurement of viral titers by plaque assay on vero cells. For experiments in which interferon pre-treatment was used, cells were treated with 100 U/mL (HaCaT) or 1000 U/mL (HFF-1) of interferon beta, gamma, or both. Interferon was removed from cells after 16 hours of incubation, washed with DPBS, and infected as described above.

Liquid chromatography-mass spectrometry

Total protein from each sample was reduced, alkylated, and digested using single-pot, solid-phase-enhanced sample preparation with sequencing grade modified porcine trypsin (Promega) (117). Tryptic peptides were trapped and eluted on 3.5um CSH C18 resin (Waters) (4mm x 75um) then separated by reverse phase XSelect CSH C18 2.5 um resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted at a flow rate of 0.300uL/min using a 60 min gradient from 98% Buffer A (0.1% formic acid, 0.5 % acetonitrile):2% Buffer B (0.1% formic acid, 99.9% acetonitrile) to 95:5 at 2.0 minutes to 80:20 at 39.0 minutes to 60:40 at 48.0 minutes to 10:90 at 49.0 minutes and hold

until 53.0 minutes and then equilibrated back to 98:2 at 53.1 minutes until 60 minutes. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 50 x 12 Th isolation windows at 15,000 resolution, normalized AGC target 500%, maximum injection time 40 ms) (118). A second DIA was acquired in a staggered window (12 Th) pattern with optimized window placements. Following data acquisition, data were searched using Spectronaut (Biognosys version 19.1) against the UniProt *Homo sapiens*, UniProt *Chlorocebus sabaeus,* UniProt HHV1 strain17, and NCBI HHV1 McKrae (April 2024) respectively, using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (119). The data was normalized using VSN and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (120–125). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

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CHAPTER FOUR

THE PRODUCER CELL TYPE OF HSV-1 ALTERS VIRAL GENE EXPRESSION OF MULTIPLE VIRAL STRAINS

The central dogma of molecular biology states that genetic information flows from DNA to RNA and finally to protein. This is a rule with few exceptions in nature, and HSV-1gene expression falls within the purview of central dogma (1). This means that HSV-1 mRNA is transcribed from the genes of the dsDNA viral genome, where they are then translated into functional proteins within the cytoplasm of infected cells. The data presented in chapter 3 led us to conclude that the producer cell type of HSV-1 alters the infectious properties of the virions that are produced. This was tested by quantifying viral protein production and the output of infectious virions from infected cells. The expression of viral proteins is required to form an infectious virion that can deliver the viral genome to new cells. While translation of viral proteins is vital to replication, this does not mean that producer cell-dependent changes to infection begin with the translation of viral proteins. The experiments in chapter 3 did not assess whether HSV-1 gene expression is altered in a producer cell-dependent manner. This is important, as viral gene expression is the first step in lytic replication once the genome has entered the nucleus of the cell. In addition, the enhanced translation of viral proteins by HaCaT-HSV relative to Vero-HSV and HFF-HSV may be directly related to enhanced transcription of viral genes. HSV-1 gene expression is tightly regulated and categorized into three stages: immediate-early (IE), early (E), and Late (L) gene expression (Fig. 4 Chapter 1) (2).

We hypothesize that HSV-1 gene expression is altered by the producer cell type. The aims of the experiments presented in chapter 4 are to determine whether the producer cell type of

HSV-1 alters aspects of HSV-1 gene expression by monitoring both transcription and late protein production. In addition, these experiments seek to address whether producer cell-dependent effects on HSV-1 replication is conserved across strains. The experiments in chapter 3 that tested whether the infectious properties of virions change in a producer cell-dependent manner were conducted using HSV-1 strain McKrae. The experiments in this chapter introduce an additional strain that is commonly used for HSV-1 research, strain 17. Both strains are commonly used laboratory strains. Genome sequencing of each strain has shown that there are 11 viral proteins that exhibit a less than 98% sequence conservation between the two strains (3). The divergence in the sequences of these 11 proteins have not been specifically linked to differences in viral infectivity. However, HSV-1 McKrae is generally associated with more severe disease in animal models compared to other strains including strain 17. Infection with HSV-1 Mckrae generally results in higher rates of reactivation from latency in neurons and induces high rates of lethal encephalitis (4–8).

Producer cell-dependent effects on the viral transcription of HSV-1 strain Mckrae

To determine whether HSV-1 gene expression kinetics change based on the producer cell type, target cells were infected with HSV-1 Mckrae derived from HaCaT, vero, or HFF-1 cells. qRT-PCR was used to quantify viral transcripts of ICP27 (an IE gene), ICP8 (an E gene), and VP5 (an L gene) relative to cellular 28s rRNA. Each of these genes are critical to HSV-1 replication. The IE protein ICP27 regulates both host and viral transcription. It can prevent 3’ end formation of many cellular genes, causing production of improperly terminated transcripts that accumulate in the nucleus (9–11). ICP27 also recruits RNA polymerase II to viral replication

sites, exports viral mRNA from the nucleus to the cytoplasm, and interacts with translation initiation factors to promote translation of viral transcripts (12–15). The E protein ICP8 is required for the formation of viral replication compartments in the nucleus and viral genome replication (16–18). The L protein VP5 is a major structural unit of the viral capsid. Capsids do not form without VP5, meaning the virus cannot produce infectious virions (19,20).

HaCaT-HSV produced significantly more ICP27 transcripts at 1 hpi (HaCaT and vero) and 3 hpi (vero only) relative to Vero-HSV and HFF-HSV (Fig. 1). At 3 hpi, VP5 transcripts were more abundant in HaCaTs infected with HaCaT-HSV, but not ICP27 or ICP8. However, all three of these genes were expressed at greater quantities by HaCaT-HSV in vero cells at 3 hpi. No significant difference in HSV-1 gene expression was observed in either vero or HaCaT cells by 5 hpi. These results show that the abundance of viral transcripts synthesized by HSV-1 McKrae is producer cell-dependent, and that the effect on transcription can depend on the infected cell type and the specific gene that is tested.

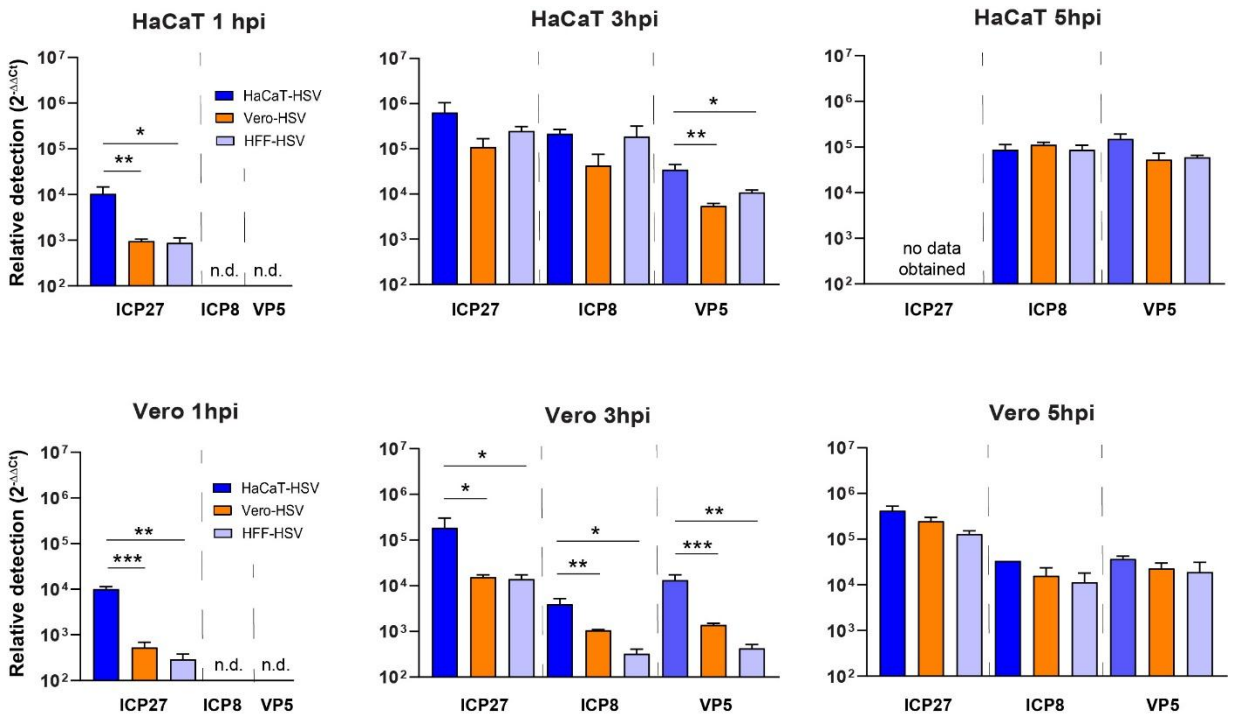


Figure 1. Producer cell-dependent effects on HSV-1 gene expression. HaCaT and vero cells were infected with HSV-1 McKrae at an MOI of 10. Infected cells were harvested at 1, 3, and 5 hpi for RT-qPCR of ICP27, ICP8, and VP5 transcripts. Relative quantification of RNA was performed using the ΔΔCt method. 28S rRNA was used as an endogenous control. qRT-PCR data are representative of three biological replicates with error bars representing SEM.

Producer cell-dependent effects on the viral gene expression of HSV-1 strain 17

To further elucidate whether HSV-1 gene expression kinetics change based on the producer cell type, cells were infected with a recombinant HSV-1 strain 17 (OK15). OK15 expresses eYFP under the regulation of a human cytomegalovirus promotor during HSV-1 IE gene expression. The kinetics of eYFP expression were previously shown to match the kinetics of immediate-early HSV-1 gene expression. In addition, OK15 expresses HSV-1 VP26 fused to an mCherry fluorophore during late gene expression. The expression of VP26-mCherry allows us to determine the timing of late gene expression and quantify the amount of VP26 that is

produced based on fluorescent intensity (21). VP26 is a structural protein involved in capsid assembly, packaging of viral genome into capsids, and capsid transport post-entry (22,23). Cells were infected with OK15 at an MOI of 1 or 10 and monitored for the timing and intensity of eYFP and VP26-mCherry expression using live time-lapse fluorescent microscopy.

When HaCaT cells were infected with an MOI of 10, HaCaT-OK15 exhibited greater fluorescent intensity of eYFP and VP26-mCherry throughout infection (Fig. 2a & 2b). This was consistent when comparing HaCaT-HSV OK15 protein expression in HaCaT cells at both MOI 1 and 10 relative to Vero-HSV and HFF-HSV (Table 5). This same trend was observed in vero cells with one exception. Vero-HSV produced a higher quantity of eYFP and VP26-mCherry in vero cells when infected at MOI 10 when compared to HaCaT-HSV. However, infected HFF-1 cells showed contrasting results to HaCaT cells and previous experiments. Infection of HFF-1 cells with HFF-HSV or Vero-HSV resulted in higher quantities of eYFP and VP26-mCherry production relative to HaCaT-HSV, with one exception, in which HaCaT-HSV produced more eYFP and VP26-mCherry compared to HFF-HSV at an MOI of 10. These results show that HSV-1 OK15 produced from HaCaT cells express eYFP and VP26-mCherry at greater quantities in HaCaT cells but that producer cell-dependent effects on eYFP and VP26-mCherry expression in vero or HFF-1 cells are condition dependent.

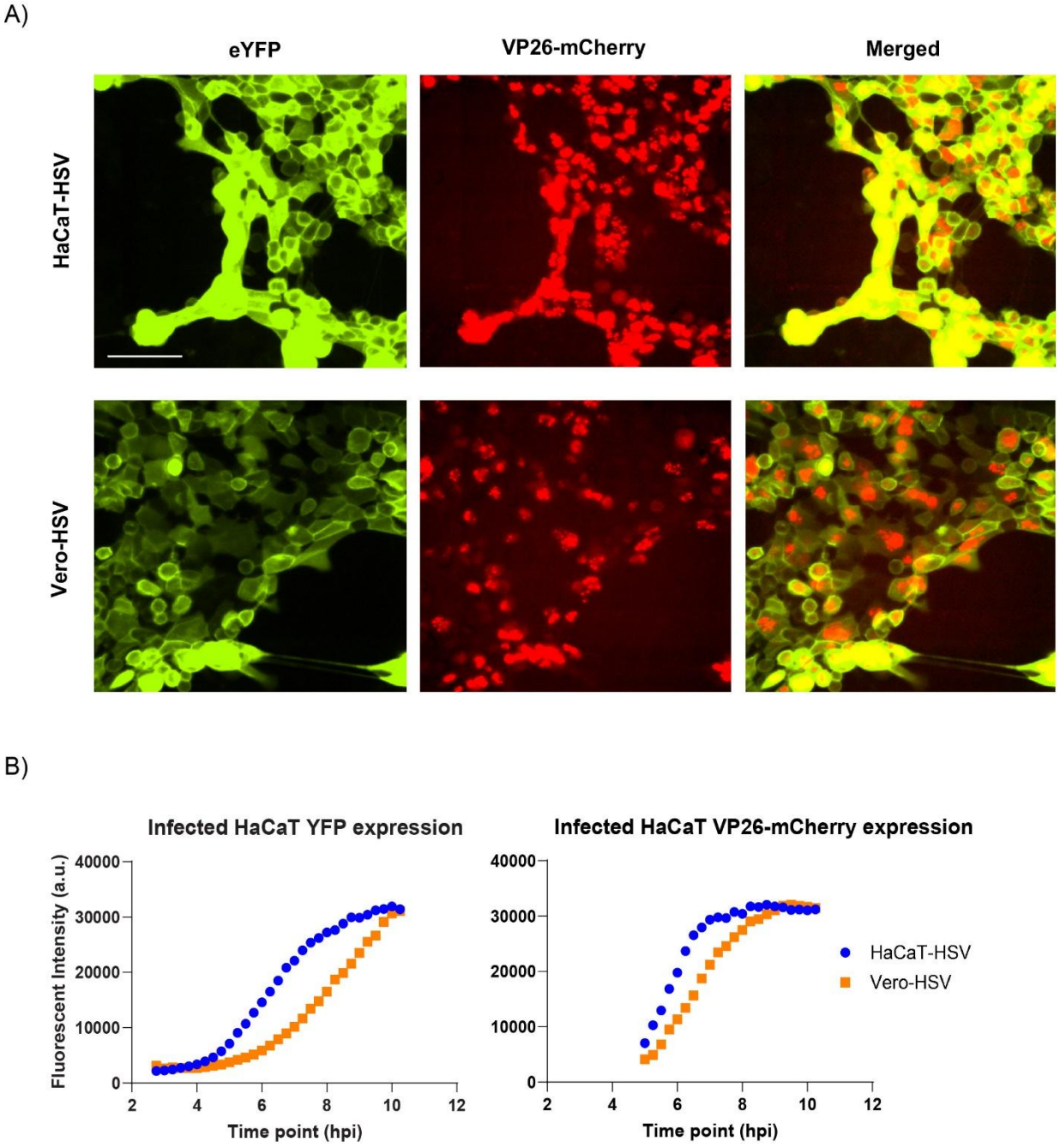


Figure 2. A dual reporter HSV-1 exhibits more robust protein expression in HaCaTs infected with HaCaT-derived virus. (A) HaCaT cells were infected at an MOI of 10 with a dual reporter HSV-1 (OK15) that expresses immediate-early YFP and VP26-mCherry. Images of HaCaTs infected with HSV-1 OK15 derived from HaCaT or vero cells at 4.5 hpi were acquired with equivalent exposure time and power and are represented with equal channel look-up table (LUT) parameters. Scale bar = 200 μm. (B) Quantification of fluorescent intensity of immediate-early

YFP and HSV-1 VP26-mCherry in HaCaTs infected with HSV-1 OK15 over time as described in A. Quantification of fluorescent intensity was performed using FIJI Image J after subtraction of background fluorescence. **** indicates $p < 0.0001$ using 2-way ANOVA with mixed effects analysis.

Table 5. Producer cell-dependent effects on HSV-1 OK15 immediate-early gene expression and VP26 protein production.

HSV-1 producer cell comparison	Infected cell type	MOI	Producer cell-HSV YFP expression over time	Producer cell-HSV VP26-mCherry over time
HaCaT, HFF-1	HaCaT	1	HaCaT > HFF-1 ****	HaCaT > HFF-1 ****
HaCaT, HFF-1	HaCaT	10	HaCaT > HFF-1 ****	HaCaT > HFF-1 ****
HaCaT, HFF-1	vero	1	HFF-1 > HaCaT **	HaCaT > HFF-1 ****
HaCaT, HFF-1	vero	10	HaCaT > HFF-1 ****	HaCaT > HFF-1 ****
HaCaT, HFF-1	HFF-1	1	HFF-1 > HaCaT ****	HFF > HaCaT ****
HaCaT, HFF-1	HFF-1	10	HaCaT > HFF-1 ****	HaCaT > HFF-1 ****
HaCaT, vero	HaCaT	1	HaCaT > vero ****	HaCaT > vero ****
HaCaT, vero	HaCaT	10	HaCaT > vero ****	HaCaT > vero ****
HaCaT, vero	vero	1	n.s.	HaCaT > vero ****
HaCaT, vero	vero	10	vero > HaCaT ****	vero > HaCaT ****
HaCaT, vero	HFF-1	1	n.s.	vero > HaCaT ****
HaCaT, vero	HFF-1	10	vero > HaCaT ****	vero > HaCaT ****

This table compares HSV-1 OK15 immediate-early YFP and VP26-mCherry expression over time when the virus is produced from HaCaT, vero, or HFF-1 cells. ** indicates $p < 0.01$, **** indicates $p < 0.0001$ using 2-way ANOVA with mixed effects analysis. n.s. means not significant.

Discussion

The data presented in this chapter shows that the producer cell type of HSV-1 strain McKrae and strain 17 alters viral gene expression. Strain McKrae derived from HaCaTs produced a higher quantity of viral transcripts depending on the infected cell type and the specific gene that was tested. This was shown to be true of the IE gene ICP27, E gene ICP8, and L gene VP5. HSV-1 strain 17 derived from HaCaTs generally exhibited more robust IE and L

gene expression, but this phenotype was dependent upon the infected cell type and the MOI used for infection. The results from these experiments bolster the results from chapter 3. Taken together, we have concluded that infection of cells by HaCaT-HSV will generally lead to a higher abundance of viral transcripts, proteins, and infections virions that are produced relative to Vero-HSV and HFF-HSV.

The analysis of producer cell-dependent gene expression of viral genes offered somewhat differing results when comparing HSV-1 strain McKrae and strain 17. In conditions where a producer cell-dependent effect on viral transcription was observed, HaCaT-HSV of the McKrae strain always produced a higher abundance of viral transcripts. This matches the trends observed in chapter 3 in which HaCaT-HSV consistently produced a higher quantity of viral proteins and infectious virions. Even though HSV-1 OK15 generally exhibited more robust gene expression within infected cells when derived from HaCaT cells, this was not consistently observed, particularly in HFF-1 cells. The differences in producer cell-dependent gene expression that were observed between the two strains may be caused by several factors. First, we relied on two different assays to monitor gene expression in each strain. Specifically, qRT-PCR was used to measure the abundance of specific viral genes when monitoring HSV-1 McKrae gene expression. The analysis of gene expression for HSV-1 OK15 was measured by quantifying the fluorescent intensity of reporter proteins expressed by the virus using live imaging. Second, it is possible that there are strain-dependent features that cause variability in producer cell-dependent effects on HSV-1 virions. Although the producer cell-dependent differences in the genome to PFU ratio of HSV-1 OK15 did not reach statistical significance as previously described for HSV-1 Mckrae, the HaCaT-OK15 produced the lowest genome to PFU ratio, matching the overall trend of the

HSV-1 Mckrae genome to PFU ratio (Table 6). As a result, it seems unlikely that the observed producer cell-dependent differences in replication between the two strains can be explained by the genome to PFU ratio. However, The LC-MS/MS results discussed in chapter 3 clearly indicate that viral and cellular proteins are differentially incorporated in a strain-dependent manner, which could mean that producer cell-dependent effects change based on the strain that is tested (Fig. 4 Chapter 3, Table 4 Chapter 3, Sup. Table 1). HSV-1 strain McKrae and strain 17 each contained hundreds of unique proteins when produced from HaCaTs. The differential incorporation of viral proteins was particularly notable. HSV-1 McKrae uniquely incorporated pUL2, pUL6, pUL34, and ICP27 whereas strain 17 uniquely incorporated ICP47, gN, and pUL55. As mentioned in the introduction, ICP27 is heavily involved with many aspects of viral gene expression. This makes ICP27 the most likely protein candidate that would impact viral gene expression in a strain-dependent manner, potentially leading to differences in producer cell-dependent replication. Regardless of the inconsistencies, these results show that producer cell-dependent effects alter the infectious properties of virions across two different strains of HSV-1.

Table 6. Producer cell-dependent effects on the HSV-1 genome to PFU ratio.

HSV-1 producer cell and strain	**Average genome:PFU ratio**	**HSV-1 infectious MOI when normalized to HaCaT-HSV genome content**
HaCaT-HSV McKrae	4.6 ± 0.8	10.0
Vero-HSV McKrae	14.4 ± 1.8 **	3.0
HFF-HSV McKrae	17.0 ± 3.4 **	3.0
HaCaT-HSV OK15	9.3 ± 3.1	10.0
Vero-HSV OK15	14.2 ± 4.8	7.0
HFF-HSV OK15	40.6 ± 14.9	2.0

The genome to PFU ratio of HSV-1 McKrae and OK15 derived from HaCaT, vero, or HFF-1 cells. Genome and PFU content was determined using qPCR to quantify genome content and plaque assay to quantify PFUs, respectively, with values being represented with ± the SEM. Analysis of the statistical significance was performed relative to HaCaT-HSV using unpaired t-test. ** indicates $P<0.01$.

Materials and methods

Fluorescence microscopy

Epifluorescence imaging was performed on a Nikon Ti-Eclipse inverted microscope (Nikon Instruments) equipped with a Spectra X LED excitation module (Lumencor) and fast-switching emission filter wheels (Prior Scientific). Fluorescence imaging used paired excitation/emission filters and dichroic mirrors for DAPI, yellow fluorescent protein (YFP), green fluorescent protein (GFP) and tetramethylrhodamine (TRITC) filters (Chroma corp.). All brightfield images were acquired using phase-contrast configuration at 4x or 20x optical magnification. Imaging of immunofluorescent stained samples were set to the same exposure and power when comparing treatments within the same sampling group as determined by cell type and staining time post infection.

Live imaging was performed on the microscope using a stage-top incubation system (Quorum Scientific). Cells were incubated at 37 °C in 5% CO_2. Images were acquired starting 1.5 hpi every 15 minutes. Exposure times were set to 100 ms (YFP) and 500 ms (TRITC) at 50% power.

Quantification of HSV-1 OK15 eYFP and VP26-mCherry fluorescence

Cells were infected with HSV-1 OK15 using an MOI of 1 or 10. Cells were washed with DPBS 1 hpi and replaced with DMEM supplemented with 2% FBS and 1% pen/strep Quantification of eYFP and mCherry fluorescence was quantified using Fiji ImageJ. Because HaCaT cells display significant cytopathic effect and movement throughout infection, ROIs were defined using a grid of cell-sized squares encompassing the entirety of the image. The onset and intensity of eYFP and mCherry fluorescence were determined using the max pixel intensity.

Values that were equivalent to background signal were excluded from the analysis. The background signal was subtracted from ROI values.

Quantification of viral transcripts by qRT-PCR

Cells that were infected with HSV-1 at an MOI of 10 were harvested in 0.4 mL of Trizol reagent and immediately stored at -20 °C. RNA was isolated and purified according to the Trizol manufacturer's protocol and resuspended in molecular biology-grade water. Isolated RNA was treated with DNAse to assure that sample were free of DNA contamination using the DNA-free Kit (Invitrogen AM1906) in accordance with the manufacturer's protocol. RNA concentration and quality were assessed by 260/280 nm absorbance using an ND1000 UV/Vis NanoDrop (S/N 6612). 1 µg of RNA was reverse-transcribed using M-MuLV Reverse Transcriptase (NEB MO253L) in accordance with the manufacturer's protocol. No RT control reactions were performed to ensure the absence of contaminating DNA in isolated RNA preparations. cDNA was stored at -20 °C. qPCR was performed using the Power Sybr Green kit following the manufacturer's protocol (appliedbiosystems 4367659). Primers targeted viral ICP27, ICP8, and VP5 and cellular 28S rRNA was targeted as an endogenous control.

qPCR primers:

28S fwd 5'- GGG CCG AAA CGA TCT CAA CC −3'

28S rev 5'- GCC GGG CTT CTT ACC CAT T −3'

ICP27 fwd 5'- AGA CGC CTC GTC CGA CGG −3'

ICP27 rev 5'- AGG CGC GAC CAC ACA CTG T −3'

ICP8 fwd 5'- CAT CAG CTG CTC CAC CTC GCG −3'

ICP8 rev 5’- GCA GTA CGT GGA CCA GGC GGT−3’

VP5 fwd 5’- TGG ATG GTA TGG TCC AGA TGC −3’

VP5 rev 5’- GCA CAA CGG CGC TGC TCT −3’

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CHAPTER FIVE

CONCLUSION

The producer cell type of several viruses, including HSV-1, alters the macromolecular composition and infectivity of the virions that are produced. It is important to delineate the impact that the producer cell type has on HSV-1 virions both scientifically and medically. The lack of a consensus cell line used to propagate HSV-1 could result in altered experimental outcomes and interpretations. This could also lead to problems with experimental reproducibility in the field. The effect that the producer cell type has on virions could be medically significant. HSV-1 can infect a wide array of tissues in the body. In some cases, this results in devastating sequalae including blindness, encephalitis, and systemic viral dissemination. Studying the effects of the producer cell type on HSV-1 macromolecular composition, infectivity and immune evasion may provide us with a better understanding of how the virus spreads within the body and between hosts. This could lead to the development of therapeutics to prevent and treat disease. The work outlined in this dissertation sought to determine whether the producer cell type of HSV-1 alters the infectious properties and macromolecular composition of the virions that are produced.

Chapter two reviewed the phenomenon of producer cell-dependent effects on viral macromolecular composition, infection and immune recognition. These effects have been observed for several viruses including those of the *Coronaviridae*, *Flaviviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Retroviridae*, and *Togaviridae*. Producer cell-dependent alterations to virions can include changes to the lipid composition of viral envelopes, glycosylation patterns of viral

envelope proteins, virion protein content, and the selection for genetic mutations. Some of these alterations have been linked to changes in cell tropism, immune recognition, and overall infectivity of virions. It can also alter the contents of viral inoculum beyond just macromolecular changes to the virion. The way in which the producer cell alters HSV-1 virion and inoculum content is not well studied, but previous work that was summarized in chapter 2 showed that the producer cell can alter both the HSV-1 virion and L-particle proteome, as well as the quantity of L-particles that are secreted from infected cells. However, these studies did not determine whether these producer cell-dependent changes result in changes to HSV-1 virion infectivity or immune recognition.

The experiments presented in chapter three sought to determine the extent to which the producer cell type alters the infectious capacity of HSV-1. This work was performed by propagating HSV-1 from HaCaT, vero, and HFF-1 cell lines. HaCaT and HFF-1 cells were selected as producer cells because they are human cell lines that are more representative of a cell type that HSV-1 would naturally infect. vero cells were selected because they are likely the most prevalent cell line used for the propagation of HSV-1 in research. The replication capacity of HSV-1 derived from each cell type was compared using fluorescence microscopy-based assays that measured viral protein production, as well as plaque assay to measure the output of infectious virions from infected target cells. The infection of target cells was performed at an MOI of 1 or 10 as determined by PFU/cell. The genome:PFU of HaCaT-HSV was significantly lower than NF-κB Vero-HSV and HFF-HSV, meaning that normalizing the MOI to genome/cell rather than PFU/cell would have resulted in far more infectious virions being applied to target cells when infecting with HaCaT-HSV. Previous work has shown that higher MOI infections by

HSV-1 result in enhanced replication of HSV-1. This means that infection of cells based on genome content rather than PFUs would have almost certainly exacerbated the replication phenotypes that were observed in Chapter 3. Overall, HaCaT-HSV produced a higher quantity of viral proteins and infectious virions. HFF-HSV generally produced the lowest quantity of viral proteins and an equivalent amount of infectious virions from infected target cells when compared to Vero-HSV. Interestingly, HaCaT-HSV was less sensitive to the pre-treatment of cells with type I and type II IFN, meaning it had a greater capacity to replicate in cells that have entered an antiviral state. Virions that were produced from each cell line were purified away from cellular material and analyzed for protein content using LC-MS/MS. Several viral proteins were differentially incorporated into HSV-1 virions, and over 100 cellular proteins were uniquely incorporated into either HaCaT-HSV or HFF-HSV.

The experiments in chapter 4 build upon the results from chapter 3. These experiments assessed whether the producer cell type of HSV-1 alters specific aspects of viral gene expression. This was tested on HSV-1 strain McKrae by measuring IE ICP27, E ICP8, and L VP5 transcription using qRT-PCR. Overall, a higher abundance of viral transcripts were produced by HaCaT-HSV relative to Vero-HSV and HFF-HSV at early time points in both vero and HaCaT cells. Viral gene expression was also assessed using the recombinant HSV-1 OK15, which served as a strain 17 based reporter virus for IE and L gene expression. Unlike experiments that relied on HSV-1 McKrae, producer cell-dependent effects on OK15 gene expression did not always result in HaCaT-OK15 eliciting the most robust expression in comparison to Vero-HSV and HFF-HSV. Instead, the producer cell-dependent effects on OK15 gene expression were partially dependent upon the infected cell type and the MOI of infection.

Future Studies

Further work is required to determine how the producer cell type of HSV-1 alters the infectious capacity of the virions. We hypothesize that the enhanced replication capacity of HaCaT-HSV relative to Vero-HSV and HFF-HSV was caused by producer cell-dependent alterations to the virion proteome. Indeed, LC-MS/MS analysis of HSV-1 virions resulted in detection of many viral and cellular proteins, and the protein quantity detected by the analysis was largely altered by the producer cell type. The methods used to purify HSV-1 in chapter 3 were derived from a previous study that was the first to characterize the HSV-1 virion proteome by mass spectrometry (1). These methods are meant to separate virions from cellular contaminants based on particle density, but follow-up experiments are needed to validate whether the proteins that were detected are truly virion-incorporated. With the shear quantity of proteins that were detected, the best way to do this would likely be to select protein candidates of interest and verify their incorporation into virions using immunogold labeling of proteins and visualization with electron microscopy. Upon validation of protein incorporation into virions, further experiments can be performed to determine whether these proteins alter the replication capacity of HSV-1.

Viral proteins that were detected at a significantly greater abundance in HaCaT-HSV relative to both Vero-HSV and HFF-HSV include gG, gK, ICP0, and ICP10. Research into gG and ICP10 has been scarce, and more insight into the function of these two proteins is needed to assess how their differential incorporation into virions may alter virion infectivity. gK has several functions upon expression within the cell, but virion-associated gK functions specifically in viral entry (2,3). Although there could be quicker entry kinetics for HaCaT-HSV due to a greater

incorporation of gK, the difference in entry kinetics is likely to be subtle and unlikely to explain the differences in virion replication capacity. ICP0 seems to be the best protein candidate for follow-up experiments to test our hypothesis. The differential incorporation of ICP0 verifies previous work showing that ICP0 is incorporated into virions in a producer cell-dependent manner (4). This protein has several pro-viral functions that include inhibition of antiviral responses and the ubiquitination of cellular proteins (5–8). The higher abundance of ICP0 may explain the dampened sensitivity of HaCaT-HSV replication to IFN pre-treatment, as mutations or deletion of ICP0 cause HSV-1 to become hypersensitive to IFN pre-treatment relative to wild type HSV-1 (9–12).

Vero-HSV and HFF-HSV each incorporated pUL24 and pUL42 at greater abundance relative to HaCaT-HSV. Both proteins bind the p50 and p65 subunits of the transcription factor NF-κBNF-κB and inhibit its translocation to the nucleus (13,14). Although NF-κB nuclear translocation can elicit anti-viral responses, some HSV-1 genes possess kB binding sequences that co-precipitate with NF-κB (15–21). NF-κB nuclear translocation has been found to enhance viral replication in epithelial cells (16,17). The higher quantity of pUL24 and pUL42 in Vero-HSV and HFF-HSV may be inhibiting NF-κB from nuclear translocation and causing dampened viral replication. A potential flaw in this hypothesis is that ICP0 also inhibits NF-κB nuclear translocation (5). The multi-functional nature of pUL24, pUL42, and ICP0 raises the question of whether these proteins perform all of their known functions upon viral entry, prior to the onset of their expression by the virus. Much of our knowledge about HSV-1 protein function is within the context of active viral gene expression or overexpression of a single viral protein in cells. It is possible that the limited amounts of these proteins that are brought into the cell by virions

narrows their functionality relative to what is observed during active expression of the protein. Ultimately, the role of these proteins as it relates to producer cell-dependent effects on viral replication needs to be assessed in infected cells that have yet to express these particular proteins. This will require several experiments, but the first experiments will likely need to use an immunoprecipitation assay combined with LC-MS/MS to determine which host proteins these viral proteins are directly interacting with after viral entry.

SiRNA knockdown and CRISPR knockouts can both be employed to study the effects of protein incorporation. These methods will be described further when cellular proteins are discussed later in this chapter. An alternative approach to consider in the study of differential incorporation of viral proteins would be to introduce synonymous mutations within codons of the viral protein of interest, known as codon pair deoptimization (22). This technique has been used to attenuate viruses with the goal of vaccine design (23–25). It results in rapid degradation of mRNA and decreased translation efficiency, which causes a reduction in protein production overall. It is believed that the attenuation of these viruses is likely caused by a decrease in viral protein incorporation into the virions due to a lower protein availability within the cell. Interestingly, this somewhat aligns with our hypothesis that the producer cell-dependent alterations to the infectious capacity of HSV-1 virions is being caused by variability in protein incorporation.

The viral proteins that have been discussed are enticing candidates to test our hypothesis. However, the cellular proteins may ultimately be the cause of producer cell-dependent alterations to virion replication. Before discussing differential incorporation of cellular proteins, it is worth noting that there were many commonalities in virion composition between HaCaT-HSV and

HFF-HSV. Analysis of cellular proteins by DAVID showed protein enrichment in several cellular pathways, regardless of the producer cell (table 7) (26,27). The reason as to why the virion may be incorporating proteins associated with specific pathways can be speculated based on how HSV-1 infects and replicates in cells. For example, the pathway that had the highest representation of cellular proteins in HSV-1 virions is the endocytic pathway. HSV-1 has two mechanisms of entry that are dependent upon the cell type in which the virus infects. One of these mechanisms of entry is endocytic uptake of the virion, and HSV-1 may be incorporating cellular proteins that are required for endocytosis (28).

Table 7. Human cell proteins that were incorporated into HSV-1 virions are associated with various cellular pathways.

	HaCaT-HSV		**HFF-HSV**	
Kegg pathway category	**Protein count**	**P-value**	**Protein count**	**P-value**
Endocytosis	32	1.0E-07	30	7.3E-07
Spliceosome	18	1.3E-02	11	4.4E-01
Nucleotide metabolism	15	1.4E-05	14	5.0E-05
Ubiquitin-mediated proteolysis	15	3.2E-03	15	2.6E-03
RNA degradation	13	1.1E-04	12	3.9E-04
Biosynthesis of cofactors	13	3.2E-02	14	1.2E-02
SNARE interactions in vesicular transport	11	7.5E-07	10	5.9E-05
AMPK signaling pathway	11	3.8E-02	11	3.3E-03

This table displays the quantity of proteins that are associated with specific cellular pathways that were detected by LC-MS/MS analysis of HSV-1 virions. Pathways containing less than 10 proteins that were identified in virions were excluded. The assignment of detected cellular proteins to pathways was performed using the DAVID bioinformatics software. Adjusted p-values were obtained using Fisher's Exact test with Benjamini correction and represent the probability that the identified proteins are associated with each specific pathway.

Alternatively, some of these endocytic proteins that were detected may be involved in the construction of the virion. Several HSV-1 glycoproteins are initially incorporated into the plasma membrane of the cell. These glycoproteins must be endocytosed from the plasma membrane to

reach the site of secondary envelopment and become virion associated (29–33). This applies to the viral glycoprotein B (gB), which contains a YTQV motif (29). YTQV motifs interact with the adaptor protein complex AP-2, resulting in endocytosis of the protein (34). To date, interaction between viral glycoproteins and AP-2 has not been shown to occur directly. However, the AP-2 complex was detected in our LC-MS/MS analysis of purified virions. It is possible that AP-2 incorporation is mediated by a direct interaction with viral glycoproteins that is required for their endocytic uptake and eventual assemblage onto virions.

Determining the role of cellular proteins that are incorporated into virions is particularly challenging, as many of these proteins are not well studied in the context of HSV-1 infection. This problem is not specific to HSV-1 but instead extends to most viruses that have been shown to incorporate cellular proteins. The first mass spectrometry-based analysis of HSV-1 virions was performed by Loret et. al in 2008. 49 cellular proteins were identified by this analysis, and a follow-up study confirmed that a subset of these proteins exhibit pro-viral functions (1,35). This follow-up study relied upon siRNA knockdown of cellular proteins to inhibit incorporation of those proteins into virions. While several of the proteins that we identified in our LC-MS/MS analysis were found to be important for viral replication, none of them were differentially incorporated in a producer cell-dependent manner with the exception of Rab11a. SiRNA knockdown could be an approach used to determine if differential incorporation of certain proteins, both viral and cellular, impacts viral replication, as siRNA knockdown does not typically knockdown 100% of a protein. This may result in reduced incorporation instead of non-incorporation. However, it would be beneficial to determine if incorporation of certain cellular proteins impact virion infectivity at all. To do this, a CRISPR knockout of that gene could be

used to prevent expression of the candidate protein. Virions harvested from the knockout cells would lack the protein, and replication of virions derived from knockout cells could be compared to replication of virions derived from wild type cells.

As mentioned, Rab11a has been previously shown to be important for the replication capacity of HSV-1 virions. Virions derived from Rab11a siRNA knockdown cells suffered a 50% reduction in the output of infectious virions from target cells (35). Rab11a was detected at a higher abundance in HaCaT-HSV relative to HFF-HSV, meaning that Rab11a is a protein of interest that should be validated and tested for producer cell-dependent alterations to virion replication. The protein is associated with trafficking of vesicles from the trans-golgi network to the plasma membrane (36). This protein also recruits motor proteins required for transport along actin and the microtubule network (37,38). Rab11a has been identified as a protein associated with secretory vesicles that contain Pseudorabies virus virions, an alphaherpesvirus commonly used to model HSV-1 infection, that egress from the infected cell (39). The association of Rab11a with alphaherpesvirus virions in various studies, combined with the fact that HSV-1 virions lacking Rab11a have a reduced replication capacity, indicates that Rab11a may be involved with more than virion exocytosis and could be important for establishing infection in cells.

Even though Rab11a was differentially incorporated in a producer cell-dependent manner, it was still detected in both HaCaT-HSV and HFF-HSV. Over 100 cellular proteins were uniquely incorporated into virions derived from each cell type. Contactin-1 (CNTN1) is a cellular protein that was in the top 10 most abundantly detected proteins unique to HaCaT-HSV in our LC-MS/MS analysis. This protein is associated with the cellular membrane and acts as an adhesion protein. The function of CNTN1 has been tested in cells infected with Influenza A virus

(40). During infection, CNTN1 induces degradation of the mitochondrial antiviral-signaling protein (MAVS). MAVS functions downstream of double-stranded RNA (dsRNA) detection by PRRs, and its activation leads to the production of type I IFN secretion and the induction of antiviral responses. dsRNA can be detected by PRRs during HSV-1 infection due to the formation of secondary structures by viral mRNA, leading to activation of MAVS and subsequent type I IFN signaling (41). Interestingly, CNTN1 was also shown to promote HSV-1 replication (40). Expression of CNTN1 resulted in a 6-fold increase in the transcription of the viral thymidine kinase (TK). Transcription of TK was the only method used to test HSV-1 replication in this study, but this result warrants further experiments to test whether CNTN1 is responsible for the increased infectious capacity of HaCaT-HSV.

Several target cells were used to compare the replication capacity of HSV-1 derived from each cell type. Although the results were consistent, future experiments are needed to determine whether the producer cell type alters the outcome of HSV-1 infection in peripheral neurons. Neuron infection is arguably the most critical aspect of HSV-1 infection in humans. Neurons are the only cell type in which HSV-1 can establish latency, and because HSV-1 establishes life-long latency within the trigeminal ganglion, the virus can persist within the body in the absence of active replication. Reactivation of the virus allows for HSV-1 to spread both within the body and between other people (42). Our lab cultures superior cervical ganglion (SCG) from mice to model HSV-1 infection in neurons. The mechanism by which HSV-1 establishes latency is currently unknown and under a great deal of scrutiny. It is possible that the producer cell type of HSV-1 impacts both lytic replication and the establishment of latency within neurons. Repeating

the experiments from chapter 3 in SCG neurons could provide critical insight into HSV-1 spread and disease.

If producer cell-dependent differences in lytic replication are observed in neurons, follow-up experiments would be warranted to determine why this is the case. For example, dampened lytic replication could be a result of changes to the efficiency of virion trafficking within neuronal axons. When HSV-1 infects neurons, virions must enter the neurons through axons and travel in a retrograde direction towards the nucleus (43). Changes in virion trafficking could be observed with live fluorescence microscopy of viral capsids that express VP26-mCherry. qPCR is an additional tool that could be used to verify enhanced or hindered virion trafficking. Pre-treatment of neurons with cycloheximide prevents HSV-1 protein expression and therefore prevents viral genome replication. Isolating the neuronal nuclei from the cell body would allow for quantification of viral genome in the nucleus and cell body, which could provide further evidence for alterations in virion trafficking in combination with fluorescent microscopy. Of course, enhanced or dampened lytic replication in neurons could be a result of producer cell-dependent alterations to virions that change the propensity of the virus to establish latency. This same qPCR assay would be needed to quantify the amount of viral genome in the nucleus of neurons that are latently infected. In addition, qRT-PCR would be required to quantify the levels of LAT relative to expression of viral lytic genes to parse out latency establishment from other phenotypes such as abortive infection.

It has been long established that the outcome of HSV-1 infection can be dependent upon the type of cell it infects. However, few studies have been performed that test how the producer cell type alters HSV-1. Continued research into producer cell-dependent effects on HSV-1

replication and spread is ultimately required to further our understanding of the virus. The alteration of viruses by the producer cell provides an interesting avenue for drug discovery. This research could further illuminate commonalities between viruses that replicate in similar tissues, leading to the development of broad-spectrum antivirals that target these commonalities.

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