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## First Impressions-the Potential of Altering Initial Host-Virus Interactions for Rational Design of Herpesvirus Vaccine Vectors

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### Abstract

**Purpose**—The earliest host-virus interactions occur during virus attachment and entry into cells. These initial steps in the virus lifecycle influence the outcome of infection beyond delivery of the viral genome into the cell. Herpesviruses alter host signaling pathways and processes during attachment and entry to facilitate virus infection and modulate innate immune responses. We suggest in this review that understanding these early signaling events may inform the rational design of therapeutic and prevention strategies for herpesvirus infection, as well as the engineering of viral vectors for immunotherapy purposes.

**Recent Findings**—Recent reports demonstrate that modulation of Herpes Simplex Virus Type-1 (HSV-1) entry results in unexpected enhancement of antiviral immune responses.

**Summary**—A variety of evidence suggests that herpesviruses promote specific cellular signaling responses that facilitate viral replication after binding to cell surfaces, as well as during virus entry. Of particular interest is the ability of the virus to alter innate immune responses through these cellular signaling events. Uncovering the underlying immune evasion strategies may lead to the design of live-attenuated vaccines that can generate robust and protective anti-viral immune responses against herpesviruses. These adjuvant properties may be extended to a variety of heterologous antigens expressed by herpesviral vectors.

### Keywords

HSV; Vaccine; herpesvirus; gK; herpesvirus vector; VC2

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#### Compliance with Ethics Guidelines

#### Conflict of Interest

Dr. Kousoulas declares a pending patent for vaccines against genital herpes simplex infections (United States Patent Application 20170266275 pending).

Paul J.F. Rider, Farhana Musarrat, Rafiq Nabil and Shan Naidu declare that they have no conflict of interest.

#### Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors

## Introduction

Herpes simplex viruses type-1 and type-2 (HSV-1; HSV-2) are responsible primarily for orofacial and genital infections, respectively. Importantly, HSV-1 is the leading viral cause of blindness in developed countries, and both HSV-1 and HSV-2 can cause severe fatal encephalopathies in adults and children (1). HSV-1 and HSV-2 seroprevalence is estimated at 67 percent (0–49 years of age) and at 11 percent (15–49 years of age) respectively, reflecting their ability to replicate and spread throughout the human population very efficiently (2, 3). Currently, despite many efforts over the last 20 years, there is no vaccine for HSV-1 or HSV-2, and drug-based inhibition of viral replication can often lead to the emergence of mutations that render these drugs ineffective (4, 5). As such, an HSV vaccine is desirable.

Approaches to the development of a vaccine(s) against herpes simplex viruses include subunit vaccines, as well as attenuated and replication-defective live viruses (6, 7). Currently, there are several ongoing clinical trials of subunit, DNA and replication-defective approaches to the development of a herpes simplex virus vaccine (7). Challenges include the limited efficacy of subunit vaccines, as well as the establishment of a latent infection by many attenuated and replication-defective vaccine strains. Most approaches to the generation of live virus vaccines have been to delete or mutate genes involved in important aspects of the viral life cycle, such as immune evasion genes ( $\gamma$ 34.5), or DNA replication genes (UL5, UL9, UL29). More recently, HSV vaccine candidates harboring deletions or mutations in virion envelope glycoproteins have exhibited exceptional promise and will be highlighted in this review.

The initial interaction of virus with the host cell initiates a cascade of events that determines the outcome of infection at both the cellular and organismal levels. While traditionally these signaling pathways have been viewed from an anti-viral perspective, it is now understood that they represent complex combinations of host anti-viral signaling pathways, as well as pathways that are initiated to benefit viral infection. Recognition of viral proteins by pattern recognition receptors (PRR) such as TLR-2 can result in signaling events that initiate anti-viral pathways resulting in induction of inflammatory and/or interferon responses (8–12). Additionally, fusion of the viral envelope with cellular plasma membranes during virus entry has been shown to lead to the induction of innate immune signaling (13). Importantly, the mechanisms by which herpes simplex viruses modulate cellular signaling processes during virus entry contribute to the establishment of latency in neurons, and the ability of the virus to successfully infect a variety of different cell types (14, 15).

Herpes viruses encode an array of gene products that have been shown to modulate pathways involved in innate and adaptive immunity, differentiation, cell division, apoptosis, autophagy and metabolism (1, 16, 17). For example HSV-1 infection has been shown to induce a biphasic activation of NF $\kappa$ B, where attachment (18) and entry as well the viral UL37 protein induce (19) activation of NF $\kappa$ B pathways initially. In this regard what has been described as the herpesvirus “signalosome” (20) includes both temporal and spatial control of cellular signaling pathways initiated by both host and virus. The outcome of this complex modulation has been shown to have profound impacts on virus entry, tropism,

establishment of either a lytic or latent states, as well as immunogenicity and pathogenesis (1, 20, 21).

Herein, we emphasize that an understanding of the molecular and cell biology of viral entry and associated intracellular signaling may inform the rational design of live-attenuated vaccine candidates. Utilization of these engineered viral vectors for the expression of heterologous antigens specified by other viral, bacterial, or parasitic pathogens may generate significant immune responses against these infections. Similarly, expression of tumor antigens may augment anti-tumor immune responses. Details of the signaling pathways affected during the early stages of viral replication are beyond the scope of this review, but are the subject of excellent recent reviews to which the reader is directed (20, 22). In this review, we examine specific pathways affected by virus infection and provide examples from recent work in the field highlighting underlying cellular signaling mechanisms that lead to significant enhancement of anti-viral immune responses. The review is primarily focused on the prototypic human alphaherpesvirus, herpes simplex virus type 1 (HSV-1) with selected relevant reference to other herpesvirus species where relevant. Overall, it is anticipated that the ideas discussed here may be readily applied to other viruses.

## Early events in Herpesvirus infection

Herpesviruses are among the largest and most complex virus families. Herpesvirus entry can occur by fusion and/or endocytosis and is a complex, coordinated process involving multiple virus-specified membrane proteins present in the virus envelope (23, 24). These viruses infect multiple cell types and dependent on cell type, infection can result in lytic, latent or abortive programs. The minimal membrane fusion complex for HSV includes four envelope glycoproteins: gB, gD, gH, and gL, which are necessary and sufficient to promote membrane fusion (25). Further appreciation for the complexity of the HSV fusion process is that the sole fusogenic glycoprotein gB, unlike other type III fusion proteins, is regulated by complex interactions with other viral glycoproteins and membrane proteins (26–31).

Glycoprotein D (gD) is the main receptor binding protein of HSV. Binding of the receptor by gD (discussed in greater detail below) alters its conformation. This gD conformational change is thought to change the conformation of the gH/gL heterodimer, which ultimately triggers a conformational change in gB from a non-fusogenic to a fusogenic state (23, 24). Gaps in current knowledge include available 3-dimensional structure of only the prefusion structure of gB, as well as lack of mechanistic and structural details of the interactions between gD-gH/gL or gH/gL and gB. HSV also enters a variety of cells via endocytosis (32–35). Discussed in greater detail below, the molecular mechanisms facilitating the endocytosis of HSV are beginning to be understood (35–38). While endocytosis has been demonstrated to be critical for the pathogenesis of cytomegalovirus (39–41), it is however not clear what contribution endocytosis makes to the pathogenesis of HSV infection.

## Signaling Pathways affected by Herpesvirus Glycoproteins

The most obvious opportunity for induction of intracellular events is via receptor-mediated cellular signaling. Multiple cellular receptors have been identified for HSV entry (23, 42).

HSV-1 gD binds multiple cellular receptors to facilitate entry including: Nectin-1, Nectin-2, HVEM, and 3'-O sulphated heparan sulphate (3-OS) (43–46). gB has also been shown to bind to PILR $\alpha$ , myelin associated glycoprotein (MAG) and non-muscle myosin heavy chain IIA (NMHCIIA) to facilitate entry (47–49). While it is important to keep in mind the possible contribution of alternate HSV receptors to the virus-host interaction, for purposes of this review, we will focus on HSV entry via Nectin-1 and HVEM. Nectin-1 and HVEM double knockout mice were not susceptible to infection with HSV-2 indicating these receptors are sufficient for viral infection of mice whereas other receptors may serve secondary functions during virus infection, leastways in mice (50).

The structures of HSV-1 gD bound to nectin-1 and HVEM have been determined (51, 52). Nectin-1 is considered the primary receptor for HSV entry. Intravaginal HSV-2 infection of nectin-1 deficient mice led to decrease in infectivity and spread to neural tissue (50). HSV-2 infection of CaSki cells in which nectin-1 was silenced using siRNA led to greatly reduced calcium release (53). HVEM, a member of the tumor necrosis family, is expressed on many tissues and cellular ligands for HVEM include CD160, BTLA, and LIGHT (54, 55). Dependent on which ligand is bound, HVEM ligation results in either a pro- or anti-inflammatory signaling (56). Binding of soluble gD to HVEM has been shown to activate NF $\kappa$ B pathways and this interaction has been exploited in subunit vaccine design (18, 57–59). Intravaginal infection with HSV-2 in mice deficient for HVEM expression does not alter pathogenesis or establishment of latency (50). However, more recent work has identified HVEM as an important determinant of ocular pathogenesis in experimental infections of mice with HSV-1 (60–62). Specifically, HVEM KO mice were less susceptible to ocular pathogenesis after ocular infection with HSV-1 (61).

While HSV gB has not been shown to bind integrins a number of herpesvirus gBs have been found to bind integrins (63, 64). Two groups have shown that HSV-1 gB binds to TLR-2 with one group demonstrating activation of TLR-2 after binding (11), while a second group did not report activation (65). PILR $\alpha$ , to which gB has been shown to bind, possesses an immunoreceptor tyrosine based inhibitory motif (ITIM) (66). Clustering of PILR $\alpha$  after ligand binding has been shown to negatively regulate neutrophil inflammation by *cis* regulation of  $\beta$ 2 integrin activation (67). gH is a membrane protein found as heterodimer with gL and is essential for herpesvirus fusion (1, 23, 24). No membrane fusion activity has been identified for gH/gL and its function in entry is not entirely clear. Recently gH/gL specified by different alphaherpesviruses have been found to bind to integrin receptors, and may function as tropism determinants (35, 68, 69). Several reports have demonstrated the role of calcium ion fluxes and HSV entry (38, 53, 70, 71). HSV-2 gH interaction with  $\alpha$ v $\beta$ 3 is responsible for calcium release from the endoplasmic reticulum (ER) and subsequent phosphorylation of focal adhesion kinase (FAK) during virus entry (69). In support of an important role for the gH-integrin interaction it was shown that siRNA knockdown of  $\alpha$ v $\beta$ 3 prevents HSV entry (69). Consistent with these results, work from the Campadelli-Fiume laboratory has demonstrated an important role for integrins in HSV infection (8, 10, 35, 72–74). They have proposed that these integrins serve as co-receptors. A mechanism includes the finding that  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8 integrins can promote a conformational change in gH that displaces gL to promote entry (72). Further evidence to support a role in HSV entry for these integrins includes data that they facilitate endocytosis of HSV in a variety of cell types (35).

For equine herpesvirus 1 (EHV-1), another alphaherpesvirus, it has been shown that binding of gH to  $\alpha 4\beta 1$  integrin induces calcium signaling and that abrogation of this pathway redirects entry to the endocytic pathway (38). Multiple groups have shown that gH-integrin complex formation requires the presence of other fusion complex members, such as gD and gB to function (10, 71), implying that either gD receptor binding, or fusion complex formation is important for subsequent gH-integrin complex formation.

A role for gH binding to  $\alpha v\beta 3$  integrin and activation of innate immune signaling pathways has been demonstrated (8, 10). gH was found to bind TLR-2 directly and simultaneous binding of  $\alpha v\beta 3$  creates a bridge for TLR-2 activation (73). Binding of gH to TLR-2 and  $\alpha v\beta 3$  was shown to activate inflammatory (NF $\kappa$ B), as well as type I IFN signaling pathways (8, 73). A second pathway induced by  $\alpha v\beta 3$  independent of TLR-2 was found to target interferon regulatory factors -3 and -7 (8).

As mentioned earlier, the process of membrane fusion between the HSV-1 viral envelope and cellular plasma membranes during virus entry induces antiviral signaling pathways (13). Induction of a type I interferon response by HSV virus-like particles (VLPs) was shown to depend on phosphatidylinositol-3-OH kinase (PI3K). Inhibition of PI3K, but not calcium release from the ER, blocked the induction of interferon stimulated gene (ISG) expression, placing PI3K activation upstream of calcium release in this process. Interestingly, the induction of ISG by fusion of the viral envelope with cellular plasma membranes was dependent on STING, typically thought of as a sensor of cytoplasmic DNA. Recently, it was also shown that prior to entry, HSV-2 induces an innate antiviral response at the epithelium by an unknown mechanism requiring viral O-linked glycans (75).

In addition to the demonstration that PI3K is important for the induction of the innate response to HSV fusion, several groups have shown that the PI3K pathway is important for HSV entry and cell-to-cell fusion (36, 70, 76, 77); reviewed in (78). It has been reported that PI3K activation is important for actin polymerization critical to virus entry into neuronal cells (79). PI3K inhibitors were shown to block RhoA, a cellular protein involved in cytoskeleton regulation after HSV infection of fibroblast cells (76). There is an emerging role for cytoskeletal dynamics and the sensing of intracellular pathogens (80). Specifically, aberrant RhoA activation has been shown to result in the activation of NOD 1 (81, 82). It is interesting to speculate that manipulation of the cellular cytoskeleton by HSV during entry is not only to facilitate viral infection, but also to alter global cellular host responses.

## Tropism

Herpesviruses have the capacity to infect a variety of cell types. It has been shown by a number of groups that herpesviruses encode distinct gene products that facilitate infection of different cell types (83–88). Interestingly, in those herpesviruses for which a tropism determining protein complex has been identified, gH/gL is the common denominator. HCMV, a betaherpesvirus, possesses distinct protein complexes which facilitate entrance into differing cell types. A protein complex of gB, gH/gL and gO mediates fusion in fibroblast cell types where a second protein complex, a pentamer, composed of gB, gH/gL, and UL128, 130 and 131 facilitates entry into epithelial and endothelial cells (89). A second



## Current HSV vaccine strains or HSV-derived vectors containing mutations expected to affect signaling pathways and tropism

### Glycoprotein D

The Herold group has pursued an HSV vaccine that is deleted for gD (HSV-2 gD<sup>-/+gD-1</sup>) (110–112). This vaccine protects rodents from lethal infection with both laboratory and clinical strains of HSV-1 or -2 (110–112). Produced in complementing cells that express HSV-1 gD, the vaccine is competent only for an initial entry event after which only virus particles which lack gD, and hence are non-infectious, will be produced. The HSV-2 gD<sup>-/+gD-1</sup> vaccine elicited antibody dependent cellular cytotoxicity (ADCC) as its major mechanism of action (112). How the absence of gD elicits a humoral response characterized by cytotoxicity and little neutralizing activity is an interesting question. The authors speculate that perhaps the gD-HVEM interaction is important in producing a humoral response dominated by neutralizing antibody and that when gD is absent after one round of replication, the ADCC response predominates. Alternatively, they suggest the presence of gD could mask viral antigens and prevent the development of protective antibody responses. Interestingly, similar to the HSV-2 gD<sup>-/+gD-1</sup> virus Royer et al. found that FcRn was important for vaccine candidate HSV-2 0 NLS efficacy as siRNA depletion of FcRn in mouse eyes abrogated protection upon corneal challenge (113). This group also identified a role for the complement system in mediating protection from ocular challenge. Specifically, vaccinated C3 deficient mice cleared virus more slowly after ocular challenge than vaccinated wild type mice (113).

The Cohen laboratory has generated an HSV-2 vaccine candidate, HSV2-gD27, which contains mutations that abrogate interaction between gD and Nectin-1, the primary receptor for entry into neurons (96, 114). As such, this virus should be unable to infect neurons and establish latent infection boosting its safety profile. The mutations in gD were found to be remarkably stable and HSV2-gD27 was attenuated in ocular, intracranial and intravaginal routes of infection (114). Importantly, HSV2-gD27 exhibited superior protection of mice after vaginal challenge as compared to mice vaccinated with a gD subunit vaccine (114). The authors describe, similar to HSV-2 gD<sup>-/+gD-1</sup>, that while more protective than the gD subunit vaccine, HSV2 gD-27 elicited low titers of neutralizing antibodies compared to mice vaccinated with gD subunit (114). While this is not entirely surprising given that HSV2 gD-27 may not be expected to select for many gD-nectin-1 neutralizing antibodies, it would be interesting to test the ADCC activity or determine if there is a similar dependency on FcRn or FC $\gamma$ RIII for HSV2 gD-27 efficacy as was shown for HSV-2 gD<sup>-/+gD-1</sup>. Finally, although infection of neurons via nectin-1 is considered the primary route of infection HSV2-gD27, as well as challenge viral DNA could be found in DRGs (114). Thus this virus would be expected to establish, maintain, and although not examined in this study perhaps reactivate from latency.

### Glycoprotein K(gK)

gK is a four transmembrane domain virion envelope protein with the amino and carboxyl termini located within the extracellular and intracellular spaces. gK functions in close association with the virally encoded UL20 membrane protein, which assumes a similar

membrane topography with the exception that the amino and carboxy termini are located intracellularly and extracellularly, respectively. Our working hypothesis supported by our published results suggests that the gK/UL20 heterodimer may regulate gB's fusogenicity through direct interactions with gB, as well as regulating additional functions carried out by gH/gL and gM. In this regard, it is important to note that syncytial mutations that cause extensive virus-induced cell fusion, a phenomenon thought to be highly similar to fusion between the viral envelope and cellular plasma membranes during virus entry, are predominantly located in the carboxyl terminus of gB and amino termini of gK and UL20 (115–125). We have shown that the amino terminus of gK binds to the amino terminus of gB, while the amino terminus of UL20 binds to the carboxyl terminus of gB. In addition, the gK/UL20 complex binds gH and gM glycoproteins. (94, 126–129).

Both gK and UL20 are highly conserved among all alphaherpesvirus species (87, 130). For other herpesvirus subfamilies (beta and gamma) multiple complexes mediating entry have been identified. HCMV, EBV, and HHV-6 have all been shown to use multiple distinct protein complexes to facilitate entry. As mentioned above, these complexes facilitate entry into differing cell types and mediate distinct signaling events. For HSV-1 this has not been shown. We have hypothesized that gK is component of a distinct alphaherpesvirus entry complex that facilitates virus entry and downstream cellular signaling. Primary evidence supporting a role for gK as a tropism determinant is our finding that deletion of 38 amino acids from the amino terminus of gK renders the virus incapable of entry into neurons via axonal termini (87).

VC2 is a recombinant HSV-1 (F)-strain in which the amino terminal 38 amino acids of gK have been deleted in combination with the 18 amino acids in the N-terminus of UL20 (131). We have shown that intramuscular immunization with VC2 protected mice from lethal intravaginal infection with either HSV-1 or HSV-2 (131). Additionally, VC2 is immunogenic in rhesus macaques and protects guinea pigs from a lethal infection with HSV-2 (submitted and (132)). The gK mutation renders the virus unable to enter into neurons via the axonal termini *in vitro* and no virus is present in trigeminal ganglia of vaccinated mice (131). This mutation also leads to the hyper-induction of proinflammatory pathways upon entry into fibroblasts or epithelial cells *in vitro*. In human corneal epithelial cells infected with wild type or virus with a deletion of amino acids 31–68 in gK, we found I $\kappa$ B $\alpha$  to be more rapidly degraded in cells infected with mutant virus compared to cells infected with parental virus (*Rider and Kousoulas*). Furthermore, we found that inflammatory mRNAs associated with induction of NF $\kappa$ B activity were more highly induced after infection with mutant virus compared to cells infected with parental virus (*Rider and Kousoulas*). The way in which this adjuvant-like activity of VC2 affects the development of a protective response to subsequent challenge with HSV is the subject of active investigation in our laboratory.

We have recently found that deletion of the first 38 amino acids of gK abolishes the ability of the virus to enter into cells via fusion of the viral envelope with cellular plasma membranes, while allowing the virus to enter via clathrin-mediated endocytosis (*Musarrat et al. manuscript submitted*). It is increasingly appreciated that distinct signaling pathways are activated by identical receptors via endosomes versus at the plasma membrane (133). These endosomal signaling events are important mediators of a variety of important



biological processes including innate and adaptive immunity (133–135). We have hypothesized that VC2 enhanced immunogenicity and elicitation of protective immune responses may be associated with the inability of the virus to enter via a fusion mechanism, and conversely, that forced entry through endocytosis may eliminate certain immune evasion strategies allowing the production of robust immune responses. A differential host response to vaccination by VC2 versus parental virus is reflected in the finding that ocular challenge with highly pathogenic strains of HSV-1 results in no ocular pathogenesis in VC2-vaccinated mice compared to mice vaccinated with parental virus (*Naidu et al., manuscript in preparation*). The mechanisms are currently under investigation in our laboratory.

## Conclusions and Future Directions

There has been a great deal of progress in the herpes simplex virus vaccine field (7). With recent FDA approval for the first oncolytic virotherapy (TVEC, Amgen, Inc), an HSV-1 derived virus, safety has been demonstrated for HSV-1 derived vectors. Despite significant progress in the understanding of protective immune responses against HSV infections, there are no available vaccines. A comprehensive analysis of the ways in which HSV vaccine candidates affect the herpesvirus “signalosome” as part of their efficacy is lacking. It is to be expected that mechanisms of protection such as ADCC and complement activation seen with HSV-2  $gD^{-/+gD-1}$  and HSV2-gD27 are influenced by early stages of virus-host cell interaction of a potential live-attenuated vaccine strain, as well as with the subsequent challenge with wild-type virus. Overall, the ways in which affected signaling pathways translate to protective innate or adaptive immune responses need to be elucidated. A greater understanding of the role that diverse receptor usage plays during infection and pathogenesis and how they affect downstream cell signaling and innate immune responses will lead to greater opportunities to engineer effective live-attenuated vaccines.

Precluding a complete analysis of HSV-1 signaling activity at attachment and entry is the dearth of information regarding infection of neurons. This information may be most clearly relevant to the rational design of vaccine strategies to abrogate infection of neurons and thus block the establishment or maintenance of latent infection. The difficulty of experimental approaches to the analysis of biologically relevant signaling events during entry into axons needs to be overcome before a complete picture can emerge. Significant progress regarding neuronal culture and single cell transcriptomics will help here. The predilection of HSV to enter into neurons and establish latency suggests that intracellular signaling events in neurons are of specific interest and may be more complicated than those elicited in other cell types. Ultimately, understanding of neuronal signaling in viral infection may shed light of how the virus prepares neurons for the establishment of latency and reactivation from latency, and the role of the immune system in these phenomena. Finally, initial encounter of virus in the host is by polarized epithelial cells which are expected to differ greatly from widely used tissue culture models of infection. A greater emphasis on established and using polarized epithelial models of infection for signaling and tissue culture studies may yield additional biologically meaningful data from which to rationalize future vaccine design.

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- Of major importance

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