ABSTRACT

Title of Document:	CREATING LIVE ATTENUATED HSV-2 VACCINE CANDIDATE STRAINS THROUGH CODON PAIR BIAS DEOPTIMIZATION
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Herpes simplex virus 2 (HSV-2) is the primary causative agent of genital herpes, a disease characterized by painful recurrent anogenital lesions, higher risks for HIV and other STD transmissions, and potential childbirth complications. Despite the large medical burden of HSV-2, no vaccines are currently available. Most HSV-2 vaccine candidates have been subunit vaccines due to safety concerns, but live attenuated vaccines (LAVs) should be considered. Codon pair bias (CPB) is the phenomenon that specific codons pairs appear more frequently than expected. CPB deoptimization (CPBD) has been shown to significantly attenuate polio, influenza, and *Streptococcus pneumoniae,* indicating that CPBD is a viable attenuation method and LAV strategy. However, CPBD has never been used to attenuate DNA viruses, so its ability to attenuate these viruses remains unknown. This thesis describes the usage of CPBD to deoptimize specific HSV-2 genes to create potential HSV-2 LAV candidates.

CREATING LIVE ATTENUATED HSV-2 VACCINE CANDIDATE STRAINS THROUGH CODON PAIR BIAS DEOPTIMIZATION

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2015

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Dedication

I dedicate this thesis to my mother, who has been my rock of support and love throughout my tumultuous journey through graduate school. She has both comforted me in times of doubt and sadness as well as cheered me on in times of excitement and accomplishment. Without her steady and steadying presence, I have no doubt this masters would still be a dream instead of reality.

Acknowledgements

A graduate degree, while only conferred on one person, is really done by a village of people: people who mentor you through long experiments, people who love and support you when experiments inevitably fail, people who push you when you get discouraged, and people who inspire you with love of exploring your field and asking hard, but fascinating questions. I have a whole village of people to thank for shaping me into the scientist I currently am and the educator that I wish to be.

First to thank are my lab families of the DeStefano and Cohen labs. Dr. DeStefano allowed me to work in his lab during a difficult time in my life, showing me patience and understanding when I most needed it. Dr. Irani Ferreira and soon-to-be Dr. Vasudevan Achuthan provided guidance with experiments as well as laughter, chocolate, and endless patience when I would blow off steam from TAing. Dr. Cohen took a chance on me when he had not had a graduate student before and entrusted me to build this project and make it my own, which I appreciate very much. Dr. Wang was my direct mentor in the Cohen lab and as such, taught me how to be a good scientist and also taught me a lot about myself. Dr. Culver and Dr. McIver so graciously accepted to be on my committee despite their busy schedules.

Of course, I must also thank everyone else who had a hand in developing this project. Dr. Mariam Quinones provided bioinformatics help with the bam file containing deep sequencing data of R519. Dr. Susanna Lamers and Dr. Ruchi Newman provided HSV-2 alignments. Dr. Steffan Mueller helped to develop SAVE and synthesized the CPBD *U_L30*, *U_L31*, and *U_L34*.

Next to thank are my family. To my mother who has been an irreplaceable bastion of support for me throughout this journey, thank you. To my father, who instilled in me a love of science and education from a very early age, who has helped me weather this storm with his humor, thank you. To my aunt Michelle and her wife Debbie who have been two of my loudest cheerleaders, thank you. To my goddaughter Emily, who has inspired me to be the very best model of what women can accomplish in the STEM fields so she will know that women can do anything, thank you. To my wonderful boyfriend, Matt, who has been so patient and supportive and loving throughout this sometimes joyful, sometimes painful experience, thank you. To all of my aunts, uncles, and cousins who have universally supported me in this endeavor, thank you. To my cat Sherlock, who tolerates my hugs even though he hates them and who has offered many cuddles when I was stressed writing this thesis, thank you. You all have showered me with love and support through this experience and even if I said thank you a thousand times, it would not be enough.

Last but certainly not least to thank are my friends, teachers, and all others who inspired me to pursue my dreams of being a scientist. To my high school English teacher Mrs. Juda who gave me my intense love of grammar and subsequent skill of editing, thank you. To the sisters of the Gamma Sigma chapter of Delta Delta Delta at Colgate University, who accepted me unconditionally and gave me the beautiful gift of true sisterhood, thank you. To Jen, Hilary, Gillian, and Jillian, who have brightened my life so much with their love and laughter, thank you; I would be truly lost with your friendship.

To my nature camp friends at the Eleanor Buck Wolf Nature Center – Danielle, Sarah, Claudia, Chris, Cassie, Tom, Sam, and Nate – who filled my summers with joy and excitement, thank you. To the Subbarao lab, especially Dr. Kanta Subbarao and Dr. Andrew Broadbent, who gave me my first lab experience and instilled in me a passionate love for influenza and vaccine development, thank you. To the lovely staff at the Ratchford Eye Center – Dr. Ratchford, Dr. Chin, Dr. Kjellen, Diane, Carrie, Pat, Lori, Maddie, Tatianna, and Jackie – who are such big supporters of mine, thank you.

To my friends in the BISI-MOCB program – in particular, Joe, Sarah, Randi, Prarthana, Bess, Carey, Sara, and Steve – and all my fellow TAs – in particular, Tiffany, Brian, Shahnawaz, Phil, and Gregg – in BSCI 223, who have been constants in a life that was chaotic at times, thank you. To the wonderful family I have met through Matt – Nicki, Ally, Sima, Carolyn, Joe, Andrew, and Zac – who enrich my life with hilarity and support, thank you. To Matt's friends who embraced me from the beginning – Steve, Gina, Dave, Courtney, Zac, Paul, Josh, Brian, Rachel, Megan, Michelle, and many others – who have made me feel so welcome and accepted, thank you.

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Section 1: HSV-2 Characteristics

Viral Family. Herpesviridae is a large viral family of linear double-stranded, nuclear-replicating DNA viruses that infect a wide variety of species that includes mollusks, fish, birds, and mammals (Akhtar and Shukla 2009; McAllister and Schleiss 2014). *Herpesviridae* is further divided into three subfamilies – *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* – all of which contain animal and human pathogens that cause various diseases (Akhtar and Shukla 2009; McAllister and Schleiss 2014). These viral subfamilies arose between 220-180 million years ago, allowing these viruses to carefully adapt to their specific host species and evade immune responses (Ellerman-Eriksen 2005; Grinde 2013).

Multiple herpesviruses pose significant medical problems for veterinary science and farming such as suid herpesvirus 1 or pseudorabies virus (PRV) and equid herpesvirus 1 (EHV-1) (Tischer and Osterrieder 2010). In pigs, PRV causes Aujeszky's disease, which is characterized by abortions, stillbirths, seizures, and fever (Pomeranz, Reynolds, and Hengartner 2005); the mortality rate for piglets is almost 100%, resulting in large financial losses for swine farmers (Pomeranz, Reynolds, and Hengartner 2005). EHV-1 can cause abortions in pregnant mares as well as neurological problems, which are costly for farmers and ranchers (Ma, Azab, and Osterrieder 2013). Live attenuated vaccines (LAVs) for PRV, bovine herpesvirus 1, and Marek's disease already exist, but vaccines for animal herpesviruses should continue to be developed to prevent economic loss (Dropulic and Cohen 2012).

Some herpesviruses, particularly gallid, equid, and primate herpesviruses, have been shown or are suspected to pose a zoonotic threat (Tischer and Osterrieder 2010). *Cercopithecine* herpesvirus 1 or Herpes B virus, which naturally infects macaques and presents the same symptomatic and viral lifecycle profile as herpes simplex virus type 1 (HSV-1) does in humans, has been classified as a bioterrorism agent due to its ability to cause severe encephalitis in infected patients (Tischer and Osterrieder 2010).

Currently nine human herpesviruses (HHV-1, 2, 3, 4, 5, 6a, 6b, 7, and 8) have been discovered (Suazo et al. 2015). These herpesviruses cause a wide range of diseases with different symptoms, such as the alphaherpesviruses herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), which cause oral and genital herpes, respectively (Grinde 2013); the gammaherpesvirus Epstein-Barr virus (EBV), which causes infectious mononucleosis and chronic active EBV (Grinde 2013); the alphaherpesvirus varicella-zoster virus (VZV), which causes chickenpox and shingles (Grinde 2013); and the betaherpesvirus cytomegalovirus (CMV), which causes birth defects including mental retardation and deafness (Grinde 2013). Thus, herpesviruses constitute a substantial disease burden that affects infants, adults, and the elderly, driving multiple vaccine efforts for these viruses.

Virion Structure. Herpesviruses have a large, linear dsDNA genome ranging from 124-230 kbp with a unique long (U_L) and unique short (U_S) region flanked by inverted repeats (Akhtar and Shukla 2009; Dolan et al. 1998; McAllister and Schleiss 2014). The 155 kbp HSV-2 viral genome is enclosed in the nucleocapsid, which is surrounded by the tegument, an area between the capsid and the envelope that contains many viral proteins important for initiating viral genome replication and inhibiting the host

immune responses (Figure 1) (Akhtar and Shukla 2009; Batterson and Roizman 1983; Ellerman-Eriksen 2005; Read and Frenkel 983; Zhou et al. 1999). The tegument is then surrounded by the envelope, which is studded with multiple glycoproteins that serve different functions (Akhtar and Shukla 2009; Ellerman-Eriksen 2005; McAllister and Schleiss 2014; Nicola and Strauss 2004). For example, gD mediates virion attachment to host cells, gB facilitates successful fusion of the viral envelope and the host cell membrane, and gH/gL activates gB by inducing conformational changes (Akhtar and Shukla 2009; Ellerman-Eriksen 2005; Nicola and Strauss 2004). These glycoproteins, specifically gD and gB, have been popular for use in subunit vaccines due to their exposure to the immune system and their essential functions to successful viral infection.

Viral Lifecycle. HSV-2 has a complex lifecycle that spans across two different types of susceptible host cells as gD has the ability to bind two different receptors, giving HSV-2 a dual tropism of both epithelial and neuronal cells (Figure 3). During primary infection, the virus lytically infects epithelial cells surrounding the genital mucosa via the attachment of gD to its receptor herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor family (Akhtar and Shukla 2009; Carfí et al. 2001; Connolly et al. 2005; Heldwein and Krummenacher 2008; Montgomery et al. 1996; Suazo et al. 2015). Receptor attachment prompts conformational changes to activate a complex of two other surface glycoproteins, gH/gL, which then induces conformational changes in gB to activate its fusogenic properties to successfully fuse the viral and host cell membranes (Akhtar and Shukla 2009; Coleman and Shukla 2013; Heldwein and Krummenacher 2008; Suazo et al. 2015). HSV-2 can also enter a host cell through the

endocytosis pathway (Akhtar and Shukla 2009; Clement et al. 2006; Coleman and Shukla 2013; Suazo et al. 2015).

Once inside the host cell, the tegument releases dozens of proteins that perform many different functions such as transport, immune evasion, apoptosis, and host cell translation inhibition (Coleman and Shukla 2013; Suazo et al. 2015). The capsid is transported to the nuclear membrane by the microtubule system via the tegument proteins VP1-2 and UL37 (Akhtar and Shukla 2009; McAllister and Schleiss 2014; Suazo et al. 2015). The capsid docks onto the nuclear pore complex (NPC) to inject the viral DNA into the nucleus so it can be transcribed by host cell RNA polymerase II (McAllister and Schleiss 2014; Suazo et al. 2015). Viral gene transcription is sequential in which the protein products of earlier transcribed genes initiates transcription of later genes (Suazo et al. 2015). Immediate early (IE) or alpha genes are transcribed first as they are important for immune evasion and decreasing host cell translation (Ellerman-Eriksen 2005; McAllister and Schleiss 2014; Suazo et al. 2015). Early (E) or beta genes are transcribed second as they are necessary for viral DNA replication (Ellerman-Eriksen 2005; McAllister and Schleiss 2014; Suazo et al. 2015). Early late (EL) or gamma-1 as well as Late (L) or gamma-2 genes are transcribed last as they encode virion capsid, tegument, and surface structural components (Ellerman-Eriksen 2005; McAllister and Schleiss 2014; Suazo et al. 2015).

Once capsid proteins are made, they migrate into the nucleus to enclose viral genomes and acquire various tegument proteins (Suazo et al. 2015). Capsids are enveloped by the nuclear inner membrane as they exit the nucleus, but almost immediately lose this envelope by fusing with the nuclear outer membrane (Suazo et

al. 2015). Therefore, the teguments are re-exposed upon the entry of the capsids into the cytoplasm to allow additional tegument protein acquisition before re-envelopment at the *trans*-Golgi network (Suazo et al. 2015). The mature virions are then released from the cell via vesicles where they can continue to lytically infect epithelial cells or infect neuronal cells to establish latency (Suazo et al. 2015).

The virus travels to neuronal cells innervating the area and infects them via nectin-1, a member of the immunoglobulin (Ig) superfamily (Connolly et al. 2005; Lopez et al. 2001; Spear, Eisenberg, and Cohen 2000). Once in the neurons, the virus travels up the axons to establish a state called latency in the nuclei of the cells of the dorsal root ganglia (DRG) (Chentoufi and BenMohamed 2012; Coleman and Shukla 2013; Grinde 2013; McAllister and Schleiss 2014). Latency is characterized by viral replication suppression mediated by low level transcription of pro-latency genes such as the latency-associated transcript (LAT) (Chentoufi and BenMohamed 2012; Egan et al. 2013). Unfortunately, not much is known about how the virus establishes or maintains latency or what factors control reactivation (Egan et al. 2013). However, it is assumed that both viral and host characteristics and interactions play a role in determining the duration of latency as well as the frequency of reactivation (Egan et al. 2013). Latency is also thought to be evolutionarily advantageous as it permanently shields the virus from the host immune system between outbreaks (Chentoufi and BenMohamed 2012; Egan et al. 2013; Ellerman-Eriksen 2005; Grinde 2013). Latency most definitely provides obstacles to both prophylactic and therapeutic HSV-2 vaccine development.

Periodically, triggers such as physical or chemical stress, immunosuppression, or UV light will reactivate the virus from its latent state in the neurons to initiate recurrent outbreaks of lesions (Chentoufi and BenMohamed 2012; Chentoufi et al. 2012; Coleman and Shukla 2013; McAllister and Schleiss 2014). The virus is also capable of spontaneous reactivation independent of triggers (Chentoufi and BenMohamed 2012). Genome replication and transcription of pro-lytic genes, viral protein production, and virion assembly occur in the cytoplasm of infected neuronal cells (McAllister and Schleiss 2014). Virions travel back down the axons into the surrounding epithelia to initiate viral replication (Coleman and Shukla 2013; McAllister and Schleiss 2014). Symptomatic outbreaks may occur from 4-15 times annually with lesions present for 3-12 days (Wald et al. 1997); HSV-2 can be isolated from these lesions 1-4 days after onset, giving a relatively wide window for transmission to sexual partners (Wald et al. 1997). Reactivation is a risky process for the virus considering lytic replication will induce a host immune response, but it does allow the virus opportunities to be transmitted to new hosts.

The HSV-2 lifecycle is not only confined to latency inspired by symptomatic outbreaks of lesions. Subclinical shedding is defined by frequent episodes of viable HSV-2 virions being “shed” or released into the genital mucosa without symptoms (Coleman and Shukla 2013; Egan et al. 2013; Mertz et al. 1992; Wald et al. 1997; Wald et al. 1995). Subclinical shedding significantly contributes to continued HSV-2 transmission because no identifiable familiar symptoms of a herpetic outbreak are present, which might convince individuals that they are HSV-2 negative and/or to engage in unprotected sex (Chentoufi and BenMohamed 2011; Coleman and Shukla

2013; Egan et al. 2013; Johnston, Koelle, and Wald 2011; Wald et al. 2000). Mathematical models have shown subclinical shedding episodes are frequent and overlapping with widespread bilateral shedding throughout the genital mucosa, but also short (Crespi et al. 2007; Mark et al. 2008; Schiffer et al. 2009; Tata et al. 2010); reactivations last a median of about 13 hours as the constant low leak of virus from the DRG is rapidly cleared by the host immune system (Crespi et al. 2007; Mark et al. 2008; Schiffer et al. 2009; Tata et al. 2010). Widespread bilateral shedding throughout the genital mucosa suggests that distinct reactivations from different ganglia are occurring simultaneously, but factors such as the titer of virus and persistence of HSV-2-specific CD8+ T cells in the mucosa determine which reactivations are cleared and which progress to lesions (Crespi et al. 2007; Tata et al. 2010; Zhu et al. 2007).

Wald and colleagues showed that subclinical viral shedding is quite common in that the median positive HSV-2 shedding day percentage was 24% with a range as wide as 0% to 75% (Wald et al. 1997); that is, the average HSV-2-positive woman sheds HSV-2 24 days out of 100 days, but some women may not shed at all and other may shed more than 75 days out of 100 days (Wald et al. 1997). The titers of HSV-2 shed also widely varied among women, with higher average titers seen when lesions were present as would be expected (Wald et al. 1997). On HSV-2-culture-positive days, the presence of lesions had no effect on the amount of HSV-2 DNA found in the mucosa, indicating that lesion-causing levels of HSV-2 DNA can be shed without any symptoms which would increase transmission risk on those days (Wald et al. 1997). Another study found that asymptomatic HSV-2 infected individuals shed virus on 10% of days,

showing that asymptomatic individuals are silent carriers that can still transmit the virus to individuals who may develop symptoms (Tronstein et al. 2011).

Section 2: HSV-2 Disease

Genital Herpes. Herpes simplex virus type 2 (HSV-2) is the primary causative agent of genital herpes (Wald et al. 1997). As described previously, HSV-2 is spread through intimate contact initially by lytically infecting epithelial cells in the genital region and then establishing latency in the DRG, thereby establishing a lifelong infection in the host (Akhtar and Shukla 2009; Suazo et al. 2015). The virus then periodically reactivates in the neurons, traveling down neuronal axons into the surrounding epithelial tissue to cause ulcers in symptomatic individuals (Akhtar and Shukla 2009; Suazo et al. 2015). These ulcers may present on the anal, penile, and labial tissues in different numbers and sizes (Wald et al. 1997). The severity, number, and duration of subsequent outbreaks in symptomatic individuals also vary, which can provide prognostic difficulties (Wald et al. 1997).

Unfortunately, multiple societal and viral characteristics contribute to the continued transmission of HSV-2. The societal stigma against herpes and the accompanying shame and distress prevents many symptomatic individuals from seeking treatment as well as informing their sexual partners (Dropulic and Cohen 2012; Green and Kocsis 1997). Both symptomatic and asymptomatic infected individuals frequently have subclinical shedding of the virus, meaning that these individuals can pass on their infection despite showing no symptoms (Johnston, Koelle, and Wald 2011; McAllister and Schleiss 2014; Mertz et al. 1992; Wald et al. 1997). Over 80% of infected individuals do not know they are infected and can transmit the virus to others

(Johnston, Koelle, and Wald 2011; McAllister and Schleiss 2014; Wald et al. 2000; Xu et al. 2002). Lack of symptoms in infected individuals allows the virus to potentially infect their sexual partners, contributing to the staggering 23 million new infections every year (Johnston, Koelle, and Wald 2011; McAllister and Schleiss 2014).

HSV-2 genital herpes affects about 536 million people worldwide, though cases of HSV-1 genital herpes are on the rise due to increased transmission from unprotected oral sex (Hofstetter, Rosenthal, and Stanberry 2014; Johnston, Koelle, and Wald 2011; Looker et al. 2008; Ryder et al. 2009). In the United States alone, about 20% of women and 40% of non-Hispanic blacks are HSV-2 seropositive, which underscores the high prevalence of HSV-2 infection in different subpopulations (Awasthi and Friedman 2014; Chentoufi and BenMohamed 2011). Many of these subpopulations, especially women, are also at higher risk for contracting HIV or other STIs such as human papillomavirus, which is associated with cervical cancer and genital warts, due to a number of factors (Abu-Raddad et al. 2008; Chentoufi and BenMohamed 2011; Milligan, Bernstein, and Bourne 1998). Such factors include but are not limited to location (Rajagopal et al. 2014; Kagee et al. 2011); socioeconomic status (Kagee et al. 2011); no or little access to healthcare and health education (Kagee et al. 2011; Kalichman and Simbayi 2004); inability to pay for such healthcare or transport to clinics due to being poor, uninsured, underinsured, or not insured at all (Kagee et al. 2011; Kagee, Le Roux, and Dick 2007); cultural or religious beliefs (Kagee et al. 2011); and gender roles, sexual expectations of women, relationship dynamics and power inequality, and domestic violence (Jewkes and Morrell 2010). Sub-Saharan Africa has staggering rates of HSV-2 infection – as much as over 80% of people over 35 in some

regions, especially in women and subpopulations at higher sexual risk – which continues to perpetuate the HIV/AIDS epidemic there (Johnston, Koelle, and Wald 2011; Paz-Bailey et al. 2007; Rajagopal et al. 2014; Smith and Robinson 2002). Despite HSV-2 itself not usually being life-threatening, being HSV-2 seropositive puts individuals – especially immunocompromised individuals such as newborns, HIV/AIDS patients, and patients on immunosuppressive drugs – at higher risk for both severe HSV disease and other infections that can be fatal (Akhtar and Shukla 2009; Chentoufi and BenMohamed 2011; Egan et al. 2013; Herget et al. 2005).

HSV-2/HIV Co-Infection. HSV-2 co-infection is highly correlated with human immunodeficiency virus (HIV) infections in both developing and developed countries (Awasthi and Friedman 2014; Rajagopal et al. 2014). Sub-Saharan Africa bears a disproportionate burden of HSV-HIV co-infection with up to 90% of HIV-positive people also testing HSV-2-positive (Abu-Raddad et al. 2008; Rajagopal et al. 2014).

HSV-2 lesion disruption of the genital mucosa and the frequency of subclinical shedding recruit HIV-susceptible immune cells such as CD4+ T cells to these areas and induce long-term persistence in the mucosa (Corey et al. 2004; Freeman et al. 2006; Johnston, Koelle, and Wald 2011; Koelle et al. 1998; Zhu et al. 2009). This long-term persistence of CD4+ T cells enables HIV to more successfully establish infection in these individuals, resulting in HSV-2 seropositive people having a 2-4-fold increase in HIV acquisition risk (Corey et al. 2004; Freeman et al. 2006; Johnston, Koelle, and Wald 2011; Zhu et al. 2009).

Conversely, HIV/AIDS increases the severity and duration of HSV-2 lesions, which again leave these individuals more susceptible to acquiring other STIs (Corey et

al. 2004; Freeman et al. 2006). Due to HIV/AIDS patients' decreased immunity and HSV-2's neurotropism, HSV-2 can infect their spinal cords to cause visceral disease (Britton et al. 1985). HSV-2/HIV-double-seropositive people also have a 2-5-fold increase in transmitting HIV to sexual partners compared to HIV-positive, HSV-2-negative individuals (Abu-Raddad et al. 2008; Gray et al. 2001; Latif et al. 1989). This increase in risk may be due to the fact that HSV-2 increases HIV transcription *in vitro*, leading to high levels of HIV RNA in genital lesions and plasma (Albrecht et al. 1989; Golden et al. 1992; Mole et al. 1997; Schacker et al. 1998; Schacker et al. 2002). HIV also increases HSV-2 mucosal shedding, thus increasing the transmission risk of HSV-2 to sexual partners (Augenbraun et al. 1995; Mbopi et al. 1999; Mostad et al. 2000).

Neonatal Herpes. Babies born to mothers with primary HSV-2 infection not controlled by antiviral therapy can develop disseminated neonatal herpes, which has a mortality rate as high as 85% if not treated (Gardella and Brown 2011; Kimberlin 2004; Whitley et al. 1980). Neonatal herpes is seen in approximately 1 out of 3200 live births, averaging about 1500 cases per year in the United States (Gardella and Brown 2011; Kimberlin 2004). The neonate can contract HSV-2 in three ways: *in utero*, which occurs about 5% of cases; peripartum, where the neonate contracts HSV-2 by its contact with herpetic lesions as it passes through the birth canal and vagina, which occurs in 85% of cases; and postnatal, which occurs in 10% of cases (Gardella and Brown 2011; Kimberlin 2004; Whitley et al. 1980). Disseminated HSV-2 infection usually is seen in 33-50% of all babies born with neonatal herpes (Gardella and Brown 2011; Kimberlin 2004); disseminated HSV-2 is characterized by encephalitis and vesicular rashes with death onset being due to liver and pulmonary dysfunction

(Gardella and Brown 2011; Kimberlin 2004). One third of babies with HSV-2 infection develop CNS disease, which presents with seizures, tremors, irritability, and temperature instability, with death onset being due to extensive brain damage (Gardella and Brown 2011; Kimberlin 2004).

Section 3: HSV-2 Immunology

A discussion of immunology is necessary for any vaccine development field because a vaccine candidate's success is almost completely based on its ability to induce an immune response that satisfies a certain endpoint such as preventing infection or disease. Gaps in knowledge about what immune responses must be elicited to confer immunity or eliminate disease symptoms obstructs successful vaccine creation, allowing continued transmission of the agent as well as maintaining or increasing its disease burden.

Innate Immunity. The innate immunity system is the first arm of the immune system engaged in response to a pathogen invasion. This first innate immune system response is required for the later engagement of the adaptive immune system if the innate immune system cannot eliminate the pathogen on its own (Iwasaki and Medzhitov 2015). This arm of the immune system responds very rapidly to a pathogen, but this response has relatively low pathogen specificity and stores no memory of any encountered pathogens (Iwasaki and Medzhitov 2015); therefore, subsequent reinfections caused by the same pathogen results in the same innate immune response without any maturation that might improve pathogen neutralization and clearance (Iwasaki and Medzhitov 2015).

Innate immune responses may be broken in the two major defenses of anatomical barriers such as skin and cytokine-cell interactions initiated by certain receptors that activate the many subpopulations of innate immune cells. Anatomical barriers such as skin obviously protect organs and blood from pathogens by providing a physical shield to prevent contact between them (Boyton and Openshaw 2002; Oranges, Dini, and Romanelli 2015; Ribet and Cossart 2015; Shin and Iwasaki 2013). Openings into the body are also usually protected by various secretions such as oral and vaginal mucus, ear wax, and saliva that can trap or degrade pathogens to prevent viable cells or virus from proceeding further into the body of the host (Boyton and Openshaw 2002; Ribet and Cossart 2015). However, skin cells themselves are hosts to a number of pathogens including HSV-2, so skin cannot provide a completely impenetrable barrier to all pathogens (Bonazzi and Cossart 2011). Also, pathogens can still bypass barriers by entering wounds or normal openings such as the mouth, nipples, tear ducts, nose, and ears (Boyton and Openshaw 2002; Oranges, Dini, and Romanelli 2015; Ribet and Cossart 2015). Some pathogens can evade or downregulate mucosal defenses in the nose, mouth, ears, vagina, and urethra (Boyton and Openshaw 2002; Ribet and Cossart 2015).

When physical barriers are breached, the cells of the innate immune system are engaged via cytokine production caused by pathogen material binding to various extracellular and intracellular receptors (Iwasaki and Medzhitov 2015). These extracellular and intracellular pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), various pathogen-only essential molecular structures such as peptidoglycan for bacteria, lipopolysaccharide (LPS) for

Gram-negative bacteria, triacyl and diacyl lipopeptides for mycobacteria, zymosan for fungi and yeast, and dsRNA and DNA:RNA hybrids for viruses (Iwasaki and Medzhitov 2015; Kawai and Akira 2010; Wu and Chen 2014). PPRs can be both displayed on the host cell membrane to bind to extracellular PAMPs and on membranes on organelles to bind to intracellular PAMPs (Iwasaki and Medzhitov 2015; Kawai and Akira 2010). Considering PAMPs are exclusive to pathogens, there is little if any possibility for misidentification of host or self proteins as non-self PAMPs unlike in adaptive immunity (Iwasaki and Medzhitov 2015; Kawai and Akira 2010).

Once PRRs are activated, they stimulate cytokine production of interferons (IFNs), tumor necrosis factors (TNFs), interleukins (ILs), and colony-stimulating factors (CSFs) (Iwasaki and Medzhitov 2015; Kawai and Akira 2006; Uyangaa, Patil, and Eo 2014). These many cytokines then cause an array of downstream effects such as immune cell maturation, or stimulation of immune cells into an more active state which may include heightened cytokine production, phagocytic activity, or activation of other immune cells; differences in secretion levels of hormones or cytokines to activate or deactivate immune cells; differences in surface membrane receptor composition and number to increase or decrease ability to interact with other immune or infected host cells; and heightened ability for migration out of blood vessels to wound sites (Brinkman et al. 2004; Iwasaki and Medzhitov 2015; Hermesh et al. 2010; Kawai and Akira 2006; Thomas; Uyangaa, Patil, and Eo 2014). Some of these cytokines are responsible for activating professional antigen-presenting cells (APCs), which then can engage the adaptive immune system by “presenting” antigen, or

antibody-generating molecules, to T cells (Iwasaki and Medzhitov 2015; Uyangaa, Patil, and Eo 2014).

There are both membrane-bound and cytosolic PRRs that recognize PAMPs and activate signal cascades to create anti-pathogen cell states and alert other components of the immune system. Toll-like receptors (TLRs), one type of membrane-bound PRR expressed on most phagocytic innate immune cells, are particularly important to innate immune system activation as they can sense a wide variety of pathogens and activate multiple innate immune defenses (Iwasaki and Medzhitov 2015; Kawai and Akira 2006; Kawai and Akira 2010; Uyangaa, Patil, and Eo 2014). Once TLRs bind to their pathogen ligand, they initiate signal cascades that activate the NFκB and MAPK pathways (Uyangaa, Patil, and Eo 2014). NFκB is a widely acting transcription factor that helps initiates transcription for many immune response genes such as iNOS, TNFα, and IL-12 (Iwasaki and Medzhitov 2015; Kawai and Akira 2010). TLRs also activate interferon (IFN), which induces a myriad of immune responses aimed at reducing viral replication and shedding (Suazo et al. 2015). Cytosolic PRRs include Nod-like receptors (NLRs) and RIG-I like receptors (RLRs), which also activate NFκB as well as caspase 1 and 9, which leads to production and secretion of the inflammatory cytokine IL-1 and apoptosis, respectively (Iwasaki and Medzhitov 2015; Wu and Chen 2014).

Many subpopulations of white blood cells (WBCs) with complex and sometimes overlapping jobs participate in the innate immune system (Uyangaa, Patil, and Eo 2014). Granulocytes and monocytes are two preceding cell types that can further differentiate into more specialized cells (Egan et al. 2013); granulocytes

differentiate into basophils, eosinophils, and neutrophils while monocytes differentiate into macrophages (MΦ) and dendritic cells (DC) (Egan et al. 2013). Neutrophils and MΦs engulf pathogens via phagocytosis and destroy them in the resulting vesicles with different enzymes (Ellerman-Eriksen 2005; Egan et al. 2013). Basophils, eosinophils, and mast cells target parasites and are responsible for allergic reactions by releasing histamine (Davoine and Lacy 2014). Natural killer (NK) cells kills infected host cells by releasing granules to form holes in the infected cells' membranes (Ellerman-Eriksen 2005; Egan et al. 2013; Chan et al. 2011; Uyangaa, Patil, and Eo 2014). DCs are professional APCs, meaning they process pathogen peptides into recognizable fragments for the adaptive immune system via major histocompatibility complexes (MHC), which will be elaborated on later (Egan et al. 2013; Uyangaa, Patil, and Eo 2014).

HSV-2 Innate Immunity. Little is known about the innate immune response against HSV-2 besides that a lack of such a response, usually immunocompromise due to mutations in genes encoding certain proteins such as STAT1 or TRAF3 or to NK cell deficiencies, can lead to severe HSV-2 infections and encephalitis (Dropulic and Cohen 2011; Dropulic and Cohen 2012; Sancho-Shimizu et al. 2011).

Interferon responses are particularly important for controlling HSV-2 infection. Activation of TLR3 and TLR9 is important for inducing interferon in response to HSV-2 infection (McAllister and Schleiss 2014). Interferon has multiple downstream effects such as anti-viral state induction in cells, DC maturation which is important for activating CD8+ T cells, NK maturation and recruitment, and CD8+ T cell recruitment to infection sites (McAllister and Schleiss 2014; Uyangaa, Patil, and Eo 2014);

therefore, stimulating interferon production seems to be instrumental in controlling HSV-2 infection (McAllister and Schleiss 2014; Uyangaa, Patil, and Eo 2014). IFNα, IFNβ, and IFNγ have been implicated in inhibiting HSV-1 replication, which strongly suggests these IFNs would inhibit HSV-2 replication as well due to these viruses' similarity (Leventon-Kriss et al. 1987; Sainz and Halford 2002). Sainz and Halford showed that pre-treatment with IFNα/β with IFNγ inhibited plaque formation in Veros and IFNβ/γ treatment reduced ocular lesions and latency in the trigeminal ganglia (TG) (Sainz and Halford 2002). This logic is further supported by reduction of lesions in HSV-2 seropositive individuals who apply topical IFNα to affected areas (Shupack 1992).

Because the innate immune system has no memory of pathogen encounters, activation of this system by a HSV-2 vaccine might be considered somewhat less important than its effect on the adaptive immune system considering that humans are challenged by multiple exposures of HSV-2 during their lifetimes. However, this logic would be detrimental to HSV-2 vaccine development since the innate immune response is required for adaptive immunity, a HSV-2 vaccine candidate should be evaluated on its ability to elicit a strong innate immune response such as interferon production to mature professional APCs such as DCs (McAllister and Schleiss 2014). Producing many professional APCs that are presenting various epitopes from different viral proteins would ensure a robust activation of T cells, which then would lead to a robust activation of B cells that would recognize more epitopes (McAllister and Schleiss 2014). Therefore, adjuvants in particular should be evaluated on their ability to stimulate the innate immune response (de Veer and Meeusen 2011). Since a previous

study by Khodai and colleagues showed that including the TLR9 agonist cPG significantly increased innate immune responses compared to the TLR4 agonist MPL, choosing adjuvants that stimulate TLR3 and TLR9 may produce more effective subunit vaccines (Khodai et al. 2011).

Adaptive Immunity. Adaptive immunity is the second arm engaged in response to a pathogen invasion. Its two subarms, humoral immunity mediated by antibodies and cell-mediated immunity mediated by T cells, illustrate the many differences between adaptive and innate immunity's ways of controlling infection (Uyangaa, Patil, and Eo 2014). The adaptive arm cannot be engaged without the innate immune system, so it cannot react as quickly to an infection as the innate immune response (Uyangaa, Patil, and Eo 2014). However, it can adapt, hence its name, to the pathogen encountered, tailoring its humoral and cellular responses to specificities in pathogen epitopes and lifecycles (Flajnik and Kasahara 2010). These specialized responses are remembered by the adaptive immune system so when subsequent infections occur, these responses can further specialize to speed pathogen clearance and minimize or eliminate symptom duration (Flajnik and Kasahara 2010; Neuberger et al. 2000). The memory B and T cells of the adaptive immune response, which will be elaborated on later, are more rapidly activated as each subsequent infection occurs (Flajnik and Kasahara 2010; Neuberger et al. 2000). This phenomenon is called an anamnestic response since the adaptive immune system is engaged earlier and produces a more robust response during each subsequent infection because it already remembers the pathogen (Neuberger et al. 2000).

Humoral Immunity. The humoral subarm is controlled by B cells, whose main job is to produce antibodies which are incredibly important for controlling infection (Irani et al. 2015). Antibodies can neutralize pathogens by binding to them to prevent them from infecting host cells by occluding viral attachment proteins (Irani et al. 2015). Antibodies can opsonize pathogens by binding to them, thus making these pathogens more visible to innate phagocytic immune cells like neutrophils and MΦ (Irani et al. 2015). Antibodies are also important for activating the complement pathway (Sörman et al. 2014; Suazo et al. 2015). Antibodies are also important for antibody-dependent cellular cytotoxicity (ADCC) by binding to pathogens and NK cells (Lubinksi et al. 2011). Due to these three major jobs, a vaccine candidate's ability to elicit antibodies is intimately tied to its success as a viable vaccine.

Antibodies consist two light and two heavy chains which form a classic Y-shape (Flajnik and Kasahara 2010; Litman et al. 1993; Liu and May 2012). The composition of these light and heavy chains depends on VDJ recombination in immunoglobulin (Ig) genes in immature B cells in the bone marrow before they have encountered antigen (Borghesi and Milcarek 2006; Flajnik and Kasahara 2010; Litman et al. 1993; Lou, Casali, and Xu 2015; Neuberger et al. 2000; Papavasiliou and Schatz 2002); VDJ recombination along with junctional diversity yields a myriad of different antibodies theoretically capable of binding virtually any antigen (Borghesi and Milcarek 2006; Litman et al. 1993; Neuberger et al. 2000; Papavasiliou and Schatz 2002). The tips of the arms of the Y (F_{ab}) are two variable regions that undergo somatic hypermutation to better bind antigen while the tail of the Y (F_c) is a constant region that binds to immune cells to effect certain functions or changes (Neuberger et al. 2000; Papavasiliou and

Schatz 2002; Sörman et al. 2014). Somatic hypermutation, or increased mutation rate in these antigen-binding regions in response to an antigen encounter, increases subsequent antibodies' affinity for these antigen (Neuberger et al. 2000; Papavasiliou and Schatz 2002). This process of affinity maturation occurs during each subsequent infection to generate antibodies with higher affinity for their antigen, which can prevent illness from recurring during these subsequent infections (LeBien and Tedder 2008; Neuberger et al. 2000). Antibodies can be made in five different isotypes – IgM, IgG, IgA, IgE, and IgD – via class switching of the constant heavy chain so they can interact with different types of immune cells and activate different cytokine pathways (Flajnik and Kasahara 2010; Irani et al. 2015; Lou, Casali, and Xu 2015; Neuberger et al. 2000).

During primary infection, B cells with antibodies that successfully bind antigen are stimulated by both antigen encounter and help from T cells to proliferate into plasma B cells or memory B cells (Bortnick et al. 2012; Kurosaki, Kometani, and Ise 2015; Neuberger et al. 2000; Shlomchik and Weisel 2012). The pathways and signals that determine B cell differentiation into plasma or memory B cells are mostly unknown, although new research has pinpointed the transcription factors XBP1, IRF4, and Blimp1 as important (Lou, Casali, and Xu 2015; Nutt et al. 2011; Piccaluga et al. 2015).

Plasma B cells make large quantities of antibodies whose antigen affinity increases during the course of infection as somatic hypermutation occurs (LeBien and Tedder 2008; Lou, Casali, and Xu 2015; Nutt et al. 2015). Once the infection is cleared, most plasma B cells die by apoptosis and the large amounts of antibodies secreted by these cells declines (Bortnick et al. 2012; Nutt et al. 2015). Some long-lasting plasma

B cells still reside in the bone marrow where they also produce antibodies, leading to an increase in baseline antibody titers which contributes to the faster anamnestic response during later infections (Bortnick et al. 2012; Neuberger et al. 2000; Nutt et al. 2015; Shlomchik and Weisel 2012).

Memory B cells already have class-switched, affinity-matured antibodies from their encounter with antigen during the primary response, but do not replicate or secrete antibody until they re-encounter their antigen (Kurosaki, Kometani, and Ise 2015; Neuberger et al. 2000; Shlomchik and Weisel 2012). Memory B cells wait in the lymph nodes and spleen to be rapidly activated and differentiate again into plasma and memory B cells upon subsequent antigen encounters (Kurosaki, Kometani, and Ise 2015; Shlomchik and Weisel 2012). Plasma and memory B cells created by secondary infections undergo further class switching as well as further somatic hypermaturation, which leads to even greater affinity maturation of their antibodies (Kurosaki, Kometani, and Ise 2015; Nutt et al. 2015; Shlomchik and Weisel 2012). Recreation of memory B cells during each infection ensures pathogen memory continues to be maintained (Kurosaki, Kometani, and Ise 2015; Shlomchik and Weisel 2012). Obviously, a HSV-2 vaccine that induces long-lasting immune memory by creating a large pool of memory B cells with highly affinity-matured antibodies would be ideal considering that humans are exposed to HSV-2 throughout their lifetimes.

Cell-Mediated Immunity. The other subarm of adaptive immunity, cellular immunity, is controlled by T cells, which perform a variety of roles such as activating B cells and killing infected host cells. The main two subclasses of T cells are helper T (CD4+) cells and cytotoxic T (CD8+) cells, which can differentiate into memory CD4+

and CD8+ T cells (O'Shea and Paul 2010; Zhang and Lakkis 2015; Wherry and Ahmed 2004). Helper T cells "help" other immune cells become activated via cytokine secretion and receptor-ligand binding while cytotoxic T cells directly kill infected host cells (O'Shea and Paul 2010; Wherry and Ahmed 2004).

Unlike B cells whose receptors can recognize virtually any epitope, T cells can only recognize small linear peptides that are presented on major histocompatibility complexes (MHCs) (Blum, Wearsch, and Cresswell 2013; Flajnik and Kasahara 2010). MHC Class I are displayed on almost every host cell, which is recognized by CD8+ T cells (Blum, Wearsch, and Cresswell 2013); MHC Class II are displayed on professional APCs, which is recognized by CD4+ T cells (Blum, Wearsch, and Cresswell 2013). Once the respective T cell receptors have bound to their MHCs, co-stimulation by CD28 or other molecules is required to fully activate the T cells (Chen and Flies 2013). Once CD4+ T cells are activated, they can bind to B cells that have encountered the same antigen via CD40-CD40L binding (Lou, Casali, and Xu 2015; O'Shea and Paul 2010). CD4+ T cells then secrete various cytokines to activate B cells and promote their proliferation (Lou, Casali, and Xu 2015; O'Shea and Paul 2010). Naïve CD8+ T cells have strict activation requirements as they can kill other cells by lytic granules, which include presentation of antigen via MHC Class II from APCs and CD4+ T cell help by cytokine secretion (Gutcher and Belcher 2007; Wherry and Ahmed 2004).

Both CD4+ and CD8+ T cells can differentiate into both central memory (T_{CM}) and effector memory (T_{EM}) T cells (Shin and Iwasaki 2013; Willinger et al. 2005; Zhang and Lakkis 2015). T_{CM} cells wait in the lymph while T_{EM} cells patrol the

periphery of the body via the bloodstream (Shin and Iwasaki 2013; Wherry and Ahmed 2004; Zhang and Lakkis 2015). Both types of memory T cells are rapidly activated when they reencounter their antigen, which causes a faster immune response to and clearance of the pathogen (Shin and Iwasaki 2013; Wherry and Ahmed 2004; Zhang and Lakkis 2015).

HSV-2 Adaptive Immunity. Not much is known about the adaptive immune response to HSV-2, though it is thought that antibodies are important for preventing infection and cellular responses are important for preventing disease. However, it is clear that HSV-2 IgG placental transfer is particularly important in reducing neonatal herpes risk during perinatal transmission (Brown et al. 2003; McAllister and Schleiss 2014; Prober et al. 1987).

Cell-mediated immunity, especially tissue resident T cells, is crucial in controlling infection as seen from the heightened risk of severe HSV infection when a host has a suppressed immune system due to medical treatment or HIV infection (McAllister and Schleiss 2014; Posavad et al. 1997; Zuckerman and Limaye 2013; Zhu et al. 2007; Zhu et al. 2009). Both CD4+ and CD8+ T cells play important roles during HSV-2 infection (Zhu et al. 2007; Zhu et al. 2009). Recruitment of CD8+ T cells to lesions is important for viral and infected host cell clearance (Koelle et al. 1998; Zhu et al. 2007); viral clearance from lesions is correlated to a rapid infiltration of HSV-2-specific CD8+ T cells (Koelle et al. 1998; Zhu et al. 2007). Also, an inverse correlation between lesion severity and CD8+ T cell numbers shows that more CD8+ T cells lessen the severity of viral reactivation, probably due to rapid clearance of infected host cells to limit viral spread (Schiffer et al. 2013). CD4+ T cells are also important for

controlling infection, but recruitment of these cells to genital lesions increases the risk of HIV transmission (Zhu et al. 2009).

Section 4: HSV-2 Vaccine History

Current Problems for HSV-2 Vaccine Development. Despite interest in developing both prophylactic and therapeutic HSV-2 vaccines as well as expensive clinical trials for various subunit vaccine candidates, unfortunately no HSV-2 vaccine has been licensed despite initial promise in animal models. Two major obstacles – unknown correlates of protection (CoP) and lack of non-human primate (NHP) animal models – contribute to the continuing failure in developing an effective human HSV-2 vaccine.

Correlates of Protection. Unknown correlates of protection pose a significant challenge to any vaccine's development. CoP describe the immune response necessary to induce or confer a particular immunological phenotype or endpoint (Plotkin and Gilbert 2012). Such endpoints are sterilizing immunity, or the ability to completely protect from infection and thus the gold standard for any prophylactic vaccine; disease immunity, or the ability to protect from symptomatic disease despite not protecting from infection and thus the gold standard for therapeutic vaccines; or disease reduction, or the ability to at least reduce the recurrence and duration of symptoms (Plotkin and Gilbert 2012). CoP may be further divided into mechanistic CoP (mCoP) and nonmechanistic CoP (nCoP) to further define how they contribute to immunity (Plotkin and Gilbert 2012). As defined by Plotkin and Gilbert, mCoPs are the direct immune responses that are responsible for conferring immunity while nCoPs are immune responses that highly correlate with protection, but do not provide the protection themselves (Plotkin and Gilbert 2012). Most vaccines protect through antibody

induction, but cellular immune responses or a combination of the two may be required to protect individuals for certain pathogens (Plotkin 2008).

Establishing a mCoP for a vaccine is therefore more desirable, but establishing nCoPs may be easier due to available analytical assays, immune cells, and tissues (Plotkin and Gilbert 2012). Thus nCoPs can be more useful than mCoPs in measuring immunity (Plotkin and Gilbert 2012). For example, the CoP of the zoster vaccine are both antibodies and cellular responses as they both correlate with protection from shingles (Plotkin and Gilbert 2012). However, cellular responses were statistically stronger in their correlation, making them a mCoP and antibodies a nCoP (Plotkin and Gilbert 2012). Considering that measuring antibodies is easier and more straightforward than quantifying cellular responses, the nCoP is actually more useful to measure immunity in this case (Plotkin and Gilbert 2012). Knowing both mCoPs and nCoPs for pathogens would be advantageous for vaccine development as scientists would know what type and magnitude of an immune response – such as a specific titer of antibodies to a specific epitope – must be elicited for specific endpoints like sterilizing immunity or disease protection (Plotkin 2008; Plotkin and Gilbert 2012).

Defining CoPs for vaccines is a complicated process considering how many variables exist in differences in individual immune systems, vaccine schedules and administrations, and exposure times. Each variable will be discussed in detail below.

CoP Variables Based on Immune Responses. CoPs for infection may be different from CoPs from disease protection as is for the smallpox vaccine, so the desired endpoints of a vaccine must be assessed according to the proper CoPs (Plotkin 2008). For example, to make a prophylactic smallpox vaccine, a vaccine candidate must

be evaluated on its ability to evoke antibodies (Plotkin 2008). To make a therapeutic smallpox vaccine, a vaccine candidate must be instead evaluated on its ability to induce both antibody production and CD4+ and CD8+ T cell responses (Plotkin 2008). Also, vaccination may achieve one or more CoP, but not necessarily the CoP wanted. For example, the measles vaccine does induce protective antibody titers, but the amount produced determines whether the CoP of sterilizing immunity or disease immunity is met (Plotkin 2008). Titers above 200 mIU/mL protect against infection whereas titers between 120-200 mIU/mL only protect from disease (Plotkin 2008). Therefore, the endpoints of a clinical trial or animal test determines what CoP must be met to determine success.

CoP Variables Based on Vaccine Characteristics. The properties of the vaccine itself also influence CoP, specifically their quality and ability to accurately measure. The challenge dose used for a clinical or animal trial affects CoP quality and quantity, so the dosage must be chosen carefully to induce a high enough quality CoP of a high enough quantity to achieve the desired endpoint (Plotkin 2008). For example, an attenuated polio vaccine blocked viral intestinal shedding in only 30% of vaccinees after high dose challenge despite blocking such shedding in 80% of vaccinees after low dose challenge (Onorato et al. 1991; Plotkin 2008). These results show that the dose of the vaccine induce enough immunity to protect the majority of people from small viral titer exposures, but not large viral titer exposures (Onorato et al. 1991; Plotkin 2008). Based on these data, the dosage should be increased, but considering that it is an attenuated polio vaccine, certain risks such as reversion to wild-type virulence come with higher doses (Onorato et al. 1991; Plotkin 2008). Therefore, multiple dosages

should be tested to determine which dosage, if any, both achieves the desired CoP and remains safe.

Lack of NHP Animal Models. Current animal models used for HSV-2 vaccine candidates have certain limits in their similarity to HSV-2 pathogenicity in humans. No NHP models currently exist for HSV-2, which is problematic because vaccine candidate results in NHP usually better correlate with results in humans compared to results in mice (Dropulic and Cohen 2012). Unfortunately, vaccine candidates that looked promising in mice and/or guinea pigs failed in large-scale, expensive human trials.

Mice, particularly the inbred BALB/c and C57BL/6 strains, are by far the most popular animal model for HSV-2 for their cost, short generation time, well-characterized immune system, small size for easy handling, and smaller housing requirements (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012). Mice can be easily inoculated intravaginally after treatment with medroxyprogesterone, which thins the vaginal mucosa to facilitate successful infection (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012). Blood, mucosa, and DRGs can be easily taken or harvested to measure antibody and cellular responses (Dropulic and Cohen 2012). These mice then develop acute disease, viral shedding, and latency in the DRG quite similarly to humans, so the mouse model yields good evaluation of potential prophylactic vaccine candidates' effectiveness on preventing infection, acute disease, or latency (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012).

Two major problems with mice, namely lack of spontaneous reactivation and heightened viral virulence, prevent mice from being a near perfect model for HSV-2.

Mice do not have spontaneous reactivation and subsequent disease episodes that humans do, so latently infected mice need to be stimulated with UV light or heat to induce reactivation (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012; LeBlanc et al. 1999; Sawtell and Thompson 1992). Therefore, mouse models are not useful for evaluating therapeutic vaccine candidates' ability to prevent recurrent disease via long-lasting immune responses (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012). Intravaginal inoculation, the preferred method of injection for HSV-2 vaccine candidates, can lead to viral invasion of the sacral ganglia, causing bladder control loss, hind limb paralysis, and death (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012). Considering that HSV-2 does not cause these symptoms in humans during acute infection, chronic mouse HSV-2 pathogenesis and to a lesser extent acute pathogenesis do not mirror either of those in humans (Dasgupta and BenMohamed 2011).

Guinea pigs are the gold standard small animal model for HSV-2 in part because they do have spontaneous reactivation of HSV-2 like humans do, so they are useful for evaluating the effectiveness of both prophylactic and therapeutic vaccines (Dasgupta and BenMohamed 2011; Scriba and Tatzber 1981; Stanberry et al. 1982; Stanberry et al. 1985; Stanberry 1991). During acute infection, guinea pigs shed virus and develop latency in the sacral and dorsal root ganglia, both of which can be measured to see if shedding is reduced or latency is prevented by the vaccine candidate (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012; Stanberry 1991). Antibody titers can be easily measured, but cellular responses in have not been well studied due to lack of available immune reagents and inbred animal strains (Dasgupta and BenMohamed

2011; Dropulic and Cohen 2012). Considering that cellular responses are theorized to be important for controlling recurrent disease, the limitations on available immune reagents as well as a lack of knowledge of the guinea pig immune system hamper scientists' ability to find CoPs responsible for blocking recurrent disease in a spontaneously reactivating model (Dasgupta and BenMohamed 2011).

Cotton rats, rhesus macaques, and owl monkeys have also been used as models for HSV-2, but their usefulness is reduced compared to mice and guinea pigs for various reasons. Cotton rats have spontaneous reactivation and do not need medroxyprogesterone treatment before intravaginal inoculation, which confer two big advantages over mice (Yim et al. 2005). Despite cotton rates also having good acute HSV-2 infection and spontaneous reactivation, lack of immune reagents, lack of knowledge about their immune system, and their difficulty to work with has blocked any rise in their popularity as models (Dasgupta and BenMohamed 2011; Yim et al. 2005). Rhesus macaques, a popular NHP model for many other diseases, unfortunately have low symptomatic infection rates with HSV-2, averaging about only 10% of animals developing lesions (Crostarosa et al. 2009: Dropulic and Cohen 2012). Therefore, rhesus macaques are not a practical model for HSV-2 considering the number of animals that would be needed per study to generate usable data, which would significantly increase the already expensive costs of NHP studies (Crostarosa et al. 2009). On the other hand, owl monkeys develop lethal HSV-2 infections, which is beneficial for demonstrating live attenuated vaccine (LAV) safety (Melendez 1969; Meigneir 1990). However, their low 50% lethal dose (LD_{50}), or the number of virions

needed to kill off 50% of infected hosts, prevents them from being challenged with high doses that vaccine efficacy studies would require.

Several factors about HSV-2 can explain why this lack of NHP animal models exists and why the smaller animal models of mice and guinea pigs do not always provide an accurate prediction of a vaccine candidate's efficacy in humans. HSV-2 as well as the other human herpesviruses have coevolved with humans for millions of years, so the virus has specifically adapted to evade the human immune system, which leads to immune response discrepancies in animal models (Ellerman-Eriksen 2005; Dropulic and Cohen 2012; Grinde 2013). The one challenge model used in animal studies also does not mirror the multiple exposures that humans have over their lifetimes, so little information is known about vaccine candidates' ability to induce long-lasting immunity to protect from infection years after immunization (Dropulic and Cohen 2012). However, to do long-term studies to study memory immune responses and antibody titers over time would be quite expensive due to the number of animals needed, extended housing and veterinary care needs, and potentially more personnel needed to run experiments and analyze data (Dropulic and Cohen 2012). Additionally, animals are challenged at their highest immune response to the vaccine candidate, which is a highly unlikely scenario to happen in humans (Dropulic and Cohen 2012).

HSV-2's evolved specificity to evade a single species' immune system has led to several consequences for the virus itself as well as our inability to create a HSV-2 vaccine. This specificity means that HSV-2 has a narrow host range as seen in its inability to cause significant disease in rhesus macaques, which has prevented development of a NHP animal model (Dropulic and Cohen 2012). Humans ethically

cannot be used in challenge studies of new vaccine candidates with unknown safety profiles, so new vaccine candidates must be tested in inbred mice or guinea pigs. However, these two models have substantial differences in their innate, mucosal, and adaptive immune systems including different major histocompatibility complexes (MHC) alleles and mucosal immune cell distribution compared to outbred humans (Dasgupta and BenMohamed 2011; Dropulic and Cohen 2012). HSV-2's immune evasion proteins such as ICP47, which downregulates MHC Class I, and gE, which binds to human IgG Fc, have significantly more activity in humans compared to mice, which can falsely amplify their effectiveness (Ahn et al. 1996; Johansson et al. 1985). Since guinea pig T cells cannot identify human leukocyte antigen (HLA)-restricted T cell epitopes, this model cannot be used to evaluate any HSV-2 T-cell-based vaccines unless transgenic guinea pig strains are created (Dasgupta and BenMohamed 2011).

Finally, vaccine viruses are replicated in cell culture where they may acquire attenuating mutations, further diverging them from the non-attenuated HSV-2 strains that circulate in humans (Szpara et al. 2010). HSV-2 is mainly grown in the African green monkey kidney cell line Vero since it has been approved to grow vaccine viruses and produce other biologics for use in humans (Barrett et al. 2009). Since this cell line is not human, vaccine viruses may acquire mutations specific to promote replication and downregulate antiviral states in Veros which could impact their ability to elicit immune responses in humans (Dropulic and Cohen 2012).

Subunit, Whole Virus Inactivated, and Replication-Defective HSV-2 Vaccines. Almost all previous HSV-2 vaccine candidates have been subunit protein vaccines, usually consisting of HSV glycoprotein D (gD) and/or gB, two major surface glycoproteins

which are crucial for virus attachment and fusion to cells, respectively (Chentoufi et al. 2012; Zhu et al. 2014). These two glycoproteins are also the two most highly antigenic HSV proteins, which make them desirable as vaccines as they can induce high neutralizing antibody titers and CD4+ and CD8+ T cell responses, which as discussed before can usually confer immunity (Chentoufi et al. 2012; Cohen et al. 1984; Kim et al. 2008; Kohl et al. 2000; Zhu et al. 2014). The popularity of these subunit vaccines stems from their obvious safety advantages since protein vaccines cannot accidentally infect and are extremely unlikely to harm vaccines (Chentoufi et al. 2012; Dropulic and Cohen 2012; Zhu et al. 2014). Also, the high similarity shared between both HSV-1 and HSV-2's gB and gD glycoproteins hopefully could lead to cross-protection after vaccination (Chentoufi et al. 2012; Dropulic and Cohen 2012). However, none of these subunit vaccines adequately protected trial participants from HSV-2 acquisition or disease, which clearly indicates that other vaccine strategies for HSV-2 should be investigated as well (Chentoufi et al. 2012; Dropulic and Cohen 2012; Zhu et al. 2014).

Whole virus and replication defective vaccine candidates have different benefits and disadvantages compared to subunit vaccines. These two types of HSV-2 vaccines are not nearly as popular as subunit vaccines primarily for safety concerns, but they do offer key advantages that may help them become more widely considered as viable HSV-2 vaccine options as vaccine production quality standards have tightened and virus manipulation techniques continue to evolve. Few whole virus HSV-2 vaccines have been made for HSV-2, primarily due to concerns that inactivation methods would not completely inactivate all virions and allow viable virions to establish infection in vaccines (Dropulic and Cohen 2012).

The same concern stands for replication defective vaccines, which use virions that are incapable of completing more than one round of replication due to mutation or deletion of essential viral genes (Dropulic and Cohen 2012; Zhu et al. 2014). If these virions reverted back to wild-type, the vaccine virus could infect vaccinees instead of protecting them from infection although this risk is mitigated via complete or at least partial deletion compared to simple point mutations. However, whole virus and replication-defective vaccines present the entire repertoire of viral epitopes to the immune system instead of just one or two glycoproteins (Dropulic and Cohen 2012; Zhu et al. 2014). Considering that studies have found antibodies and T cells responses to other surface glycoproteins, tegument and capsid proteins, and viral DNA replication machinery proteins, exposing the immune system to many epitopes as opposed to just one or two may elicit combinations of immune responses that protect or reduce disease (Dropulic and Cohen 2012; Koelle et al. 1998; Koelle et al. 2001; Laing et al. 2010; Zhu et al. 2007; Zhu et al. 2014). Replication-defective vaccines further this benefit by undergoing at most a single round of replication, which can elicit a more robust immune response (Dropulic and Cohen 2012).

Subunit Vaccines. Previous subunit HSV-2 trials have mainly been a mixture of gD. gB, and various adjuvants to try to induce a potent enough immune response to protect from infection and disease (Chentoufi et al. 2012; Zhu et al. 2014). While many of these subunit vaccines looked promising in animal models, all of them have failed to meet their specified endpoints in human clinical trials, suggesting that one or two glycoproteins may not be enough to elicit protective immune responses (Chentoufi et al. 2012; Zhu et al. 2014). For the following discussion, a quick nomenclature

explanation is needed in regards to specific glycoprotein names: glycoproteins from HSV-2 are denoted with a 2 after the letter (gD2) whereas those from HSV-1 are denoted with a 1 (gD1).

In the 1980s and 1990s, different combinations of multiple viral proteins and glycoproteins were tested as both prophylactic and therapeutic vaccines. A double-blind study of a therapeutic vaccine candidate consisting of DNA-free viral antigens isolated from infected human cells in symptomatic individuals with frequent outbreaks showed that this vaccine not only induced poor immunity but also did not significantly decrease disease in these individuals compared to controls despite being highly immunogenic in animals (Dropulic and Cohen 2012; Kutinová et al. 1988). Another subunit vaccine composed of gB2, gC2, gD2, gE2, and gG2 purified from infected chick embryo fibroblasts (CEFs) was tested for its ability to protect HSV-2 seronegative people in HSV-2 serodiscordant couples where their partners had frequent recurrent HSV-2 lesions (Dropulic and Cohen 2012; Mertz et al. 1990). This double-blind, placebo-controlled clinical trial showed that the HSV-2 seronegative partners were not significantly protected from developing genital herpes compared to placebo (Dropulic and Cohen 2012; Mertz et al. 1990); vaccinees' antibody titers to gD and gB were very low compared to their partners', suggesting that the lack of protection from transmission may have been due to insufficient antibody titers (Dropulic and Cohen 2012; Mertz et al. 1990). Another vaccine candidate made with formalin-inactivated HSV-1 glycoproteins extracted from cells did not reduce outbreak frequency in individuals with frequent recurrent genital outbreaks, but did statistically significantly reduce number and severity of lesions (Dropulic and Cohen 2012; Skinner et al. 1997).

These studies all suggested that viral proteins alone were unable to induce sufficient immunity, so future subunit vaccine candidates needed adjuvants (Dropulic and Cohen 2012).

As mentioned above, more recent HSV-2 subunit vaccine candidates use gD, gB, and different adjuvants such as MF59 and aluminum hydroxide (alum), which can be combined with other adjuvants such as monophosphoryl lipid A (MPL), to boost immune responses (Zhu et al. 2014). Even with these adjuvants, subunit gD and gB vaccine candidates have not been successful in humans, suggesting that additional immune boosting techniques are needed (Chentoufi et al. 2012; Dropulic and Cohen 2012; Zhu et al. 2014). A second adjuvant could be added to a vaccine candidate, though having two or more adjuvants does not seem to be common in at least previous vaccine candidate studies. Chemical interactions between two or more adjuvants potentially could cause problems either with safety of the vaccine formula, stability of the antigen proteins, or the composition and magnitude of the immune response. However, if adjuvant combinations can be certified as safe and immunogenic, they could be utilized to boost subunit vaccine efficacy since due to their safety, subunit HSV-2 vaccines will continue to remain popular.

The most probable reason why no subunit gD and gB vaccine has met endpoints in human trials is inclusion of only one or two epitopes of a complex virus that encodes over eighty proteins, which probably has prevented the needed range of both humoral and cellular immune responses for sterilizing immunity or disease protection (Dropulic and Cohen 2012; Zhu et al. 2014). Studies have shown that HSV-2-specific CD4+ and CD8+ T cells respond to a wide variety of viral proteins, including tegument, IE, and

capsid proteins (Dropulic and Cohen 2012; Koelle, Frank, et al. 1998; Koelle et al. 2001). CD8+ T cells are responsible for destroying infected host cells, so a vaccine that contains multiple popular viral proteins recognized by CD8+ T cells would elicit a robust adaptive cell-mediated immunity as these CD8+ T cells would recognize many HSV-2 peptide fragments displayed on MHC Class I molecules of infected host cells. One study by found that 40% and 20% of HSV-2 seropositive individuals had T cells that recognized gD and gB, respectively, which shows that while these two surface glycoproteins were widely recognized by T cells, these people still were not protected from disease (Posavad et al. 2010). However, HSV-2 immune seronegative (IS) individuals, or people who has no signs of HSV-2 infection but show HSV-2 specific T cell activity, had high percentages of HSV-2 T cells that recognized IE proteins such as UL39, ICP0, and ICP4 (Posavad et al. 2010); few IS individuals had T cells that recognized either gD and gB (Posavad et al. 2010). This distinct skewing of recognized T cell epitopes between HSV-2 seropositive and IS individuals strongly suggests that T cells that recognize IE proteins are more important for protection from infection than T cells that recognize gD or gB (Dropulic and Cohen 2012; Posavad et al. 2010).

These CD4+ T cells would activate B cells that also recognize these more hidden epitopes. These interior viral proteins are expressed earlier in the viral lifecycle, so T cell responses to these IE proteins may be more effective in controlling viral replication as they would stop viral downregulation of immunity and host cell translation (Braun, Payne, and Dong 2006). This logic is supported by a study by Braun, Payne, and Dong that found that higher CD4+ T cell responses to IE proteins like ICP4 and ICP0 correlated to low recurrence HSV-2 seropositive individuals

(Braun, Payne, and Dong 2006). In contrast, higher CD4+ T cell responses to gD correlated to high recurrence HSV-2 seropositive individuals (Braun, Payne, and Dong 2006). Therefore, gD2-specific CD4+ T cells do not seem to protect against disease at all (Braun, Payne, and Dong 2006). Subunit vaccines that only contain surface antigens that are mainly produced late in the viral lifecycle will not activate T and B cells that recognize these interior viral epitopes that would be able to shut off viral replication earlier.

A prophylactic vaccine candidate of gD2 and gB2 in adjuvant MF59, a squalene-containing oil-in-water emulsion, was tested in two randomized, double-blind, placebo-controlled studies in which HSV-2 seronegative partners of serodiscordant couples and attendees of a STI clinic at high risk for HSV-2 infection (Corey et al. 1999; Dropulic and Cohen 2012). While vaccinees' HSV-2 infection rate was 50% lower than controls five months post-vaccination, this protection was undetectable one year post-vaccination and thus indicated that this vaccine candidate did not evoke long-lasting immunity (Corey et al. 1999; Dropulic and Cohen 2012). No reduction in symptomatic HSV-2 disease was seen despite the vaccine candidate inducing higher neutralizing antibody titers than natural infection, strongly suggesting that antibody titers alone cannot protect against infection (Corey et al. 1999; Dropulic and Cohen 2012). Pre-existing HSV-1 immunity did not reduce HSV-2 acquisition rate, but did increase the number of asymptomatic HSV-2 infections, confirming that some immune cross-protection exists between HSV-1 and HSV-2, which would be expected considering their high level of similarity of 83% identity in genomic coding regions (Chentoufi et al. 2012; Corey et al. 1999; Dolan et al. 1998).

Another prophylactic vaccine candidate of gD2 with alum-MPL was tested in two populations of HSV-1/HSV-2 double seronegative individuals or women with any HSV serostatus whom had partners with genital herpes (Dropulic and Cohen 2012; Stanberry et al. 2002). Neither population was protected from genital disease, showing 38% and 42% efficacy, respectively (Dropulic and Cohen 2012; Stanberry et al. 2002). Interestingly, subanalyses of the vaccine indicated 73% and 74% efficacy in these two populations in preventing disease in double seronegative women, but not in HSV-1 positive/HSV-2 negative women or in men with any HSV serostatus (Dropulic and Cohen 2012; Stanberry et al. 2002). Perhaps the preexisting immune response to HSV-1 precluded a robust enough immune response to protect against HSV-2 (Coleman and Shukla 2013). Another reason could be that anti-HSV-1 antibodies and T cells do not have high enough cross protection with HSV-2 despite high similarity between the viruses to adequately protect, indicating that maybe these anti-HSV-1 immune responses target very specific epitopes that are not shared between HSV-1 and HSV-2. The fact that no men were protected suggests that vaginal mucosal immunity was important for protecting double-seronegative women as all men would not have such mucosal surfaces where anti-HSV antibodies could be deposited to intercept virions contacting the mucosal surface (Coleman and Shukla 2013).

This same gD in alum-MPL formulation was tested in a large scale clinical trial (Herpevac) in over 8000 double seronegative women because the results from the previous study showed high efficacy in preventing HSV-2 disease in this population (Belshe et al. 2012). Vaccinees presented high antibody titers and CD4+ T cell responses to gD2, but this vaccine did not protect these subjects from HSV-2 disease

or infection, indicating that these anti-gD2 responses were unable to protect from infection or disease and further suggesting that exposing the immune system to more epitopes of the virus is necessary to induce a protective immune response (Belshe et al. 2012; Dropulic and Cohen 2012).

This discrepancy between the two trials has not been fully explained, though differences in the test populations may be to blame as the first trial had women whose partners had confirmed genital herpes whereas the Herpevac trial only had women deemed at risk for HSV infection and disease (Belshe et al. 2012). Perhaps the first trial showed efficacy because these women most definitely had repeated exposures to HSV-2 either from symptomatic outbreaks or subclinical shedding (Belshe et al. 2012; Dropulic and Cohen 2012). These repeated exposures may have boosted the vaccine's effectiveness since vaccinees were most likely challenged soon after vaccination during peak immune response and repeated challenges maintained higher levels of antibody and T cells (Belshe et al. 2012; Dropulic and Cohen 2012). Therefore, its ability to generate long-lasting immunity despite infrequent exposures was unable to be critically evaluated (Dropulic and Cohen 2012). Women in the Herpevac trial may not have been challenged as soon after vaccination, so if the vaccine was not able to generate a robust memory response, they would not be protected from infection or disease when challenged months or years after vaccination (Dropulic and Cohen 2012). Interestingly, the vaccine did show a 35% reduction in infection and 58% reduction in genital disease caused by HSV-1, which was the more common etiologic agent of genital herpes in the Herpevac control group (Awasthi, Belshe, and Friedman 2014).

Whole Virus Inactivated Vaccines. Whole virus vaccines were popular in the 1940s to 1960s, but problems with inactivation, poorly controlled studies, and lack of positive results have severely reduced whole virus HSV-2 vaccine candidate production (Dropulic and Cohen 2012). Whole virus was grown in fertilized eggs or cell culture, which unfortunately could introduce attenuating mutations into vaccine viruses which may change epitopes (Dropulic and Cohen 2012). The three methods of inactivation – heat, chemicals like formaldehyde or formalin, and UV light – run the risk of not fully inactivating all virions (Dropulic and Cohen 2012). In 1964, the first double-blind, randomized, placebo-controlled trial for a HSV-2 vaccine was conducted by vaccinating individuals with recurrent disease with formaldehyde-inactivated whole virus (Dropulic and Cohen 2012; Kern and Schiff 1964). Unfortunately, reduction in lesions in vaccinees and the placebo group was similar, indicating that this therapeutic vaccine candidate failed (Dropulic and Cohen 2012; Kern and Schiff 1964). No other properly controlled, double-blinded studies have been done for inactivated whole virus vaccines (Dropulic and Cohen 2012). However, inactivated vaccines should be revisited as an option if formulated with newer, more immunogenic adjuvants.

Replication-Defective Vaccines. Live HSV-2 vaccines have garnered more attention with the development of the replication defective *dl*5-29 virus, a HSV-2 strain with the essential U_L5 and U_L29 ORFs deleted (Da Costa et al. 2000; Hoshino et al. 2005). This replication-defective strain cannot undergo a full round of replication in the host, limiting the possibility of latency establishment while still evoking a protective immune response (Da Costa et al. 2000; Hoshino 2005). Viral stocks are grown in complementing 5/29 Vero cells that express these two proteins, which

eliminates the manufacturing concern that this vaccine virus would be difficult to mass-produce (Da Costa et al. 2000). This vaccine candidate has reduced latency capacity in both mice and guinea pigs (Da Costa, Jones, and Knipe 1999; Hoshino 2005). Both vaccinated mice and guinea pigs had less viral shedding and better survival when challenged with wild-type HSV-2 (Da Costa, Jones, and Knipe 1999; Hoshino 2005). Guinea pigs vaccinated with this vaccine candidate also were better protected from wild-type HSV-2 challenge compared to those vaccinated with gD, showing higher neutralizing antibody titers, fewer HSV-2 genomes in DRGs, and less viral shedding (Hoshino et al. 2009). Currently this vaccine candidate is undergoing a clinical trial in humans.

Other studies on different ways to attenuate HSV-2 have been published, such as deleting part or all of certain immune evasion genes such as ICP0, which inhibits the interferon response, or mutating crucial residues on gD that prevent the virus from binding nectin-1, the receptor it uses to enter neurons (Halford et al. 2011; Wang et al. 2012). While these methods have produced viable attenuated potential vaccine candidates, other attenuation methods should be investigated, especially those that preserve as many epitopes as possible.

Live Attenuated HSV-2 Vaccines. Live attenuated vaccines (LAVs) have been licensed for varicella, influenza, measles/mumps/rubella (MMR), rotavirus, polio, and vaccinia (Minor 2015). LAVs usually give a more potent, durable, and complete immune response since the vaccine virus still replicates in the vaccine recipient and possesses all the potential epitopes that the immune system would encounter during a natural infection (Dropulic and Cohen 2012; Minor 2015). Taking into account that the only

licensed, available human herpesvirus vaccine currently available is an attenuated Oka strain of varicella-zoster, LAVs for other herpesviruses like HSV-2 should be seriously considered (Dropulic and Cohen 2012; Yamanishi 2008).

Recent data of HSV-2 LAV candidates in mice further recommend LAVs as a viable choice for HSV-2 vaccines. Different immune evasion genes such as ICP0 or genes necessary for viral spread like gE have been deleted to generate LAV candidates (Halford et al. 2011; Wang et al. 2012). ICP0 is an interferon antagonist, so by partially deleting certain domains like the nuclear localization signal (NLS) would make a strain that is less able to evade host immune responses (Halford, Püschel, and Rakowski 2010). Halford and colleagues deleted the NLS from ICP0 from HSV-2 (ICP0ΔNLS) to test its ability to protect mice from lethal challenge after intraocular or intravaginal challenge (Halford, Püschel, and Rakowski 2010). Both intraocularly and intravaginally ICP0ΔNLS-vaccinated mice with this strain were protected from lethal challenge (Halford, Püschel, and Rakowski 2010); intravaginally ICP0ΔNLS-vaccinated mice were better protected from mortality, generated higher neutralizing antibody titers, and had reduced vaginal shedding compared to mice vaccinated with gD2 in alum+MPL (Halford et al. 2011). Considering that severely immunocompromised mice died after vaccination with ICP0ΔNLS, ICP0ΔNLS is not attenuated enough to bring to clinical trials (Halford, Püschel, and Rakowski 2010). Other attenuation methods should be used on ICP0ΔNLS to further attenuate it such as deletion of more immune evasion genes like ICP47, which inhibits MHC Class I presentation, and gC, which inhibits complement activation.

Despite previous success in mice, LAVs for HSV-2 have not been seriously considered due to the risk that the vaccine virus will still be able to establish latency in the neurons of vaccine recipients and potentially reactivate and expose their intimate partners (Dropulic and Cohen 2012). Therefore, any live attenuated HSV-2 vaccine candidates must be subjected to rigorous safety testing to ensure these viruses replicate sufficiently to induce a protective immune response, but are unable to replicate to high enough titers or are unable to infect neurons to establish latency (Dropulic and Cohen 2012). If completely preventing latency is unattainable, having a vaccine strain establish latency would be acceptable as long as it does not cause disease in vaccinees.

Current LAVs for HSV-2 have only utilized common attenuation methods of missense mutations or deletions of viral genes. Despite their demonstrated ability to attenuate viruses, both missense and deletion mutations pose some problems that potentially could impact the effectiveness of vaccine candidates made using these mutations. Missense mutations can potentially revert to wild-type, especially if made in genomic areas of high variability or in essential protein moieties; however, this concern is somewhat lesser for DNA viruses such as HSV-2 compared to RNA viruses, which on average have higher mutation rates. Missense mutations also may change epitopes, which may prevent the resultant immune response from being able to recognize wild-type viral exposures. For example, Wang and colleagues formulated a HSV-2 gD mutant (HSV-2 gD27) that has three point mutations at amino acids 215, 222, and 223 to ablate nectin-1 but not HVEM binding so the virus can still lytically replicate in epithelial cells but not infect and establish latency in neurons (Wang et al. 2012). While reducing the ability of HSV-2 vaccine candidates to establish latency is

crucial, mutating these residues may destroy epitopes that if recognized by the immune system during wild-type, nectin-1-binding-competent HSV-2 natural infection, may prevent latency. Full or partial deletions of genes are safer as the chance of reversion is much lower, but they completely remove or more severely distort epitopes compared to missense mutations. Therefore, preserving as many epitopes as possible between the wild-type and vaccine strains is crucial, so attenuation methods such as codon bias deoptimization (CBD) and codon pair bias deoptimization (CPBD) should be more frequently used to make LAV candidates for both viral and bacterial pathogens.

Section 5: Codon Pair Bias Deoptimization

Codon Pair Bias Deoptimization (CPBD) As An Attenuation Method. Codon bias (CB) and codon pair bias (CPB) have been observed in many sequenced genomes. Both such phenomena are possible due to the degeneracy of the genetic code whereas multiple codons can encode the same amino acid. CB refers to the phenomenon that certain codons for a particular amino acid are used more frequently than expected by chance (Buchan, Aucott, and Stansfield 2006). For example, the codons UCU and UCC both encode serine, but in a specific genome, the codon UCU is used significantly more often than UCC to code for serine in polypeptides (Coleman et al. 2008). CPB refers to the fact that certain codons are found next to each other in pairs more often than expected by chance (Buchan, Aucott, and Stansfield 2006; Coleman et al. 2008). For examples, the codon pairs UCUGUU and UCCGUU encode the dipeptide serine-valine, but UCUGUU is used significantly more frequently to encode serine-valine than UCCGUU. Different species and viruses exhibit different CBs and CPBs (Boycheva et al. 2003; Buchan, Aucott, and Stansfield 2006; Coleman et al. 2008; Gutman and

Hatfield 1989; Moura et al. 2005). Both CB and CPB have been shown to affect translational elongation rates and fidelity *in vivo* (Buchan, Aucott, and Stansfield 2006; Curran et al. 1995; Irwin et al. 1995; Folley and Yarus 1989; Smith and Yarus 1989).

The CPBs of pathogen genomes can be taken advantage of by a relatively new method called synthetic attenuated virus engineering (SAVE) first described by Coleman and colleagues for polio (Coleman et al. 2008). SAVE identifies the CPB of viral genomes and then recodes them by substituting rarely used codon pairs in for frequently used codon pairs without changing the amino acid sequence (Coleman et al. 2008). SAVE therefore can make hundreds of silent mutations throughout a viral genome without disrupting or altering potential epitopes, a process called codon pair bias deoptimization (CPBD) (Coleman et al. 2008). Therefore, any potential vaccine virus made with SAVE would theoretically induce the same humoral and cellular immune response as a wild-type strain (Coleman et al. 2008). The vast number of mutations also drastically reduces the chance for reversion, making CPBD a safe attenuation method (Coleman et al. 2008). SAVE can quickly analyze viral genomes to generate deoptimized sequences, which then can be customized according to researchers' wishes on how much and what parts of the genome should be deoptimized (Coleman et al. 2008).

The mechanism of attenuation of SAVE and CPBD is currently unknown (Wang et al. 2015). Problems with transcription and/or translation of the deoptimized genome could contribute to the attenuated phenotype of pathogens. Transcription issues could include decreased ability of the template to bind transcription factors and RNA polymerase, which could result in poor initiation or processivity of RNA

polymerase; inability to modify the mRNA in certain ways such as capping or polyadenylation; and other issues stemming from the presumably very different secondary structures formed by different chemical interactions due to the many nucleotides changes in the deoptimized mRNA (Wang et al. 2015). A recent study by Tulloch and colleagues claims that changes in CpG and UpA dinucleotide frequencies make viral pathogens more recognizable by the cell's anti-viral defenses, which prevents the virus from replicating (Tulloch et al. 2014); therefore, defects in replication not due to decreased viral fitness but to increased recognition by host cells may also be at play (Tulloch et al. 2014). Another recent study with codon pair recoded dengue virus also supports this mechanism of attenuation (Simmons et al. 2015).

Translation issues mainly would stem from differences in tRNA concentrations, which is currently the prevailing idea of how SAVE attenuates pathogens (Wang et al. 2015). Frequently used codon pairs would have higher concentrations of their corresponding tRNAs whereas rarely used codon pairs would have lower concentrations, so translation of the deoptimized mRNAs would take longer and not produce as many protein products compared to wild-type mRNA, thus leading to the attenuated phenotype (Wang et al. 2015). Considering significantly reduced levels of corresponding deoptomized proteins by Western blot were seen in two studies, a defect in translation is probably at least contributing to the attenuated phenotype of CPBD pathogens (Mueller et al. 2010; Yang et al. 2013). Based on the current compilation of data, the mechanism of attenuation is probably a combination of all these factors.

Previous Literature on CPBD Human Pathogens. Considering that SAVE and CPBD is still only recently described, few papers have been published on its ability to

successfully attenuate human pathogens and protect animals from challenge. The first paper published describing SAVE by Coleman and colleagues in 2008 deoptimized parts of the polio genome to create different "Min" viruses to characterize *in vitro* and *in vivo* (Coleman et al. 2008). These "Min" viruses had 0.5 to 3 log lower titers than wild-type polio when grown in cell culture (Coleman et al. 2008). Two "Min" viruses were tested for safety and protection in mice; "Min" viruses had a 10 fold higher 50% paralysis lethal dose (PLD_{50}), or the amount of virus needed to paralyze 50% of the mice, indicating an observable attenuated phenotype *in vivo* (Coleman et al. 2008). Mice vaccinated with these "Min" viruses were protected from paralysis and death when challenged with a lethal dose of wild-type polio, showing proof of concept that viruses deoptimized with SAVE could generate successful vaccines even for RNA viruses which have higher mutation rates (Coleman et al. 2008).

SAVE's ability to attenuate influenza has been evaluated in two papers. Mueller and colleagues deoptimized the PB1, HA, and NP influenza genes of the H1N1 PR8 lab strain to generate the resultant $PB1_{min}$, NP_{min}, HA_{min}, and $PB1/NP/HA_{min}$ PR8 viruses, which grew to 10-fold less end-titers *in vitro* (Mueller et al. 2010). PB1 is one of the three subunits of the viral RNA polymerase, HA is the surface spike protein hemagglutinin that aids in viral attachment and fusion, and NP is the nucleoprotein that packages the viral RNA genome (Mueller et al. 2010). The rationale for deoptimizing these three genes was to target different stages of the viral lifecycle – attachment for HA, genome replication and transcription for PB1, and genome packaging for NP – to see if crippling different stages led to more severely attenuated phenotypes (Mueller et al. 2010).

The comparable end-titers between the deoptimized and wild-type influenza viruses show good manufacturing potential (Mueller et al. 2010). Western blots of the corresponding deoptimized proteins showed significantly decreased concentrations, which suggests that the mechanism of attenuation of CBPD is at least in part due to defects in translation (Mueller et al. 2010). Mice infected with the triple deoptimized PR8 virus did not die or exhibit weight loss through 16 days post-infection (DPI) whereas mice infected with wild-type PR8 all died before 6 DPI, showing that the triple deoptimized virus was safe and did not cause symptoms in mice (Mueller et al. 2010). Lung samples of the mice infected with the triple deoptimized virus had a peak titer of $10^{5.5}$ PFU/mL at day 3 post-infection compared to the wild-type PR8's peak titer of 10^{8} PFU/mL (Mueller et al. 2010); this discrepancy in peak titers shows that the triple deoptimized virus does not replicate as well as the wild-type, so it was cleared faster by the murine immune system (Mueller et al. 2010).

A later paper by Yang and colleagues showed that a double deoptimized H1N1 PR8 virus with both spike proteins of hemagglutinin and neuraminidase (NA) deoptimized had significantly less HA protein and NA RNA at 6 hours post-infection (HPI), which now suggests that maybe the mechanism of attenuation for CPBD is due to defects in both transcription and translation (Yang et al. 2013). Interestingly, mice vaccinated with this HA/NA_{min} virus were somewhat protected from heterologous challenge with two H3N2 strains, A/Aichi/2/1968 and A/Victoria/3/75 (Yang et al. 2013); these mice had less weight loss and lower mortality rates than unvaccinated mice which correlated to higher vaccination doses of the HA/NA min virus (Yang et al. 2013). Considering that A/Aichi/2/1968 and PR8 have only 42% amino acid

similarity between their HA and NA proteins, it is intriguing that this PR8 HA/NA_{min} vaccine would elicit cross-protective antibodies that would recognize heterologous strains (Yang et al. 2013). Perhaps these two proteins were somehow degraded, allowing the murine immune system to recognize cryptic and conserved epitopes, especially of the HA stem, which could explain the vaccine's low cross 50% protective dose (PD_{50}) of 147 PFU/mL against A/Aichi/2/1968 and 237 PFU/mL against A/Victoria/3/75 (Yang et al. 2013). These data indicate that vaccine candidates made with SAVE could potentially confer cross-protection to heterologous challenge, which would be ideal for pathogens such as influenza that have myriad strains that undergo antigenic drift and antigenic shift.

A paper published in 2013 by Martus and colleagues studied SAVE's effect on HIV, whose extremely high mutation rate might overcome the attenuating effect of SAVE's many silent mutations through rapid reversion. A HIV virus with its entire gag protein deoptimized was attenuated in MT-4 cells, a human T cell line that is very sensitive to HIV infection, and peripheral blood mononuclear cells (PBMCs), which include lymphocytes and monocytes (Martus et al. 2013). However, almost all isolates of six deoptimized HIV virus strains recovered wild-type replication capacity after 15 serial passages in MT-4 cells; deoptimized isolates showed a 1.5 to 12-fold higher mutation frequency compared to wild-type HIV (Martus et al. 2013). Sequencing revealed multiple deoptimizing mutations reverted to wild-type as well as incorporation of nonsynonymous mutations that mitigated the negative CPB of the viral genes (Martus et al. 2013). Therefore, SAVE may not been a safe method for retroviruses due

to the concern that the vaccine virus could revert to wild-type in the host to cause disease rather than induce immunity.

A recent study on four codon pair deoptimized respiratory syncytial virus (RSV) vaccine candidates by Le Nouën and colleagues were attenuated *in vitro* and *in vivo* (Le Nouën et al. 2014). The four deoptimized RSVs made were Min A, which had NS1, NS2, M, P, N, and SH genes deoptimized (Le Nouën et al. 2014); Min B, which had the G and F surface glycoprotein genes deoptimized; MinFLC virus, which was completely deoptimized except for the M gene; and Min L, which only had the L gene deoptimized (Le Nouën et al. 2014). Min FLC was the most temperature sensitive *in vitro* whereas Min B grew the least efficiently *in vitro*, indicating that deoptimization of the G and F genes were the most attenuating *in vitro*, probably because reduced surface glycoprotein concentrations led to incorrect molar ratios with other proteins, which could have generated less infectious virions capable of successfully spreading in cell culture (Le Nouën et al. 2014). Levels of deoptimized gene mRNA and protein were reduced compared to wild-type RSV (Le Nouën et al. 2014).

These deoptimized RSV vaccine candidates were tested in both mice and African green monkeys (AGMs). In BALB/c mice, only 1 mouse out of 5 mice had replicating MinA or Min L virus in their nasal turbinates compared to wild-type RSV 1 and 5 DPI (Le Nouën et al. 2014); titers of these two viruses in the lungs were between 2 to 10-fold lower compared to wild-type RSV on 1 and 5 DPI (Le Nouën et al. 2014). Min FLC did not replicate in either the nasal trubinates or lungs throughout the experiment (Le Nouën et al. 2014). In AGMs, significantly reduced viral shedding as well as reduced duration of shedding in the upper and lower respiratory tracts was seen

for Min A, Min L, and Min FLC when compared to wild-type RSV (Le Nouën et al. 2014). However, Min A and Min L vaccination induced similar antibody titers compared to wild-type RSV and all three Min viruses showed similar attenuation to two RSV strain currently in human clinical trials, suggesting that they could be used as vaccine candidates for humans (Le Nouën et al. 2014).

While most of the limited papers on CPBD and SAVE have been on viral pathogens, this technology can also be applied to bacterial pathogens. Since the genomes of bacteria are usually much bigger than viruses, SAVE would potentially not be as cost-effective especially as the desired deoptimized percentage of the genome increases as deoptimized sequences must be customly synthesized according to the CPB of the organism. A *Streptococcus pneumoniae* paper from Coleman and colleagues deoptimized the pneumolysin gene, the protein product of which is a virulence factor for this species as it creates pores in target cells (Coleman et al. 2011). Mice vaccinated with this deoptimized strain had better survival against wild-type challenge, which can be attributed to their significantly higher antibody titers against whole bacteria, pneumolysin, and pneumococcal capsule lipopolysaccharide (Coleman et al. 2011). This paper shows proof of concept of CPBD and SAVE's ability to successfully attenuate bacterial pathogens to create vaccine candidates.

This method theoretically could attenuate protozoan and fungal human pathogens as well by deoptimizing virulence genes, though the number of fully or partially deoptimized virulence genes required to acquire an attenuated phenotype probably will vary between pathogens. Deoptimizing other genes required for cell wall synthesis, membrane transporters and pumps, metabolism, DNA replication and repair,

transcription, translation, and protein modification probably would confer a more severely attenuated phenotype if there are safety concerns, but these strains might not grow well *in vitro* and thus would be difficult to upscale their manufacture.

SAVE could also be used to attenuate animal pathogens to create veterinary vaccines, which would take less time to get to market as they are easier to license due to looser requirements for safety. A recent paper attenuated vesicular stomatitis virus (VSV), which is both a veterinary and human viral pathogen that typically causes gastrointestinal disease, by partially deoptimizing the N-terminal segment of the L polymerase gene (Coleman et al. 2015). This L_{min} virus was nonviable in cell culture and produced very little L protein, showing that they overattenuated the virus and thus should not deoptimize as much of the L protein or deoptimize other genes instead (Coleman et al. 2015).

Summary of Projects. No papers using CPBD and SAVE to attenuate DNA viruses have been published, so SAVE's ability to attenuate DNA viruses is unknown. Given the previous failures of subunit HSV-2 vaccines in clinical trials and the fact that the only herpesvirus vaccine available is a LAV for varicella-zoster virus, LAVs for HSV-2 should be more seriously considered. Therefore, this thesis seeks to create HSV-2 LAV candidates by deoptimizing three essential viral genes – the polymerase (*U_L30*) and two nuclear egress complex proteins (*U_L31* and *U_L34*). These three genes were chosen because targeting genes important for viral genome replication and egress would maximize the possibility of generating an attenuated phenotype in the resultant mutants while still allowing full expression of other viral proteins. These deoptimized ORFs will be inserted into the HSV-2 strain R519 bacterial artificial chromosome

(BAC). Mutations will be made in this BAC by a two-step recombineering process called *galK*-DOG double selection (Warming et al. 2005). The galactose operon of *E. coli* contains *galE, galT, galK,* and *galM* which enable the cell to utilize galactose as an only carbon source (Warming et al. 2005); *galK* encodes galactokinase, which is responsible for phosphorylating galactose to galactose-1-phosphate to begin the galactose degradation pathway (Alper and Ames 1975; Warming et al. 2005). These three wild-type ORFs will be replaced by a *galK* cassette by homologous recombination, allowing transformants to be selected by growth on M63 minimal media with galactose. The *galK* cassettes will then be replaced with the CPBD genes using a 2-deoxy-galactose (DOG) selection to kill any *galK*-BAC-containing bacteria as galK catalyzes DOG into 2-deoxy-galactose-1-phosphate, a toxic intermediate compound that cannot be metabolized (Figure 4). Once the mutant viruses are rescued, their replication kinetics in multiple cell lines and their vaccine potential in mice will be characterized.

Mice will be monitored for primary infection symptoms such as lesion size, severity, number, and duration; hind limb paralysis; ruffled fur; lethargy; and death. Vaginal swabs will be analyzed by plaque assay to determine the titer of shedding virus. DRGs will be harvested for qPCR to quantify how many HSV-2 genomes established latency in the mice. Since mice do not have spontaneous reactivation like humans do, challenged mice should try to have reactivation induced to see how well the vaccine candidates reduce subsequent outbreak severity and duration. UV light has been used to reactivate latent ocular herpes, so perhaps UV light could also reactivate latent genital herpes. If reactivation cannot be achieved for mice, these experiments should

be repeated in guinea pigs to measure any effects on subsequent outbreak frequency, duration, and severity.

The CoP for HSV-2 are unknown, which makes vaccine development difficult in that researchers do not know what immune responses their vaccine candidates must induce to meet endpoints of infection prevention or disease reduction. Various monoclonal gD and gB antibodies (mAbs) have been shown to be effective at neutralizing HSV-2 *in vitro* and *in vivo*, suggesting that antibodies are important for preventing infection. First, literature 50% inhibitory concentration (IC_{50}) values, or the amount of mAb needed to inhibit viral infection by 50%, for specific gD and gB monoclonal antibodies will be confirmed. Mice will be injected with different doses of these antibodies and challenged with wild-type HSV-2 to analyze how well these antibodies protected from infection and disease. The same tests described for CPBD mice studies will be done for these mice as well to determine latency, shedding, and disease reduction.

Chapter 2: Methods

Section 1: Basic Molecular Biology Techniques

In accordance with proper sterile technique and BSL-2 safety rules, all protocols involving live cells and virus were completed in a certified biosafety cabinet with the researcher wearing a lab coat and gloves. Excess cells and unneeded virus were killed by submergence in a 10% bleach solution for at least 20 minutes. All used cell culture items such as flasks and plates were discarded in biohazard waste to be autoclaved. All large pipets used with pipet guns were disinfected in a Wescodyne solution prepared by autoclave staff.

Eukaryotic Cell Culture. Media requirements for Vero cells were DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% 100x Penicillin-Streptomycin-Glutamine (PSG, Gibco). To start a new cell culture, one cryovial of Vero cells was flash-thawed in a bath of 37 ^{o}C water before being resuspended in either 15 or 25 mL of pre-warmed 37 ^{o}C complete DMEM for a T_{75} or T_{175} flask, respectively. Flasks would be seeded at different confluencies as desired using the assumption that seeding 7 x 10^6 cells in a T_{75} flask and 1.2 x 10^7 cells in a T_{175} flask would result in 100% confluency in 24 hours. Vero cells were split every 3 to 4 days to maintain adequate confluency. To split cells, complete DMEM, 0.05% trypsin (Gibco), and 1x PBS (Gibco) were pre-warmed in a 37 ^{o}C water bath. Complete DMEM would be removed from the flask with a 25 mL pipet and the flask would be washed once with 10 mL 1x PBS to maximize trypsinzation efficiency. Then 5 mL of trypsin was added to the flask, which was incubated at 37 ^{o}C, 5% CO_2 until the cells would sluff off when

flask was tilted. Cells were resuspended by flooding them down the flask bottom with a 5 mL or 10 mL pipet multiple times until most cells were individual under the microscope. Then 0.1 mL of cells was added to 0.3 mL of complete DMEM to make a 1:4 dilution. Fifteen microliters of cells were flooded onto a clean hemocytometer with a clean coverslip. Cells in the four 4x4 squares were counted, added up, and multiplied by 10^4 to determine cells/mL. Flasks were reseeded with the desired number of cells and made up to 25 mL with complete DMEM. Flasks were incubated at 37 ^oC, 5% CO_2.

Preparation of Cell-Free HSV-2 Viral Stocks. T_{175} flasks were seeded with 1.2 x 10^7 Vero cells in 25 mL complete DMEM the day before infection and incubated at 37 ^oC, 5% CO_2 overnight. If flasks were not 100% confluent, they would be incubated at 37 ^oC, 5% CO_2 until they were. Complete DMEM was removed with pipet and 4 mL of fresh complete DMEM was added back to the flask. Flasks would be inoculated with different µL amounts of virus from a 1 x 10^7 PFU/mL HSV-2 viral stock depending on what multiplicity of infection (MOI) was desired. Flasks were incubated at 37 ^oC, 5 % CO_2 for 1 hour with tilting every 15 minutes before 21 mL of complete DMEM was added to the flask. Flasks were incubated at 37 ^oC, 5% CO_2 for two days or until when 100% cytopathic effect (CPE) was observed. Cells were removed from the flask bottom with a cell scraper and transferred to a 50 mL Falcon tube. If the rest of the protocol could not be immediately completed, the 50 mL Falcon was thoroughly vortexed before storing at -80 ^oC. Cells were spun at 800 rpm at 4 ^oC for 5 minutes. Media was aspirated off pellet until 3 mL were left and the remaining media and pellet were immediately frozen on dry ice. Tube was thawed in a 37 ^oC water bath for 2-3 minutes before being thoroughly vortexed for 30 seconds, a procedure called freeze-thawing. Tube was

freeze-thawed 3 times before spun at 2000 rpm at 4 ºC for 10 minutes. Supernatant was transferred to new 50 mL Falcon and transferred to cryovials in 0.2-0.5 mL aliquots. Aliquots were frozen on dry ice and stored at -80 ºC.

Plaque Assay. Plaque assays were stained using crystal violet, which was made in 1 L batches by combining 100 mL formaldehyde, 50 mL acetic acid, 600 mL methanol, 250 mL water, and 10 g crystal violet powder in a chemical fume hood. The appropriate number of 6-well plates were seeded at 5 x 10^5 cells/well in 2.5 mL complete DMEM per well and incubated at 37 ºC, 5% CO_2 the day before infection. Complete DMEM media was aspirated via low-power vacuum pipetting from all wells. The first well had 550 µL of complete DMEM added and all other wells had 500 µL complete DMEM added. Then 5.5 µL of HSV-2 viral stock would be added to the first well to create a 1:100 (10^{-2}) dilution that was mixed by either pipetting up and down or tilting the plate back and forth. Then 55 µL from this well would be added to the next well and mixed to create a 1:1000 (10^{-3}) dilution. This process was repeated for all wells with a new pipet tip to prevent inaccuracy in the 1:10 dilution scheme ranging from 10^{-2} to 10^{-7}. After the complete DMEM in the last well was mixed with 55 µL from the previous well, 55 µl would be discarded from the last well. All infected plates would be incubated at 37 ºC, 5% CO_2 for 1 hour with tilting every 15 minutes to ensure adequate viral contact with the Vero cell monolayer. During incubation, overlay medium containing human γ-globulin (intravenous immunoglobulin, IVIG) was made by adding 2.5 µL 10% IVIG per mL of complete DMEM needed. Viral inoculum was aspirated via low-power vacuum pipetting and 2.5 mL overlay medium per well was added to further inhibit viral spread. All plates would be incubated with overlay media

for 2 days at 37 ^{o}C, 5% CO_2. Overlay media was aspirated via low-power vacuum pipetting and at least 1 mL of crystal violet solution was immediately added to wells. All plates were incubated at room temperature (RT) for 1 hour. Plates were washed by repeatedly carefully submerging them in cold water and lifting them until water ran clear. Plates were dried upside down on paper towels. Plaques were first counted by eye using a pen to mark already counted plaques and number was recorded on side of corresponding well. Then plaques were confirmed via microscope with any additional plaques added to the number obtained by eye. Titer in PFU/mL was calculated as follows:

$$\text{PFU/mL} = (\#\ \text{plaques})(\text{dilution factor})(2)$$

Agarose Gel Electrophoresis. To make desired agarose percentage, an appropriate amount of ultrapure agarose was dissolved in the appropriate amount of 1x TBE buffer in a 500 mL glass flask. Flask was weighed on balance. Flask was microwaved for 2 minutes and left to cool until comfortable to touch. Flask was reweighed to determine mass lost due to evaporation; ddH_2O was added to make up to original weight as well as 5 μL ethidium bromide (EtBr) for nucleic acid staining. The liquid agarose was poured into a clean gel tray with its ends sealed with tape and the appropriately sized combs in place. Gel was allowed to set at RT after any bubbles were popped and debris removed. Tape was removed before placing the gel in the Horizon 11.14 BRL Horizontal Gel Electrophoresis Apparatus (Life Technologies, Gibco). Apparatus was flooded with 1x TBE until top of the gel was completely covered. A test mixture of 5 parts TE mM NaCl and 1 part 6x loading dye was used to ascertain well integrity. Each well to be used had 4 μL of text mixture pipetted in it to ensure there were no leaks or

breaks in the well. Text mixture was removed by pipetting 1x TBE up and down into the well. Each tube of DNA to be run had 2 μL 6x loading dye added to track their movement through the gel. About 4-6 μL of a DNA ladder of appropriate size, usually 1 kb, were added to the first well. About 15-25 μL of each tube of DNA were added to the next wells. Before running, another 10 μL EtBr was added to the 1x TBE buffer for better staining. Gels were run at constant voltage at appropriate voltages for the known or presumed lengths of the DNA fragments (≤1 kb fragments were run at 5 V/cm, 1-12 kb were run at 4-10 V/cm, and ≥12 kb were run at 1-2 V/cm). Gel would be run for several hours at higher voltages or overnight at low voltages.

Section 2: galK-DOG Double Selection Cloning Process

Description of HSV-2 Strain R519. HSV-2 Strain R519 is a plaque-purified strain from HSV-2 strain 333, which was isolated from a genital lesion by W. Rawls at Baylor College of Medicine in Houston, Texas and strictly passaged in human cells, presumably to prevent attenuating or non-human-species-specific adaptive mutations (Westmoreland and Rapp 1976). This strain also does not produce syncytia (Wang et al. 2012). Strain R519 was passaged in Vero cells for all following experiments.

Bacterial Cells. H312-1B-S1 was transformed in SW102 cells, a strain derived from DY380 so it has the λ prophage recombineering system. For detailed information about the creation of the H312-1B-S1 BAC using Cre-Lox recombination and *galK*-DOG double selection, please see Wang et al. 2012.

Media, Antibiotics, and Plates Used for Bacterial Culture. Chloramphenicol, an antibiotic that inhibits protein synthesis, was used as the selector for transformants. To make 12.5 μg/mL stock, 50 mg chloramphenicol was dissolved in 20 mL 100% ethanol

(EtOH) in a 50 mL Falcon tube; stocks were stored at -20 ºC when not in use and kept on ice when in use. Luria-Bertani (LB) media was made by combining 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 1 L of ddH_2O. LB agar (LA) was made by adding agar to 1.5% final concentration to the appropriate amount of LB media. LB and LA were autoclaved on P3 cycle to sterilize. LB was cooled to RT and stored on bench at RT for later use. LA was cooled to 56 ºC in a 56 ºC water bath before pouring. The appropriate amount of chloramphenicol to make a final concentration of 12.5 μg/mL was added to LA (LA-C) before plating 20 mL LA-C per plate. Plates were left at RT individually stacked for about 30-40 minutes to solidify. Plates were parafilmed and stored at 4 ºC until later use. Plates were placed at RT for several hours before use to reduce condensation and increase absorption. M9 media was made by adding 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, and 0.5 g NaCl to 1 L molecular grade water. 1x M63 minimal media was made by adding 2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, and 0.5 mg $FeSO_4 \cdot 7H_2O$ to 1 L molecular grade water, adding KOH to adjust pH to pH 7. After autoclaving on P3 cycle, sterilized M9 and M63 media were stored at RT. For M63 minimal media plates, the following solutions were made: 1 M $MgSO_4 \cdot 7H_2O$ solution by adding 24.67 g $MgSO_4 \cdot 7H_2O$ to 100 mL molecular grade water that was 0.22 μm sterile-filtered before storage at RT; 0.2 mg/mL d-biotin in molecular grade water that was dissolved by stirring on a stir plate and then 0.22 μm sterile-filtered into 10.5 mL aliquots in 15 mL Falcon tubes before storage at 4 ºC; 20% galactose or DOG in molecular grade water that was 0.22 μm sterile-filtered in aliquots stored at RT; 20% autoclaved glycerol in molecular grade water; and 10 mg/mL L-leucine in warm molecular grade water to dissolve leucine with stirring on a stir plate, then cooled and

0.22 μm sterile-filtered into 9.5 mL aliquots in 15 mL Falcon tubes for storage at -20 ^{o}C. To make M63 minimal media plates, 1 L 1x M63 media with 15 g agar (M63-A) was autoclaved on P3 and left to cool until comfortable to touch. Then 1 mL 1 M $MgSO_4 \cdot 7H_2O$, 10 mL galactose or DOG, 5 mL biotin, 4.5 mL leucine, and 500 μL 12.5 μg/mL chloramphenicol was added to M63-A mixture. After pouring 20 mL per plate, plates were left to solidify at RT before storage at 4 ^{o}C.

Transformation of DH5α Cells with galK *Plasmid*. The *galK* cassette was contained in a pBluescript SK (-) plasmid backbone that contains ampicillin resistance (Figure 3). The orientation of the f1 origin in the plasmid allows expression of antisense ssDNA. Two premade 100 μg/mL carbenicillin plates were thawed at RT. Two empty microcentrifuge tubes were chilled on ice. Control pUC19 plasmid and 78 ng/μL *galK* plasmid stock were thawed on ice with intermittent tapping to ensure dissolving. The galK plasmid stock was diluted to 1 ng/μL in a chilled microcentrifuge tube. Competent DH5α cells were thawed on ice; 50 μL were pipetted into each of the chilled empty microcentrifuge tubes. Then 0.5 ng of control pUC19 and 1 ng of galK plasmid were added to their respective tubes. Tubes were gently tapped to mix and incubated on ice for 30 minutes. Tubes were heat shocked at 37 ^{o}C for exactly 20 seconds and immediately returned to ice. After tubes were chilled for 2 minutes on ice, 0.95 mL LB media were added to the tubes. Tubes were incubated at 37 ^{o}C, 225 rpm for 1 hour for recovery. After recovery, 100 μL was plated on one half of each plate and 10 μL was plated on the other half of each plate. After spreading the cells, plates were left at RT to allow culture absorption. After culture was fully absorbed, plates were incubated upside down in a plastic bag at 37 ^{o}C overnight.

galK *Plasmid Purification*. Selected transformant colonies were picked with a sterile toothpick. Inoculated toothpicks were added to 5 mL LB with 0.1 mg/mL ampicillin. Tubes were incubated at 37 ºC, 225 rpm overnight. The galK plasmid was isolated using the QIAprep Spin Miniprep kit (QIAGEN). Bacterial cells were harvested at 8000 rpm at RT for 3 minutes. Supernatant was decanted and pellet resuspended in 250 µL Buffer P1. Resuspended cells were transferred to new microcentrifuge tubes before adding 250 µL Buffer P2. Tubes were mixed by inverting 4-6 times. Then 350 µL Buffer N3 was added to the tubes, which were immediately inverted 4-6 times. Tubes were centrifuged at 13000 rpm at RT for 10 minutes. Supernatants were transferred to new tubes and respun at the same conditions. Supernatants were added to QIAprep spin columns and centrifuged at 13000 rpms at RT for 60 seconds. Flow-through in the collection tubes was discarded. QIAprep spin columns were washed with 0.5 mL Buffer PB and centrifuged at 13000 rpm at RT for 60 seconds. Flow-through in the collection tubes was discarded. QIAprep columns were washed with 0.75 mL Buffer PE and centrifuged at 13000 rpm at RT for 60 seconds. Flow-through was discarded before additionally centrifuging the tubes at 13000 rpm at RT for 1 minute. QIAprep columns were placed in a new microcentrifuge tube. Plasmid DNA was eluted in 200 µL water by adding water to QIAprep spin columns, incubating at RT for 1 minute, and centrifuging at 13000 rpm at RT for 1 minute. Plasmid concentration was determined by nanodrop. Tubes were stored at -20 ºC.

Restriction Enzyme Digestion of Purified galK *Plasmid*. To confirm the galK plasmid isolated had the same restriction enzyme digestion pattern as the original stock used in the transformation, a double digestion was performed using NotI, which would cut at

position 1252, and HindIII, which would cut at position 4161, to create a 1267 bp fragment and a 2909 bp fragment. Tubes were made with 150 ng plasmid; 15.35 µL of a master mix consisting of 1 µL 10 U/µL NotI, 1 µL 20000 U/mL HindIII, and 2 µL 10x Buffer 3 (NEB) per tube; and water up to a total of 20 µL. Tubes were incubated at 37 ºC for 1 hour. DNA along with a 1 kb ladder was run on a 1% agarose gel at 90 V for 2 hours. Pictures of gel were taken in closed UV box.

Transformation of DH10B strain SW102 Cells with H312-1B-S1 BAC. Cells were thawed on ice and kept on ice at all times. Once thawed, 25 µL of cells were transferred to a chilled microcentrifuge tube on ice and then mixed with 1-5 µg H312-1B-S1. Mixture was pipetted into the slot of a 0.1 cm chilled cuvette, taking care to not create bubbles. The cuvette was electroporated at 25 µF, 1.75 kV, and 200 Ω with a shock value of at least 4.5. The cuvette was immediately removed after the machine beeped. One mL of recovery medium was pipetted up and down into the slot to resuspend the cells. Recovery medium with cells was transferred to a 10 mL round bottom tube and incubated at 37 ºC at 200-250 rpm for 1 hour. A LA-C plate was warmed at RT before 100 µL of culture was plated on one half of the LA-C plate. The remaining 900 µL was centrifuged at 13000 rpm for 1 minute to form a cell pellet. About 800-850 µL of supernatant was aspirated off and the pellet was resuspended in the remaining 50-100 µL media before being plated on the other half. Plate was incubated upside down at 37 ºC in a plastic bag to avoid drying out after culture was absorbed by agar.

BAC Purification. Any missing materials for the media were ordered as follows: d-biotin (Supelco) and DOG (MP) stored at 4 ºC; L-leucine, sodium phosphate dibasic anhydrous (Na_2HPO_4), and iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Sigma-

Aldrich) stored at RT; restriction endonuclease DpnI (NEB) stored in restriction enzyme ice block at -20 ^{o}C; and Expand High Fidelity PCR kit (Roche) stored at -20 ^{o}C. All BACs were purified using Macherey-Nagel's NucleoBond BAC 100 kit and protocol for low-copy plasmid purification with minor changes. SW102 cells transformed with the appropriate BACs were grown up in 100 mL cultures with 12.5 μg/mL chloramphenicol at 30 ^{o}C, 225 rpm overnight. Cells were pelleted at 6000g at 4 ^{o}C for 15 minutes and then resuspended in 24 mL of Buffer S1 with RNase A. After adding 24 mL Buffer S2 while swirling the tubes, they were gently inverted 6-8 times to mix and left at RT for 2-5 minutes. Then 24 mL of chilled Buffer S3 were added to tubes while swirling them, which were immediately inverted 6-8 times to mix and incubated on ice for 5 minutes. While incubating, NucleoBond BAC 100 columns were equilibrated with 6 mL of Buffer N2. Columns were emptied by gravity flow with flow-through being collected into a tray. Folded filters were placed in funnels placed on top of each column and wet with a few drops of Buffer N2. Contents of the now lysed cultures were loaded into the wet filters and allowed to flow through the columns by gravity. Filters were gently squeezed to remove more liquid. Columns were washed twice with 18 mL of Buffer N3. Buffer N5 was preheated to 50 ^{o}C to increase BAC yield. BACs were eluted from the columns with 15 mL warmed Buffer N5 into Oakridge tubes. BACs were precipitated by adding 11 mL RT isopropanol to the tubes and centrifuging at 15000g at 4 ^{o}C for 30 minutes. After supernatants were discarded in 50 mL Falcons in case the pellets were lost, 1 mL RT freshly prepared 70% ethanol was added to the pellets. Tubes were vortexed briefly before centrifugation at 15000g at RT for 10 minutes. Ethanol was removed from the pellets via pipetting in

microcentrifuge tubes in case pellets were lost. Pellets were allowed to dry for 10-20 minutes before resuspending them in the desired amount of ddH_2O. Pellets were checked every 5 minutes while drying to ensure they did not over-dry. Pellets were resuspended in 100 µL water by swirling tube as pipetting could shear the BACs. Tubes were put in 65 ^oC water bath for 1 hour to facilitate dissolving. Water containing resuspended pellets was transferred to empty microcentrifuge tubes. Another 100 µL of water was added to Oakridge tube to wash and dissolve any remaining BAC DNA and transferred to the corresponding microcentrifuge tubes. BAC DNA concentration was determined by nanodrop before storage at 4 ^oC.

galK-*DOG Double Selection*. As this procedure is quite long, it has been broken down into subsections. This entire process has been adapted from the procedure detailed in Warming et al. 2005 with minor changes.

Confirmation of R519-Specific Mutations. Any R519-specific mutations in and around *U_L30*, *U_L31*, and *U_L34* were found via alignments of multiple HSV-2 strains to reference HSV-2 strain HG52 that were compiled by Dr. Susanna Lamers of Bioinfoexperts, LLC and Dr. Ruchi Newman formerly of the Broad Institute and now of the Ragon Institute of MGH, MIT, and Harvard. Mutations were confirmed by analysis of a deep sequencing file of R519 to ensure mutations were genuine (Table 1).

PCR of galK *cassette*. Primers (IDT) were designed as follows: a 50 bp homologous region to flanking areas of *U_L30*, *U_L31*, and *U_L34* attached to 20-25 bp of *galK* sequence to make 70-75mers (Table 2). Lyophilized primers were dissolved in the appropriate amount of sterile molecular grade water to make 100 µM stock solutions which were stored at -20 ^oC when not in use. Stock solutions were used to

make a 1:20 dilution for 5 μM working concentration solutions by adding 1 μL stock solution to 19 μL sterile molecular grade water. galK plasmids were diluted to 10 ng/μL in molecular grade water. PCR was performed on the *galK* cassette with the above primers. Each PCR tube was made as follows: 1 μL 10 ng/μL galK plasmid, 3 μL 5 μM forward primer, 3 μL 5 μM reverse primer, 4.5 μL 10x EHF buffer, and 33.5 μL molecular grade water. A master mix of dNTPs and DNA polymerase was made to have the following volumes of each reagent per tube: 0.5 μL 10x EHF buffer, 1 mM dNTP, 0.35 μL EHF polymerase, and 3.15 μL molecular grade water. Mixtures were run under the following conditions: 96 ºC for 5 minutes; 30 cycles of 94 ºC for 15 seconds, 60 ºC for 30 seconds, and 72 ºC for 1 min; 72 ºC for 7 minutes; and 4 ºC indefinitely. Each tube had 5 μL master mix added when the first 30 seconds at 60 ºC was hit. After PCR run was done, 2 μL of 6x loading dye was added to 6 μL of each PCR reaction. All PCR reactions along with a 1 kb ladder were run on premade 1% agarose gel at 90 V for 2 hours. Remaining PCR reactions were stored at -20 ºC. This PCR reaction was done twice and so corresponding PCR products were pooled together after confirmation on gel that all PCR products from both runs were the appropriate length of 1.3 kilobases (kb) without unknown bands or contamination.

DpnI Clean-Up of galk *Cassette PCR Products*. Clean-up was performed by adding 2 μL DpnI per 50 μL PCR products, which only cuts methylated DNA and thus will degrade any template plasmid DNA left. Mixtures were incubated at 37 ºC for 1 hour before being run on a premade 0.8% agarose gel at 25 V overnight.

Gel Purification of Dpn1-Cleaned-Up PCR Products. Gel extraction of these cleaned PCR products was performed using the QIAquick Gel Extraction kit

(QIAGEN). A scalpel was used to cut out the UV-illuminated bands on the gel. Gel fragments were placed into microcentrifuge tubes and weighed. Three volumes of Buffer QG were added to 1 volume of gel; for example, one gel fragment weighed 270 mg, so 3 x 270 = 810 μL Buffer QG was added to it. If fragments weighed more than 400 g, multiple spin columns were used. Tubes were incubated at 50 ^{o}C for 10 minutes until the gel fragments were dissolved. The colors of the tube were confirmed to be yellow. One gel volume of isopropanol was added to each tube. After mixing, the solutions were pipetted into QIAquick spin columns placed in collection tubes and centrifuged at 13000 rpm at RT for 1 minute. Flow-through was discarded and 0.5 mL Buffer QG was added to each QIAquick spin column to remove all agarose. QIAquick spin columns were centrifuged at 13000 rpm at RT for 1 minute again. Flow-through was discarded before the spin columns were washed with 0.75 mL Buffer PE. Columns incubated at RT for 5 minutes with Buffer PE before being centrifuged at 13000 rpm at RT for 1 minute. Flow-through was discarded before an additional spin at the same conditions was performed to remove all ethanol. Spin columns were placed into empty microcentrifuge tube. DNA was eluted into 30 μL molecular grade water. Spin columns were incubated at RT for 1 minute after water was added. After centrifugation at 13000 rpm at RT for 1 minute, 2 μL of purified DNA along with a 1 kb ladder was run on a premade 0.8% agarose gel at 90 V for 90 minutes. Approximate DNA concentration was determined by intensity and size of the bands compared to the 60 ng of the 1000 kb band in the 1 kb ladder. Eluted DNA was stored at -20 ^{o}C.

Transformation of H312-1B-S1 with galK Cassette. Cleaned PCR products were diluted to make 5 ng/μL working concentration stocks, which were stored at -20

^{o}C until use. A vial of SW102 bacteria containing the H312-1B-S1 BAC was placed on dry ice. Three 5 mL aliquots of LB with 12.5 μg/mL chloramphenicol in 50 mL Falcon tubes were inoculated with SW102 glycerol stock by scraping off some frozen stock with a loop and swirling it in the LB media. Glycerol stock was immediately returned to -80 ^{o}C after use. Tubes were loosely capped and incubated at 30 ^{o}C, 225 rpm overnight. Ultracentrifuge was set to 1 ^{o}C with the appropriate JA-20 rotor attached. Two ice buckets were prepared with one being used to chill ddH_2O and 0.1 cm cuvettes and the other being used as an ice-water slurry for making competent cells. M63-galactose plates were left at RT to warm up. A 25 mL LB with 12.5 μg/mL chloramphenicol was inoculated with 0.5 mL of the cloudiest overnight culture. Culture was shaken in a 30 ^{o}C water bath at 120 rpm for 3-4 hours until OD of culture was between 0.55-0.6. Then 10 mL of this culture was transferred to a 50 mL Falcon tube, which was then heat shocked for 15 minutes in a 42 ^{o}C water bath to induce recombineering activity. Another 10 mL aliquot of culture was left at 30 ^{o}C as uninduced negative control. Both cultures were cooled in the ice-water slurry, transferred to Oakridge tubes, and centrifuged at 8000 rpm at 1 ^{o}C for 5 minutes. The following process was repeated twice: supernatants were decanted, pellets resuspended in 1 mL ice-cold ddH_2O by swirling the tubes in the ice-water slurry; once resuspended, 9 mL ice-cold ddH_2O were added to the tubes before centrifugation at 8000 rpm at 1 ^{o}C for 5 minutes. Supernatants were decanted and tubes inverted on paper towels to soak up excess supernatant. Pellets were kept on ice and resuspended in 80 μL of ddH_2O. Microcentrifuge tubes for electroporation were made as follows: 50 μL either induced or uninduced control cells with 4 μL of 5 ng/μL *U_L30*, *U_L31*, or *U_L34*-galK

PCR products. These mixtures were transferred to chilled 0.1 cuvettes which were electroporated at 25 μF, 200 Ω, 1.75 kV for a duration of at least 4.8. Cuvettes were immediately removed from electroporation machine and 1 mL LB media was pipetted into their slots to resuspend the transformed cells. Transformed cells were transferred to new microcentrifuge tubes and were recovered for 1 hour in a shaking 30 ^{o}C water bath. Cells were pelleted at 13000 rpm at RT for 30 seconds. After supernatants were decanted, pellets were twice resuspended in 1 mL 1x M9 media and re-centrifuged again at 13000 rpm at RT for 30 seconds. Supernatants were decanted and pellets resuspended in 1 mL 1x M9 media for plating on pre-warmed M63-Galactose plates. For uninduced controls, 100 μL of cells were plated on whole M63-Galactose plates. For induced cells, 10 μL were plated on one half of plates and 100 μL on the other half of plates. The remaining volumes of the transformed cultures were plated on separate whole M63-Galactose plates. All plates were incubated inverted and in a plastic bag at 30 ^{o}C for 3 days. Colonies were chosen with a loop and restreaked on new M63-Galactose plates every 3 days for 3 rounds of colony purification. All previous round plates were parafilmed and kept at 4 ^{o}C once the next round began.

Section 3: HSV-2 Neutralization Assay by Monoclonal gD and gB Antibodies

Antibodies. gD and gB antibody hybridomas were grown up and resultant purified gD and gB antibodies were supplied by Lawrence Lance. Four antibodies to gD – DL11, 1D3, MC5, and MC2 – and one antibody to gB – C226 – were used in this experiment. Antibodies were aliquoted in 1-1.5 mL per microcentrifuge tube. Aliquots for immediate use were stored at 4 ^{o}C to avoid freeze-thawing while all others were stored

at -20 ^{o}C. These antibodies' epitopes and other characteristics have been previously described (Bender et al. 2007; Lazear et al. 2012).

Neutralization Assay. Vero cells were seeded in 6-well plates at 8 x 10^5 cells per 2.5 mL complete DMEM per well. Plates were incubated at 37 ^{o}C, 5% CO_2 overnight. Antibodies were diluted as follows: 100 µL of 4 mg/mL MC2 and 1D3 were added to 100 µL 2x PBS to make 200 µL 2 mg/mL solutions. Then 100 µL of 2 mg/mL MC2, 1D3, DL11, and C226 were added to 100 µL sterile water to make 200 µL 1 mg/mL solutions. For controls, premade 1:10 IVIG (1%, 10 mg/mL IVIG) and 1x PBS made by diluting 100 µL 2x PBS in 100 µL sterile water were used. Antibodies and controls were diluted in 24-well plates by using a multichannel pipet to add the appropriate volume of PBS to all columns. For the experiment shown in Figure 6, 267.84 µL 1x PBS was added to the first column for MC2, DL11, 1D3, and 1x PBS to start a 1:2 dilution series with a 1:250 dilution as the first dilution. For C226 and 1:10 IVIG, 243 µL 1x PBS was added to the first column to start a 1:2 dilution series with a 1:20 dilution as the first dilution. All other columns were filled with 135 µL 1x PBS. Then 2.16 µL of MC2, DL11, 1D3, and 1x PBS were added to their first column wells and serially diluted by pipetting 135 µL from the previous well to the next well after mixing by pipetting up and down. After mixing the last well's dilution, 135 µL were discarded. This same process was repeated for C226 and 1:10 IVIG with 27 µL instead of 2.16 µL being added to the first well. HSV-2 R519 virus with a titer of 3.29 x 10^5 pfu/mL was thawed on ice. It was then diluted in cold complete DMEM so as to produce around 120 plaques per 135 µL per well. For this experiment, a total of 4.68 x 10^4 pfu were needed in 5.265 mL complete DMEM for 39 wells, so viral stock needed to be diluted

to 8.89 x 10^2 pfu/mL. Therefore, 19.6 µL of 3.29 x 10^5 pfu/mL viral stock were added to 7.3 mL complete DMEM. Then 135 µL of diluted virus was added to each well of antibody dilution made in the 24-well plates. The virus-Ab mixtures were incubated at RT for 1 hour with tilting every 20 minutes. Complete DMEM was removed from 6-well Vero plates via vacuum pipeting and replaced with 200 µL pre-warmed complete DMEM with Repipetter. All virus-Ab mixtures were transferred to Vero cells and incubated at 37 ºC, 5% CO2 for 1 hour with tilting every 15 minutes. Inoculum was removed via vacuum pipetting and replaced with 2.5 mL overlay medium per well. This overlay medium is identical to that used in the plaque assay described above. Plates were incubated at 37 ºC, 5% CO_2 for 2 days. Overlay media was removed from wells via vacuum pipetting. Wells were stained with 1 mL plaque staining solution and incubated for 1 hour at RT. Plates were washed in water by submerging and lifting until water ran clear. Plates were dried on paper towels before plaque counting as described in the plaque assay protocol above. The IC_{50} values of antibodies and 1:10 IVIG control were calculated in Prism.

Calculation of IC_{50} in Prism Software. After opening the Prism software, the option "Enter and plot a single Y-value for each point" was selected. Dilution values were entered in the X column as x-values and plaque numbers were entered in the Y column as y-values. Next, the "Analyze" option was chosen to bring up a menu screen; the option "Transform" was chosen from this menu screen to drop down a small options list. After the option "Transform X values using" was chosen, the option "X = log(X)" was chosen and confirmed by clicking "Okay." After the "Analyze" option was selected again, the "Nonlinear regression" option was chosen and confirmed by

clicking “Okay.” After confirmation, the option “Standard Curves to interpolate” was selected and then the “sigmoidal, 4PL, X is log(concentration)” option was selected. The “Constrain” option was selected to set top and bottom constraints. Top constraint was set to the average number of plaques – in this experiment, 61.8 – found in the 1x PBS negative control. Bottom constraint was always set to 0 plaques. The constraints were confirmed by clicking “Okay.” The resulting graph was generated with a list of statistics such as IC_{50} values, log(IC_{50}) values, degrees of freedom, R square values, and absolute sum of squares values. IC_{50} values given by Prism are unitless since dilution was used as the independent variable. To find IC_{50} values in concentrations, the original concentration of the mAbs – 1 mg/mL – were divided by the IC_{50} calculated.

Chapter 3: Results

First Step of galk-DOG Double Selection

PCR Products. PCR was performed on the *galK* gene with primers that had 50 bp homology to the flanking regions of U_L30, U_L31, and U_L34 to create the corresponding *galK* cassettes (Figure 5). When products were run on a gel, all bands were 1.3 kbp, which corresponded to the expected 1331 bp length of *galK* (Figure 5A). No strong bands at other lengths were seen, but leftover template DNA probably was still present. PCR products were then cleaned with DpnI, which cleaves methylated DNA, to remove any traces of pGalK template DNA. These cleaned products were again run on a gel, showing the same length of 1.3 kbp as before with no bands at 4.2 kbp, which is the size of pGalK. These bands were gel purified and run again on a gel to assess approximate concentration (Figure 5B). Since the 1 kb band is 60 ng, U_L30 and U_L31 are about 130 ng/μL and U_L34 is about 65 ng/μL.

Transformation of H312-1B-S1 *with* galK *Cassette*. All plates with transformants grew on the M63-Galactose plates, indicating initial success that the corresponding galK cassettes replaced U_L30, U_L31, and U_L34 in the H312-1B-S1 BACs. After three rounds of colony purification, no colony "dropped out," meaning that it suddenly didn't grow in the next round, which would indicate that it was a hitchhiker WT BAC-containing colony. No confirmation other than the fact that they grew on M63-Galactose media, indicating that they were able to break down galactose presumably due to expression of *galK*, to confirm correct *galK* cassette insertion was done for this thesis, so please see future directions for more reliable confirmation experiments.

Neutralization Assay of Monoclonal gD and gB Antibodies

IC_{50} Values of gD and gB mAbs. A single neutralization assay experiment determined the IC_{50}s for three gD mAbs – MC2, DL11, and 1D3 – and one gB mAb C226 compared to a 1:10 dilution of IVIG and a PBS-only negative control (Figure 6). These three gD antibodies' epitopes and structure have been previously described by Lazear and colleagues (Lazear et al. 2012). MC2 is a HSV-2-specific antibody that recognizes a discontinuous epitope (aa 246) on the fusion activation face of gD (Lazear et al. 2012). IC_{50} values were generated based on the concentration of antibody needed to neutralize 50% of HSV-1 strain KOS plaques and HSV-2 strain 333 plaques on Vero cells 2 DPI. MC2 required over 25 μg/mL to neutralize HSV-1 compared to 0.781 μg/mL for HSV-2 (Lazear et al. 2012). DL11, 1D3, and C226 are all common-type antibodies, meaning they recognize both gD1 and gD2 (Bender et al. 2007; Lazear et al. 2012). DL11 recognizes a discontinuous epitope (aa 132. 140) on the receptor binding face of gD (Lazear et al. 2012). DL11 had low IC_{50}s for both strains, needing only 0.004 ug/mL to neutralize HSV-1 and 0.312 μg/mL to neutralize 50% of HSV-2 strain 333 (Lazear et al. 2012). 1D3 recognizes a continuous epitope (aa 10-20) on gD (Lazear et al. 2012). 1D3 has slightly better neutralizing activity on HSV-1 with an IC_{50} of 0.390 μg/mL for HSV-1 strain KOS and 6.2 μg/mL for HSV-2 strain 333 (Lazear et al. 2012). C226 recognizes a pseudocontinuous epitope (aa 234-472) in the FR2 domain of gB to block viral fusion (Bender et al. 2007).

All three gD mABs had lower IC_{50}s than that of IVIG, indicating that they were more effective than 1% IVIG at inhibiting HSV-2 infection (Table 3). MC2 had a IC_{50} of 548.6, DL11 had a IC_{50} of 1448, and 1D3 had a IC_{50} of 1126. Therefore, when 1

mg/mL is divided by these values, the IC_{50} concentrations are as follows: 2 μg/mL for MC2, 0.7 μg/mL for DL11, and 0.9 μg/mL for 1D3. The 1:10 IVIG dilution had an IC_{50} of 105.7, so when 1 mg/mL is divided by this value, an IC_{50} concentration of 9 μg/mL is given. Therefore, MC2 was about 2.5-fold less effective at HSV-2 R519 neutralization compared to its literature value of 0.781 μg/mL for HSV-2 333. DL11 was also about 2-fold less effective at R519 HSV-2 neutralization compared to its literature value of 0.312 μg/mL for HSV-2 333. On the other hand, 1D3 was about 7-fold more effective at neutralizing HSV-2 R519 since its literature value for HSV-2 333 neutralization was 6.2 μg/mL. C226 had a very low IC_{50} of 46.9, giving its IC_{50} concentration as 20 μg/mL. Bender and colleagues found C226's IC_{50} to be 1.2 μg/mL, showing that this antibody was about 17-fold less effective at neutralizing virus than its literature IC_{50} concentration (Bender et al. 2007). The PBS-only control did not show any neutralization activity, indicated by the relatively straight best-fit line made by its points versus the sigmoidal lines made by the mAbs and IVIG.

Chapter 4: Discussion

Section 1: Discussion of Results

Viability of galK *clones of* H312-1B-S1. A high number of transformant colonies for each CPBD gene (named *galK-U_L30, galK-U_L31,* and *galK-U_L34)* were colony purified at least three times to minimize the probability of mistakenly growing up hitchhiker colonies containing the wild-type H312-1B-S1 BAC. The colonies for *galK-U_L31* and *galK-U_L34* seemed consistently smaller than the colonies for *galK-U_L30*, so colonies of both small and large colony phenotypes were purified. Due to no colony "dropping out" during the three rounds of purification, it is reasonable to assume that all isolated transformant colonies of each CPBD gene were successfully transformed with their respective *galK* cassette. However, it is possible that the *galK* gene could have been inserted non-homologously into a different site in the BAC HSV-2 genome than expected, resulting in *E. coli* containing different BAC clones that grow at different rates.

Monoclonal gD and gB Antibody IC_{50} values. The neutralization assay shows that the gD antibodies MC2, DL11, and 1D3 had IC_{50}s five to ten times that of IVIG, whose IC_{50} of 105.7 or 9 μg/mL is around what is normally expected of IVIG. The IC_{50}s were 2 μg/mL for MC2, 0.7 μg/mL for DL11, and 0.9 μg/mL for 1D3, showing that all these gD antibodies were highly effective at neutralizing HSV-2 strain R519. The gB antibody C226 had a low IC_{50} of 46.9, meaning its IC_{50} was 20 μg/mL, which is in stark contrast to its published IC_{50} of 1.2 μg/mL (Bender et al. 2007). C226 was found to be about 17-fold less effective in this experiment compared to its literature IC_{50}.

Though it may seem that IVIG should be the best at neutralizing HSV-2 as it is pooled IgG from thousands of human donors, there could be several reasons as to why IVIG was less effective than all by one mAb. Since IVIG contains antibodies against multiple pathogens, the concentration of HSV-2-specific antibodies might be relatively low. Also, the affinity of these HSV-2-specific antibodies might be low as well, so they cannot neutralize HSV-2 due to poor binding to epitopes. Epitope competition between antibodies may also play a role in low neutralizing activity; if multiple antibodies bind the same epitope, fierce competition for these common epitopes could lead to obscuration of more cryptic epitopes of antibodies that might be more potently neutralizing. Another scenario may be IVIG just contains non-neutralizing antibodies which cannot neutralize virions at all.

The large discrepancy between C226's IC_{50} value in the literature and this neutralization assay needs to be resolved. Repeating this neutralization assay would confirm if these data were incorrect especially since only one neutralization experiment was performed due to time constraints. Other factors may contribute to this inconsistency such as the age of the antibody, how it was stored, and how many freeze-thaws it went. As it stands, Bender et al.'s reported literature IC_{50} value is quite low, which may be due to the use of fresh, newly isolated antibody that had not undergone freeze-thaws, which causes antibodies to lose activity (Bender et al. 2007). The antibodies used in this experiment were sent from another lab that might have stored them differently than Bender's lab. Aliquots were stored at 4 ^{o}C for over a month before use in this experiment, but perhaps 4 ^{o}C was not cold enough to retain antibody activity

despite the lack of freeze-thawing. In addition, the IC_{50} values of the gD mAbs should be reconfirmed with more repetitions of neutralization assays.

The differences between the literature and experimental IC_{50}s also could be due to differences in the epitopes recognized by these antibodies between HSV-2 strain 333 and R519. Even though R519 is a plaque-purified strain of 333 so the nucleotide and amino acid differences between the two strains should be minimal, it is possible that some mutations had been introduced into the R519 genome during plaque purification that have changed the epitopes on gD and gB. These changes to the epitopes could have decreased or increased these antibodies' affinity for their epitopes, which would explain why some antibodies such as C226, DL11, and MC2 were less effective while other antibodies such as 1D3 were more effective at virus neutralization.

Section 2: Future Directions

Cloning. Purified BACs for all transformants need to be digested to see if they show the correct restriction enzyme digestion pattern on an agarose gel for H312-1B-S1. Primers were already made to PCR the *U_L30*, *U_L31*, and *U_L34* regions to see if the *galK* cassette was inserted with the expected lengths for non-insertion (3856 and 745 bp for *U_L30*, 1083 bp for *U_L31*, and 1077 bp for *U_L34*) and *galK* insertion calculated. PCR will be run on the colony purified bacteria's BACs and products run on gels to see if the correct *galK*-BAC lengths of 1464 bp for *U_L30*, 1497 bp for *U_L31*, and 1577 bp for *U_L34* were seen. If so, it would be reasonable to assume that these colonies do contain the BACs with *galK* instead of spontaneous gain of the ability to grow on M9 media. However, it is possible that the *galK* gene might have undergone non-homologous recombination into BAC vector, so the desired construct might not have been obtained.

One way to further confirm the correct insertion of *galK* in the desired loci is to sequence the *U_L30*, *U_L31*, and *U_L34* regions from purified BACs isolated from these colonies. To confirm that H312-1B-S1 has not undergone major alterations during insertion of the *galK* cassette and subsequent amplification in bacteria, restriction enzyme digestion of the BAC after *galK* cassette insertion should be done.

Because only the first step of *galK* insertion was completed for this thesis, the second step of replacing the *galK* gene with the CPBD *U_L30*, *U_L31*, and *U_L34* genes must be completed next. These three genes would be inserted into their proper place and orientation into their respective BACs via the same transformation protocol described in the Methods section, thereby creating three BACs each containing a single CPBD gene – H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34. Transformed *E. coli* cells will be plated on media containing DOG to select for H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34 transformants as cells still containing BACs with *galK* insertions will die since galK catalyzes DOG into a toxic intermediate. Considering this second selection step is relatively leakier than the first step, more than three rounds of colony purification on DOG-containing media should be done to minimize hitchhiker *galK*-BAC-containing colonies.

Purified colonies containing H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34 will be grown up in LB media containing chloramphenicol to maintain the BACs, as described above. Considering that these BACs are extremely low copy number compared to plasmids due to their large size, much troubleshooting has determined that at least 100 mL of culture needs to be used during the BAC purification process to get visible pellets and usable amounts of BAC. Therefore,

multiple purified colonies will be grown up in sizes of at least 100 mL and undergo BAC extraction using the Macherey-Nagel BAC 100 kit to confirm these colonies indeed have H312-1B-S1-U_L30, H312-1B-S1-U_L31, or H312-1B-S1-U_L34.

Purified H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34 will be nucleofected into Vero cells using Lonza's Nucleofector kit and Amaxa protocol for Vero cells. Transfection of BACs by conventional methods of lipofectamine or Trans-It has low efficiency due to their much larger size than plasmids. Nucleofected Vero cells will be added to T_{175} flasks with 25 mL complete DMEM and incubated at 37 ^oC until cytopathic effect (CPE) is 90-100% before the resultant viruses are harvested. Considering that these resultant viruses might show some attenuation in cell culture, the amount of incubation time needed until 100% CPE may vary between the H312-1B-S1-U_L30, H312-1B-S1-U_L31, or H312-1B-S1-U_L34 nucleofected Vero cell flasks. Cell-free virus will be made from harvested infected Vero cells and titered by plaque assay to determine viable virus content in PFU/mL. When not in use, cell-free virus will be stored in cryovials at -80 ^oC to preserve viability. The viruses will be characterized by restriction enzyme analysis to ensure no genome rearrangements occurred during their rescue from bacteria and to confirm the codon deoptimized sequences are stable.

The H312-1B-S1-U_L30, H312-1B-S1-U_L31, or H312-1B-S1-U_L34 viruses will first be characterized in cell culture before testing them in mice. Growth curves will be done for each virus to ensure they grow to reasonably comparable titers to wild-type virus. If these viruses are too attenuated to grow to high enough titers, replacement of the fully CPBD *U_L30*, *U_L31*, and *U_L34* with partially CPBD *U_L30*, *U_L31*, and *U_L34*

should be done to see if it rescues the low titer phenotype *in vitro*. If they grow to comparable titers to wild-type *in vitro,* they show that they have potential as vaccine candidates from a manufacturing standpoint and should be evaluated for safety and efficacy in mice, respectively.

CPBD H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34 Vaccine Candidate Animal Studies. Evaluating these viruses as vaccine candidates first requires confirming their safety in mice. BL/6 female healthy 6-week-old naïve mice will be inoculated intravaginally with a range of doses to determine the mouse lethal dose 50 (MLD_{50}), or the number of virions required to kill off 50% of infected mice, of the viruses. The dosage scale should be calculated based on known dosages of wild-type virus that mice can and cannot survive. The dosage scale should incorporate very low dosages that mice theoretically should be able to survive, so any deaths or euthanasia due to weight loss or hind limb paralysis in these low dosage groups would immediately indicate if these viruses have very low LD_{50}s and thus are unsafe. Conversely, the dosage scale should also include very high dosages that mice theoretically should not be able to survive to make sure the LD_{50}, which should be higher for H312-1B-S1-U_L30, H312-1B-S1-U_L31, and H312-1B-S1-U_L34 as they contain CPBD genes, is not missed. Obviously, this safety study should be controlled with mice receiving the same dosages of parent wild-type strain R519 as a positive control and receiving only the vaccine formulation without virus as a negative control to check for toxicity of non-viral materials.

Mice will be monitored for primary infection symptoms such as genital lesions, lethargy, ruffled fur, hind limb paralysis, and death. Number, severity, and duration of

lesions should be assessed to see if there is any noticeable difference compared to wild-type infected mice. Bleeds should be done to look at antibody titers via neutralization assays and T cell responses via flow cytometry.

The major concern would be to determine if the vaccine viruses were able to establish latency in the DRGs, which can be assessed in three major ways. First, periodical vaginal swabbing over at least several weeks and preferably longer to better mimic viral shedding in humans should be done for plaque assays to assess frequency and amount of viral shedding, which would suggest that the vaccine virus established latency in the DRGs. Further analysis of vaginal mucosal immunity should be done via neutralization assays using vaginal swabs to see how much antibody is traversing the mucosal layer of the vagina. However, since mice do not shed for more than 7-10 days after vaccination, these swabs would only inform about acute vaginal shedding; chronic vaginal shedding over long durations would have to be assessed in guinea pigs. Second, stimulation of HSV-2 reactivation in vaccinated mice would show if recurrent lesions formed, which could only happen if latency was established in the DRGs. Since UV light has been used to reactivate latent ocular herpes, UV light should be used to try to stimulate reactivation of latent genital herpes. If reactivation is not achieved in mice, this information will be gathered from vaccinated guinea pigs, which do have spontaneous reactivation. Third, all mice should have their DRGs harvested for quantitative PCR (qPCR) to determine how many HSV-2 genome copies, if any, are in the DRGs. Ideally, these vaccine candidates should not establish latency in the DRGs and consequently have little to no sustained viral shedding in the genital mucosa while still inducing a robust enough antibody and T cell response to satisfy the unknown

CoPs to protect from infection for a prophylactic vaccine or from disease for a therapeutic vaccine.

If any mice died during the safety study, especially at the lower dosages, their antibody response in particular should be compared to mice who survived to see if there is any difference between type and number of antibodies generated. This scenario may indicate that the viruses are not attenuated enough to be safe, which means that the viral BACs should be reconstructed with more CPBD genes or other attenuation methods like missense mutations or deletions. For example, any combination of the three CBPD genes – *U_L30/U_L31*, *U_L31/U_L34*, *U_L30/U_L34*, or *$U_L30/U_L31/U_L34$* – could be inserted together into the BAC to further attenuate the virus. On the other hand, if very little antibody response was generated at the higher dosages, these viruses may be too attenuated to elicit an immune response that would protect them from infection or disease. These BACs would then have to be reconstructed with only partially CPBD *U_L30*, *U_L31*, and *U_L34* to partially rescue the attenuated phenotype to boost viral replication to induce a more robust immune response.

After safety of these vaccine candidates have been confirmed in mice, vaccination with viral challenge studies should be conducted to analyze their ability to either prevent infection or protect from disease, hopefully allowing the deduction of the CoP for HSV-2. Again, BL/6 female healthy 6-week-old naïve mice will be vaccinated intravaginally with different doses chosen based on data from the safety studies. Any primary infection symptoms caused by the vaccine viruses should be noted, especially if these symptoms were not seen in the safety studies.

Then these mice should be challenged with different doses of wild-type HSV-2 to determine the extent of protection they provide. Challenge doses should be chosen based on the MLD_{50} of the wild-type virus; a low dose that the vaccine virus should be able to protect against, a medium dose that mimics the average challenge viral dose that humans encounter during natural infection, and a large dose that theoretically should overwhelm the immune response should be included. Multiple challenges over several weeks to months and even longer to mimic the multiple exposures to HSV-2 humans have during their lifetimes should be done to see if the vaccine candidates established long-lasting immunity in the hosts. During these long-scale challenges, bleeds and vaginal swabs of the mice should be done to look at antibody titer as well as T cell changes over time to determine what overall and mucosal immune responses are necessary to protect from infection or disease.

Lack of protection at the low and medium doses would indicate that the vaccine is not viable whereas protection at the high dose indicates that the vaccine induces a very robust immune response that probably exceeds that induced by natural infection, so it should be a good vaccine candidate for humans. The severity, duration, and number of lesions in addition to mouse-specific HSV-2 symptoms such as hind limb paralysis, lethargy, and ruffled fur should be monitored during the challenges. Vaginal swabs should be done to determine frequency and amount of viral shedding, neutralization assays using vaginal swabs should be done to look at musical antibody titers, and DRGs from these mice also should be harvested to determine if the challenge virus established latency. If these vaccine candidates show promise in all of these areas, they potentially could be effective in humans.

If intravaginal vaccination of mice with these vaccine candidates has yielded good results, a number of further analyses using different animal models and inoculation routes should be used. A HSV-2 vaccine would not be widely accepted or used if it required intravaginal vaccination for humans considering cultural norms and social stigma surrounding STIs and sex, especially in areas of high HSV-2/HIV co-infection such as sub-Saharan Africa (Rajagopal et al. 2014); inability to intravaginally inoculate men as they obviously do not possess vaginal mucosal areas; individual embarrassment and unwillingness to expose intimate areas to doctors; fear of harassment or retaliation, especially for young, unmarried, sexually active women; and fear of pain or discomfort of receiving the vaccine, more conventional vaccination routes such as intramuscularly (i.m.) or intradermally (i.d.) must be evaluated (Zhu et al. 2014). Therefore, these vaccine candidates should be tested in mice at least i.m. with all the above variables studied to see if it generates a comparable immune response to intravaginal inoculation. Then the same studies detailed above should be performed in guinea pigs since they do have spontaneous reactivation of HSV-2 like humans, so the vaccine candidate's ability to stop reactivation can be assessed.

Monoclonal gD and gB Antibody Analysis. Monoclonal gD and gB antibodies will be further assessed for neutralization activity against wild-type HSV-2 in neutralization assays to confidently determine their IC_{50}s. Based on data already gathered by Bender, Lazear and colleagues, the antibodies I have chosen to focus on are MC2, DL11, 1D3, and C226 because of their low ID_{50} for HSV-2 (Bender et al. 2007; Lazear et al. 2012). After analysis of these mAbs, more antibodies described by Bender, Lazaer, and

colleagues should be examined *in vitro* by neutralization assay, especially common-type antibodies that recognize HSV-1 and HSV-2.

Monoclonal gD and gB Antibody Animal Studies. In an effort to determine the CoPs of HSV-2, BL/6 6 week old naïve female mice will be pre-treated with different doses of the monoclonal gD and gB antibodies listed above before being challenged with different doses of wild-type HSV-2 to see how well they protect the mice from infection and disease. Experiments elaborated for the CPBD vaccine candidates would be done in these mice as well.

ORF	Nucleotide Position	Ref HG52 Base	R519 Base	AA Change?	Total Read Count	R519 Base Count	Genome Position
U_L30	633	G	A	silent	8999	A : 8966 (99.6%)	63897
U_L31	594	C	T	silent	8678	A : 8641 (99.6%)	67256
U_L34	776	C	T	aa change A>V	6599	T : 6492 (98.4%)	70894

Table 1: Confirmation of R519-Specific Mutations. Before CPBD with SAVE, any mutations in the genome must be identified to ensure the deoptimized sequence encodes the correct amino acid changes. The ORFs of *U_L30*, *U_L31*, and *U_L34* of R519 were aligned to multiple HSV-2 strains to identify mutations compared to the reference HSV-2 strain HG52. Only three mutations were found in these three genes: two silent mutations in *U_L30* and *U_L31* and one missense mutation in *U_L34*, changing an alanine to a valine. These mutations were confirmed from a deep sequencing file of R519. Total Read Counts of the bases in question indicates how many times that base was sequenced whereas R519 Base Counts indicates how many times the R519 base was read in those sequences. Out of 8999 reads of the G>A mutation in *U_L30*, 8966 reads read an A in that position; out of 8678 reads of the C>T mutation in *U_L31*, 8641 reads read a T in that position; and out of 6599 reads of the C>T mutation in *U_L34*, 6492 reads read a T in that position. Therefore, these bases were read as the R519 expected mutated bases 99.6%, 99.6%, and 98.4% of time, indicating that these mutations were genuine.

Name	Primer Sequence
Primer Name	Primer Sequence
galKUL30F	5'-GAGGCGGTGGTGGGAGGGGGCGGCGTATAGCAGGACA ACGACCGGCGGCG**CCTGTTGACAATTAATCATCGGCA**-3'
galKUL30R	5'-GGCCGCAGACATTTATTGCAAGATGCGGGACATCAGCT TCGACGGGGGGC**TCAGCACTGTCCTGCTCCTT**-3'
galKUL31F	5'-CAGACGACACTCGATCTCTCTCCTGTCCCTGGAGCACAC CCTCTGTACCT**CCTGTTGACAATTAATCATCGGCA**-3'
galKUL31R	5'-CGTGGCCGCGCGGCTCAGGGCCGCGGGGTTCGGGCCG GCGGGGGCCGGCG**TCAGCACTGTCCTGCTCCTT**-3'
galKUL34F	5'-TTTTACATAACAGTAGGGGGTGGGGGAACGCGCACCCT TGCCCGGTCGCG**CCTGTTGACAATTAATCATCGGCA**-3'
galKUL34R	5'-TCGCGGGCGTCTGGGATGCACTGGCGGGCGGGACGGTG GCTCGCCCCCTT**TCAGCACTGTCCTGCTCCTT**-3'

Table 2: *galK* Primers for U_L30, U_L31, and U_L34. For the *galK*-DOG double selection process, primers need to have 50 base pairs homology to flanking areas connected to 20-mers of *galK* sequence. The bold letters are the *galK* sequence 20-mers.

mAb	Literature IC_{50} for HSV-2 333	Experimental IC_{50} for HSV-2 R519
MC2 (gD mAb)	0.781 μg/mL	2 μg/mL
DL11 (gD mAb)	0.312 μg/mL	0.7 μg/mL
1D3 (gD mAb)	6.2 μg/mL	0.9 μg/mL
C226 (gB mAb)	1.2 μg/mL	20 μg/mL

Table 3: Comparison of literature IC_{50}s to experimental IC_{50}s of gD and gB mAbs. The control 1:10 IVIG had an IC_{50} of 9 μg/mL. The literature IC_{50}s of MC2 and DL11 are relatively close to experimental values with both MC2 and DL11 being found as between 2-2.5-fold less effective compared to literature IC_{50}s reported by Lazear et al. 2012. The experimental IC_{50} for 1D3 was a little less than 7-fold more effective than its literature IC_{50} reported by Lazear et al. 2012 whereas the experimental IC_{50} for C226 was about 17-fold less effective than its literature IC_{50} reported by Bender et al. 2007. This experiment was only performed once, so discrepancies between literature and experimental IC_{50}s may be resolved with more repeats of this neutralization assay.

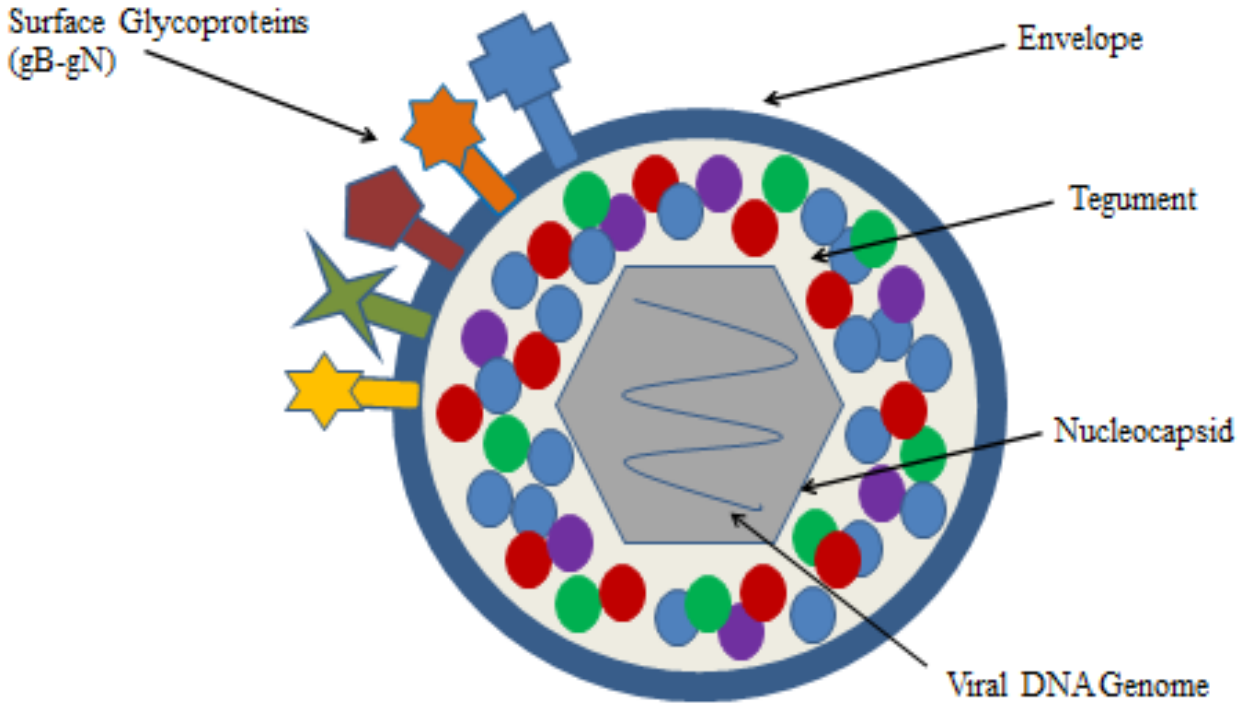


Figure 1: HSV-2 Virion Structure. The viral DNA genome is contained in the nucleocapsid, which is surrounded by the tegument. The tegument is the area between the nucleocapsid and envelope that contains various viral proteins important for immune evasion. The envelope is studded with multiple glycoproteins such as gD, which mediates attachment; gB, which mediates fusion; and gH/gL, which activates gB. The entire envelope surface is covered with glycoproteins; only a few glycoproteins are shown on this diagram so as not to affect labeling of other viral structures.

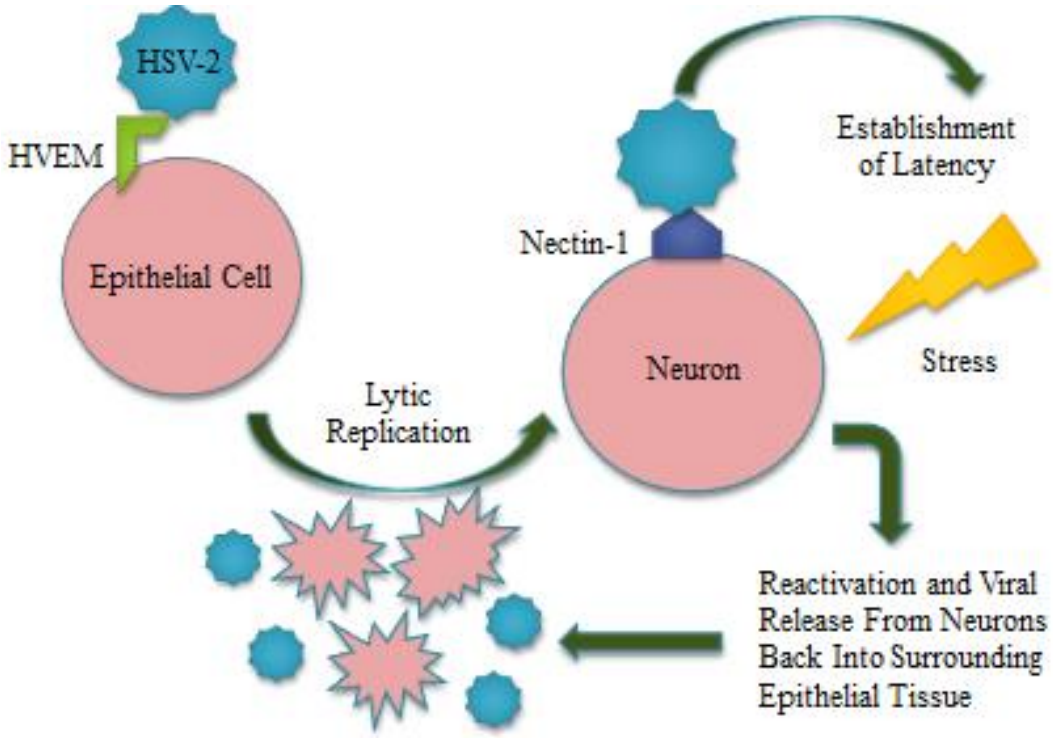


Figure 2: HSV-2 Lifecycle. HSV-2 has a complex lifecycle that includes two tropisms. During primary infection, HSV-2 infects epithelial cells of the genital mucosa though binding of gD to herpesvirus entry mediator (HVEM), which is expressed on both epithelial and neuronal cells. HSV-2 undergoes lytic replication in the epithelia to cause the characteristic lesions of symptomatic genital herpes. The virus then infects innervating nerves in the area through binding of gD to nectin-1. Once HSV-2 has successfully infected the nerves, virions travel up the axons to the nuclei where they establish latency in the DRG. Not much information is known about the latent state except that the viral genome is not actively replicating, but latency-associated transcripts (LATs) are made. Latent HSV-2 cannot be detected by the immune system as no epitopes are being presented, providing quite a big barrier to a cure as well as a vaccine. Triggers such as stress or UV light reactivate the virus from latency. Virions are synthesized in the nerves, travel back down the axons, and lytically infect the epithelia to create new lesions.

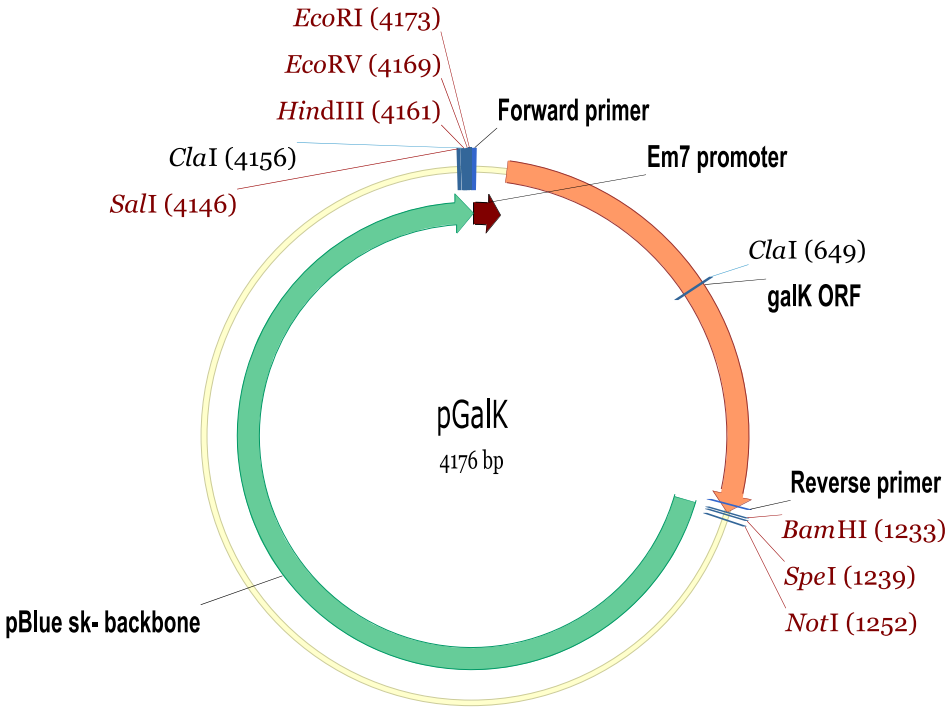


Figure 3: *galK* Plasmid Map. pGalK is a 4176 bp plasmid in the pBlue Sk- backbone, which contains two *ori*s and an *AmpR* gene under the AmpR promoter as a selectable marker. *galK* is under the Em7 promoter, a synthetic promoter derived from the T7 bacteriophage promoter. The *galK* ORF was isolated using HindIII and BamHI restriction endonucleases. This plasmid map was generated by Dr. Kening Wang and has been reproduced here with permission.

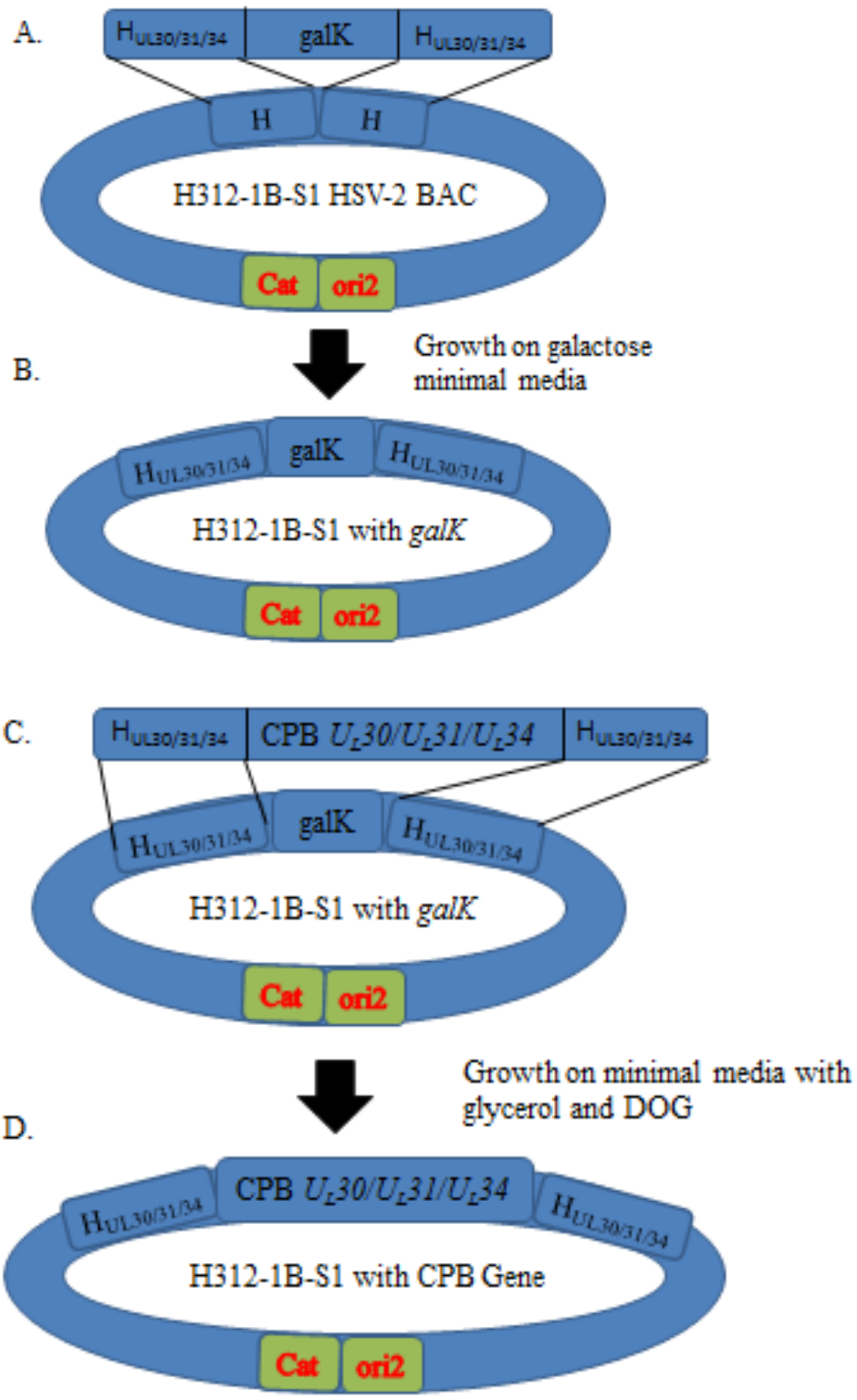
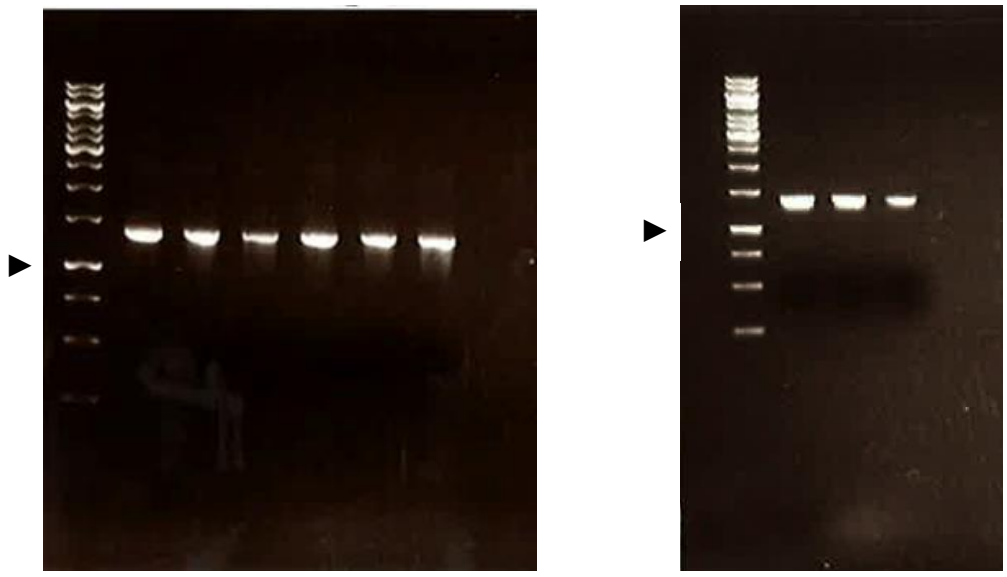

A.
$H_{UL30/31/34}$
galK
$H_{UL30/31/34}$
H
H
H312-1B-S1 HSV-2 BAC
Cat
ori2
B.
Growth on galactose minimal media
$H_{UL30/31/34}$
galK
$H_{UL30/31/34}$
H312-1B-S1 with *galK*
Cat
ori2
C.
$H_{UL30/31/34}$
CPB $U_L30/U_L31/U_L34$
$H_{UL30/31/34}$
$H_{UL30/31/34}$
galK
$H_{UL30/31/34}$
H312-1B-S1 with *galK*
Cat
ori2
D.
Growth on minimal media with glycerol and DOG
$H_{UL30/31/34}$
CPB $U_L30/U_L31/U_L34$
$H_{UL30/31/34}$
H312-1B-S1 with CPB Gene
Cat
ori2

Figure 4: *galK*-DOG Double Selection BAC Recombineering Procedure. Primers with 50 bp homology to the flanking areas of the site of interest are used to amplify the *galK* gene from pGalK by PCR. SW102 cells containing H312-1B-S1 are made competent and transformed with the *galK* cassette. The galK cassette replaces the wild-type U_L30, U_L31, or U_L34, giving the transformant cells the ability to grow on minimal media containing galactose. After colony purification, these transformants are transformed again with the CPB U_L30, U_L31, and U_L34. These transformants are plated on M63 minimal media with DOG as all *galK*-BAC-containing cells will die because galK will phosphorylate DOG to a toxic intermediate. After colony purification, these BACs will be purified and nucleofected into Vero cells to rescue the resultant deoptimized viruses. This cloning process has been adapted from Warming et al. 2005.



A B.

Figure 5: *galK* PCR Products. (A.) The *galK* gene in pGalK was amplified by two PCR experiments denoted *galK* #1 and *galK* #2 using the primers shown in Table 1. This PCR created the *galK* cassette needed for the first step of the *galK*-DOG double selection protocol by attaching homology arms for U_L30, U_L31, or U_L34 to the *galK* gene. Bands are about 1.3 kb, which is the expected length of the product. Lane 1: 1 kb ladder with 1 kb band marked with ►; Lane 2: *galK* #1-U_L30 PCR product; Lane 3: *galK* #1-U_L31 PCR product; Lane 4: *galK* #1-U_L34 PCR product; Lane 5: *galK* #2-U_L30 PCR product; Lane 6: *galK* #2-U_L31 PCR product; Lane 7: *galK* #2-U_L34 PCR product (B.) The *galK* #1 PCR products were cleaned with DpnI, which degrades methylated DNA, and run on a gel. These bands were gel purified and rerun to estimate concentration. Bands are still at 1.3 kb as expected, but have crisper borders. Lane 1: 1 kb ladder; Lane 2: *galK* #1-U_L30 DpnI-cleaned PCR product; Lane 3: *galK* #1-U_L31 DpnI-cleaned PCR product; Lane 4: *galK* #1-U_L34 DpnI-cleaned PCR product

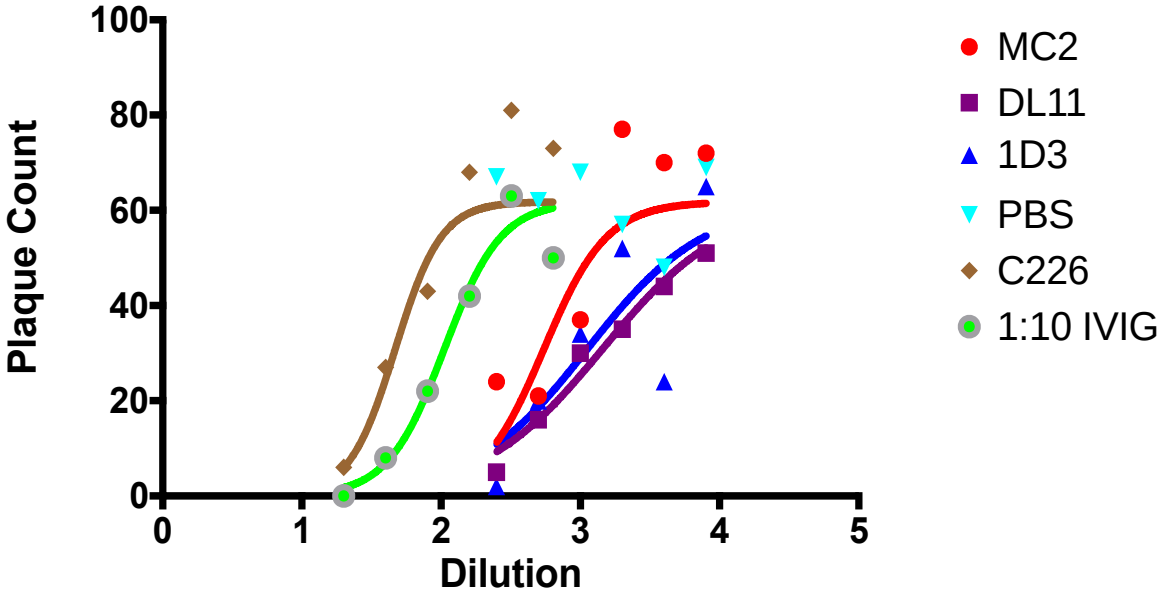


Figure 6: Neutralization Assay of gD and gB mAbs. Different dilutions of gD and gB mAbs were incubated with HSV-2 R519 and plated on Vero cells to determine plaque count. All gD mAbs had IC_{50} values lower than that of 1:10 IVIG, indicating that they were more effective at neutralizing HSV-2 than 1:10 IVIG. The gB mAb C226 had a higher IC_{50} value than 1:10 IVIG, meaning that it was less effective at neutralizing HSV-2, which opposes its literature IC_{50} value reported by Bender et al. 2007. This experiment was only performed once, which may explain the discrepancies between the literature IC_{50}s reported by Lazear et al. 2012 and Bender et al. 2007 and the experimental IC_{50}s reported here. For exact IC_{50} values, please see Table 3.

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