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Developing Methods to Study Pathogenesis and Immune Response to HSV-2 in the Guinea Pig Model

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Developing Methods to Study Pathogenesis and Immune Response to HSV-2 in the Guinea Pig Model

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

The University of Texas Medical Branch

March, 2018

Acknowledgements

This thesis project was made possible with financial support from grants provided to Dr. Milligan. Special thanks also goes to Dr. H. Schafer for his generous gift to the lab of a number of B-cell lines, without which much of this work would not be possible. I would like to express gratitude to Dr. Milligan for taking me into his laboratory and supporting me for the short period of time that I was there and for encouraging to do what was best for me in the long run. I would also like to express special gratitude to my mentors in the HPTM program, Drs. Judith Aronson and Mark Hellmich for working closely with me especially during my final months in the program and providing me guidance.

I am also very grateful to my lab mates Brianne Banasik and Clarice Perry for showing me the ropes in the lab, answering so many questions, and making my time in the lab that much more enjoyable. I am very fortunate to have been in such a warm and welcoming environment. Many thanks also go to the laboratories of Dr. Richard Pyles and Dr. Nigel Borne who provided plenty of help, resources, and equipment throughout my time here.

In addition I would like to thank my family and the many friends that I've made here at UTMB that have supported me through throughout this time. They have been a source of advice and have acted as a soundboard for me countless times over the past few years, and for that I will forever be grateful.

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Publication No._____

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The University of Texas Medical Branch, 2018

Supervisor: Gregg Milligan

In order to develop an effective vaccine for HSV-2 it is important to adequately understand the immune response in the proper animal model. Guinea pigs fully recapitulate human HSV-2 disease, but reagents and assays available for this animal are limited. Consequently, many reagents and assays used must be developed and optimized in lab. We utilized several different hybridoma lines received from a partnering lab which produced guinea pig IFNγ detecting antibodies. To evaluate the sensitivity and assay applicability of these antibodies, I generated and purified antibody from two Hybridoma lines; NG3.5 and VE-4. To generate enough antibody for immunological assays, I grew large quantities of antibody-producing hybridoma cells and collected the supernatant. From this, I isolated and purified the antibody for future assays. We evaluated the ability of the antibodies to detect intracellular IFNγ production for flow cytometry (FC) analysis. The purified antibody was conjugated to a fluorochrome and applied to IFNγ producing guinea pig (gp) spleen cells post stimulation with PMA. FC data analysis showed this antibody-fluorochrome conjugation was not suitable for flow cytometry. Upon changing the fluorochrome, cell stimulant, and IFNγ antibody (NG3.55 to VE-4) we still were unable to develop a working FC intracellular cytokine staining

protocol. Contrasting the disappointing results with FC, we developed an ELISPOT which used the NG3.5 antibody to detect IFNγ secretion from stimulated guinea pig cells. We also used a modification of this assay to quantify antibody secreting cells in immunized guinea pig cells. Tissues were isolated from the spleen, inguinal lymph node, and bone marrow of infected and uninfected male and female guinea pigs. Antibody secreting cell numbers were highest in the spleen and bone marrow of both male and female guinea pigs. Interestingly, we also found the magnitude of ASC response to total HSV-2 glycoprotein was similar to that of gD alone. This was true in all three tissues. The assays optimized here will provide future tools with which to better understand which protective immune responses are necessary for an effective therapeutic/prophylactic vaccine.

In short, here we outline the development and optimization of assays to quantify 2 important immune responses to HSV-2 infection in guinea pigs. These studies will provide a platform from which other immune assays can be developed to better understand immunity against HSV-2. For example, there is still a need to better understand the protection provided by effector CD4 and CD8 T cells as well as important sites of their localization in a prophylactic and therapeutic setting. Further immune assay development will expand our knowledge of necessary immune correlates of protection for an effective vaccine against genital HSV-2 infection.

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BM	Bone Marrow
ConA	Concavalin A
DC	Dendritic Cell
dMPL	Deacyl monophosphoryl lipid A
FC	Flow Cytometry
FBS	Fetal Bovine Serum
gD	glycoprotein D
gp	guinea pig
HSV	Herpes Simplex Virus
ICS	Intracellular cytokine staining
IFN	Interferon
ingLN	inguinal lymph node
i.m.	intramuscular
LAT	Latency Associated Transcript
MPL	monophosphoryl lipid A
ODN	oligodeoxynucleotides
pDC	plasmacytoid dendritic cell
polyIC	polyinosinic:polycytidylic acid
polyICLC	polyinosinic:polycytidylic acid stabilized with poly-l-lysine
sc	subcutaneous
ASC	Antibody secreting cell

CHAPTER 1: INTRODUCTION TO HERPES SIMPLEX VIRUS 2 (HSV-2)

HSV-2 Prevalence and Incidence

HSV infections are distributed worldwide and have been reported in both developed and developing countries, including remote Brazilian Indian tribes.[22] In fact, herpes simplex viruses are among the most ancient diseases described to affect humans and currently HSV-2 infection infects nearly 20% of the population worldwide and it is estimated that nearly 20 million new cases arise each year in the US, at a cost to the nation's health care system of close to $16 billion a year.[23,24,168] HSV-2 prevalence varies depending on the region of the world and can range from around 5% for countries such as Spain, up to around 70% for Sub-Saharan Africa.[24,26,27] However, it is important to note that the populations evaluated in these studies can vary significantly, as well as the methods used for determining seroprevalence (e.g., enzyme-linked immunosorbent assay vs. immunoblot).[25] In the US, a government study showed that 16% of Americans between the age of 14 and 49 are infected with HSV2. This equates to over 50 million people infected with differences of infection rates among the sexes: 25% of women vs 20% of men being infected with the disease.[22]

Many people infected with HSV-2 are not aware that they have the disease and may spread it during subclinical episodes of viral shedding.[29] Subclinical shedding of the virus is a serious issue of HSV-2 and likely increases transmission since this is a period

of time where people are unaware that they are infectious and therefore believe it to be safe to engage in sexual activities. Studies found that subclinical shedding occurs on about 20% of days in symptomatic patients and 10% of days in asymptomatic patients, though the amount of virus shed during these shedding events was similar.[162] Because of this, transmission of the virus cannot be decreased simply by informing patients to abstain from sexual activities while they are symptomatic. This could likely be why the virus is also still highly prevalent among those with low to moderate levels of sexual activity. For instance, the HSV-2 seropositive rate among women in the U.S. with only 2-4 sexual partners in their lifetime is still 18.8%.[28] Notably, the risk of HIV-1 acquisition is 3-fold higher among HSV-2–seropositive persons; nearly 50% of HIV infections can be attributed to the prevalence of HSV-2 in places where 80% of the population is seropositive.[29,30] Additionally it is important to realize that because most infections are subclinical, disease incidence and prevalence data underestimate the impact of HSV infection.[12]

Notably, HSV-2 also results in significant physical pain and emotional distress for those infected. Additionally, for those with compromised immune systems, HSV-2 is associated with significantly exacerbated disease and serious complications. For example, patients that are receiving chemotherapy or those that have an acquired immunodeficiency syndrome are susceptible to HSV-induced meningitis.[166]

A serious complication of genital herpes is the transfer of HSV-2 infection from mother to infant during birth and women who become infected with genital HSV-2 during pregnancy having the highest risk of transmitting to newborns.[31] The estimated incidence of neonatal herpes varies widely, from 4 to 31 in 100,000 live births.[29,33]

However, babies infected with HSV-2 during birth often experience long-term neurologic sequelae and mortality.[32] Additionally, in 2015 a study by the CDC determined that the mean excess cost for newborns admitted with neonatal herpes was $40,044, representing a significant burden financially for both parents and the health care system.[167] Neonatal HSV infection is categorized as skin, eye, and/or mouth (SEM), disseminated, or central nervous system (CNS) disease. [163] These categories are also predictive of morbidity and mortality. CNS disease in newborns can be either diffuse (blood-borne transmission) or focal (neuronal transmission), with the incidence of both diseases being approximately 1500 to 2000 cases annually.[164] Untreated disseminated disease is accompanied by a mortality rate of 85%, and neonates with untreated encephalitis face a mortality rate of almost 50%.[164] Additionally, a minimum of 65% of survivors of disseminated disease or encephalitis have severe neurologic sequelae if they are not treated.[165] HSV-2–associated meningitis is normally seen during genital HSV-2 infection, however this comorbidity can also occur in adults experiencing recurrent genital herpes infections as well. Severe CNS infections associated with this virus also occur in adults.[166] Although rare this is still a significant health issue and contributes to rising costs of health care for treatment of HSV-2 and the associated complications.

The data and statistics described here underscore the need for an effective intervention such as a prophylactic and/or therapeutic vaccine to address infection and transmission rates of the virus. Prophylactic vaccines would provide protective immunity against genital HSV-2 infection prior to exposure, with a possible secondary effect of prevention of HIV infection in high risk populations. Therapeutic vaccines, on the other hand, would reduce genital lesions shedding in HSV-2 seropositive individuals, having

direct effect on transmission rates providing both personal and public health benefit. Because of this, the pursuit for prophylactic and therapeutic vaccines for HSV-2 is a significant endeavor.

Virion and Genome Organization

Herpes simplex virus type 2, also known as Human Herpes Virus 2, is a member of the virus order Herpesvirales subfamily Alphaherpesvirinae, and belongs to the Simplexvirus genus. The Herpesviridae family includes over 200 members, capable of infecting different species, and among them are eight currently known to cause disease in humans.[34] Based on the genome structure, tissue tropism, cytopathologic effect, site of latent infection, pathogenesis, and manifestations of the disease, the human herpesvirus (HHV) are grouped into three subfamilies; Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae.[22,35] Interestingly, viruses included in the subfamily Alphaherpesvirinae are characterized by their short reproductive cycle, ability to spread rapidly between cells and to lyse infected cells efficiently, and the establishment of latent infections in sensory ganglia.[22]

HSV-2 is an enveloped virus with a linear double-stranded DNA genome enclosed by an icosahedral capsid composed of 162 capsomeres.[39] This capsid is wrapped in a lipid bilayer known as the envelope and is attached to said envelope via the tegument. The virion is spherical and is 186 nm in diameter with glycoprotein spikes anchored to its envelope.[36]

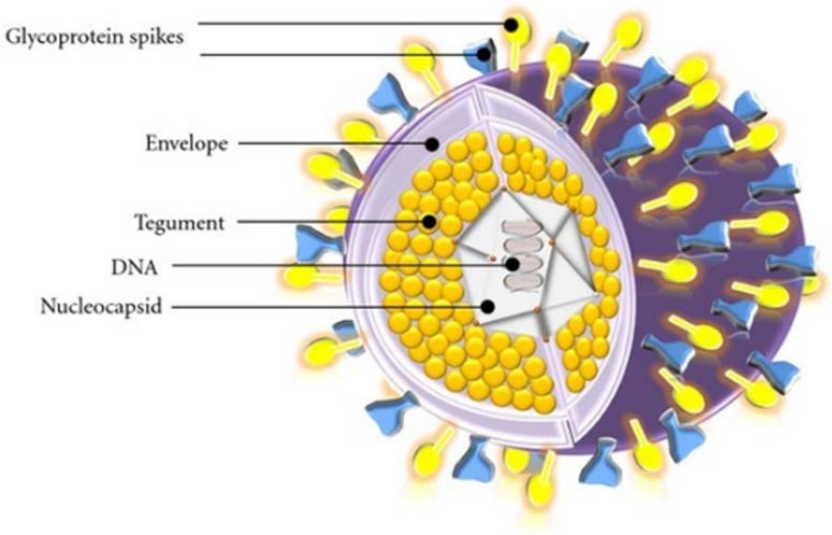


Illustration 1: Physical structure of the HSV-2 virion. Adapted from Elbadawy et al. 2012[215]

HSV-1 and HSV-2 contain at least 74 genes,[37] however taking into account the 94 ORFs assumed in each virus, there are as many as 84 separate protein coding genes.[38] These genes encode diverse proteins that perform a functions necessary for viral life such as forming the viral capsid, the surrounding tegument, and encasing envelope. They also control virus replication and infectivity.

The HSV-2 genome is 155kbp and contains 2 longer regions of unique sequences (U_L and U_S), both of which are contained by 2 terminal or internal repeat elements known as TR_L-IR_L and IR_S-TR_S.[40,41] U_L plus its flanking repeats is termed the long (L) region, and U_S with its flanking repeats is termed the short (S) region.[42] A depiction of this organization is shown below in Figure 1. The long region contains 56 genes, while the short region contains 12 genes.[43]

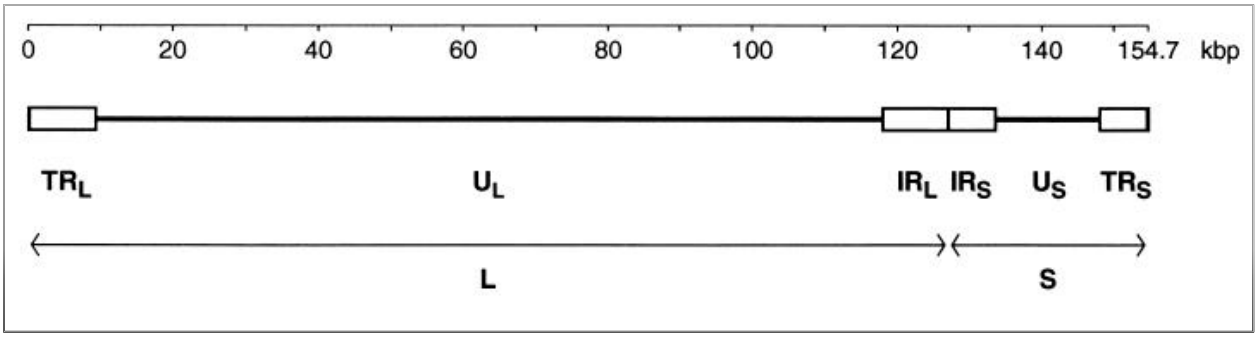


Figure 1: Overall organization of the genome of HSV-2. For each pair of terminal and internal repeats (TR; IR) the two copies are in opposing orientations. (Adapted from Dolan et al. 1998)

Structural proteins, such as the capsid, tegument, and envelope proteins of HSV-2 as well as genes that control viral the replication processes and infective ability are transcribed by both U_L and U_S regions of the genome. The genes that make up HSV-2's genome can be classified into immediate-early, early, early late, and late viral genes. During replication these genes are not expressed synchronously but instead in four consecutive rounds of transcription. First, immediate early genes are transcribed, many of which encode for proteins contributing to immune evasion and control cell translation.[169] These genes include ICP4, ICP0, ICP27, ICP22, and ICP47. Immediate-early genes also encode for the transcription of proteins that regulate the expression of early and late genes, and these proteins are also part of the tegument that enters the cell after the capsid. For example, the ICP0 gene encodes VHS, the virion host shutoff protein. The functions of VHS include shutting off host cell protein synthesis, degrading host mRNA, assisting with viral replication, and regulating gene expression of other viral proteins.[171] Early genes, which are expressed after the immediate-early genes, control the biosynthesis of enzymes involved in DNA replication, as well as the production of various glycoproteins that comprise the virion envelope. HSV-2 early late and late genes primarily encode for proteins that form the capsid and tegument to subsequently form the virion particle.[43] True HSV late genes, require DNA replication for any appreciable accumulation of their

mRNAs. Some late genes, however, are expressed to a degree in the absence of DNA replication but require DNA synthesis for maximal expression.[170]

Importantly, newly found micro RNAs (miRNAs) of HSV-2 play an important role in pathogenesis. HSV-2 miRNAs are able to target both cellular and viral mRNAs and seem to have a role in cell proliferation regulation, apoptosis, evading host immunity and regulating lytic and latent infection.[172] Once latency is established in the host by the virus (discussed in later sections) the only transcription produced by the virus is the latency associated transcripts (LATs). These LATs readily transcribe miRNAs which are able to modulate viral latency. ICP4, an immediate early gene of HSV-2 that upregulates early and late genes, negatively regulates the expression of these LAT miRNAs.[173] Notably, the downregulation of LAT and its miRNA drives the virus towards lytic infection. Studies have also observed that, HSV-2 miRNAs efficiently silence the expression of the neurovirulence factor ICP34.5 and a key transactivator ICP0. ICP0 expression promotes the entry of HSV-2 into the replication cycle and subsequently this protein has a major role in lytic infection and virus reactivation.[174] Taken together, current data suggest that it is very likely that the regulation of LAT-encoded miRNAs contribute to HSV-2 latency and reactivation. Of note, HSV-2 encodes for 18 stem-loops that result in the production of 24 mature miRNAs. Though the function of the majority of miRNAs remain largely unknown, the functions that have been revealed relate HSV-2 miRNAs to latency regulation.

IMMUNOGENIC TARGETS

The HSV-2 virion consists of a capsid enclosing the DNA genome, the tegument, and an envelope containing the envelope glycoproteins which consist of glycoprotein B (gB), gC, gD and gH/L. Upon infection, viral replication is broken into immediate early, early, and late gene transcription. The immediate early and early viral genes encode the non-structural and enzymatic early proteins. This is followed by late gene transcription in which the structural proteins are transcribed. All of these transcribed proteins are potential targets for both CD4 and CD8 T cells. Late HSV structural proteins, especially gD and gB, capsid protein VP5, and tegument protein UL49 are mainly recognized by CD4 T cells.[44,45] The most abundantly expressed glycoprotein on the virion and on the surface of virus-infected cells is gD. Furthermore, the majority of the antigen specific neutralizing antibodies found in HSV-2 patients are directed towards gD and gB.[46,47] On the contrary, CD8 T cells in patients are able to recognize a wide variety of viral proteins including immediate early and early proteins, meaning that a proper vaccine candidate needs to target both CD4 and CD8 T-cell effectors via different repertoires of antigens and adjuvants.[19,48] Importantly, it has been reported that individuals with asymptomatic infection have exhibited T cell responses against specific HSV-2 antigens not observed in symptomatic individuals.[154] So far all prophylactic and therapeutic vaccines targeting HSV-2 have failed in clinical trials despite generating high titers of neutralizing antibodies. The induction of a robust CD8 T cell response by potential vaccines is largely unreported which could be an important underlying factor to vaccine success or failure.

In general, T-cell targets include HSV proteins present in large quantities in the virion such as viral envelope, tegument, capsid, and regulatory proteins, as well as enzymes.[175] HSV-specific CD4 T-cells are typically multifunctional for Th1/Th0-like

cytokines and, after expansion, have cytolytic potential.[177] Cytotoxic lymphocytes (CTLs) recovered from peripheral blood mononuclear cells (PBMC) recognized a diverse array of HSV-2 viral antigens which calls for future analyses of HSV-2 CD8 T cell antigens to span the whole proteome.[179] Studies so far have found that HSV-2-specific CD8 T-cells in herpetic skin lesions are predominately directed to tegument proteins which confirm the antigenicity of immediate early (IE) proteins and glycoproteins deduced from studies using blood.[176] Additionally, HSV-2 specific cytotoxic lymphocytes (CTLs) located in infected tissues have been found to be reactive to tegument proteins VP13/14 and VP22 and the immediate early protein ICP0.[178] Moreover, the contact of HSV-infected cells with naive T cells has been shown to inhibit and alter future T cell signaling.[190]

CD8+ T cell responses in humans are inhibited by the viral protein ICP47 which acts by blocking peptide movement into MHC class I molecules via interactions with the transporter associated with antigen processing (TAP).[207] Empty MHC class I molecules are degraded in the cytoplasm, which prevents mounting an effective CD8+ T-cell response against HSV. ICP47 also downregulates HLA-C expression on HSV-2-infected dendritic cells to render dendritic cells susceptible to natural killer cells.[207] HSV-2 specific T-cells localize to sites of primary and recurrent infection such as skin, cervix, and sensory nerve endings.[191] Moreover, in humans, CD4 T cells along with CD8 T-cells show prolonged localization to the dermo-epidermal junction after HSV-2 healing and have an activated and antiviral phenotype.[180, 181]

Finding potential T cell targets for HSV-2 remains an important endeavor, especially in light of the virus' ability to evade antibody-dependent cell mediated cytotoxicity. This evasion has been demonstrated by a number of studies. For instance B-

cell deficient mice infected with HSV-2 are able to ultimately resolve infection.[182] Moreover, in this study, the presence of high titers of HSV-specific IgG in vaginal secretions failed to protect T-cell-depleted HSV-immune B6 mice against intravaginal challenge with HSV-2. Another group found that HSV-2's ability to evade antibody-dependent cell cytotoxicity was mediated by its production of several viral glycoproteins that competitively bound to IgG antibodies.[183] HSV-2 glycoprotein C (gC2) is an immune evasion molecule that inhibits complement. HSV-2 glycoprotein E (gE2) functions as an immune evasion molecule by binding the IgG Fc domain.[184]

More information on immunogenic targets in vaccines currently in preclinical and clinical trials is discussed further in the 'Prophylactic vs Therapeutic Vaccines' section.

HSV-2 Pathogenesis

Infection with HSV-2 is initiated by the adsorption of the glycoproteins found within the viral envelope to glycosaminoglycan chains on the surface of target cells. Heparan sulfate, a common glycosaminoglycan on the surface of many cells, is considered the main HSV-2 binding receptor. This receptor also has a key role in the adherence of glycoproteins to the surface of cells.[18] Initial attachment of HSV-2, which is mediated by gB and gC, is easily displaced until glycoprotein D attaches to receptive cells via its receptors which include herpesvirus entry mediator (HVEM), the nectins 1 and 2, and a specific form of HS called 3-O-sulfated heparan sulfate (3-OS HS).[49] gD binding to cell surface receptors, triggers a conformational change in its polypeptide chain enabling the HSV-2 heterodimer gH/gL (also located on the viral surface) to

interact with the cell. This exposes the fusion domains of both the heterodimer and gB facilitating to fusion of the viral envelope with the plasma membrane of the cell.[18]

Once the envelope fuses with the cell, the tegument and capsid are released into the infected cell. UL41 gene protein, VHS, is released into the cytoplasm at this time. VHS is a virion-associated host shutoff protein and functions to shutoff of host protein synthesis and degrade host mRNA. During this time, the viral capsid and other viral proteins travel directly to the nucleus to inject the viral genome through the nuclear membrane. Once inside the nucleus, viral DNA reconfigures itself into a circular form, allowing for replication. VP16 initiates HSV-2 gene expression during lytic infection by forming a complex with the Oct-1 and HCF-1 proteins in the host cell.[188] This results in the recruitment of general transcription machinery to viral immediate early (IE) gene promoters, triggering lytic gene expression. Following the production of HSV immediate-early regulatory proteins, a number of early enzymatic activities, and an array of early and late structural proteins, virions assemble and bud from infected cells (causing their lysis) and then spread to neighboring cells for further propagation.[144] The mechanical scarification of the superficial layer of the epidermis provides a pathway for the virus to enter epithelial cells and replicate; a necessary condition for initial HSV-2 infection.[212] Virus antigens are then taken up by local skin-resident DCs, which migrate to the lymph node to present antigens to CD4+ T cells.[212] Importantly, T lymphocytes induced by DCs differentiate into three kinds of memory cells, central memory, effector memory and resident memory T cells. Only the resident memory T cells take up residency in the skin (at the previous site of virus infection) and near the latently infected ganglion. Upon consequent infection by HSV-2, these cells provide the bulk of the protection even

though effector memory T cells can also be recruited to the site from systemic circulation.[212]

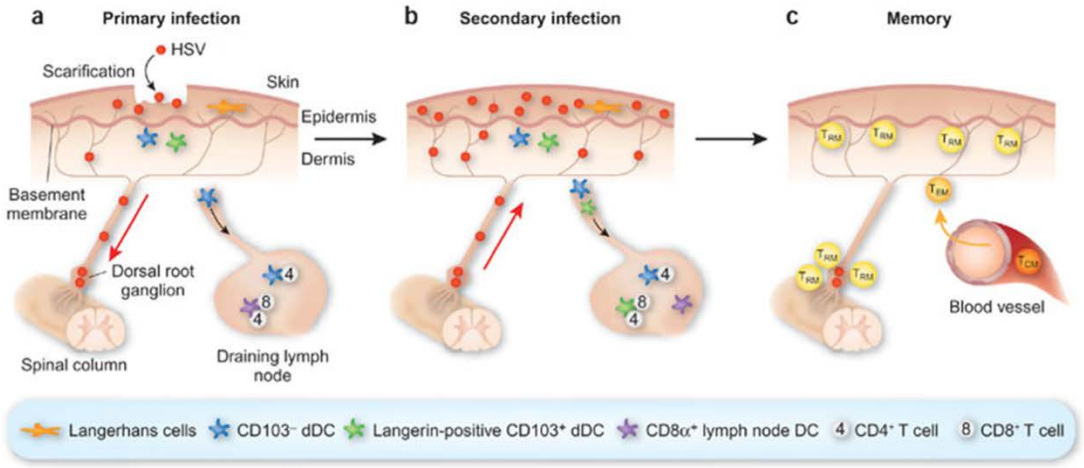


Illustration 2: Pathogenesis during Primary and Secondary HSV-2 infection along with the memory response. Adapted from (Iwasaki 2009)

HSV infects epithelial cells in the skin and mucosal surfaces during primary infection. At the primary infection site, the virus goes through several rounds of replication, after which, intact HSV-2 virions or their nucleocapsid released from infected cells enter neuronal axons and are transported via retrograde axonal flow to the dorso-sacral ganglia where latency is established.[18] The virus establishes latent infection when conditions that normally provide an efficient and organized lytic environment are insufficiently supported. Specifically, it is thought that the extensive trafficking of capsids through long axons results in the inefficient transport of tegument proteins, such as VP16, to the nucleus.[187] This in turn leads to insufficient transcriptional activation of IE proteins, resulting in an infection that favors genome repression by epigenetic regulation.[187] Latency establishment is associated with the circularization of the viral genome to form an episomal DNA element which is then packed in histones.[185] During

latency, the viral DNA is copied by cellular DNA polymerases, along with the chromosomes, preferably when the cell engages in mitosis.[186] Additionally, during latency, the hosts natural epigenetic mechanisms such as methylation and packaging in histones silences the viral genes. However not all genes are silenced. During latency, the only transcript produced by the virus is the latency associated transcript (LAT). These transcripts are able to block apoptosis in the cell and are thought to facilitate the survival of latently infected neurons.[189] LATs play a major role in regulating latency by producing miRNAs that interfere with the production of lytic genes. Although epithelial cells are destroyed during lytic HSV replication, neuronal cells are not destroyed and are able to provide a reservoir for latent virus.[28] Under certain immunosuppressed conditions (physical or emotional stress, fever, exposure to UV light, menstruation, or hormonal imbalance[50]), the virus is able to reactivate from this latent reservoir. Reactivation of the latent virus in the ganglion results in the anterograde migration of infectious virions to the skin and infection of the epithelial cells throughout the dermatome innervated by the ganglion. During this time virus is detectable at the epithelial surface; this is known as viral shedding. This shedding can be either symptomatic and associated with lesions and ulcers in the genital area or asymptomatic.[19]

HSV-2 Immunology

INNATE IMMUNE RESPONSE TO HSV-2

Through interactions with TLR9, TLR2, and TLR3 on plasmacytoid dendritic cells (pDCs) and other cells, HSV stimulates the innate immune system to produce IFN-

α and other type 1 IFNs.[51-54] Focus has been given mainly to TLRs 3 and 9 following studies showing no protection was seen with TLR-2 and TLR-4 ligands.[61,62] TLR3 is an intracellular receptor which responds to double stranded RNA. Activation of TLR3 results in the activation of NFkB and IRF3 via a MyD88-independent pathway. These transcription factors are potent stimulators of an antiviral response specifically via the induction of IFN-α and IFN-β genes.[192] Activation of TLR3 also leads to the secretion of Th1-type cytokines and chemokines, and can result in the production of IFN-γ. TLR-9 is expressed intracellularly in endosomes, and its stimulation leads to the production of immunomodulatory cytokines in vitro and in vivo. These immunomodulatory cytokines include the production of IL-6 and IL-12 by B cells; IL-6 and IFNγ by CD4 T cells; and IFN-γ by NK cells.[193] The production of type 1 IFNs creates an antiviral state in the cell and at the same time activates several different cell types, including NK cells and pDCs which are crucial for both controlling initial infection and priming adaptive immune responses.[60] STING alongside other cytoplasmic DNA sensors mediates the production of IFN during the response to HSV infection.[55] During infection, the production of type 1 IFNs is necessary in order to activate innate immune cells to provide early control of viral infection. The most important of these innate effector cells include natural killer (NK) cells, which are involved in cytokine production and the recognition and killing of virally infected cells.[60] Plasmacytoid dendritic cells (pDCs), which are primarily responsible for Type I IFN production, are also important for the same functions.[60] These activated NK cells induce antiviral activity by mediating apoptosis of virally infected cells through the release of perforin and granzyme B, and are also an important source of early IFNγ.[65]

In line with this, innate immune system agonists have been shown to have profound effects on HSV-2 resistance in the host. Specifically, in a controlled, randomized trial, topical resiquimod 0.01% gel (TLR 7 and 8 agonist) decreased HSV-2 genital shedding and stimulated the production of cytokines that promoted a Th1 immune response.[56] It was also shown that polyinosine-poly(C) (PIC), a TLR3 agonist, provided effective protection against HSV-2 disease.[57] Conversely, single nucleotide polymorphisms in TLR2 are associated with an increased frequency of HSV-2 shedding and genital lesions.[58] Interestingly, type 1 IFN gene transcription is actually not detected in biopsies of genital HSV-2 lesions. This suggests that despite the ability of HSV-2 to trigger IFN-α production in the innate immune system, there may be local defects in the immune responses in situ.[59]

TLR agonist usage in vaccines as adjuvants is useful because they can trigger a nonspecific broad antiviral state in the absence of infection necessary for activation and differentiation of innate and adaptive immune cells, therefore, providing mucosal immunization to HSV-2.[60] Studies have shown that optimal protection (with adjuvants such as CpG and polyIC), defined as decreased pathology and viral titers, and increased survival, require adjuvant administration between 24 hours prior to[63] and 6 hours after[64] viral challenge.

The immune response directed against HSV-2 is immense, and, rationally, the virus has developed a variety of immune system evasion mechanisms. Immediate early proteins ICP0 and ICP27 produced by HSV-2 both modulate the immune response in several ways. ICP0 is able to inhibit the activation of interferon stimulating genes by interfering with nuclear translocation of IRF3.[194] Meanwhile, ICP27 is both necessary and

sufficient for inhibiting interferon-induced STAT1 phosphorylation and is partially sufficient for the inhibition of STAT1 translocation to the nucleus.[195] Additionally, ICP27 has been shown to inhibit the production of type 1 IFNs via inhibition of NF-κB and IRF3 activation.[196] VHS, the host shut off protein mentioned earlier, also interferes with IFN signaling through inhibition of JAK/STAT signaling and IRF7 expression.[197] IRF7 is essential for development of an effective antiviral *in vivo* response against HSV.[198]

Innate immunity to HSV-2 also shows a complex relationship with the adaptive immune system. IFN-γ stimulated from HSV-specific memory T-cells has shown to cause potent innate cell activation and subsequent protective immunity. The innate cells here included phagocyte, dendritic cells, and NK/NKT cells, which were activated to induce a strong program of differentiation including the expression of effector cytokines and microbicidal pathways.[67]

ADAPTIVE IMMUNE RESPONSE TO HSV-2

Cellular Immune Response

An increasing amount of evidence highlights the importance of adaptive immune modalities in controlling HSV-2 infection including neutralizing antibodies as well as both CD4 and CD8 T cells. Though it has been established that the innate immune system is necessary in controlling initial viral infection, the adaptive immune response has been shown to be crucial for clearance of the virus. In light of this, an HSV-2 prophylactic vaccine will likely have to induce a stronger humoral and cellular immune responses than are elicited by natural infection in order to prevent the establishment of latency. In an

immunotherapeutic setting, a treatment for HSV-2 will need to prevent shedding and disease outbreaks.[19]

With the exception of herpes zoster vaccines, T-cell responses have not been identified as critical correlates of protection in humans. However, there is ample evidence of the importance of T cells in HSV immunity. The severity of HSV-2 disease and/or shedding inversely correlates with the number of HSV-2 specific CD8 T cells in both immunocompetent and immunocompromised patients.[19] The presence of a large CD8+ cytotoxic T lymphocyte population has been detected in genital lesions, where greater levels of cytolytic activity from these infiltrating lymphocytes correlated with viral clearance.[45] Post infection, HSV-2 specific memory CD8 T cells accumulate in the skin near sensory nerve endings in both mice and humans and are able to quickly control shedding of HSV-2 from nerve endings as well as control infection of epithelial cells.[75,76] Additionally, priming of HSV-2 specific CD8 T cells in both lymph nodes and tissues has shown to be critical for HSV-2 immunity.[74] Given this information, a vaccine enhancing the establishment of tissue resident memory CD8 T cells in the genital epithelium could serve as an effective defense against primary HSV-2 infection and may also prevent seeding of nerves that leads to latent infection in the dorsal root ganglion.[77]

CD4 T cells have also proved to be of great importance for protection against HSV-2 disease. We know that broad and persistent mucosal CD4 T cell responses to HSV-2 exist in the female genital tract of HSV-2+ women, suggesting that a population of resident memory T cells are resident at the site of infection.[79] Also, in recurrent HSV-2 genital lesions, CD4 T cells, monocytes, and PDCs infiltrate first to fight infection with CD8 T cells following step a few days later. Here, the IFNγ production, mainly by CD4+

T cells, is important for clearance.[73] IFNγ impedes some mechanisms of HSV-2 evasion of the immune system such as the downregulation MHC-I expression in infected keratinocytes. This allows CD8 T cells to recognize and kill infected cells.[48] IFNγ also stimulates MHC-II expression on keratinocytes, allowing recognition by CD4 T cells.[48] HSV-2-experienced CD4 T cells have also been shown to coordinate NK cell activation and their presence during HSV-2 antigen presentation is required to activate these cells to produce IFNγ.[78] The IFNγ produced by these CD4 T cells has also been shown to be critical for the transport of HSV-2 specific antibodies to cross neuronal barriers. After memory CD4 T cells migrate to the dorsal root ganglia and spinal cord in response to infection with HSV-2 and enter these neuronal tissues, CD4 T cells secrete IFNγ and mediate local increase in vascular permeability, enabling antibody access for viral control at this site.[81]

Tregs are a subset of CD4+ T cells that suppress immune responses and can be identified by expression of interleukin 2 receptor α (IL-2RA), Foxp3 and the lack of CD127 expression.[80] Although the role of regulatory T cells has been controversial in the past it has recently been shown that regulatory T cells are essential to promote proper CD4 T-cell priming upon mucosal infection.[121] Soerens and his colleagues showed that HSV-2-specific CD4+ T-cells fail to accumulate in the vagina in the absence of Tregs.[81,121] They also saw that Treg presence during T-cell priming is critical for subsequent accumulation of antigen-specific CD4 T-cells in the infected tissues. Interestingly, there also seems to be a direct correlation between Treg density and HSV-2 severity. This association of a high Treg to conventional T cell ratio with high viral

shedding suggests that the balance between regulatory and effector T cells influences human HSV-2 disease.[80]

Though there is ample evidence of the importance of T cell responses in HSV-2 immunity, and T cell responses have been identified as correlates of protection for Herpes Zoster, T-cell responses have not been identified as critical correlates of vaccine mediated protection for HSV-2.[19] This could be because candidate vaccines have either not sufficiently stimulated T-cell responses or the appropriate responses have not been measured. Additionally, how these T-cell responses are regulated in the lymph nodes and at the site of infection remains poorly understood. Understanding the role of these T cells, especially at biologically relevant sites such as the vagina and cervix, will be central to the elucidation of adaptive immune mechanisms involved in controlling HSV-2 disease.[79]

Humoral Response

Genital infection with HSV-2 results in the production of HSV-2 specific immunoglobulin G (IgG) and immunoglobulin A (IgA) in the genital tracts of humans and mice, with IgG appearing to be dominant over IgA.[82, 83] The humoral immune response is important in both the prophylactic and therapeutic vaccine setting. In the prophylactic setting, antibodies are important for the prevention of infection or decrease in infection rates. Therapeutically, virus specific antibodies are important in the clearance of reactivated virus from the genital epithelium thereby decreasing the severity and rate of genital lesions. Historically, a number of studies have pointed to the importance of the humoral immune response to HSV-2.[157-159] It is known that neutralizing antibodies can bind the viral envelope glycoproteins that are necessary for viral entry during infection[157] and importantly, maternal antibodies against HSV-2 reduce neonatal transmissions of the

virus.[158] Also, it was demonstrated that antiserum limits the extent of mucosal and dorsal root ganglia infection as well as the recurrence rate in the guinea pig model, if antibody is delivered in high quantities during the first 24 hours of infection.[159] However, clinical trials exploiting the production of antibody against HSV-2 glycoproteins have not shown protection against HSV-2 infection or development of disease, despite their induction of high titers of neutralizing antibody. However it is still possible that a different effector function such as antibody-dependent cell-mediated cytotoxicity is important for protection. Subsequently, vaccine efforts have been geared more toward the induction of a robust cell mediated response.

Because of this, unfortunately, relatively little is known about the polyclonal antibody repertoire induced by HSV-2 glycoproteins during natural infection or how these antibodies affect virus neutralization during infection.[84] In order to learn more about what constitutes the humoral response to HSV-2, studies examining the IgG content isolated from human serum have found that human anti-HSV-2 neutralizing IgG is predominantly against gD and gB.[84,85] These studies also found that even though there are several key HSV-2 neutralizing epitopes within gD and gB that are commonly targeted by human serum IgG, there are others epitopes within these same glycoproteins that fail to induce consistent responses.[84,85] This is particularly important for researchers considering designing HSV-2 vaccines by synthetically manufacturing and targeting specific antigen epitopes.

Finally, studies investigating the importance of the humoral response in the murine model have been controversial. One study showed that immunized B-cell deficient mice experienced greater levels of viral protein titers in the vaginal epithelium

and secretions 20 and 48 h post HSV-2 infection, when compared to their wild-type controls.[86] However more recent studies suggest this could be due to a lack of CD4 T cell activation by B cells, a protective activity independent of antibody production. Additionally it has been shown that adoptive transfer of serum IgG from HSV-2 immunized mice into the vaginal lumen of naive mice also reduced the viral load and pathological signs of disease.[202] Conversely, several studies seem to negate the importance of the humoral response during clearance and have shown that that resistance to a lethal challenge of HSV-2 in immunized mice was not dependent on B-cells or their antibodies.[87, 88]

The contribution of the humoral immune response to HSV-2 is not completely understood. Although antibodies against HSV-2 have been shown to mediate prophylactic protection in mice, B cells are not absolutely required for protection in the context of an acute infection.[160] It has been suggested that B cells more likely interact with other immune effectors such as T cells to activate them and confer protection in that way. A better understanding of the humoral arm of HSV-2 immunity is warranted for the future production and better understanding of therapeutics for genital herpes. What we do know now is that humoral immunity seems to play an early and beneficial role in primary genital HSV-2 infection but ultimately, a cellular component is required for HSV-2 clearance and protection.

Prophylactic vs Therapeutic Vaccines

For decades, researchers have been working on the development of an effective HSV-2 vaccine. There are two different approaches for vaccine formulation for HSV-2; either the development of a prophylactic or a therapeutic vaccine. Though different, both have obvious benefits and health indications worldwide. Prophylactic vaccines aim at preventing acquisition of a pathogen or preventing disease and thus must stimulate broad and durable immunity at all portals of pathogen entry in the case of HSV-2, the genital mucosa and surfaces.[19] Therapeutic vaccines on the other hand, aim to reduce recurrences or minimize disease severity and recurrence duration of those that are already infected.[19] Therapeutic vaccines also aim to decrease shedding which would directly translate to a reduction in transmission of the virus. These are important distinctions when considering vaccine development for HSV-2. The only approved vaccines that exist for a human herpes virus are vaccines against chicken pox or Herpes Zoster. The Shingrix vaccine, the latest vaccine for Herpes zoster, prevents reactivation of the chicken pox virus in infected individuals which puts it in the category of a therapeutic vaccine. One group pointed out that much of the success of the Shingrix vaccine could be attributed to the fact that Herpes Zoster is a disease of reactivation and in turn Shingrix is a therapeutic vaccine for disease.[19] The end points of therapeutic vaccines are to stimulate memory B and T cells which tend to be abundant, sensitive to vigorous restimulation, and can readily enter tissues during inflammation or indeed reside there.[19]

This in turn is less difficult a task than generating or enhancing a primary immune response which requires an antigen-specific signal to be delivered to naïve T cells along with a second costimulatory signal in order to activate these cells into effector cells. This costimulatory signal normally comes from professional antigen presenting cells,

specifically dendritic cells in this case. Because of this, it is important that a prophylactic vaccine stimulate the appropriate DCs. These requirements must be met when considering the design of a prophylactic vaccine. Additionally, the memory cells of interest in a therapeutic vaccine are more likely to be activated in the peripheral tissue where the disease occurs and do not require costimulation for activation. Because of this they can be activated by a multitude of antigen-presenting cells including keratinocytes, monocytes and inflammatory DCs.[19]

However this argument is contradicted by the fact that the previous Herpes Zoster vaccine, Zostavax, was used not only to prevent reactivation (shingles) but is also currently used at a lower dose as a prophylactic in children to prevent the acquisition of chicken pox (Varivax). Both of these vaccines are live attenuated varicella virus and the Varivax vaccine in children has shown to reduce breakthrough chickenpox infection in children down to 2.2% after the recommended 2 doses.[214] This gives some hope that there can be a similar achievement in other human herpes viruses such as the herpes simplex viruses.

DIFFICULTIES CONCERNING THE DEVELOPMENT OF AN HSV-2 VACCINE

Over the years there have been many attempts to develop an HSV-2 vaccine, however, to date, none have been approved for human use. This is despite many vaccines showing efficacy in animal models such as the mouse and guinea pig. This could be due to a number of issues, though pointed out years ago[89], still remain relevant now. For instance, herpes simplex viruses have coevolved with humans for millions of years and major histocompatibility complex alleles are different in humans than in other animals. Because of this, many HSV-2 viral proteins have likely undergone sequences changes

that limit recognition by the human immune system, which differs from the immune systems of the animals in which the virus is studied, such as the murine immune system and the guinea pig immune system.[89] Subsequently, HSV-2 encodes a number of immune evasion genes, many of which have species-specific effects making targeting the correct immune response difficult in animal models. As an example, although glycoprotein E binds the Fc domain of human IgG and blocks its activity, it does not perform this same function in either mice or guinea pigs.[90] Moreover, animals in vaccine trials are usually given a single challenge dose. This starkly contrasts with humans who are typically challenged with virus during multiple exposures. Though this single expose in animals reduces the required number of animals to show effectiveness of vaccines and improves statistical power, it differs markedly from human infection, in which most exposures to HSV-2 do not result in clinical disease.[89]

HSV-2 VACCINE CLINICAL TRIALS

Although there are a number of HSV-2 vaccines currently in the preclinical stage of development, there are only a few in clinical trials. As of now these consist solely of therapeutic vaccines. VCL-HB01, a therapeutic vaccine aimed at reducing the frequency of lesion recurrences in those with genital HSV-2 infection, is currently in a randomized, double-blind, placebo-controlled Phase 2 trial to evaluate its safety and efficacy.[91] This vaccine, developed by Vical, is a bivalent DNA vaccine encoding full-length HSV-2 UL-46 and gD antigens. In HSV-2 the UL46 gene encodes virion tegument phosphoproteins, the properties and functions of which are poorly understood. Previously, in a Phase 1/2 randomized, double-blind, placebo-controlled trial of 165 HSV-2 infected adults, VCL-HB01 demonstrated a statistically significant reduction in genital lesion rate compared to

baseline.[92] Significantly, recurrence rate is one of the most clinically meaningful endpoints for patients as it provides information on both the number and frequency of recurrences over time in this chronic disease setting.

HSV529, a live, replication-defective HSV-2 virus (deleted for UL5 and UL29) is also in clinical trials.[93] This therapeutic vaccine recently completed Phase 1 of clinical trials where it showed serum neutralizing antibody titers that were significantly increased from baseline after 3 doses of HSV529 compared to placebo ($P < 0.001$).[94] This increase persisted up to 6 months after the third dose of vaccine ($P < 0.001$). Serum and vaginal antibodies to HSV-2 gD also significantly increased after 3 doses of vaccine in group 3 subjects ($P < 0.001$ and $P = 0.012$, respectively). Interestingly, HSV529 also induced CD4 and CD8 T-cell responses in vaccine recipients. All in all, the vaccine proved to be safe, well-tolerated, and immunogenic, in addition to eliciting significant neutralizing, gD, and ADCC-mediating antibodies, and modest cellular immune responses in HSV seronegative individuals.

COR-1, another DNA vaccine, contains codon-optimized gD2 and ubiquitin-fused truncated gD2 to enhance generation of cytotoxic T cells. Previously, this polynucleotide vaccine had been shown to enhance immunogenicity and protect against lethal HSV-2 challenge in mice.[99] Recently in a Phase 1 study, COR-1 was found to be safe when given by intradermal route to HSV-1/2-seronegative participants and induced gD2-specific T cell but not antibody responses.[98] The lack of an antibody response during the study contrasts to the results the obtained in mice and was attributed to too low a dosage in humans as compared to their animal studies. However it was noted remarkable that the vaccine was able to elicit consistent CTL responses especially since needle and syringe

delivery of a DNA vaccine has not consistently given rise to measurable cellular responses in humans at such a low dose. From this study, it was concluded that COR-1 has potential to be used as a therapeutic vaccine for HSV-2 infection. A blinded, placebo-controlled, dose escalation Phase I/IIa study of COR-1 in HSV-2 seropositive and symptomatic subjects that aims to primarily assess the safety and tolerability of the vaccine and its effect on viral load commenced in late 2015, and results are therefore pending.

Though no longer in clinical trials, 2 recent HSV-2 vaccines merit mention. Both Gen-003 and HerpV showed promise in early clinical trials, however, failed to continue to later phases. Gen-003 by Genocea in a Phase Ia/II trial showed a reduction in viral shedding of greater than 50% (13.4% to 6.4%) in individuals that received the vaccine as compared to controls and lesion rates were also significantly reduced.[155] The vaccine also elicited increases in antigen binding, virus neutralizing antibody, and T-cell responses. Gen-003 was active in a Phase II trial until recently as Genocea decided to discontinue HSV-2 vaccine development.

HerpV, developed by Agenus, consists of 32 distinct 35mer peptides containing HSV-2 epitopes joined to their Hsp70 heat shock protein and adjuvanted with QS21. In a Phase II trial, this vaccine produced significant CD4 and CD8 T cell responses to antigenic peptides. It was also able to elicit a 15% decrease in viral shedding, which persisted up to 6 months after the initial vaccine series.[12,96] However as of March 2017, Agenus announced the discontinuation of the project in order to focus efforts on different projects the company was involved in.[97]

The lack of an effective HSV-2 vaccine despite many years of research and development of numerous prototype vaccines has led scientists to question why this is and what needs to be changed. One of the foremost issues to date is that HSV-2's site of primary infection is the mucosa. Because HSV-2 infects the vaginal mucosa/genital epithelium and also infects the sensory ganglia, it is necessary to recruit antibodies and immune cells to these sites of infection. Therefore, although many endpoints for vaccine research tend to be antibodies and cellular immunity in the blood, this does not necessarily reflect what immune response is like at the site of infection. Because HSV-2 consistently reactivates from the ganglia, infecting the mucosa and genital epithelium, the immune system is being constantly re-exposed to virus which is able to continue replication even in the presence of local neutralizing antibodies and T cells.[100, 101] This is important and means that an effective vaccine will need to improve on the natural and continuous exposure that HSV-2 positive patients experience.[102]

HSV-2 Vaccine Platforms

The road to the development of a vaccine for HSV-2 has led to the investigation of many different vaccine platforms for their efficacy in generating the appropriately protective immune response. There are advantages and disadvantages to each platform. This coupled with the route of vaccination (discussed in later sections) can provide a variety of protective or therapeutic immune responses. There have been a number of vaccines in the preclinical phase developed over the recent years which are discussed in the following sections.

Subunit Vaccines

The most widely used platform for HSV-2 vaccines in human clinical trials has been glycoprotein subunit vaccines.[12] Glycoprotein D is expressed abundantly on HSV-2's surface and is responsible for most neutralizing antibody activity by the immune system. Because of this, gD is a rationale target for HSV-2 vaccines and is a component of most subunit vaccines for HSV-2.[109] Currently there are a number of subunit vaccines in the pre-clinical stage of development.

One vaccine candidate, G103, developed by Odegard and colleagues, consists of three recombinantly expressed HSV-2 proteins (gD and the products of the UL19 and U25 genes) adjuvanted with glucopyranosyl lipid A (GLA), a TLR4 agonist, formulated in stable emulsion.[110] In early testing, this vaccine elicited antigen-specific antibody binding, neutralizing antibody responses, and robust CD4 and CD8 effector and memory T cell responses. Subsequently, this group evaluated the vaccine for use as both a prophylactic and therapeutic vaccine in mice and guinea pigs. In mice, prophylactic immunization completely protected against lethal intravaginal HSV-2 infection and therapeutic immunization expanded both CD4 and CD8 T cells induced by previous HSV-2 infection. Prophylactic vaccination was not evaluated in guinea pigs but therapeutic immunization showed to be about 50% effective in reducing the number of lesions per animal.

Recently, a vaccine containing glycoproteins C, D and E was tested for its ability to protect rhesus macaques and guinea pigs from HSV-2 infection.[111] Glycoprotein gD is important for virus entry and gC and gE are important for immune evasion by the virus. In macaques, this vaccine induced plasma and mucosal neutralizing antibodies along with antibodies able to block gC2 and gE2 immune evasion activities. The vaccine also

stimulated CD4 T cell responses in the macaques. In guinea pigs the trivalent vaccine was 97% efficacious in preventing genital lesions and the vaccine also showed a significant reduction in viral shedding rates.

Finally, a subunit vaccine composed of gD, gB, and tegument protein UL40 was evaluated for its efficiency as a vaccine in mice.[112] The vaccine was evaluated with several different Th1-inducing Toll-like receptor (TLR) agonists in vivo. This subunit vaccine combined with TLR9 agonist CpG oligodeoxynucleotide in a squalene-based oil-in-water emulsion promoted a more robust, functional HSV-2 antigen-specific CD8 T cell responses and high titers of neutralizing antibodies when compared to the MPL alum adjuvant.

Live Attenuated Vaccines

In principle, live attenuated vaccines have distinct advantages over subunit and inactivated vaccines, primarily because replication of the pathogen allows for the entire repertoire of pathogen-specific antigen expression.[107] However, concerns over the safety of live attenuated vaccines presents a strong disadvantage because of the possibility of conversion back into the wild-type virus. These kinds of vaccines would also not be suitable for pregnant or immune compromised individuals. In the past few years there have been several live attenuated HSV-2 vaccines in the pre-clinical stage of development. Inoculation with a live HSV-2 virus in which the nectin-1 binding domain of gD2 was altered so that the virus is impaired for infecting neural cells, but not epithelial cells provided better protection from vaginal challenge in mice with HSV-2 than that obtained with a subunit vaccine, despite inducing lower titers of HSV-2 neutralizing antibodies in the serum.[103] Compared to intranasal and subcutaneous,

intramuscular injection of this attenuated virus provided better inhibition of challenge virus replication in the vagina.

Replication Defective Vaccines

Replication-defective mutant viruses are defective for one or more functions that are essential for viral genome replication or synthesis and assembly of viral particles. As vaccines, these mutant viruses have the advantage of expressing viral antigens inside infected cells so that MHC class I and class II presentation can occur efficiently, but have the disadvantage of having a slight chance of reversion back to a replication competent virus.[106]

Subunit vaccines comprised of gD so far have failed in clinical trials. Because of this, one group hypothesized that deletion of gD-2 would unmask protective antigens. They then tested an HSV-2 virus deleted in gD-2 and complemented to allow a single round of replication on cells expressing HSV-1 gD (ΔgD(-/+gD-1)) for its ability to provide protection against lethal infection with HSV-2 in mice.[104] Subcutaneous immunization with this mutant provided 100% protection against lethal infection and also prevented latency establishment in the animals. Additionally injection into SCID mice did not produce disease while injection of wild type virus at 1000-fold lower doses were fatal. The live vaccine also elicited HSV-2 specific antibodies in the serum and vaginal washes after intravaginal challenge in the mice. These antibodies produced cell-mediated cytotoxicity, but little neutralizing activity. In further testing, prime and boost immunization (s.c.) with live, ΔgD-2 virus completely protected mice from challenge with the most virulent HSV-1 and HSV-2 virus strains from a panel of clinical isolates.[105]

Synthetic Peptide Based Vaccines

Peptide vaccines consist of synthetic peptides that are able to produce protective immune responses against HSV-2, mainly through targeting CD4 T cell and B cell epitopes, which have been shown to be protective against HSV-2.[3] Recently our lab developed and tested a synthetic peptide vaccine composed of self-assembling nanofibers presenting gB from HSV-2 adjuvanted with CpG. In preliminary studies the vaccine was shown to induce robust effector and memory CD8+ T cell responses in mice.[161] Additionally, prime-boost immunization with this peptide nanofiber vaccine combined with adjuvant resulted in the development of HSV-specific CD8+ memory T cells.

Mentioned earlier, HerpV is a peptide vaccine with 32 peptides linked to heat shock protein (HSP) and QS-21 adjuvant. Recent studies have shown the immunogenicity of this vaccine in its ability to generate CD4+ and CD8+ T cell responses in both mice and HSV-2 positive human participants.[113,114] However, unfortunately, the antibodies generated against these synthetic peptides have shown to be ineffective at conferring protection against HSV-2.[115]

Live Vector/DNA Vaccines

Adenoviruses are attractive vaccine vectors as they induce both innate and adaptive immune responses in mammalian hosts.[116] In the past, these kinds of vaccines were shown to elicit HSV-2 specific CD8 T cells after vaccination.[117, 118] However, due to the safety concerns of live virus vaccines, as discussed earlier, no major vaccine company in the US or EU is working on the development of live vector vaccines. There have been some recent investigations, though, on the effectiveness of an adenovirus vaccines in animal models.[119, 120] One study found that a replication defective

recombinant adenovirus vaccine based on glycoprotein D and truncated UL25 was able to induce specific T-cell responses and protect mice against intravaginal HSV-2 challenge compared with formalin-inactivated (FI)-HSV-2.[119] The vaccine showed higher reductions in mortality and stronger antigen-specific T-cell responses than the FI-HSV-2. Moreover, the combined gD2/UL25 adenovirus vaccine showed a significant decrease in the severity of genital lesions compared to adenovirus vaccines with either gD2 or UL25 alone. It is important to note that this study specifically studied the effect of this vaccine in acute infection. Meanwhile treatment of HSV-2 in the population would either focus on prophylaxis or therapeutically treating patients that have been infected with HSV-2 for some time and focus on decreasing shedding/recurrent disease.

HSV-2 Route of Vaccination and Adjuvants

There are many routes of delivery currently used for HSV-2 vaccines including subcutaneous, intramuscular, intranasal, intravaginal, and intradermal delivery. When considering a potential vaccine for HSV-2, the route of immunization is highly important being that different immune responses are elicited at different vaccination sites, directly impacting vaccine efficacy.[3] Comparison between intravaginal, intranasal, and intradermal vaccination saw that although all 3 routes reduced vaginal shedding and acute genital disease, intravaginal vaccination most effectively reduced primary and recurrent HSV disease.[203] Conversely, the lymphoproliferative response was highest in animals receiving intradermal vaccination. Moreover, the type of vaccine used can affect which route is best for vaccine administration. For instance, a comparison of intramuscular and footpad vaccination in mice with an HSV-2 DNA vaccine encoding gD2 showed that

intramuscular immunization induced higher levels of antibody in sera, while footpad s.c. immunization triggered a higher HSV-specific cytotoxicity response.[132] Though it is not fully understood what mechanisms govern why different vaccination routes elicit different immune responses, these considerations are important to keep in mind when developing new candidate vaccines for HSV-2. It is also important to keep in mind that serum antibodies can gain access to the vagina. Local immunization is not necessary for this but T cell residence in the vagina is complex and may require local immunization or vaginal chemokines in order to draw primed T cells in.[204]

MUCOSAL VACCINATION

The mucosal immune system is responsible for protecting the mucosal surfaces of the respiratory tract, nasal passages, and the intestines which are particularly vulnerable to infection due to their contact with the environment and thin and permeable nature.[205] There are two distinct features of the mucosal immune system. The first is that immune responses induced within one compartment are largely confined in expression to that particular compartment. The second is that lymphocytes are restricted to particular compartments by expression of homing receptors that are bound by ligands, known as addressins, which are specifically expressed within the tissues of the compartment.[205] Homing of leukocytes to various tissues is dependent on the interaction between homing receptors on leukocytes and their ligands, addressins, on endothelial cells.[206] Mucosal immunization results in homing of antigen-specific lymphocytes back to the mucosa where they first encountered the antigen.

The mucosa is the first line of defense against most infectious diseases, and so, the mucosal route of vaccination is preferred especially because of its important role in

the generation of immunity against sexually transmitted infections. Mucosal vaccination includes intranasal, intravaginal, ocular and oral delivery. This imitates the natural process of virus infection, and induces mucosal innate immune responses, which are optimal conditions for vaccine development.[3] Over the past few years, more information about the immunity induced at the mucosal sites involved in HSV-2 infection has been described. Recently it was found that Tregs in the vaginal mucosa are critical for appropriate DC trafficking from the vaginal mucosa to the draining lymph nodes. This process is necessary for effective CD4 T-cell priming, activation, and migration to infected tissues.[121] In this study, mice without Tregs failed to control viral replication, pointing to a role for Tregs in facilitating productive immune responses.

Because of the numerous natural defense mechanisms at mucosal surfaces, such as the acid and enzyme-rich environment of the stomach, and the mucus layer that coats all mucosal surfaces,[122] successful delivery of vaccines across these surfaces presents quite the challenge. Additionally, mucosal vaccines typically require adjuvants to help stimulate stronger systemic and mucosal immune responses.[123] This may be the reason why there are significantly fewer mucosal vaccines than other vaccines on the market currently.

Out of the more than 25 preventable diseases for which vaccines are available, just five are true mucosal vaccines; four are delivered via the oral route and one is delivered intranasally. These diseases include cholera, poliomyelitis, rotavirus, typhoid, and influenza respectively. Though these vaccines are currently licensed for use in humans, there currently exist no vaccines delivered by the vaginal route. Importantly, the immunity and antigen presenting cells (APCs) located in the vaginal mucosa are

distinguished from those of other human mucosa,[124] and display distinct functionality in directing both CD4+ and CD8+ T-cell responses. The human vaginal mucosa contains four major subsets of myeloid-derived APCs; LCs, CD14− LP-DCs, CD14+ LP-DCs, and Mφ.[125] To elicit desired types of adaptive immune responses in the vagina, antigens need to be delivered to these selected APC subset with adjuvants that can further promote the APC-mediated immune responses.

Alternatively, intravaginal vaccination is not the only way to create an effective genital or mucosal immune response. Recent studies showed that it was possible to recruit protective T cells to the vagina and provide protection against HSV-2 genital disease mice through intranasal vaccination.[126] Additionally, intranasal vaccination using the proper adjuvant has been shown to elicit both systemic and mucosal immune responses against HSV-2.[127] Recently, a study found intranasal immunization with adjuvanted HSV-2 gD produced significant protection against primary infection, establishment of latency, and recurrent genital herpes in guinea pigs.[152] Intranasal vaccination during this study elicited HSV-2 specific T cell proliferative responses, IFN-γ responses, and systemic and vaginal gD-specific IgG antibody responses. The systemic antibodies generated also displayed potent HSV-2 neutralizing properties and high avidity. Considering this information, it is possible that a combination vaccination, utilizing both the intranasal and intravaginal route could be optimal for an effective HSV-2 vaccine.

INTRAMUSCULAR VACCINATION

A consistent concern within the HSV-2 vaccine field is whether or not protective genital mucosal immunity can be generated via intramuscular (i.m.) vaccination.

However, the success of the human papillomavirus (HPV) vaccine does provide hope for the development an efficacious i.m. vaccine against viral pathogens infecting the genital mucosa.[128] However, to date, clinical trials involving i.m. injection of HSV-2 gB and gD in adjuvants have not been effective. It may be necessary that a different vaccine formulation be given via i.m. injection in order to elicit the proper protective immune response, but this needs to be further investigated. In preclinical studies, i.m. vaccines have been shown to confer protection in animals. A recent study investigating the replication-defective HSV-2 mutant virus dl5-29 mentioned in earlier sections, showed that dl5-29 gave a higher antibody response and better protection when given i.m as compared with subcutaneously (SC).[133] Intramuscular immunization with this vaccine also showed better protection against infection with a highly pathogenic African HSV-2; importantly showing that the vaccine could efficiently protect against HSV-2 strains from varying geographic regions.

Another study found that mice immunized i.m. with gE2-del virus all survived HSV-2 MS intravaginal challenge opposed to the 60% survival rate seen in mice immunized s.c.[134] Additionally, no mice in the i.m. group contracted disease which was not the case in the s.c. group. Interestingly, a number of studies investigating the differences between intramuscular and subcutaneous HSV-2 immunization have found that i.m. immunization outperforms subcutaneous.[133-136] It seems as though i.m. vaccinations have potential to produce protective immune responses, however more work needs to be done to understand how to confer mucosal immunity for HSV-2 in humans via this route.

DERMAL VACCINE DELIVERY

The dermis is home to a large number of DCs which play an important role in immunity against many diseases, especially HSV-2. Thusly, dermal vaccines incorporate a relatively new technology comprised of a device able to produce an array of tiny projections (microneedles) that is briefly applied to the skin or mucosa to deliver vaccines into the dermis. These microneedles are both degradable and thermostable when coated with vaccine. Most notably, they allow for large dose reductions (up to almost 1000-fold) compared with intramuscular injection, without compromising efficacy.[129, 130] Interestingly, the challenge of targeting skin resident DCs for proper antigen delivery could be solved via vaccination with the Nanopatch microneedle array. This method allows for the antigen to be delivered directly to the dermal-epidermal junction resulting in efficient antigen uptake by LCs.[130] This method of vaccination has been successfully used for HSV-2 in mice,[131] though obviously would need to be optimized for use in better animal models or humans to account for differences in epidermal thickness. Other approaches for targeting epidermal DCs during vaccination include combining a vaccine with an antibody to target a specific DC subset, or using an adjuvant that preferentially activates a particular subset of cells.[19] In summary, dermal vaccine delivery appears to be an attractive candidate for an HSV-2 vaccine platform for the induction of an effective immune response but is still in early developmental stages for this task.

VACCINE ADJUVANTS

Essential to the efficacy of a properly developed HSV-2 vaccine is the accompanying adjuvant. A few HSV-2 vaccine candidates, such as synthetic lipopeptide vaccines, have bolstered the property of being self-adjuvanting, but as a majority, the proper adjuvant is necessary in order to stimulate the proper immune response. Ideally,

the adjuvant for an HSV-2 vaccine should trigger both an effective innate immune response as well as a sufficient adaptive immune response. There are many adjuvants that have been tested in animal models though only a few have been used in humans. Adjuvants used in animals studies include alum, monophosphoryl lipid A (MPL), cholera toxin, CpG-containing oligodeoxynucleotides (CpG) and poly (I:C).[3] The appropriateness of the use of these adjuvants in humans is still an obvious consideration. Alum, which is used as an adjuvant in a majority of i.m. vaccinations has been found unfit for use in human skin due to reactogenicity.[137] Even more, alum does not produce strong T cell immunity which is an important part of HSV-2 generated immunity. Understanding the mechanism of action of adjuvants should be a point of focus for researchers and would prove beneficial in choosing the best adjuvant to complement a given vaccine and route of immunization. It has even been suggested that the differences in efficacy between Shingrix (herpes zoster vaccine) and Simplirix (HSV vaccine) could potentially be partially attributed to the mode of action of their respective adjuvants.[19] Deacyl monophosphoryl lipid A (dMPL) and saponin based adjuvants were used in both vaccines. dMPL is known for inducing strong Th1 CD4 immunity and boosting antibody titers, however, saponin-based adjuvants, are better for inducing memory CD8 T-cell responses. CD8 T cell responses are important for protection against virus reactivation, which is specifically helpful for protection against Herpes Zoster (a reactivation disease), but would not be sufficient for a prophylactic vaccine.

HSV-2 infection naturally activates both TLR 3 and 9. With this in mind CpG and poly I:C have become logical adjuvants for HSV-2 vaccination being that they stimulate TLRs 9 and 3, respectively. These adjuvants have also been modified for use in humans.

Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG ODNs) have been designed as an alternative to pure CpG DNA.[139] They have been shown to effectively stimulate cells expressing TLR9, such as dendritic cells and B cells. The use of this adjuvant ultimately improves the function of professional antigen-presenting cells and boosts the generation of humoral and cellular vaccine-specific immune responses. Importantly, CpG ODNs have the same adjuvant properties whether administered systemically or mucosally,[138] and have also shown to persist in immunocompromised hosts, which is important when considering vaccinations for those co-infected with HSV-2 and HIV.[3, 29, 30]

Poly IC is an agonist of TLR3 which is expressed in the endosomal compartments of DCs, macrophages, and on the surface of epithelial cells. Studies in mice have shown that intradermal vaccination with gD and with poly IC as an adjuvant promotes long-lasting mucosal immunity and protection from genital HSV-2 infection.[143] As noted earlier, natural HSV-2 infection stimulates TLR3, meaning polyIC would be a useful adjuvant for simulating infection. However, because of its rapid degradation, more stable derivatives of poly IC have been developed, such as poly ICLC (polyinosinic:polycytidylic acid [poly IC] stabilized with poly-l-lysine).[141] This compound is known to enhance antigen cross-presentation by DC to CD8 T cells and has been shown to induce innate immune responses similar to a live viral vaccine in humans.[140, 142] Adjuvants that activate the cells necessary for protection against HSV-2 should be considered when developing a vaccine or considering a boost schedule. Unfortunately, to date, more needs to be understood concerning HSV-2 correlates of protection and an ideal adjuvant for an HSV-2 vaccine has not been identified.

Animal Models for HSV-2 Immune Response and Vaccine Development

Because of the complications associated with HSV-2 infection and the fact that primary disease is often asymptomatic, studying the immune response to HSV-2 in humans by manipulation of immune cells isn't feasible or ethical. Instead, there are many animal models that have been used to study HSV-2 genital disease. The most commonly used include mice, guinea pigs, rabbits, and rats. The course of HSV-2 infection in an animal model depends on important experimental parameters including animal species, age, and genotype; route of infection; and viral serotype, strain, and dose.[144] The following sections will explore 2 animal models specifically; mouse models because they are the most widely characterized and have been used extensively for HSV-2 studies of immunity, pathogenesis, and vaccination and the guinea pig model as it is currently the golden standard for HSV-2 genital infection given its likeness to natural human HSV-2 genital infection.

MICE MODEL FOR GENITAL HSV-2

Because mice have been extensively characterized, are easily accessible, have an abundance of murine specific reagents for a variety of assays, and have been genetically modified for the investigation of numerous immunological pathways, these animals have been crucial in the elucidation of the immunity and pathogenesis associated with HSV-2 infection. Though mouse models have been used more extensively in studies to understand HSV-1 infection they've also been used frequently to study HSV-2 genital disease. For example, mice have been critical for the elucidation of the importance of

Type I IFNs in HSV-2 immunity. Studies have shown mice deficient in the A1 chain of the type I interferon receptor (CD118(-/-)) are susceptible to HSV-2 in the absence of medroxyprogesterone preconditioning, which is a prerequisite of murine susceptibility to HSV-2 infection.[146] Much of what happens at the onset of infection is understood as well due to work done in mice. For example, because of work done in mice, we know HSV-2 recognition and resulting TLR 3 and 9 activation causes inflammatory cytokines and type I IFNs to be released by both innate immune cells and genital epithelial cells, causing the epithelial cells to enter an antiviral state.[147] It is important to remember that the value of the mouse model lies in its extensive characterization and the availability of verified genetically modified mice. Because of this, experiments can be completed in this model that may not be as feasible in a better suited animal model.

However there are some significant weaknesses concerning the mouse model of genital HSV-2 infection. Genital infection with HSV-2 in mice requires pretreatment with progesterone in order for infection to take.[144] This treatment thins the uterine lining, significantly altering the interpretation of viral pathogenicity in this model.[148] Moreover, infection is fatal without intervention (not the case in humans) and mice do not experience spontaneous virus reactivation as do humans. This means that it is not possible to study immune modulation of virus shedding or recurrent disease in this model. Mice have much thinner skin than humans and don't show the same degree of stratification or the same distribution of immune cell subsets as humans.[144] For example, HSV-2 has been shown to initially infect epidermal γδT cells in mice, however this is not the case in humans.[145] This difference in pathology of infection has important implications when considering the applicability of results in mice to human infection.

Essentially, though there have been important discoveries in mice on HSV-2 immunity and pathogenesis, there should be caution when designing studies to develop future therapeutics and vaccines in this model given the amount of disparity between the murine and human response to HSV-2.

Despite the above, the differences between mouse and human immunity to HSV-2 have not led to a halt in preclinical studies for possible HSV-2 therapeutics and vaccines in the murine model. There are still many preclinical studies being conducted in this model for HSV-2 vaccines. One recent study suggested that an adjuvant consisting of CpG motifs could increase the effectiveness of a full length gD HSV-2 DNA vaccine to induce a stronger cell-mediated immune response.[149] Importantly this specific study only aimed to evaluate the efficacy of a HSV-2 DNA vaccine adjuvanted with a plasmid adjuvant containing CpG motifs. In this way, mice prove to be a valuable and inexpensive way to evaluate the effectiveness of a vaccine idea before validation in better models (such as the guinea pig discussed later) then clinical trials. There exists, still, the risk that a therapeutic will show ineffective in mice that would work in humans.

Recently the idea that vaccination with secreted HSV-2 glycoprotein G (sgG2) could provide prophylactic protective immunity was investigated.[150] Researchers tested this vaccine in mice and saw that 3 i.m. immunizations with sgG2 along with adjuvant (CpG motifs and alum) induced almost complete protection from genital and systemic disease after intra-vaginal challenge with HSV-2. Protection included a robust IgG response, IFN-γ producing CD4 T cells, reduction of infectious HSV, and reduction of HSV-2 DNA copy numbers in the dorsal root ganglia, spinal cord, and in serum. Again

this shows the usefulness of mice for testing a new form of vaccination in its primary stages for possible protection against HSV-2.

However, it would be irresponsible not to fully take into account the limited translatability of this model. One study recently tested the efficacy of their vaccine in both mice and guinea pigs and had varying results. The study found the vaccine to be prophylactically protective in the murine model but only report partial therapeutic protection in guinea pigs.[110] Being that guinea pigs represent a better model of human infection (discussed in the next section) this raises the question of whether vaccines results from tests in mice could realistically provide data useful for future testing in clinical trials.

GUINEA PIG MODEL FOR GENITAL HSV-2

The guinea pig model for genital HSV-2 currently represents a golden standard for genital herpes research. This is because this model recapitulates many of the most important clinical conditions of genital HSV-2 disease. Vaginal inoculation in this model results in genital lesions without hormone or other manipulation and the virus goes on to establish latency in the sensory ganglia. Infection in this model also results in spontaneous reactivation which results in asymptomatic shedding and spontaneous reactivation/lesions. Proper recapitulation of human infection and disease is necessary in an animal model as it increases the chance of positive results in animal testing translating to clinical studies. That being said, there are cons associated with this model as well. For instance, in a small percentage of animals, HSV-2 infection is associated with hind-limb paralysis. Furthermore, reagents for this model are very limited and there exist no HLA or transgenic guinea pig models for HSV-2.

Because of the lack of reagents on the market for the guinea pig model, several groups of researchers have been working to develop novel reagents for this model. Included in these groups is the Schafer lab which generated polyclonal antisera and monoclonal antibodies against recombinant guinea pig IFNγ and a number of leukocyte surface antigens. These antibodies were used to develop an ELISA assay used to detect and quantify IFNγ in vitro. This is of significant importance because of the large role IFNγ plays in the immune defense against HSV-2. Understanding which cells play a role in HSV-2 specific IFNγ secretion and how to induce this protective response is paramount in the search for a vaccine or more effective therapeutics for HSV-2. Our lab was able to utilize antibody-producing hybridoma cells produced in the Schafer lab for experiments aimed at better understanding immunology involved in the pathogenesis of HSV-2 genital disease, specifically to assess the role of HSV-2 specific CD4 and CD8 T cells in the prophylactic and therapeutic setting. These experiments are discussed in detail in the later chapters of this document.

Despite the paucity of reagents available for this model, there are still many exciting studies being completed for future HSV-2 therapeutics and vaccines in guinea pigs. Recently a study working towards the elucidation the molecular mechanisms underlying the pathology associated with genital HSV-2 infection was completed. Using next generation sequencing one group of researchers investigated alterations in the expression levels of miRNA and mRNAs associated with TLR pathways in HSV2-infected tissues.[151] They saw that the TLR pathway genes TLR1, TLR3, TLR5, TLR9, TRAF6 and TRIF were downregulated during infection with HSV-2 suggesting network regulation of mRNA by miRNA in tissues infected with HSV2 genital herpes.

In the past our lab was able to identify long lived HSV-2 specific plasma cells in the genital tract and spinal cord ganglia of chronically infected guinea pigs.[14] This shed light on the long-term maintenance of both the humoral and cellular arms of the adaptive immune response at the sites of HSV-2 latency and virus shedding. This provides an important step forward for the field; understanding the existing immune defense against long term HSV-2 infection is important for understanding which populations of cells are most important to target for the development of effective therapeutics.

Recently, a trivalent subunit vaccine containing gD, gC, and gE along with adjuvant was shown to reduce the frequency of recurrent genital lesions and vaginal shedding of HSV-2 DNA by approximately 50% in guinea pigs.[153] This vaccine also almost totally eliminated vaginal shedding of replication-competent virus in guinea pigs previously infected with HSV-2. Importantly, antibodies to gD neutralize the virus while antibodies to gC and gE block their immune evasion activities, including evading complement attack and inhibiting activities mediated by the IgG Fc domain, respectively.

Another group recently tested the efficacy of a subunit vaccine composed of the gD and gB and the novel T cell antigen and tegument protein UL40 in guinea pigs.[154] They found this vaccine successfully generated neutralizing antibodies and protective T cell responses. They also saw a suppression of vaginal HSV-2 shedding, low lesion scores, and a reduction in latent HSV-2 DNA in dorsal root ganglia to undetectable levels. Of note, this study highlights also the importance of formulating a vaccine and adjuvant combination that produces elevated T cell responses.

Though guinea pigs to date are the golden standard animal model for genital HSV-2, so far, clinical trials in humans with vaccines that showed promise in this model

have not yet been effective. One of the largest clinical trials for and HSV-2 vaccine was the Herpevac vaccine trial.[156] In preclinical studies, this vaccine showed protection against primary and recurrent HSV-2 disease in guinea pigs. However in clinical trials the vaccine showed no efficacy in preventing HSV-2 infection. Interestingly, it did show some cross protection against HSV-1 disease.

More recently, the Gen-003 trial mentioned in earlier sections showed therapeutic efficacy in guinea pigs (significantly reduced both lesions and viral shedding) **and** also showed significant efficacy in clinical trials, though it was discontinued for other reasons. In clinical trials GEN-003 induced robust and durable IgG and HSV-2 neutralizing antibodies and induced robust and sustained IFNγ, cytolytic and polyfunctional T cells.[155] Polyfunctional T cells are cells that each produce multiple immune mediators. These types of cells are considered to deliver a more effective immune response than those that only secrete one mediator. Importantly, antigen-specific T cell responses significantly correlated with lesion rate after vaccination, suggesting T cell control of HSV reduces the frequency and duration of genital lesions – an important lesson for future vaccine development and testing in animals.

In conclusion, though guinea pigs represent the best animal model for genital HSV-2 infection, there are still some issues with translating results from guinea pigs to humans. As time progresses it seems that we are making steady progress towards and effective vaccine. From the current results, it seems as though more efforts need to go into identifying and understanding the proper correlates of protection for HSV-2, properly stimulating T cell responses, and increasing the availability of reagents for the guinea pig model to facilitate proper vaccination studies.

CHAPTER 2: ANTIBODY PRODUCTION AND PURIFICATION

Abstract

Understanding the immune response to infection and utilizing a proper animal model is critical to effective vaccine development. The guinea pig model offers the best natural response to genital HSV-2 infection of all animal models thus far, but there are a very limited number of commercial assays and reagents for this model. Here we have outlined the amplification and purification of antibodies specific for guinea pig IFNγ, which is not yet available commercially. We were able to consistently grow and purify this antibody in sufficient quantities for future experiments and immune assays. These antibodies come from immortalized B cell lines received as a gift from the Schafer lab who generated antibody producing cells specific for recombinant and native guinea pig IFNγ.[16] This work is significant because consistent production of a pure supply of antibody is important for the future development of assays for analyzing immune and protective responses against HSV-2 in this important animal model.

Introduction

The guinea pig is considered the golden standard model of HSV-2 genital infection because, unlike other models, it fully recapitulates many significant pathological aspects of human genital HSV-2 disease without immunological or

pharmaceutical manipulation. Because of this, many important disease mechanisms and protective immune responses can be unveiled via experimentation in guinea pigs, however current efforts have been hindered due to a lack of availability of immunological reagents and assays for this model. Because of this, many of the reagents we use for our assays are grown and purified in lab. Help with this came from the generous donation of several B-cell lines producing antibodies able to detect guinea pig IFNγ at different levels from the laboratory of Dr. Schafer. The Schafer lab produced guinea pig (gp) IFNγ-specific antibody-generating B cell lines by immunizing mice with recombinant gp IFNγ to generate hybridoma cells. Hybridoma cell lines from these experiments were confirmed to identify recombinant and native gp IFNγ in ELISA studies.[16]

Interferon gamma (IFNγ), a type II interferon, is an important immune mediator during viral infections. Unlike Type I IFNs such as IFNα and IFNβ, the main biological function of IFNγ is the activation of cellular effector activities in cell populations carrying IFN-γ receptors.[16] These include T cells, B cells, macrophages, epithelial cells and the axon terminals of peripheral neurons.[208] The appearance of IFN-γ-secreting T lymphocytes is distinctive of TH1 responses, and the level of IFN-γ in biological samples or the enumeration of IFN-γ-producing cells is frequently used as indicator of an ongoing TH1 immune response.[16] In the context of HSV-2 infections, IFNγ is important for countering viral immune evasion, recruiting effector cells to the site of infection, and viral clearance by effector T cells.[61] Accordingly, IFNγ detecting antibodies are an extremely useful tool for analyzing the natural immune response to HSV-2 infection and analyzing protective mechanisms elicited post infection. Here we outline the

methodology involved in the production, isolation, and purification of antibodies able to recognize native gp IFNγ *in vitro*.

Methods

HYBRIDOMA MAINTENANCE PROTOCOL

Thawing: Cells were removed from the liquid nitrogen tank and placed in a 37C water bath. As the last bit of cells melted, the vial was placed on ice and allowed to rest for 2 minutes. Using aseptic technique, the vial was opened (within a biosafety cabinet) and the contents were transferred to a sterile 50mL conical tube. The sample was maintained at room temperature and 50uL of warmed growth media was added to the cells which were then allowed to rest for 1 minute. This addition and rest step was repeated with 5 additions of 100µL, 200µL, 400µL, and 800µL respectively. The sample was then allowed to rest for 5 minutes at room temperature before the addition of 23.5mL of warmed Growth Medium for a final volume of 25mL. The cells were then centrifuged at 700rpm for 10 minutes at room temperature and with the centrifuge set on slow brake. The supernatant was removed and the pellet resuspended in 10mL of warmed growth medium. The cells were then transferred to a 25cm^2 tissue culture flask. This flask was maintained at 37C with 5% CO_2. Cells were checked daily and passed at 1:5 as necessary.

Freezing: Cell suspensions ready to be frozen were centrifuged at 1300rpm for 8 minutes at 4C. Supernatant was removed and cells were resuspended in cold Growth Medium at half of the desired final volume. Slowly an equal volume of cold Freezing Medium was added dropwise. Cells were then divided into 1mL aliquots and placed into cryovials.

Vials were then placed in a Styrofoam container and then into a -80C freezer overnight. The next day, the vials were placed in the liquid nitrogen tank.

Table 1 Thawing growth medium and Freezing medium recipes

Thawing Growth Medium	**Freezing Medium**
500mL DMEM containing: **► High Glucose (4.5g/L)** **► Sodium Pyruvate** **► 2mM L-Glutamine** **Add:** **20% (130mL) FBS** **1% (6.5mL) Non-Essential Amino Acids** **1% (6.5mL) NaPyr (1µM)** **1% (6.5mL) L-Glut (2µM)** **1% (6.5mL) Pen/Strep** **(100U/mL Penicillin + 100ug/mL Streptomycin)**	50% Growth Medium 30% FBS 20% DMSO

ANTIBODY AMPLIFICATION

To amplify antibody from isolated B-cell lines, the appropriate cell line was collected from the liquid nitrogen freezer and thawed following the Hybridoma Maintenance protocol referenced later in the above section. As cells became confluent they were moved to a T25 culture flask. As they became confluent in the T25 they were moved to a T75 and then on to a T150 culture flask. During this time growth media containing 10% FBS was used. Once the cells became confluent in the 1 T150, they were passed into 6 T150 culture flasks at a 1:10 dilution with a total volume of 40mL/flask and incubated at 37C for 3-4 days until confluent. At this point, one of the 6 flasks was used to pass cells into 6 new T150 flasks and cells from the other 5 flasks were passed into roller bottles. The passing into roller bottles was done by completing the following steps:

First, all adhered cells in the culture flasks were dislodged using a cell scraper. The resulting cell suspension was the pipetted into (4) 50mL conical tubes and centrifuged at 1300 rpm for 8 minutes at room temperature. The supernatant from the tubes was then aspirated and discarded and cells were resuspended and pooled in 10mL of serum free growth media. This 10mL of cell suspension was added to 790mL of previously prepared serum free media for a total volume of 800mL. This volume was split into 2 roller bottles (400mL each) with filter caps and placed on a roller bottle apparatus on set to continuous gentle rolling setting in an incubator set to 37C. Cells were cultured in the roller bottles this way for ~7 days until confluent.

To collect supernatant from roller bottles, the 800mL of contents of both roller bottles were divided between 2 500mL polypropylene centrifuge bottles. These bottles were then centrifuged in a Beckman Coulter J Series Centrifuge on the JLA 10.500 setting using a fixed angle rotor. The samples were pelleted for 30 minutes at 4C and 3000rpm. Supernatant resulting from centrifugation was collected in 2L solid cap roller bottles and stored at 4C in the cold room. This entire process was repeated and samples pooled until the desired volume of supernatant was collected.

Table 2: Growth Media and Serum Free Media Recipes

Growth Media	**Serum Free Growth Bottle Media**
500mL DMEM **58.1ml FBS (10%)** **5.8ml Non-Essential Amino Acids (1%)** **5.8ml NaPyr (1µM) (1%)** **5.8ml L-Glut (2µM) (1%)** **5.8ml Pen/Strep (1%)**	760ml DMEM 8ml Non-Essential Amino Acids (1%) 8ml NaPyr (1µM) (1%) 8ml L-Glut (2µM) (1%) 8ml Pen/Strep (1%) 8ml Neutridoma (1%) – Add just before use

ANTIBODY PURIFICATION

In order to purify the antibody out from other proteins contained in the supernatant collected in the section above, antibody was precipitated using ammonium sulfate. Solubilized proteins remain dissolved in solution due to the higher number of hydrogen bonds between the solvent and protein. In order to precipitate, or remove the protein from the solution it is necessary to decrease the number of these bonds. This is possible by adding a high concentration of salt which acts by removing water molecules from proteins contained in the solution, thereby decreasing the amount of hydrogen bonds and increasing the likelihood of protein precipitation.

To do this, supernatant was transferred from the refrigerated roller bottles into a large 5L beaker and volume was noted to the nearest 5mL. The supernatant was stirred continuously using a stir bar while 361g of ammonium sulfate was slowly added for each liter of supernatant, allowing for each added amount to dissolve before adding more. Once the last of the ammonium sulfate was added the flask was moved to 4C overnight, continuously stirring. The following day the ammonium sulfate solution was placed in dialysis to remove excess salt prior to further purification and to allow for the refolding of the protein which was denatured during the precipitation. For dialysis, the ammonium sulfate solution was divided evenly between 500mL polypropylene bottles and centrifuged at 6000rpm for 30 minutes at 4C. The supernatant was then discarded and the protein containing pellets were resuspended and pooled in PBS (20mL PBS/L ammonium sulfate solution). 5-10 mL of PBS were saved in order to do a second rinse through of the bottles and the suspension was moved to sterile dialysis tubing which was secured on both ends, allowing space for an increase in volume. A 5L flask was filled with 5L of

PBS and the filled and knotted dialysis tubing containing the pellet suspension was added. The flask and tubing were refrigerated at 4C and stirred constantly. The PBS in the flask was replaced daily for a total 6 changes. After the last change, the solution within thee dialysis tubing was transferred to 50mL conical tubes. These tubes were centrifuged at 4000rpm for 30 minutes at 4C and the supernatant was transferred to new 50mL conical tube. Post dialysis, the renatured antibody solution will contain an excessive amount of PBS. Because of this, Centriprep 10K centrifugal filter devices were used to filter out excess PBS following the manufacturer's protocol. Filter devices were centrifuged at 3000g at 4C for 40 minutes. Once the antibody was concentrated down to the desired volume, a syringe and needle were used to collect the concentrated sample. Filtrate was used to rinse the chamber and collect additional sample. Afterwards, antibody was quantified and sterile filtered into a 15mL conical tube and stored at 4C.

After the initial quantification of all proteins in the solution (including unwanted proteins) the sample was purified using IgG/A purification columns as described by the manufacturer. After purification, protein content (which should now consist mainly of the desired antibody) was quantified again. Quantification of gp IFNγ specific antibody was determined by ELISA and percent purity was determined by comparing the antibody specific content and total protein content of the final solution.

ANTIBODY QUANTIFICATION

Antibody quantification was completed by ELISA. One day prior to quantification, CoStar E.I.A./R.I.A plates where coated with goat anti-mouse IgG2a capture antibody in ELISA coating buffer (1ug/mL, 100uL/well—1:1000 dilution). Plates

were then covered with parafilm and incubated at 4C overnight. The next day an automatic plate washer was used to wash the plates 4 times with ELISA wash buffer. Plates were then blocked with 200 uL of 2.5% BSA in ELISA wash buffer (EBB) per well and incubated at 37C for at least 1hr. During this time the mouse IgG2a antibody standards and antibody samples were prepared. The original standard stock of 1.0mg/mL was diluted down to 50ng/mL in EBB and 200uL were added to the first 2 wells (duplicates: A1 &B1). 100 uL of EBB was added across the plate (A&B 2-10) so that serial 1:2 dilutions could be performed across the plate. The last 2 wells in each row were left as blanks. All wells had a final volume of 100uL. The purified antibody samples to be quantified were prepared by first diluting the antibody down to approximately 50ng/mL. Approximations were done by calculating back from final concentrations obtained from previous antibody quantifications. 200 uL of the approximated solution of purified antibody were added to the next 2 wells in the same plate as the standards (C1 & D1). Like the standards, 1:2 serial dilutions were performed down to the 10^{th} well in 100uL of EBB. The last 2 wells in each row were left as blanks with 100uL of EBB. The plate was then covered with parafilm and incubated at 4C overnight. The next day an automatic plate washer was used to wash the coated plates 4 times in ELISA wash buffer. Afterwards, rat anti-mouse IgG2a HRP conjugated detection antibody was prepared at a 1:5000 dilution in EBB and added at 100ul/well. After addition of the detection antibody, the plate was incubated at 37C for at least 2 hours. The plate was then washed again 4 times using an automatic plate washer in ELISA wash buffer and developer was added. The developer contained 10ml/plate of room temperature citrate buffer, 10uL of 30% H_2O_2/plate, and (1) 5mg tablet of o-Phenylenediamine dihydrochloride/plate. Once the

developer was applied, the plate was incubated in the dark at room temperature for 20 minutes and then read immediately on SoftMAX plate reading software at 490nm. Comparing against the linear portion of the standard curve, optical density readings from the antibody samples were averaged to determine the final antibody concentration.

Table 3: ELISA Wash Buffer solution

3L of 10x ELISA Wash Buffer Solution:
876.6 grams NaCl **30.3 grams Na 2 HPO 4 (Sodium Phosphate)** **12.3 grams KH 2 PO 4 (Potassium Phosphate)** **2.5 Liters DI H 2**

Results

ANTIBODY PREPARATION

In order to obtain a quantity of antibody large enough for use in animal studies, it was first necessary to allow hybridoma cells to proliferate to large quantities in order to collect the supernatant they produced, containing the antibody of interest (along with other proteins). Cells were maintained and supernatant was collected as described in the methods section. Cells were maintained in serum free growth media prior to collection to ensure that there was no contamination with bovine IgG. Once a significant quantity of supernatant had been collected (~8L), the protein in the solution was precipitated using ammonium sulfate. The precipitated protein was collected, and dialyzed for 1 week as described in the methods to allow for protein refolding. The dialyzed protein solution was

then concentrated prior to total protein quantification. Total protein quantification was performed using a colorimetric protein quantification assay provided by Thermo Scientific Pierce (BCA Protein Assay Kit). Results and a flow chart of the steps involved in cell growth, supernatant collection and antibody purification is shown below.

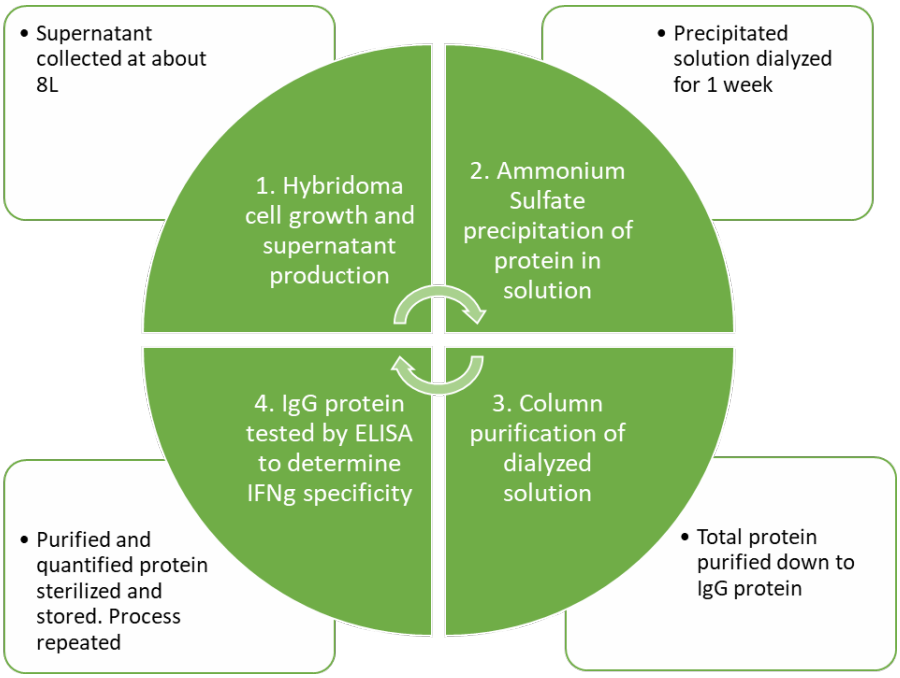
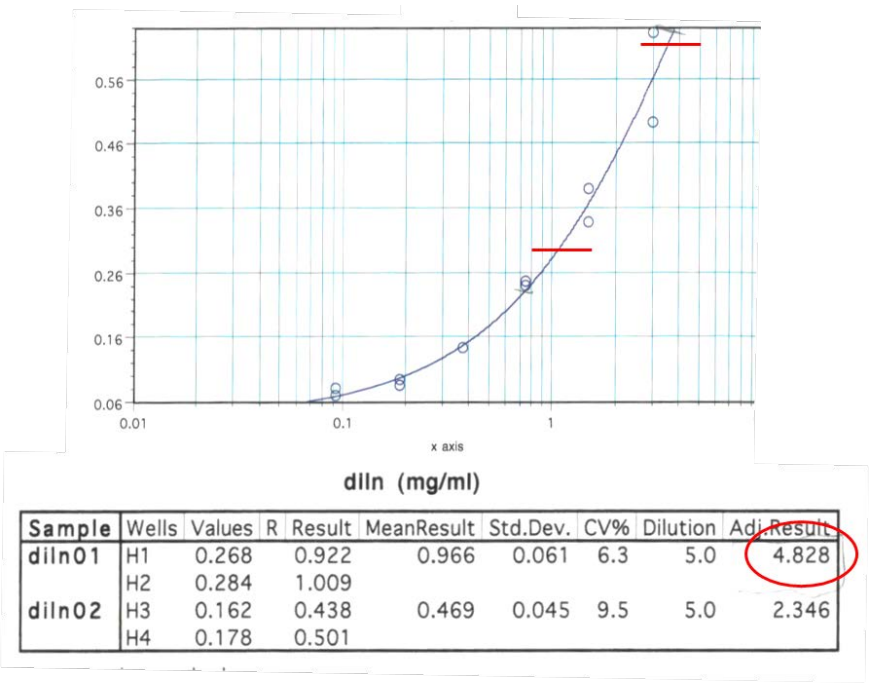


Illustration 3: Process of antibody growth and purification. This process was repeated until enough antibody had been collected in order to perform experiments. From each 8L collection of supernatant about 6-8mg of antibody were collected.

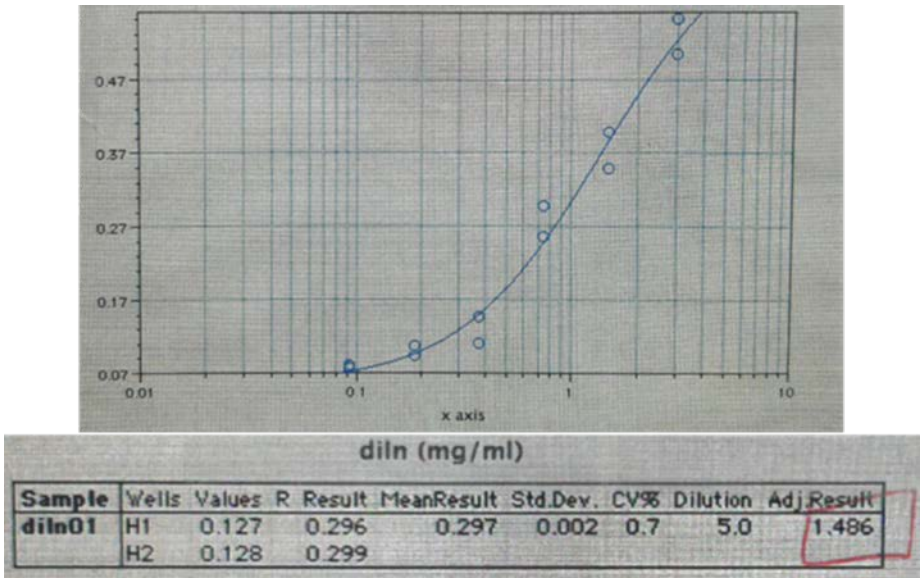


Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result
diln01	H1	0.268		0.922	0.966	0.061	6.3	5.0	4.828
	H2	0.284		1.009					
diln02	H3	0.162		0.438	0.469	0.045	9.5	5.0	2.346
	H4	0.178		0.501					

Figure 2: Total Protein Quantification: To obtain the concentration of all protein in the sample, the protein concentration values that fell within the linear portion of the standard curve graph (indicated by 2 horizontal red lines) were averaged and adjusted to account for dilutions. Example calculation: Standard curve is linear between .85 and 1.25 on the x axis (Mean Result). The "Adj. Results" of the antibody sample with mean results between .85 and 1.25 were averaged and used to represent the total protein concentration

ANTIBODY PURIFICATION

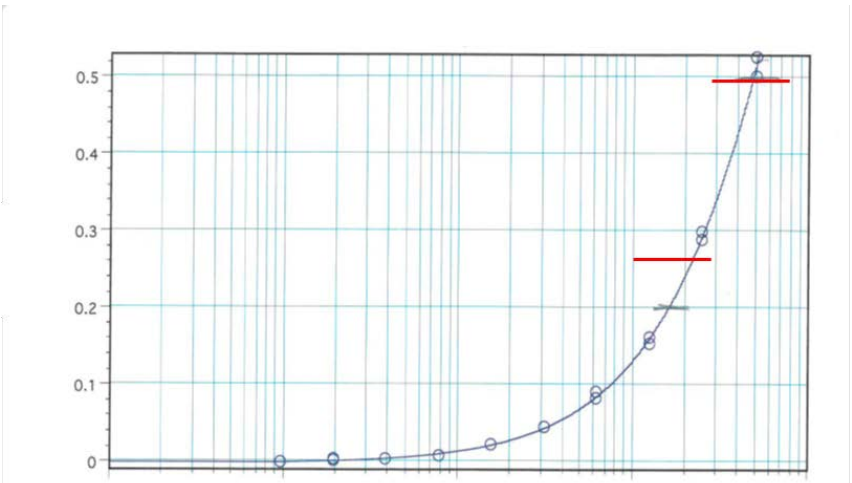
At the concentration used, the ammonium sulfate preferably precipitates out IgG protein, however other protein contaminants will also be present. To remove these unwanted proteins, post dialysis, the antibody solution was purified using IgG/A purification columns from Thermofisher Scientific and desalted according to the manufacturer's protocol. Once purified, the protein solution was assessed for total protein content again using the BCA Protein Assay Kit as described before. Theoretically the purified protein concentration should be much lower than the concentration before unwanted proteins are removed and represent the antibody with unrelated proteins removed. Results are shown below.



Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result
diln01	H1	0.127		0.296	0.297	0.002	0.7	5.0	1.486
	H2	0.128		0.299					

Figure 3 Purified IgG Quantification: IgG protein concentration was calculated in the same manner as total the total antibody quantification shown in Figure 2. The protein concentration values that fell within the linear portion of the standard curve graph were averaged (if there was more than 1 concentration that fell within the linear portion) and adjusted to account for dilutions. Here we see that the final concentration of protein after purification is 1.486mg/mL. A sample calculation of when there is more than one concentration that falls within the linear portion of the standard curve is shown to the right of the bottom of figure 4.

Afterwards, an antibody quantification ELISA was performed to confirm the concentration of the IFNγ specific antibody present in solution. Because our antibody of interest was type IgG2a, for the ELISA, goat anti-mouse IgG2a was used as the capture antibody and HRP conjugated rat anti-mouse IgG2a was used as the detection antibody. Results are shown below. Purified and quantified antibody was stored at 4C for future use. This process was repeated with protein and IFNγ-specific antibody quantification taking place for each batch of supernatant produced until the desired amount of antibody was obtained.



Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result
Pr01	E1	0.501		48.552	48.147	0.573	1.2	1.0	48.147
	F1	0.495		47.742					
Pr02	E2	0.259		21.741	21.805	0.090	0.4	2.0	43.609
	F2	0.260		21.868					
Pr03	E3	0.134		10.406	10.427	0.030	0.3	4.0	41.709
	F3	0.135		10.448					
Pr04	E4	0.062		4.590	4.693	0.146	3.1	8.0	37.547
	F4	0.065		4.797					
Pr05	E5	0.031		2.268	3.096	1.171	37.8	16.0	49.543
	F5	0.053		3.925					
Pr06	E6	0.013		1.005	1.288	0.400	31.1	32.0	41.223
	F6	0.021		1.571					
Pr07	E7	0.005		0.525	0.642	0.165	25.7	64.0	41.111
	F7	0.009		0.759					
Pr08	E8	0.001		0.280	0.323	0.061	19.0	128.0	41.401
	F8	0.003		0.367					
Pr09	E9	-0.001	R	0.112	0.252	0.198	78.6	256.0	64.502
	F9	0.003		0.392					
Pr10	E10	-0.002	R	0.077	0.270	0.272	100.8	512.0	137.985
	F10	0.004		0.462					

$\overline{X}$= 45.878ng/mL
Dilution = 1:29700
45.878ng/mL x 29700 = 1.363mg/mL

Figure 4 IFNγ specific antibody quantification: Antibody quantification ELISA was set up as described in the methods. Results were analyzed using the linear section of the standard curve as in total protein and IgG quantifications.

Finally, because the antibody would be used both as a capture and detection antibody, a "checkerboard" ELISPOT was performed in order to determine which concentrations yielded the best spot quality. The determined concentrations were used in future ELISPOT testing. Antibody used for detection was biotinylated in order to be detected later using streptavidin peroxidase.

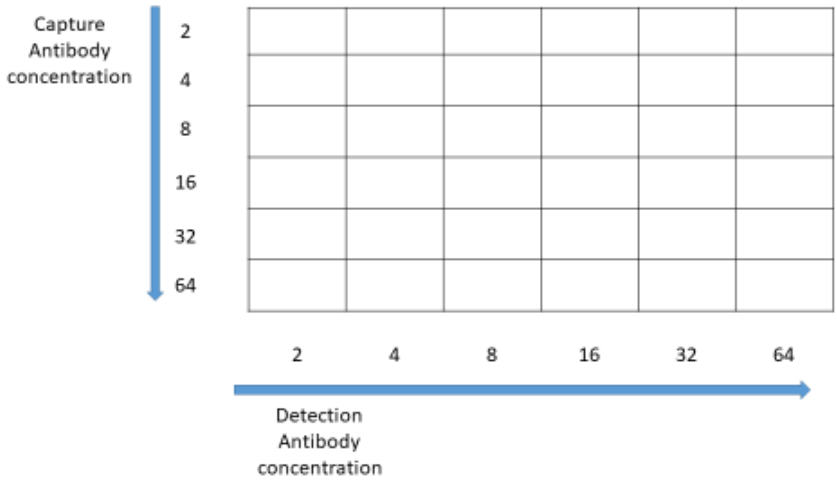


Illustration 4: Mock setup for a "checkerboard" ELISPOT

Discussion

Although the guinea pig is currently the best animal model for the study of immune responses to genital HSV-2 there is a paucity of reagents with which to conduct these studies. To address this need, we worked to produce usable quantities of guinea pig IFNγ specific antibodies for assessment of important cellular responses during HSV-2 disease. With this study we found that we were able to successfully grow up significant amounts of antibody from hybridoma cell lines and that we were able to purify the antibody to within 90%. This antibody can be sterilized for use *in vivo* in the guinea pig or used to quantify HSV-2 specific responses *in vitro* in immunized guinea pig tissues. Future studies will investigate the performance of purified antibodies in immune analysis assays such as the ELISPOT and flow cytometry.

CHAPTER 3: INTRACELLULAR CYTOKINE STAINING TO DETECT IFNγ RESPONSE IN HSV-2 INFECTED GUINEA PIG CELLS

Abstract

There is a current need in the field of HSV-2 research for immunological assays for the guinea pig (gp) model to facilitate the development of therapeutics and possible prophylactics for this disease. Here we outline an attempt to develop an intracellular cytokine staining (ICS) assay for use with flow cytometry (FC) analysis. The high throughput nature of FC allows for rapid detection and analysis of immune function during disease and treatments. We used gp IFNγ specific antibodies grown and purified in lab and investigated their ability to perform in such an assay. Guinea pig spleen cells were stimulated to activate the production of IFNγ and purified, fluorescently labeled antibody was used to detect intracellular IFNγ production. Antibody performance was compared against an isotype control to determine if significant quantification of IFNγ positive cells could be achieved. Unfortunately significant quantification was not attainable with this antibody even after several optimizing steps. Nor were we able to produce a viable assay using a different gp IFNγ specific antibody. Further investigations will need to be conducted to determine a suitable antibody or procedure for this assay.

Introduction

Because guinea pigs are the golden standard animal model for genital HSV-2, the lack of reagents and immunological assays for this model creates a significant need to be met in the field. Flow cytometry (FC) is a platform upon which several different, highly-specialized assays can be performed, and it provides the capability to perform many of these assays simultaneously. The ability to evaluate intracellular immune products, assess intracellular changes associated with activation, characterize apoptosis, and identify antigen-specific T cells makes FC a very useful tool for the characterization of immune function.[209] FC has been used to forward the field for many diseases in a variety of ways such as allowing for CD4 T-cell counts in large quantities in HIV infection studies, lineage assignment studies in leukemias and lymphomas, and assessing CD34 expression to identify stem cells for transplantation.[209] Flow cytometry allows for the identification and quantification of virus-specific leukocyte populations and subpopulations and the expanded range of monoclonal antibodies specific for lymphocyte surface antigens provides an extensive panel of reagents that facilitate complex studies. Intracellular cytokine staining (ICS) used along with FC would allow for the quantification of lymphocyte populations producing specific cytokines. With this in mind, an assay capable of utilizing flow cytometry to quantify HSV-2-specific IFNγ secreting lymphocytes in gp genital HSV-2 infection would be a valuable tool. Unfortunately, to date, reagents for such an assay do not exist for the guinea pig model. To meet this need we utilized antibodies specific for recombinant and native gp IFNγ to develop and optimize an ICS for high throughput analysis of activated immune cells in the context of HSV-2 genital infection. In previous experiments, a number of antibodies specific for recombinant and native gp IFNγ were developed and assessed for their affinity by

Western Blot and ELISA.[16] For these studies we utilized the two antibodies with the highest affinity for native IFNγ, designated antibody NG3.5 and VE4, in our attempt to develop an ICS assay.

Methods

GUINEA PIG TISSUE PROCESSING

Spleen: Guinea pig spleens were harvested and placed in a 50mL conical tube with 10mL of Hanks media (5% NBCS, 1% Pen/Strep). Spleens were then pushed through a 40 grade mesh screen to separate cells and the screen and dish were rinsed with media. The suspension was transferred to a 50mL conical and was allowed to sit at room temperature for about 5-10 minutes to allow cell debris to settle. Avoiding the cell debris, the cell suspension was transferred to a new 50mL conical and centrifuged at 1300rpm for 8 minutes at room temperature. The cells were then washed twice in 20mL of Hanks media and counted.

Lymph nodes/Bone Marrow: Lymph nodes or bone marrow were harvested and placed in a 50mL conical tube with 10mL of room temperature Hanks media. The tissue was then pushed through a 50 grade mesh screen and the screen and dish were rinsed with media. The suspension was then transferred back to the same 50mL conical and centrifuged at 1300rpm for 8 minutes at room temperature. Cells were then washed twice with 10mL of Hanks media, resuspended in 20mL of fresh Hanks media and counted.

INTRACELLULAR CYTOKINE STAINING

After the spleen cells had been washed and counted as described, $6x10^7$ cells were removed, centrifuged, and resuspended in 1mL Tcell media. 50uL of this cell suspension ($3x10^6$ cells) were added to 850uL of Tcell media in microcentrifuge tubes. Cells were then stimulated with PMA (phorbol 12-myristate-13-acetate) and ionomycin at 20ng/mL and 10 ng/mL respectively. Afterwards, cells were incubated at 37C for 2 hours before 10uL of a 1:10 dilution of Golgi Plug (Brefeldin A) was added to each tube. Cells were then incubated for 4 hours before being washed in 1mL of Hanks. The Fc receptors on the cells were blocked by resuspending them in 50uL of a 1:25 dilution of 24G2 antibody in FACS media (45mL RPMI + 5mL FBS + 200uL 25% Na Azide). The samples were incubated with the Fc block on ice in the dark for 20 minutes. After incubation, samples were centrifuged at 1000rpm for 4 minutes at 4C. Cells were resuspended in 100uL of a 1:20 dilution of FITC conjugated CD8 stain (isotype control: rat IgG1) and PE conjugated CD4 stain (isotype control: rat IgG2b) together in FACS media and incubated on ice in the dark for 30min. Afterwards cells were washed twice with 200ul/sample with FACS media and resuspended in 100uL of Cytofix/Cytoperm from BD Biosciences. At this point, the samples were incubated on ice for 20 minutes in the dark and then washed twice again with 200uL of 1X Perm Wash from BD Biosciences in order to permeabilize the membrane. Finally intracellular IFNγ was stained with 100uL of PE-Cy7 conjugated NG3.5 or VE4 IFNγ antibody (isotype control: rat IgG1) at either 1, 10, or 50ng/mL in 1X Perm Wash. VE4 was conjugated to the fluorochrome PE-CY7 using a fluorochrome conjugation kit purchased through Abcam and following the manufacturers provided protocol. Cells were incubated with the intracellular stain on ice in the dark for 30 minutes, washed twice with 200uL of 1X Perm Wash and then resuspended in 500uL of

PBS + 1% formaldehyde. Samples were stored at 4C or read via flow cytometry immediately. Data were acquired on the BD FACSCanto II (BD Biosciences) at the UTMB Flow cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

Table 4: T cell media recipe

T cell Media
ISCOVES media as base **10% FBS** **1% L-glutamine** **1% Pen-Strep** **1% Sodium Pyruvate** **1% Non-essential amino acids** **50uL/100mL 2-mercaptoethanol**

Results

ASSESSMENT OF NG3.5 DETECTION OF NATIVE IFNγ FROM STIMULATED GUINEA PIG IMMUNE CELLS

Once antibody had been purified (discussed in the previous chapter), our first goal was to assess its performance for detection of gp IFNγ in a variety of assays. Because Flow Cytometry (FC) has the benefit of being a high throughput assay, we assessed the antibody's performance in intracellular cytokine staining for this purpose. To do this, the antibody was first conjugated to the fluorochrome AMCA from Abcam. Cells were isolated from guinea pig spleens as described in the methods and stimulated with the global stimulants PMA and ionomycin to induce production of IFNγ. Cells were

stimulated for either 24 or 48 hours to determine which time frame gave the best results. Post stimulation, brefeldin A was used to block the egress of cytokines and fluorochrome-labeled antibodies were added to detect CD4 and CD8 T cells as well as live/dead cells. Cells were then permeabilized before fluorescently labeled NG3.5 antibody or isotype control was added at 3 separate concentrations to detect intracellular IFNγ production. Unfortunately there was no difference seen between the NG3.5 antibody and controls for intracellular detection of IFNγ for FC. Results are shown below.

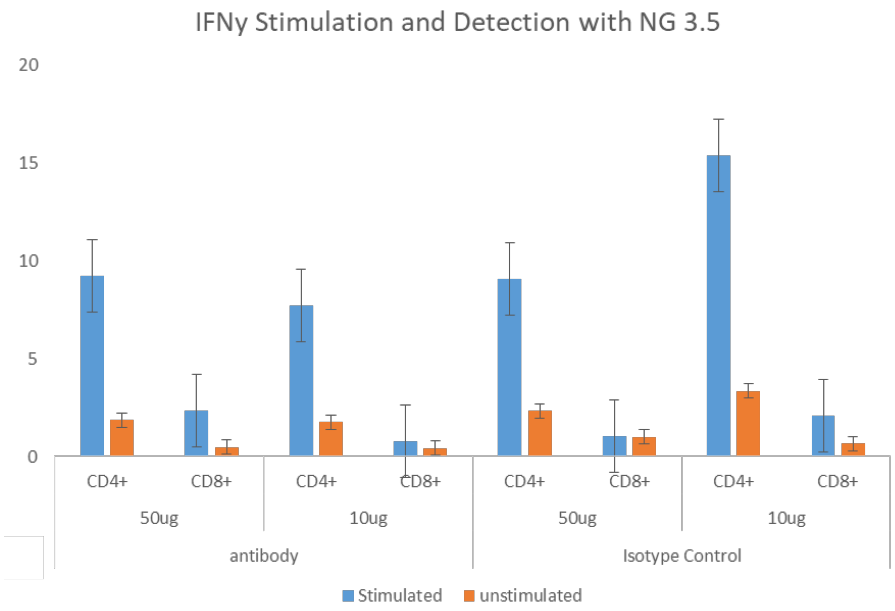


Figure 5: Results of IFNγ Stimulation and Detection with antibody NG3.5

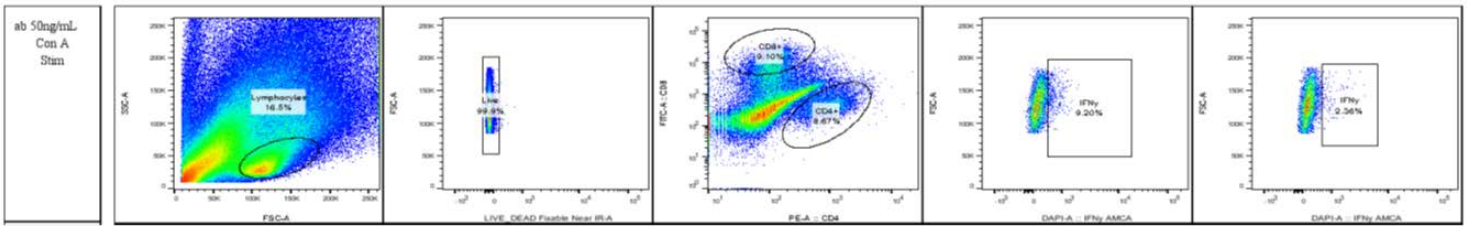


Figure 6: Sample gating methodology for selecting IFNγ positive cells

NG3.5 FC TROUBLESHOOTING

In order to determine whether the lack of signal was due to an error in the setup of the ICS FC, several parameters were adjusted and the antibody tested again. It was possible that the AMCA fluorochrome overlapped with our live/dead due to them both being a bluish stain so another fluorochrome, PeCy7, was purchased and used instead. Additionally, the T cell stimulant concavalin A (ConA) had shown, in our previous experiments, to effectively stimulate guinea pig T cells, so it was used instead of PMA and ionomycin to stimulate cells isolated form guinea pig tissues. Finally whether or not the permeability step in our process was effective was investigated by adding different colors of live/dead stain pre and post permeabilization. According to the dye's manufacturer, in cells with compromised membranes, the live/dead dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining. However, in viable cells, the dye's reactivity is restricted to the cell-surface amines, resulting in less intense fluorescence. The difference in intensity is typically greater than 50-fold between live and dead cells, allowing for easy discrimination.[213] Therefore, live cells pre-permeabilization should only have the live dead stain on the

surface. These cells after permeabilization should take up all of the second live dead stain (different color). However if the permeabilization step did not work, there would be significantly less fluorescence detected. Upon completion of this test, we saw that using the first stain it was easy to tell between live and dead stains given the distinct difference in intensity. Post permeabilization, all cells took up the second dye, meaning the permeabilization step indeed worked properly. Results are shown below. The ICS FC experiment was run again in the same way described before save the aforementioned parameters. Unfortunately, there was still no significant difference in the detection of intracellular IFNγ compared to controls (results not shown).

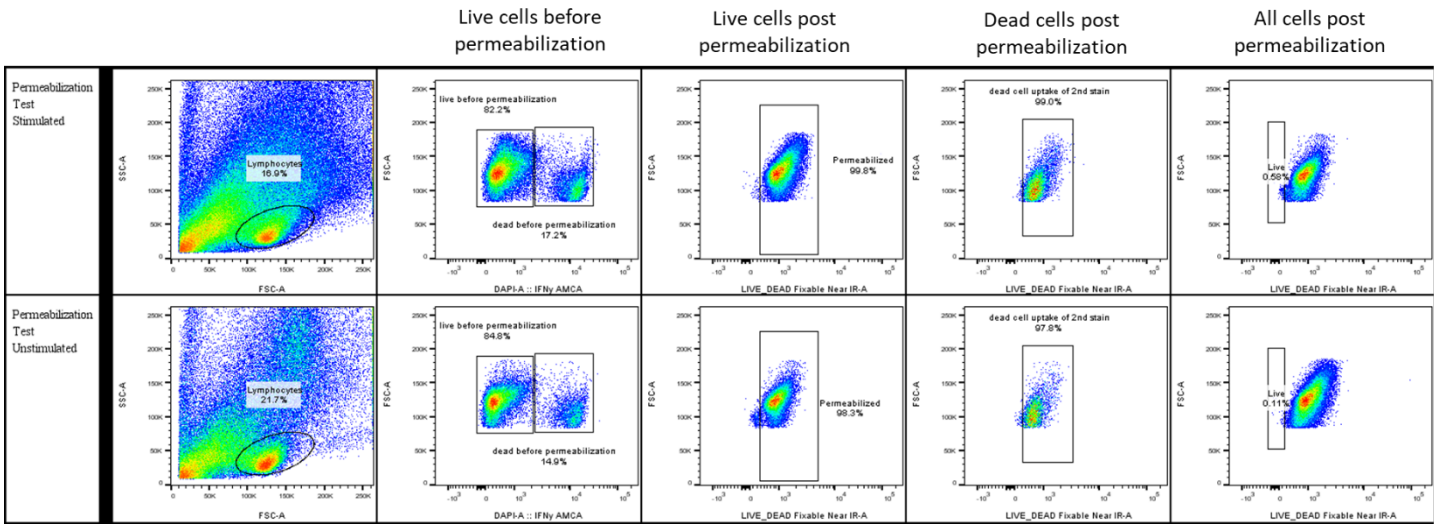


Figure 5: Test of permeabilization: The top row of panels represent stimulated cells and the bottom row of panels show unstimulated controls. First panel in each row shows gating for all lymphocytes. The second panels show live vs dead lymphocyte uptake of the first "live/dead' stain prior to the permeabilization step. The third panels show percent of second stain uptake in previously live cells after the permeabilization step. Last panels show uptake of second dye in all lymphocytes post the permeabilization step. Because we are able to see live cells in the first permeabilization step we know that our results were not due to dead cells. Because we see all cells (specifically the previously live cells) take up the second stain we know that the permeabilization step was effective.

ASSESSMENT OF VE4 DETECTION OF NATIVE IFNγ FROM STIMULATED GUINEA PIG IMMUNE CELLS

Because we were not able to see significant signaling of intracellular IFNγ with the NG3.5 antibody, we used another IFNγ-specific mAb that recognized a separate IFNγ epitope and was also shown to have high reactivity with guinea pig IFNγ, VE4. As before with the NG3.5 antibody, the VE4 purified antibody was conjugated to the PeCy7 fluorochrome. Guinea pig spleen cells were isolated and stimulated with Con A as before and were stained for extracellular T cell markers and intracellular IFNγ. Analysis by FC unfortunately still did not show a significant signal with the VE4 antibody when compared to isotype controls (results not shown).

INITIAL IFNγ SECRETING CELL RESPONSE IN HSV-2 INFECTED GUINEA PIGS TO HSV-2 GLYCOPROTEIN D STIMULATION

Although we were unable to utilize the NG3.5 IgG2b and VE4 IgG2b antibody to detect and quantify intracellular IFNγ in a high throughput flow cytometry assay we were able to use these antibodies to detect IFNγ specific immune responses to HSV-2 in infected guinea pig cells via ELISPOT in previous studies.[10] For future studies, we wanted to be able to use an optimized ELISPOT protocol to quantify memory responses to specific HSV-2 proteins in guinea pig immune cells. We therefore conducted a preliminary experiment to optimize quantification of gD-specific T cells. IFNγ responses by glycoprotein D stimulated cells from HSV-2 infected guinea pigs were measured by ELISPOT and compared to controls. Effector cells, which generate the immune response against infected target cells were isolated from spleen and antigen presenting cells were isolated from ingLN. Because guinea pigs are outbred, T cells and APC were isolated from the same animal. A diagram illustrating the relationship between effector and target cells is shown below.

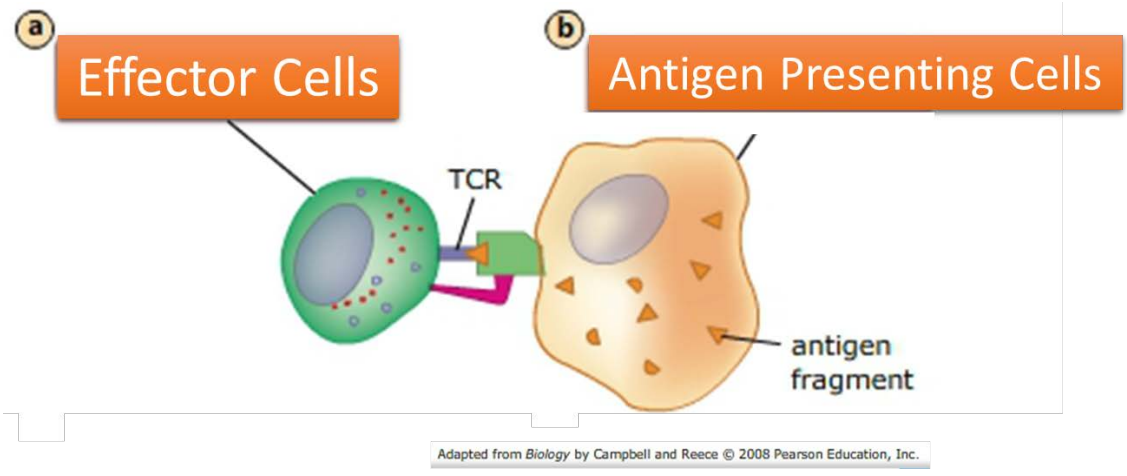


Illustration 6: Relationship between Effector and Target Cells

Spleen and inguinal lymph node (ingLN) cells were isolated from naïve and infected female Hartley guinea pigs. Effector cells were plated alone in media at 3 separate concentrations (1.0×10^6, 5.0×10^5, and 2.5×10^5 cells/well). APC were infected with HSV-2dl5-29 (a replication defective HSV-2 strain), or incubated with recombinant gD2 (rgD2) (.1, 1, or 10ug/mL), ovalbumin (.1, 1, or 10ug/mL), concavalin A (conA) (2ug/mL), or media before being added to the effector cells. IFNγ responses from effector cells exposed to HSV-2dl5-29 infected cells, UV inactivated HSV2, and ConA were used as a positive control and responses to ovalbumin and media were used as negative controls. Plates were then incubated at 37C for 24 or 48 hours. Afterwards plates were processed with detection antibody as described in the methods. Results shown below indicated that responses were most clear and had the lowest background noise with use of UV inactivated HSV-2 as the positive control. We also saw that the 1ug/ml concentration of gD and a shorter incubation time gave the clearest ELISPOT readings. From the data it was determined that we would move forward with these parameters for future studies.

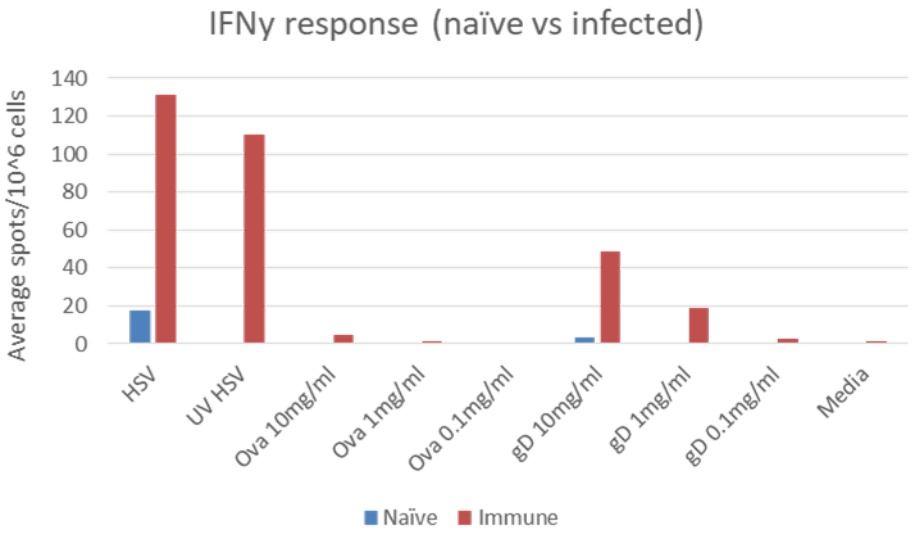


Figure 6: ELISPOT optimization using IFNγ responses. To determine which parameters produced the clearest spots for future ELISPOT testing, an experiment was performed testing the IFNγ response of cells from immune guinea pigs to restimulation with several different antigens. Responses were most clear and had the lowest background noise with use of UV inactivated HSV-2 as the positive control. We also saw that the 1ug/ml concentration of gD and a shorter incubation time gave the clearest ELISPOT readings. Though the HSV-2 infected cells and gD10mg/mL parameter gave higher readings, they also came with higher background. Incubation time here was 24 hours.

Additionally, these studies show we can quantify gD-specific T cells in immunized guinea pigs and lay the foundation for use in future studies to determine the effect of therapeutic immunizations with a gD-containing vaccine.

Discussion

Reagents for the guinea pig model are rare making it difficult to investigate specific immunological processes involved in HSV-2 genital infection in this model. Recently the Schafer lab was able to develop monoclonal antibodies able to detect gpIFNγ in culture by ELISA. However, an antibody for use high throughput immunological assays such as flow cytometry would be highly valuable for future genital

HSV-2 studies. Therefore, we tested the most reactive of the previously developed antibodies for their ability to stain intracellular IFNγ in stimulated guinea pig cells for detection by FC. Unfortunately the antibodies tested were not able to perform in these studies. Though the reason for this is not clear, there could be a number of possibilities. These antibodies have been shown reactive in ELISA and ELISPOT studies which measure extracellular cytokines. It is possible that the antibody-fluorochrome conjugation was too large to bypass the cellular membrane with ease which would hinder efficient staining. It is also possible that these particular antibodies, for some reason, were not able to bond properly to the fluorochrome, or fluorochrome binding affected their affinity which would lead to a lack of signal and results similar to the isotype control conjugated to the same molecule. Future research will continue to investigate the development of an FC ICS assay for detecting intracellular IFNγ, either by optimizing the current parameters or exploring different antibody/reagent options. Fortunately, however, we were able to use the antibodies to detect native IFNγ by ELISPOT and could use this assay to inform optimized parameters for future ELISPOT studies to quantify gp-specific HSV-2 immune responses described in future chapters.

CHAPTER 4: DETECTION OF GLYCOPROTEIN D SPECIFIC ANTIBODY PRODUCTION IN MALE AND FEMALE GUINEA PIG TISSUES

Abstract

Currently there is a lack of reagents and assays to study the guinea pig immune system in relation to HSV-2 genital disease. This is problematic because the guinea pig is the best model for genital HSV-2, and being able to characterize protective and therapeutic immune responses in this model could aid in the development of better treatments and vaccines for this disease. A prophylactic vaccine containing glycoprotein D showed efficacy in protecting guinea pigs from HSV-2 disease, however the mechanism is not clear. Understanding the immune mechanisms of protection could help us understand what immune mechanism to target in humans. Here we outline the optimization and use of an ELISPOT assay to investigate the memory immune response of infected guinea pig cells in response to stimulation with HSV-2 antigens. This was done in order to specifically develop a method to quantify vaccine-specific B cells responding to therapeutic vaccinations. Cells isolated from infected guinea pig spleen, inguinal lymph nodes, and bone marrow were plated in wells coated with either glycoprotein D alone or total HSV-2 protein. Results were compared between naïve and infected animals and in male and female animals. We saw no difference in gD- or total HSV-2 glycoprotein specific antibody response between male and female guinea pig. We also saw that the antibody secreting cell response was highest in the spleen and bone marrow. Interestingly, for each organ investigated, we saw no difference in the number of gD and

total HSV-2 protein-specific antibody secreting cells, suggesting that gD may be the dominant antigen.

Introduction

Herpes simplex virus 2 (HSV-2) causes persistent infection that cannot be eliminated completely from the body by the immune system.[1] The global burden of HSV-2 and the fact that it increases susceptibility to HIV-1,[3] legitimates the need to develop new prevention strategies, such as drugs and vaccines that are able to fight either primary HSV-2 infections or secondary reactivations. Moreover, the increasing number of HSVV-2 infected patients receiving transplants, and thus being placed on immunosuppressants, increases the number of severe HSV-2 infections in these immunocompromised individuals that are unresponsive to current therapies.[2] There is no approved prophylactic or therapeutic HSV-2 vaccine available. There is also a pressing need to understand what type of immunity protects in a vaccine setting (neutralizing antibodies, T cell response, etc.).

Immunization would be the most effective approach to control HSV-2, but prophylactic vaccines that elicit systemic immune responses against HSV-2 have failed in clinical trials.[13] Understanding how the immune system modulates HSV-2 shedding and how to properly manipulate this response with vaccines requires an animal model that accurately reflects the pathogenic events of HSV-2 as they occur in humans.[13] To date, the guinea pig is the golden standard model for HSV-2 genital infection because of its recapitulation of important disease characteristics seen in humans.

Preclinical studies have suggested that the presence of virus-specific T cells at the site of viral infection in the genital epithelia may be critical for effective protection. These cells are strategically located to protect against re-infection and to interfere with HSV-2 shedding in the genital tract thereby impacting HSV-2 transmission.[13] Because of this, it is imperative that we can detect these cells and their activation in order to understand their therapeutic effects during HSV-2 infection and reactivation. Although there are a number of commercialized assays and reagents to study the immune response in other animal models of HSV-2, such as mice, these models do not effectively recapitulate persistent infection or reactivation of latent virus normally seen in human infection.

In the studies outlined below we developed assays to detect the presence of gD specific antibody producing cells in the tissues of HSV-2 immune guinea pigs. Glycoprotein D is located on the surface of HSV-2 and is 1 of 4 glycoproteins required for host cell entry/infection (gD, gB, gH, gL). gD also plays a large roll in humoral and cellular immunity to the virus. For example, as mentioned in earlier sections, human anti-HSV-2 neutralizing IgG is predominantly against gD and gB. HSV-2 gD shares 98–99% amino acid identity among different HSV-2 strains and 82–88% amino acid identity with gD of different HSV-1 strains. gD is also a target of antibody-dependent cellular cytotoxicity, as well as of CD4+ and CD8+ T-cell responses.[200,201] Because of this it is important to be able to quantify the immune response generated from exposure to this protein, specifically in guinea pigs. [84,85]

Methods

VIRUS PRODUCTION

HSV-2 strain MS stocks were prepared on Vero cell monolayers and stored at − 80 °C as described previously.[8] The replication-defective HSV-2 strain, HSV-2 dl5-29, deleted of the HSV DNA replication protein genes UL5 and UL29, and the complementary cell line V529 expressing the UL5 and UL29 proteins[9] were a kind gift of Dr. David Knipe (Harvard Medical School, Boston, MA). Virus stocks were prepared as described previously by Xia et al.[10] and stored at − 80 °C.

GUINEA PIGS

Female and male Hartley guinea pigs were purchased from Charles River (Burlington, MA). Guinea pigs were maintained under specific pathogen free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal research center of the University of Texas Medical Branch. All animal research was humanely conducted and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch with oversight of staff veterinarians. Guinea pigs were infected by intravaginal (ivag) inoculation with 200 µl of a suspension containing 10^6 PFU of HSV-2 strain MS as described previously.[8]

ELISPOT ASSAY FOR IFNY DETECTION

The day before the ELISPOT assay was to be completed, ELISPOT plates were prepared by coating the inner 60 wells with 100uL of 15ug/mL V-E4 IFNγ capture antibody in ELISA coating buffer.[16] Plates were then covered with parafilm and incubated overnight at 4C. The next day, coated ELISPOT plates were washed 4 times with 200uL/well PBS and then blocked with 200uL/well 2.5% BSA/PBS and incubated

at 37C for at least 1 hour. Once the feeder and effector cells had been properly prepared (discussed in the Guinea Pig Tissue Processing section) they were added to the appropriate wells and incubated at 37C for 24 to 48h. At that time, the plates were washed using a vacuum aspirator to ensure thorough cell removal. The N-G3 IFNγ detection antibody in 2.5%BSA/PBS (12ug/mL, 100uL/well) was then added and the plates were covered with parafilm and incubated overnight at 4C. The next day, the plates were washed as described previously 4 times with ELISA wash buffer and developer was added. The plates were incubated in the dark at room temperature until they developed, washed with diH_2O and allowed to dry before the spots were counted. Spots were counted by ELISPOT reader and confirmed by manual counts under dissecting microscope.

Table 5 ELISPOT Developer Solution recipe

ELISPOT Developer solution
14mL 0.1 Na Acetate **1mL AEC (0.2g aminoethylcarbamazole in 50mL dimethylformamide)** ****Push solution through 30 μm filter**** **10uL 30% H_2O_2**

ELISPOT ASSAY FOR HSV-2 ANTIGEN SPECIFIC ANTIBODY PRODUCTION DETECTION

The day before the ELISPOT assay was to be completed, ELISPOT plates were prepared by coating the inner 60 wells 100uL of either HSV-2 a 1:50 dilution of total glycoprotein or 2ug/mL of gD in ELISA coating buffer. Plates were then covered with parafilm and incubated overnight at 4C. The next day, coated ELISPOT plates were

washed 4 times with 200uL/well PBS and then blocked with 200uL/well 2.5% BSA/PBS and incubated at 37C for at least 1 hour. Once the feeder and effector cells had been properly prepared they were added to the appropriate wells and incubated at 37C for 18h. At that time, the plates were washed using a vacuum aspirator to ensure thorough cell removal. Goat anti-guinea pig IgG was then added to wells at a 1:500 dilution and plates were incubated overnight at 4C. The next day, plates were washed and HRP conjugated rabbit anti-goat IgG was added to wells at a 1:2000 dilution. Plates were again incubated overnight at 4C. Plates were then washed 4 times with ELISA wash buffer and developer was added. The plates were incubated in the dark at room temperature until they developed, washed with diH_2O and allowed to dry before the spots were counted. Spots were counted by ELISPOT reader and confirmed by manual counts under dissecting microscope.

GUINEA PIG TISSUE PROCESSING

Spleen: Guinea pig spleens were harvested and placed in a 50mL conical tube with 10mL of Hanks media (5% NBCS, 1% Pen/Strep). Spleens were then pushed through a 40 grade mesh screen to separate cells and the screen and dish were rinsed with media. The suspension was transferred to a 50mL conical and was allowed to sit at room temperature for about 5-10 minutes to allow cell debris to settle. Avoiding the cell debris, the cell suspension was transferred to a new 50mL conical and centrifuged at 1300rpm for 8 minutes at room temperature. The cells were then washed twice in 20mL of Hanks media and counted.

Lymph nodes/Bone Marrow: Lymph nodes or bone marrow were harvested and placed in a 50mL conical tube with 10mL of room temperature Hanks media. The tissue

was then pushed through a 50 grade mesh screen and the screen and dish were rinsed with media. The suspension was then transferred back to the same 50mL conical and centrifuged at 1300rpm for 8 minutes at room temperature. Cells were then washed twice with 10mL of Hanks media, resuspended in 20mL of fresh Hanks media and counted.

HSV-2DL5-29 INFECTED FEEDER CELL PREPARATION

The appropriate number of cells were collected from inguinal lymph nodes of guinea pigs for each target. Because guinea pigs were outbred, target cells were not pooled and were kept separate for each sample. These cells were centrifuged and resuspended in 0.5mL of T-cell media. 100uL of HSV d15-29 or media was added to the cells and they were incubated at 37C for one hour. The cells were then washed twice in T-cell media (1300rpm for 8min at room temperature) and resuspended in 10mL of T-cell media. The cells were then counted and resuspended at the appropriate concentration in T-cell media. Targets were added to wells at 100uL/well.

Results

MALE VS FEMALE HSV-2 GD AND TOTAL GLYCOPROTEIN RESPONSE IN IMMUNIZED GUINEA PIGS

The NIH has expressed the need for experiments designed to determine the efficacy of vaccines and mechanism of immune protection against HSV-2 in both genders. To investigate the potential differences between the female and male immune response to HSV-2 we measured HSV-2-specific antibody production in infected guinea

pig cells. We also wanted to determine any difference in immune response in different immune compartments of the guinea pig; specifically the spleen, inguinal lymph nodes (ingLN), and bone marrow (BM). Cells were isolated from previously infected male and female guinea pig tissues and added to the inner 60 wells of plates that had been previously coated with either recombinant glycoprotein D or total HSV-2 glycoprotein. Cells from each tissue (spleen, ingLN, BM) were plated over both antigens at concentrations of 1.0 x 10^6, 5.0 x 10^5, and 2.5 x 10^5 cells/well and incubated for 18 hours. Sample plate setup and readout is shown in the figure below.

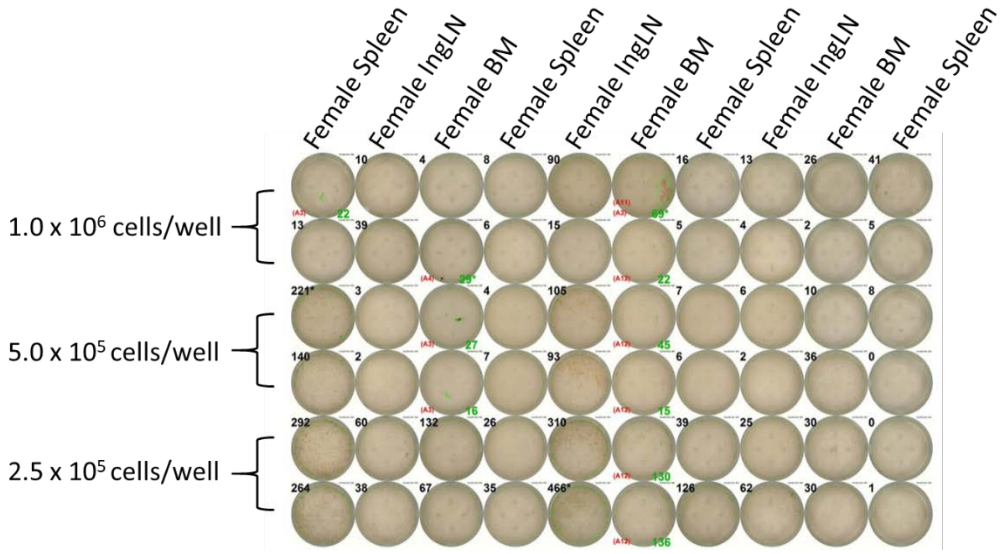


Figure 7: Sample layout for an ELISPOT plate. Plates would have been coated the day before with either total HSV-2 protein or gD. Figure also shows sample automated counting

After incubation, cells were washed and processed using goat anti-guinea pig IgG and HRP conjugated rabbit anti-goat IgG in a 2 step detection. Simultaneously during these experiments, the IFNγ secreting cell response was also measured in these animals using the NG3.5 antibody by ELISPOT (not shown).

The results of our studies, shown below, suggest that there is no difference between the male and female ASC response to stimulation by either rgD2 or total HSV-2 glycoprotein in the spleen, inguinal lymph nodes, or bone marrow of infected animals. ASC responses were similar in magnitude in guinea pig tissues of each gender and differed significantly only with that of naïve controls.

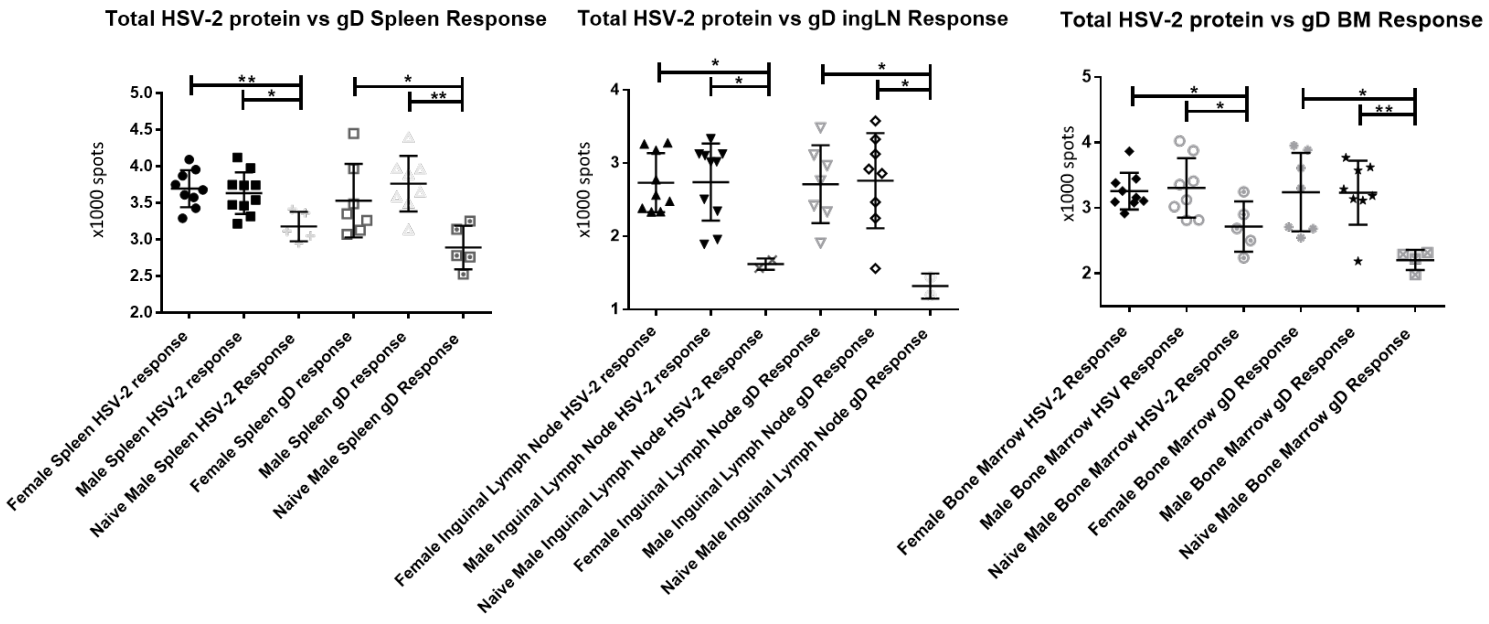


*Figure 8: Male and Female Antibody Secreting Cell Response to total HSV-2 protein and gD in the Spleen ingLN and BM. Cells were isolated from the described tissues and stimulated with either gD or total HSV-2 protein as described in the methods. Plates were incubated and spots were developed and counted. Results show no significant difference between female and male antibody secreting response to the antigens used in the tissues investigated. However we do see significant differences in stimulated vs naïve cells. Analysis was performed using ANOVA. * = p < .05, ** = p<.005*

We saw the highest antibody secreting cell (ASC) response to HSV-2 total glycoprotein in the spleen and BM with weaker antibody secretion responses in the ingLN. ASC responses to gD followed a similar pattern with highest responses in the spleen. Though the average antibody response to gD was slightly higher in the BM, it was not statistically different from the ingLN. Results are shown below. Because we saw no difference between the male and female ASC response, the following figures only show the female response for simplicity.

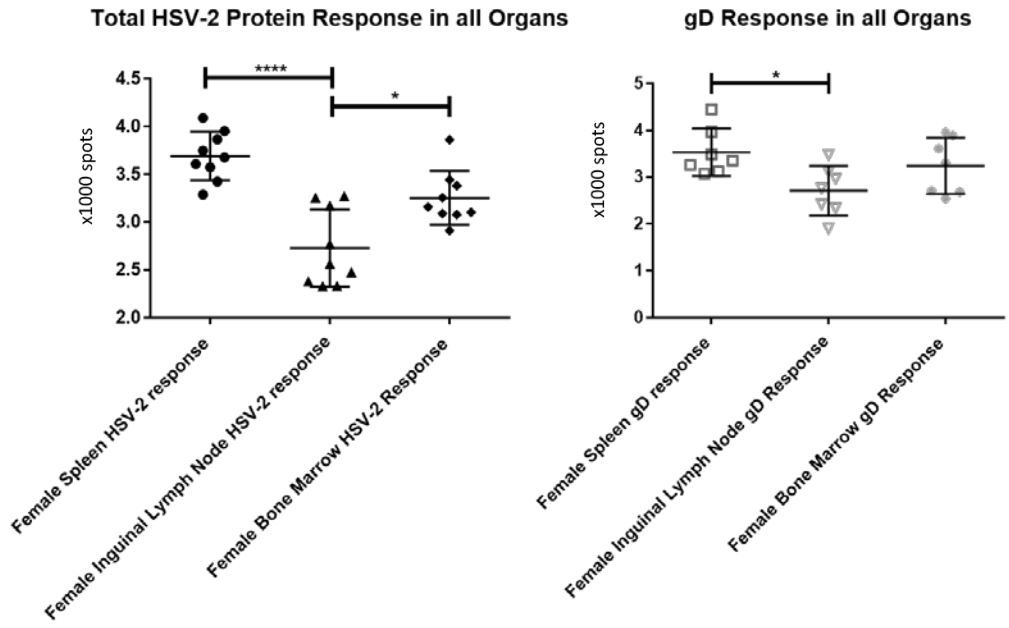


Figure 9: Organ specific differences in magnitude of ASC response to HSV-2 antigen stimulation. Comparisons were made to determine if differences in response to HSV-2 antigens existed between the organs examined. We saw that the antigen secretion response was highest in the spleen to both gD alone and total HSV-2 protein. This difference most likely occurred due to the fact nearly 50x more cells were recovered from the spleen than from the lymph node or bone marrow

Interestingly, we also saw that ASC responses to rgD2 were not statistically different from ASC responses to total HSV-2 protein in each tissue respectively. Using this method of detection, it seems as though the majority of the response is directed toward gD. Alternatively, the results seen here may be due to the fact that the total glycoprotein antigen only captures a portion of the total ASC response due to limiting amounts of each glycoprotein in the mixture.

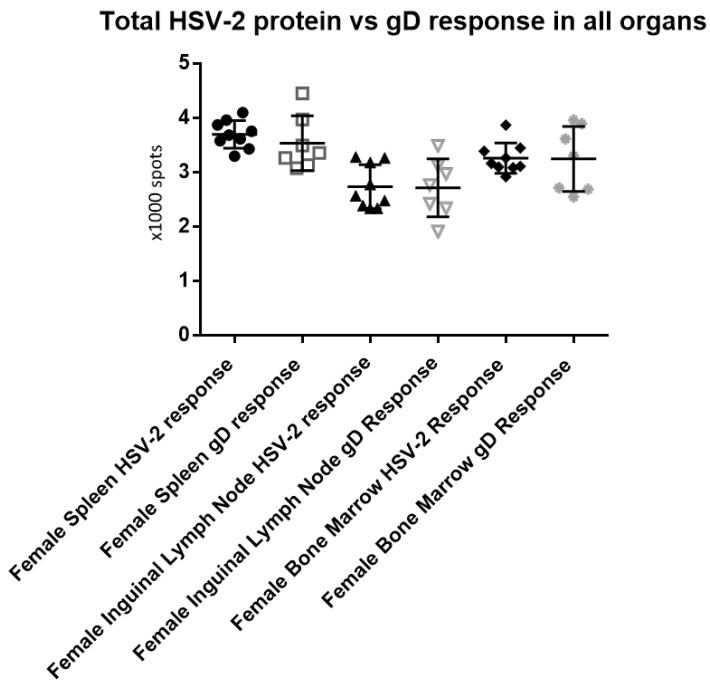


Figure 10: Comparison of total HSV-2 protein and gD specific ASC response magnitude in each tissue. ***Note****: comparisons are only being made within tissues, i.e., between the 1st, 2nd, and 3rd pair. There were no significant responses between the total HSV-2 protein response and gD response within tissues.*

Discussion

Many HSV-2 vaccine approaches utilize gD to elicit a protective immune response against the pathogen. Moreover a gD subunit vaccine was shown to protect guinea pigs from HSV-2 infection, though it failed in human clinical trials. However, though we have a vaccine that protects guinea pigs from HSV-2 infection, the mechanism of protection is not fully understood. Uncovering the specific protective immune mechanisms elicited by this vaccine would be quite useful to apply to the development of an effective human vaccine or better therapeutics. Glycoprotein D is abundant on the surface of the virus, necessary for entry, and a main target for the immune response. Quantifying and understanding the role of gD specific memory cells is paramount to our

understanding of disease pathology and resolution and can also help us better understand the mechanisms associated with the protection seen with gD vaccination in guinea pigs.

The experiments in this study outlined the development of an ELISPOT for the quantification of the HSV-2-specific antibody secreting cell response to rgD2 and total HSV-2 glycoprotein. They also explored potential immune differences between male and female animals. Our results suggest that there is no significant difference between male and female ASC response in the spleen, ingLN, or BM post stimulation with HSV-2 antigens. This has important indications for the future development of any therapeutics that will be administered to males and females. We also saw that there was a greater antibody response in the spleen and BM than in the ingLN. This pinpoints where a majority of HSV-2 specific immune cells are located in the body after infection and from where we should focus on homing cells in the case of a therapeutic vaccine. Interestingly, we also found that the antibody response was similar in magnitude to rgD2 alone as it was to total HSV-2 protein. Being that gD is only 1 of 11 surface glycoproteins on HSV-2, one would assume that a smaller fraction of cells would be specific for gD thereby producing a relatively smaller antibody response. Even if there were an equal number of isolated cells specific for gD, the concentration of gD in the total HSV-2 protein mixture is smaller than the gD solution and may therefore be limiting for capturing the entire ASC response. Essentially, using total HSV-2 glycoprotein in this ELISPOT method of ASC detection could potentially be less efficient at obtaining the entire scope of the ASC response against all HSV-2 glycoproteins due to lower spot quality or lower affinity of certain glycoprotein specific antibodies. Given these findings it would be interesting to further characterize the specificities of the antibodies generated during these responses,

particularly in the total HSV-2 protein response, and see which antibodies provide the best clearance, protection against shedding, etc. Our results suggest this could best be accomplished using recombinant glycoprotein as the capture reagent.

CHAPTER 5: GENERAL CONCLUSIONS

HSV-2 remains a pervasive pathogen worldwide and has been around for centuries, however despite its prevalence, efforts thus far have failed to provide an effective vaccine. Incremental advances in our knowledge of how natural immune control of herpes simplex virus (HSV) develops have yielded insight as to why previous vaccine attempts have only been partially successful, however, our understanding of these pathways, is still incomplete.[19] Further elucidation of the innate immune events that are responsible for stimulating these effector responses is required to accurately inform vaccine design.[19] Many attempts at vaccine development for HSV-2 genital disease have relied on the generation of neutralizing antibodies as the main correlate of protection, however, that approach has not been successful. It may be necessary to better understand the cellular mechanisms of protection. Currently, there exists a prophylactic vaccine able to protect guinea pigs against HSV-2 genital disease. Prophylactic immunization with Vaxfectin(®)-gD2/UL46/UL47 significantly reduces viral replication in the genital tract, provides complete protection against both primary and recurrent genital skin disease following intravaginal HSV-2 challenge, and significantly reduces latent HSV-2 DNA in the dorsal root ganglia when compared to controls.[211] In clinical trials however, a monovalent (gD) vaccine and a bivalent (gD + UL46) vaccine, each formulated with Vaxfectin® adjuvant was not able to provide the same protection. This could be due to a number of reasons including the subtraction of the UL47 antigen in the clinical trial vaccine. Regardless, it seems as though it is becoming more important to understand how vaccines work and then apply those immune mechanisms to what happens in humans during the same pathological processes. It is possible that mimicking the cellular

processes that protect guinea pigs and applying those same processes in humans could provide better prophylactic and therapeutic protection.

In order to fully understand the immune processes involved in a disease, it is necessary to have the proper animal model. Guinea pigs have been shown to be the best model for genital HSV-2 disease. They recapitulate the course of disease as it occurs in humans without genetic, hormonal or any other modification, unlike other models. Because of this, guinea pigs represent an excellent resource to better understand the immune mechanisms that take place during vital intervention points of HSV-2 genital disease such as initial infection, latency establishment, reactivation, and clearance. Unfortunately, reagents and immunological assays to complete these sorts of studies are almost non-existent in this animal model. This is a great misfortune, since being able to understand the mechanisms of protective immune responses to HSV-2 is vital for the development of therapeutics for this disease.

Currently we know that CD4 T cells are vital for protection against genital HSV-2 disease. Broad and persistent mucosal HSV-2 specific CD4 T-cell responses exist in the genital tract of HSV-2+ women suggesting these cells are resident at the site of infection and may play a role in clearance.[79] Also, in recurrent HSV-2 genital lesions, CD4 T cells are among the first cells to infiltrate the lesion to fight and clear the virus. Studies have also shown that CD4+ T cells are critical for the clearance of reactivated HSV-2 in the ganglia and spinal cord of infected animals.[20] Upon further investigation of the clearance mechanism associated with these cells, it was shown that without IFNγ, activated T cells did not display the same protective functions.[21]

Recently, the Shafer lab developed antibodies to detect the production of IFNγ in the guinea pig model. We aimed to use these antibodies as reagents to develop assays to investigate therapeutic immune responses in the context of genital HSV-2 disease in guinea pigs. We were able to demonstrate large scale production and purification of these antibodies for future use in detection assays for analyzing HSV-2 specific immune responses in the guinea pig model. Using these antibodies we investigated the development of 2 important assays to better understand protective immune responses to HSV-2.

The first was the development of an intracellular cytokine staining (ICS) assay to use with flow cytometry to better understand functional immunity in the context of this disease. Flow cytometry (FC) is an important tool because of its usefulness in high throughput cellular analysis and ability to perform many complex studies at once. Using FC would allow for the analysis of many important immune mechanisms that have not yet been studied in the gp model. Understanding these mechanisms can help us better understand and reinforce protective immune responses against genital HSV-2 disease. Unfortunately, thus far in our studies we were unable to produce a working ICS FC assay with our IFNγ antibodies, VE-4 and NG3.5. As mentioned earlier, these antibodies have been verified to detect gp IFNγ in a variety of assays including ELISA and ELISPOT so it is possible that fluorochrome conjugation affected their specificity. Future studies will need to investigate the mechanics of conjugation and possibly find a better suited fluorochrome for these studies. Whether or not a different gp IFNγ specific antibody would be able to perform in FC ICS should also be investigated.

The second assay we developed to better understand protective immune responses to HSV-2 was a modified ELISPOT experiment to determine the antibody secreting response to stimulation of immunized gp cells with either glycoprotein D (gD) or total HSV-2 protein. Cells from the spleen, inguinal lymph nodes (ingLN), and bone marrow (BM) from previously infected guinea pigs were isolated and plated in 96 well plates that had been coated with either the HSV-2 total protein or gD alone. We were able to show that the antibody response was strongest in the spleen and BM. Interestingly we also saw that the antibody response to total HSV-2 protein and gD alone were of similar magnitude. This was surprising since because gD only makes up a portion of the surface glycoproteins, the antibody response to gD should be just a portion of the antibody response to all the glycoproteins. That being said, studies have shown gD to be the main target of neutralizing antibodies,[47] and this could be what we are seeing here. This would be in line with previous researchers who have suggested that the immune response against gD masks potential protective immune responses against HSV-2.[104] Further studies should investigate the antibody specificities from these reactivation responses, if possible, to verify whether immunized gp humoral response to total HSV-2 protein is mainly targeted at gD. It is possible to perform the same experiment and use an HSV-2 glycoprotein mix excluding gD to determine whether the antibody responses are mostly gD specific or consist of other glycoprotein specificities. It could be possible that the HSV-2 virus as a whole prevents protective immune responses upon infection by stimulating the production of non-protective antibodies in response to infection.

The characterization of the antibodies described earlier in this work as well as future characterization and use of other assay reagents will further our ability to

understand which immune functions are most important during the body's fight against HSV-2. This is critical for the development of efficient vaccines and can provide insight into how to efficiently modulate immune functions in order to either halt disease establishment or prevent its reactivation from latency. With the development of the proper reagents and assays able to measure and modify immune responses, we will be able to better understand and quantify the protective mechanisms taking place during infection. Our results so far show that it is possible to use this reverse engineered antibody to detect native IFNγ responses in HSV-2 infected guinea pigs in ELISPOT assays. We were also able to detect HSV-2 specific antibody responses in immunized animals in various anatomical locations.

In the future, the role of CD4 and CD8 T cells in therapeutic and prophylactic settings should be investigated. Although, currently, knock out guinea pig models are not available, we do have antibodies able to deplete CD4 and CD8 T cells. Using these antibodies, we can investigate the role of CD4 and CD8 depletion on humoral and other protective responses after infection. In order to develop a proper vaccine for HSV-2, we need to know what timing schedule, route of vaccination, and which adjuvants best bolster the immune response for protection against infection or reactivation against HSV-2.

The current consensus in the field is that a therapeutic genital HSV-2 vaccine will need to increase the number of protective cell populations in virus specific areas such as the genital epithelium. Using the assays developed in these studies, we are now able to measure where protective immune responses (ASCs, IFNγ producing lymphocytes, etc.) are being generated post vaccine administration. We can also investigate the efficacy of

various adjuvants by measuring HSV-2-specific T cell proliferation after administration. Furthermore, cytokine production by these cells can be assessed via PCR.

Future studies should also use the assays developed here to further understand the protective immune responses produced with the Vaxfectin vaccine which has proven to be an effective prophylactic in guinea pigs. As stated earlier, because we know that this vaccine is effective in guinea pigs, we can understand which immune responses elicited by this vaccine are necessary for protection. For example, post vaccine administration we can determine in which tissues the ASC response is increased (spleen, serum, epithelium, etc.). We can also measure the increase in the amount of virus specific T cells generated in response to vaccination and also characterize the protective responses generated by these cells (e.g. PCR to detect cytokines). These kinds of studies will further our knowledge on what is necessary to confer protection against genital HSV-2 disease and move the field closer to the development of an effective vaccine.

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