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Development and Utilization of Novel Immunologic Reagents for Infectious Disease Studies in the Guinea Pig

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Development and Utilization of Novel Immunologic Reagents for Infectious Disease Studies in the Guinea Pig

by

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Dedication

This work is dedicated to my mother, Patty Jo Veselenak, who I miss every day and to my wife, Sonja, who keeps me honest.

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Development and Utilization of Novel Immunologic Reagents for Infectious Disease Studies in the Guinea Pig

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Genital herpes disease caused by HSV-2 infections is a worldwide public health epidemic with a global prevalence estimated at 500 million. These individuals serve as a reservoir for transmission of the virus to naïve individuals resulting in a global increase of 26 million new infections per year. Complications associated with first-episode infections acquired from horizontal transmission can include aseptic meningitis and psychosocial morbidity and neonates can be infected by virus shed into the vagina during birth leading to the development of severe sequelae or even death. Infection with HSV-2 also increases the susceptibility to HIV-1 infection more than five-fold. Antiviral chemoprophylaxis exists capable of reducing or abrogating clinical signs of primary or recurrent disease outbreaks, however long-term antiviral suppression does not clear the latent infection and cannot completely prevent transmission to the uninfected. There is currently no licensed vaccine to prevent HSV-2 infection and failures in recent clinical trials highlight the need for improved vaccine formulations containing multiple antigenic targets in order to stimulate a robust humoral and cellular immune response.

The most widely used animal model for the study of genital herpes is the mouse, however studies in this animal are limited to acute infection only. The guinea pig, a more

robust model that closely mimics human disease, can be used to study both primary and recurrent disease, however this animal model is limited due to the lack of immune reagents for this species. Development of new reagents to elucidate the immune response to infection and disease in the guinea pig would therefore benefit the field of genital herpes and other diseases for which the guinea pig is the better animal model.

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AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACV	acyclovir
APC	antigen-presenting cell
BME	β-mercaptoethanol
bp	base pairs
C_q	quantification cycle
DC	dendritic cell
DISC	disabled infectious single cycle
dsDNA	double-stranded DNA
EB	elementary body
EBV	Epstein-Barr virus
FCV	famciclovir
FCV	fold change
g	glycoprotein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GE	genome equivalents
GO	Gene Ontology
gpArray	guinea pig PCR array
GPIC	guinea pig inclusion conjunctivitis
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HIV-1	human immunodeficiency virus type 1
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSV	Herpes simplex virus
IACUC	Institutional Animal Care and Use Committee
IFN	interferon
Ig	immunoglobulin
IN	intranasally
ISRE	interferon stimulated response element
IVAG	intravaginally
LGT	lower genital tract
MG	*Mycoplasma genitalium*
MPL	3-O-deacylated monophosphoryl lipid A
NGS	next-generation sequencing
NHANES	National Health and Nutrition Examination Surveys
NK	natural killer cell
pDC	plasmacytoid dendritic cell
pDNA	Plasmid DNA
pfu	plaque forming units
PID	pelvic inflammatory disease
PMA	phorbol 12-myristate 13-acetate
qPCR	quantitative PCR

RB	reticulate body
RT-PCR	reverse transcription PCR
STI	sexually transmitted infections
TE	Tris-EDTA
TLR	toll-like receptor
T_m	melt temperature
UGT	upper genital tract
UTMB	University of Texas Medical Branch
VACV	valacyclovir
VP	viral protein
VZV	varicella-zoster virus

CHAPTER I

Herpes Simplex Virus Type 2 and Genital Herpes

TAXONOMY AND STRUCTURE

Eight viruses belonging to the family *Herpesviridae* have been identified that are adapted to humans as their primary host: Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and *Human herpesviruses* 6, 7, and 8 (HHV-6, HHV-7, HHV-8) [1]. These viruses share four common biological properties [1]:

1. The viral genome encodes a large array of enzymes for nucleic acid metabolism, DNA synthesis and protein processing.
2. Viral DNA synthesis and capsid assembly is restricted to the nucleus (although final virion processing/assembly occurs in the cytoplasm).
3. Production of infectious progeny virus results in the destruction of the infected host cell.
4. The ability to remain latent in their respective hosts.

The eight viruses have been further divided into three subfamilies based on their biological properties: the neurotropic *Alphaherpesvirinae* (HSV-1, HSV-2, and VSV), the lymphotropic *Betaherpesvirinae* (HCMV, HHV-6 and HHV-7), and *Gammaherpesvirinae* (EBV and HHV-8) [1].

HSV-2 is one of the most common sexually transmitted infections worldwide and represents a major public health concern [2, 3]. The majority of the dissertation work described herein will focus on this *Alphaherpesvirus*, and so the remainder of this chapter will focus on describing HSV-2 and its viral-host interactions.

The HSV virion is comprised of four structural elements: the core; the capsid; the tegument; and an outer envelope [4].

The core is comprised of a double-stranded DNA (dsDNA) genome that is approximately 152 kilo base pair (kbp) in length and contains a guanine and cytosine content of approximately 70% [5]. The genome may be viewed as two regions designated as Long (L) and Short (S) segments, that are flanked on each end by a pair of inverted repeat elements (**Figure 1.1**) [5, 6]. Repeat elements for the L segment are designated *ab* and *a'b'*; S segment repeats are designated as *a'c'* and *ca* [4]. The unique (U) segments, designated U_L and U_S (**Figure 1.1**) after their respective components, contain a total of 74 genes that encode distinct proteins and these genes correspond closely to the gene set for HSV-1 [5].

Figure 1.1. Structural organization of the HSV-2 genome.
The two covalently linked regions of the HSV-2 genome are denoted as L (long) and Short (S). Each region is bounded by a pair of inverted repeats that are denoted *ab* and *a'b'* for L and *a'c'* and *ca* for S. The unique sequences, denoted UL and US, code for the viral proteins. Adapted from: Roizman, B., D.M. Knipe, and R.J. Whitley, *Herpes Simplex Viruses*, in *Fields Virology*, B.N. Fields, D.M. Knipe, and P.M. Howley, Editors. 2007, Wolters Kluwer Health/Lippincott Williams & Wilkins: Philadelphia. p. 2502-2601. Reproduced with permission.

The viral capsid surrounds the core and is arranged in a T=16 icosahedral symmetry. It is contains 162 capsomers that form an outer and intermediate layer each composed of the viral proteins (VP) VP5, VP26, VP23, and VP19C [4].

The tegument fills the space between the undersurface of the lipid envelope and the capsid surface. It is an unstructured, proteinaceous layer composed of at least 20 viral proteins [4, 7]. These proteins are released into the host cell upon virion entry and mediate a number of essential functions including immune evasion, regulation of host and virion protein expression, and assembly of virions during egress from the host cell [7]. A number

of these tegument proteins have also been found to contain important T-cell epitopes that may have potential for use in vaccine development [8, 9].

The viral envelope is an outer lipid bilayer with approximately 11 different viral glycoproteins (g) embedded in its structure. These glycoproteins are designated gB, gC, gD, gE, gG, gH, gI, gL, and gM; with two additional glycoproteins, gJ and gN, that are believed to associate with the envelope although this has not been confirmed [4]. gG is the source of antigenic specificity between HSV-1 and HSV-2 and the antibody response to this glycoprotein allows the distinction between the two virus serotypes that otherwise share 83% genome sequence homology between the protein-coding regions [10]. Additionally, due to their exposure on the surface of the virion, the HSV membrane glycoproteins elicit a robust antibody response from the host. In this regard gB, the gH/gL complex and gD are particularly important with gD being one of the most potent inducers of neutralizing antibodies for either HSV serotype [11]. These four glycoproteins, gB, gD, gH and gL, are necessary for virus attachment and entry into a host cell [12] and will be discussed in more detail below.

EPIDEMIOLOGY OF GENITAL HERPES

HSV-2 is the primary cause of genital herpes infecting >500 million people worldwide and causes an estimated 23 million new infections annually [2]. Recently analysis of data collected through the National Health and Nutrition Examination Surveys (NHANES) estimated HSV-2 seroprevalence in persons aged 14-49 years in the United States to be 16.2% [3]. Data obtained from NHANES have been used to stratify HSV-2 prevalence estimates by race/ethnicity, sex and age. These analyses show that HSV-2 seroprevalence increases with age from 1.4% in persons aged 14-19 to 26.1% in persons aged 40-49 [3], likely a result of the chronic nature of HSV-2 [13]. These studies also found that persons who did not have a stable partnership had higher HSV-2 prevalence. Both observations regarding age- and relationship-related patterns were similar across race

groups; however, higher HSV-2 prevalence was found for Black men and women compared to White, Hispanic or other racial groups across ages [13]. These estimates found that Black, non-Hispanic populations had three times greater HSV-2 seroprevalence than White, non-Hispanic or Mexican Americans [3].

As with other STIs [14, 15], women bear a higher burden of genital HSV-2 infection (**Figure 1.2**), with estimates of HSV-2 seroprevalence in the United States reaching nearly 21% in women compared to only 11.5% in men [3]. While the disparity

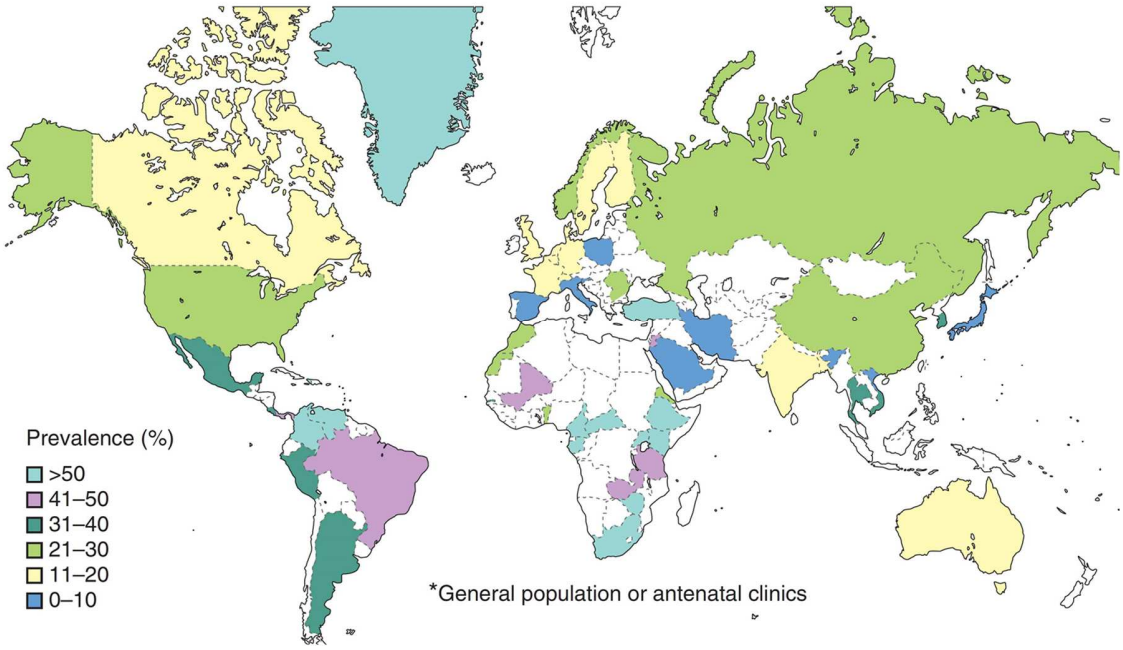

Figure 1.2. Wordwide HSV-2 seroprevalence in women.
Global HSV-2 infection in women based on serological data compiled from studies published within the last 15 years. Areas where no data were available are indicated by white. Adapted from: Schiffer, J.T. and L. Corey, *Rapid host immune response and viral dynamics in herpes simplex virus-2 infection*. Nat Med, 2013. 19(3): p. 280-90. Reproduced with permission.

is not as pronounced globally, Looker *et al*. found high HSV-2 seroprevalence for women (ages 15-49 years; 12.8%) compared to men (10.8%) across the same age range [2]. The reasons for the increased HSV-2 burden in women are not entirely clear. It has been postulated that anatomical differences between men and women increase the efficiency of

transmission in the male-to-female direction [2]. Additionally, it has been suggested that changes in sexual contact patterns between different age classes of women and men may account for the observed discrepancy [16]. This is supported by NHANES data that shows HSV-2 incidence increases with age, thus young women having contact with older men could be at a higher risk of acquiring the virus during sexual contact.

NATURAL HISTORY

Genital herpes infection normally occurs when the virus comes into contact with a susceptible mucosal surface or areas of epithelial disruption often as a result of sexual contact [17, 18]. The virus undergoes cytolytic replication at the site of inoculation that results in a primary infection in susceptible individuals (i.e. individuals without preexisting antibodies to HSV due to a previous exposure to this virus) [19].

Primary genital herpes infection can be accompanied by the development of painful lesions on the external genitalia and mucous membranes. These normally develop approximately 4-7 days after sexual exposure (**Figure 1.3**) [20]. These lesions may present in a number of forms progressing from macules and papules to vesicles and finally pustules or ulcers [21, 22]. The primary infection is normally self-limiting resolving within a few weeks in immunocompetent individuals [23]. However, it can be accompanied by complications including most severely autonomic dysfunction and aseptic meningitis [20, 24]. Less severe complications can include malaise, headache, myalgia and fever [25]. Additionally in women there is a chance for vertical transmission from the mother to the neonate [26]. Incidence of vertical transmission have been reported to be an estimated 31 out of 100,000 live births [27] and often results from the infant being exposed to HSV during delivery [28, 29]. Neonatal herpes disease can be mild particularly if it is confined to the skin and mucous membranes. However, it can also present as neurologic disease including encephalitis and as a disseminated infection in which case the infection can be

fulminating with severe sequelae including cognitive impairment, organ dysfunction, neurological disease and even death [20, 23, 30]. Importantly, although neonatal herpes

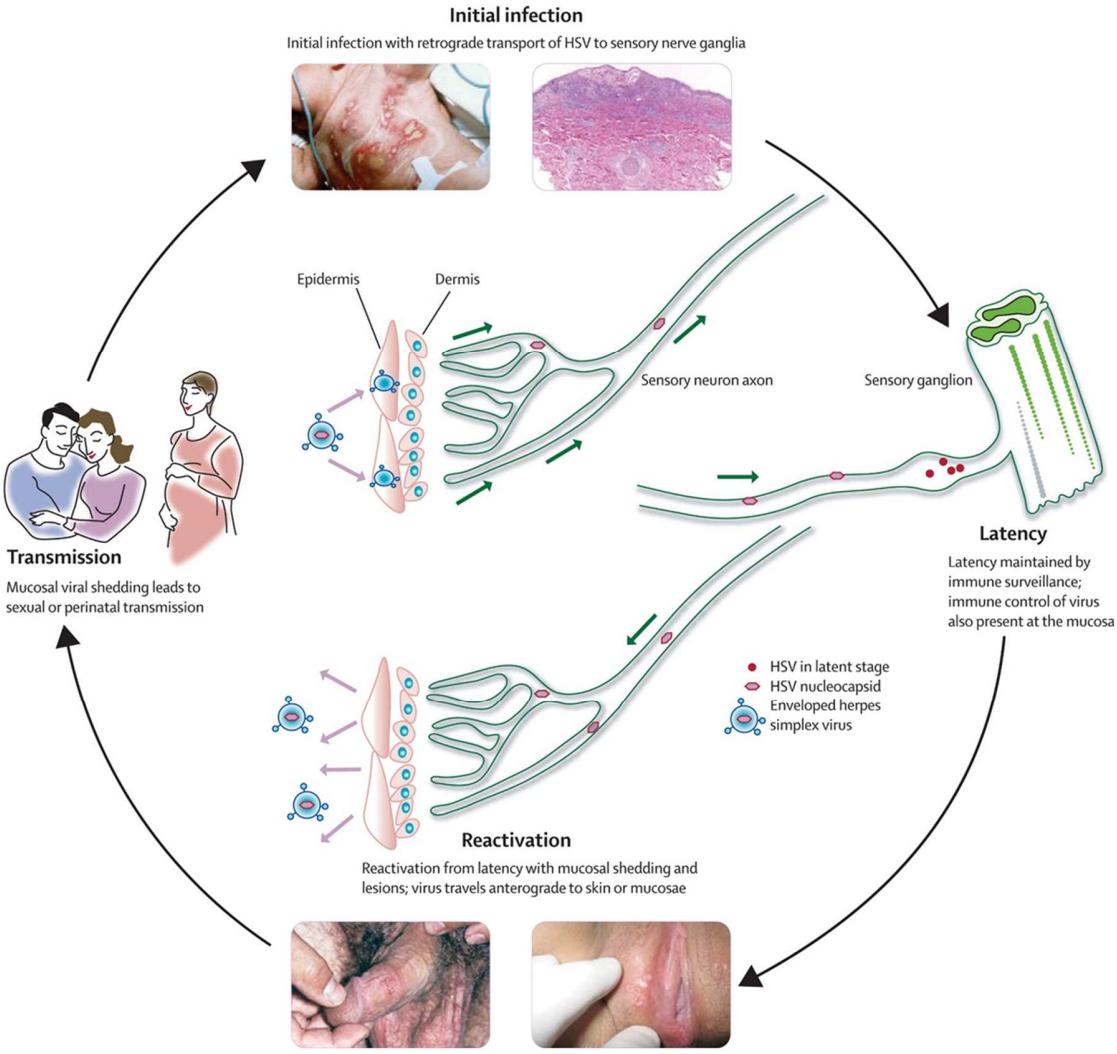

Figure 1.3. Pathogenesis of primary HSV-2 infection and latent reactivation.
Initial HSV-2 infection occurs through sexual contact or perinatal transmission and results in viral entry and replication within epithelial cells. During primary infection, the virus enters the axonal termini of neurons innervating the dermis, translocates to the nucleus in the ganglion and establishes a life-long latent infection. During latency, the virus can reactivate and produce new infectious virions that are transported back down the axon to the initial site of infection where a new round of replication can begin. Viral shedding in the presence (clinical reactivation) or absence (subclinical reactivation) of genital lesions can then occur and lead to transmission to other sexual partners. Adapted from: Gupta, R., T. Warren, and A. Wald, *Genital herpes*. Lancet, 2007. 370(9605): p. 2127-37. Reproduced with permission.

infection is most often associated with a maternal primary infection at the time of delivery or also may occur during viral reactivation that results in shedding of the virus into the genital tract.

It is important to note that many individuals that are or become infected with HSV do not experience clinical signs of disease, leaving many people unaware of their HSV infection status. In fact, between 60-80% of all HSV infections are asymptomatic or unrecognized [3, 31].

During a primary HSV infection, virus spreads from infected epithelial cells to sensory neurons innervating the site of infection through the axonal termini within the tissue (**Figure 1.3**) [32]. Once within the axon, the virus undergoes retrograde transport to the neuronal cell body where the viral DNA becomes circularized to form an episome and establishes a latent viral state and lifelong infection [33, 34]. During latency the virus can reactivate through stress or other unknown factors [35]. Reactivation leads to the production of new virions that undergo anterograde transport from the neuronal cell body back down the axon to the site of initial infection [32].

These episodes of virus reactivation may result in the development of recurrent genital lesions or ulcers but can also involve viral shedding into the genital tract in the absence of clinically recognized symptoms [20]. A study conducted on a group of HSV seropositive women showed that virus shed in the absence of symptoms, called asymptomatic shedding, occurred on over 30% of the total days sampled and over 50% of the women sampled experienced at least one asymptomatic shedding event during the 60+ days of sampling [36]. Additional studies have shown that half of HSV reactivations last ≤12 hours and over 80% of genital mucosal HSV reactivations were asymptomatic [37], a finding that was further supported by the results of mathematical modeling studies conducted by Schiffer *et al*. suggesting a near constant release of virus from the neurons innervating the genital tract [38]. Asymptomatic shedding is believed to be a major source

of virus transmission to uninfected individuals and represents a major public health concern for the control of genital herpes worldwide [39, 40].

HSV-2 Entry and Replication

The process by which HSV-2 gains entry into a host cell is complex and involves several distinct steps. Briefly, the virus must first make contact with and attach to the cell surface. Next, viral glycoproteins must encounter their specific entry receptors to proceed with internalization of the virion into the host cell. Finally, membrane fusion must occur to release the capsid into the host cell cytoplasm [41].

The first step to viral entry is the reversible binding of either of gB or gC to cell-surface heparan sulfate and chondroitin sulfate proteoglycans [41]. gC is not considered essential for this binding as gB may provide this function in the absence of gC. Next, gD must bind to one of its specific receptors, nectin-1, herpesvirus entry mediator (HVEM), or a form of heparan sulfate modified by 3-O-sulfotransferases (3-OS HS) [4]. Displacement of the C-terminus of gD triggered by receptor binding exposes a previously hidden region of gD that interacts with the viral gH/gL complex resulting in a conformational change of the complex [12]. The changed gH/gL complex then interacts with gB, up-regulating it into a fusogenic state that allows gB to extend fusion loops into the cell plasma membrane. Interactions between the ectodomains of gB and gH/gL then convert gB into a post-fusion state that leads to a successful fusion of the viral envelope with the cell membrane [12]. The virion nucleocapsid is then delivered into the cell cytoplasm and viral replication can begin. Additionally, release of the tegument proteins occurs coincident with the release of the nucleocapsid. These virus proteins migrate to locations within the host cell and prepare the infected cell for virus replication [4].

While the majority of HSV-2 cellular entry is believed to occur by the method described above, a second auxiliary pathway has also been described. This pathway requires endocytosis of the virion followed by receptor-dependent fusion of the endocytic

vesicle membrane with the virion envelope, resulting in the release of the capsid into the cell [4].

HSV replication, including transcription of the viral genome, DNA replication, and new capsid assembly, takes place in the cell nucleus [4]. The process begins with the transcription of viral DNA using the host cell RNA polymerase II in conjunction with viral factors that vary depending on the stage(s) of replication. Viral gene products are synthesized in a tightly regulated and sequentially ordered fashion as shown in **Figure 1.4** [4]. The immediate early, or alpha (α), genes are expressed first and are transcribed in the absence of *de novo* viral protein synthesis. Expression of the delayed early, or beta (β) genes, is initiated by the α gene products [4]. The products of β gene activation are comprised of DNA-binding proteins and enzymes necessary for viral DNA replication, which is accomplished by a rolling circle mechanism. Finally, the late (gamma, γ) genes are then expressed following viral DNA replication and are primarily involved in progeny virion assembly [4]. The virions are packaged and can then exit the cell either through fusion of transport vesicles with the plasma membrane or through lytic release into the extracellular space.

IMMUNE RESPONSE TO INFECTION

The immune response to HSV-2 infection involves a complex interplay between both the innate and adaptive arms of the host immune system. The initial response is the activation of innate immunity that is crucial in the early control of viral replication and dissemination [42]. Further, the innate response plays an essential role in priming the acquired immune responses necessary for controlling viral replication and resolution of disease [43]. Adaptive immunity is necessary for resolving the infection; however the acquired immune responses are insufficient without the aid of early innate immune mechanisms [44].

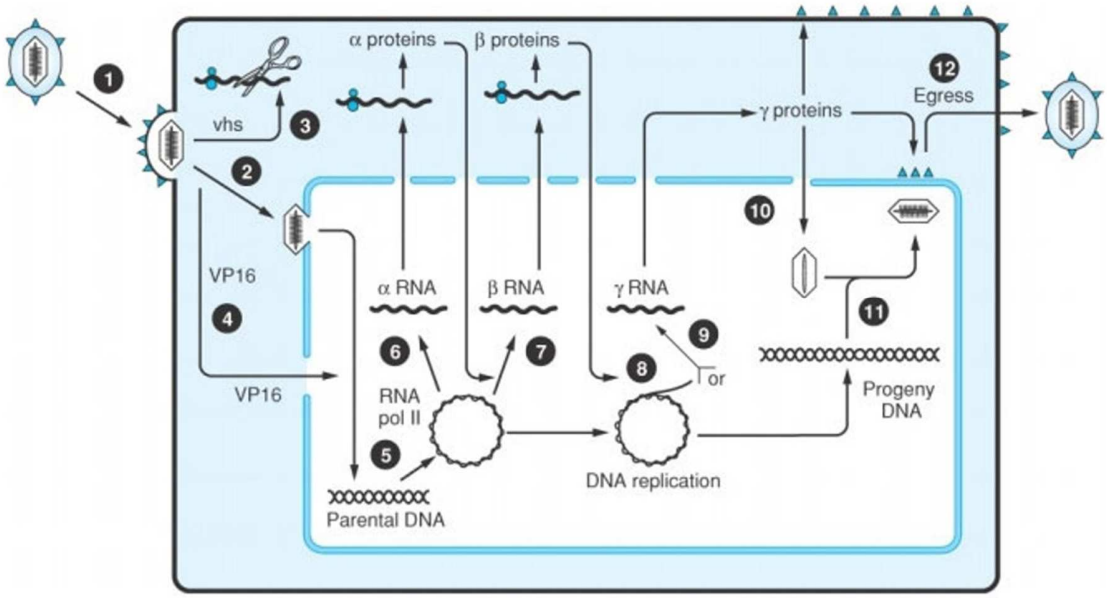

Figure 1.4. HSV-2 entry and replication cycle.
1) The virion binds to the cell membrane and initiates a series of conformational changes in gD, the gH/gL complex and gB to fuse with the cell envelope and release the capsid and tegument proteins into the cytoplasm. **2**) Transport of the capsid to the nuclear pore results in the release of viral DNA into the cell nucleus. **3**) Host mRNA is degraded by the virion shut-off (vhs) protein. **4**) VP16 is localized to the cell nucleus. **5**) The viral DNA circularizes. (**6**) VP16 stimulates virus α gene transcription by host RNA polymerase II. Nuclear viral gene expression is further mediated by 5 of the 6 immediate early (IE) virion proteins. (**7**) β gene transcription is transactivated by α proteins. **8**) The viral DNA molecule is replicated with the help of β proteins. **9**) The synthesis of virion DNA results in the stimulation of γ gene expression. **10**) New virus capsids are assembled in the nucleus with the help of γ proteins. The γ proteins also modify the nuclear and cellular membranes for use in final virion assembly. **11**) The viral DNA is placed into the capsid. **12**) The capsid containing viral DNA buds through the inner nuclear membrane forming an enveloped virion that then exits the cell. Adapted from: Roizman, B., D.M. Knipe, and R.J. Whitley, *Herpes Simplex Viruses*, in *Fields Virology*, B.N. Fields, D.M. Knipe, and P.M. Howley, Editors. 2007, Wolters Kluwer Health/Lippincott Williams & Wilkins: Philadelphia. p. 2502-2601. Reproduced with permission.

HSV-2 infects cells at the mucosal epithelial surface and is detected by toll-like receptors (TLRs) found on epithelial cells, antigen-presenting cells (APCs), natural killer cells (NKs) and plasmacytoid dendritic cells (pDCs) [44]. As shown in **Figure 1.5**, this results in activation of Type I interferons (IFNs) that initiate a positive feedback-production

loop in both an autocrine and paracrine manner [42, 45]. The protective benefits of this IFN production have been highlighted in studies conducted using TLR ligands to prime the

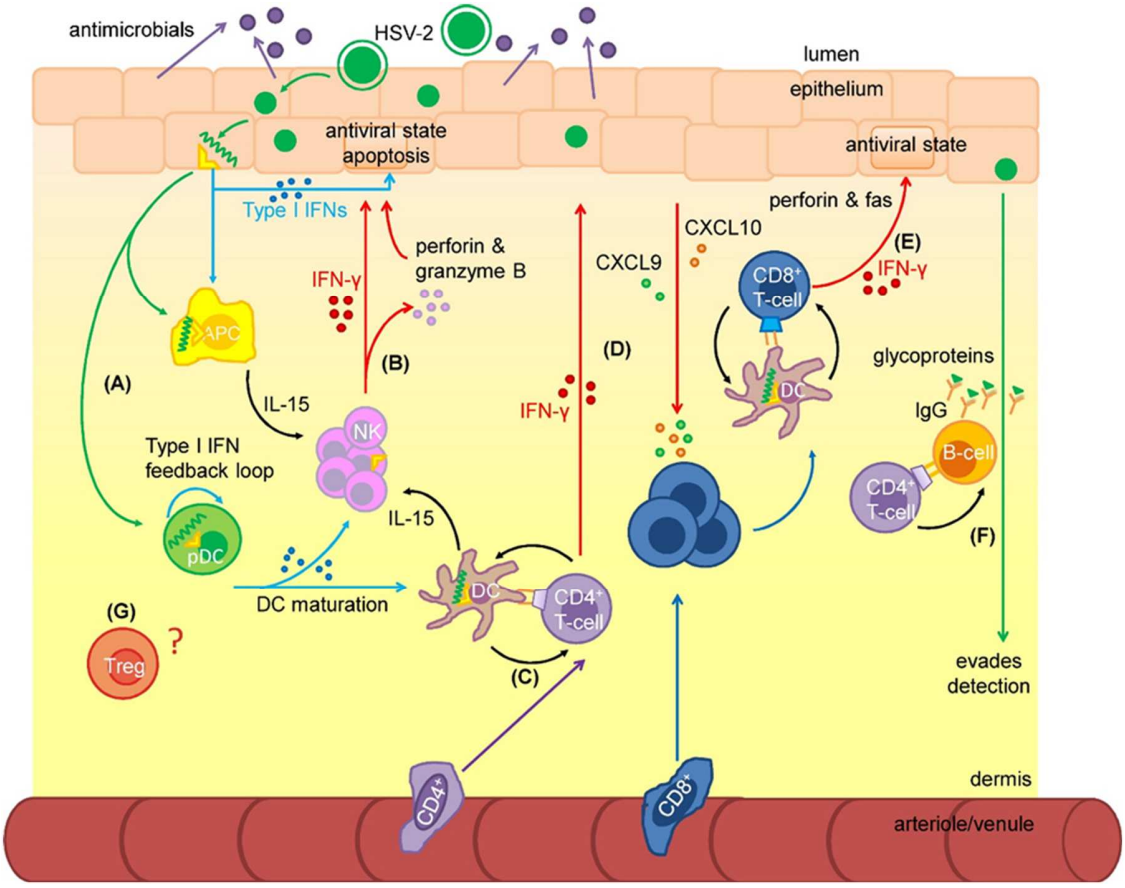

Figure 1.5. The immune response to HSV-2 infection.
A) HSV-2 infection of epithelial cells is detected by TLRs that induce Type I IFN production in a positive feedback loop. This initializes an antiviral state in the surrounding epithelial cells and drives DC maturation, IL-15 production and promotes NK proliferation and survival. **B)** The release of IFNγ by NK cells aids in the antiviral response; NK release of perforin/granzyme can induce apoptosis to kill virally infected cells. **(C)** APCs recruit and activate $CD4^+$ T-cells. **D)** Activated $CD4^+$ T-cells secrete IFNγ and induce epithelial cells to secrete chemokines to attract $CD8^+$ T-cells. **E)** HSV-2 specific $CD8^+$ T-cells additionally secrete IFNγ and utilize the fas-mediated or perforin pathways to kill infected cells. **F)** $CD4^+$ T-cells activate recruited B-cells to produce antibodies; however HSV-2 can evade antibody-mediated protection. **G)** T_{reg} cells have been found at sites of infection but their role in the response to HSV-2 infection is unclear. Adapted from: Chan, T., et al., *Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa*. J Reprod Immunol, 2011. 88(2): p. 210-8. Reproduced with permission.

immune response in animal models of genital herpes. These studies showed that pre-stimulation of the immune response using TLR-3 or TLR-9 agonists to initiate this signaling process prior to an HSV-2 infection could protect from disease and lessen viral replication [46-48]. Activation of Type I IFNs induces anti-viral gene expression to aid in the initial control of viral replication. Additionally, Type I IFNs activate innate effector cells critical in the response to HSV-2 infection, including macrophages, DCs, pDCs and NK cells (**Figure 1.5**). Once activated, DCs and macrophages process antigens for presentation to effector T-cells to prime the adaptive immune response while pDCs and NK cells can produce additional cytokines to help limit the spread of the virus [43]. Thus, while the innate immune system controls initial viral replication the antigen-specific adaptive response is critical to mediating the clearance of HSV-2 from the local site of infection [44].

Humoral immunity is an important component of the adaptive immune response. HSV-2 infection stimulates a robust antibody response that can control infection by binding to HSV-2 envelope glycoproteins responsible for entry, potentially limiting infection of the mucosae and thereby limiting the spread of virus to the dorsal root ganglia [49]. This may be accomplished by the production of non-specific natural antibodies secreted by B-cells early during the infection [50] or as a result of targeted, HSV-2 specific immunoglobulins G (IgG) and A (IgA) [44].

During a primary HSV-2 infection, T-cells are an important component of viral clearance (as demonstrated in mouse and human models of disease) and the resolution of genital herpes lesions (shown in human models of disease) [51, 52]. Further, viral containment is enhanced by T-cell responses at sites of infection [53]. IFNγ is an important cytokine secreted by both $CD4^+$ and $CD8^+$ T-cells and has been shown to be a crucial factor in these mechanisms of viral containment and control. The significance of IFNγ in this capacity has been demonstrated by the impaired resolution of HSV-2 disease seen when IFNγ was depleted prior to virus challenge in the mouse model of genital herpes [54]. $CD8^+$

T-cells have been considered the primary source of this important effector molecule and are considered the dominant adaptive immune cell that contributes to local protection against HSV-2 replication in the vaginal mucosa [43]. However, studies have shown that depletion of $CD8^+$ T-cells does not abrogate viral clearance, suggesting a compensatory role of $CD4^+$ T-cells in resolution of HSV-2 infection [43, 52]. In addition, $CD4^+$ T-cells have been shown to be the primary source of early IFNγ production during acute HSV-2 infection [55] and it is this cytokine that stimulates the secretion of two chemokines, CXCL9 and CXCL10, that are important for the recruitment of $CD8^+$ T-cells to sites of infection necessary for viral clearance [56, 57]. Taken together, these results strongly suggest that an effective immune response includes an early role for $CD4^+$ T-cells that is augmented by $CD8^+$ T-cells later during the course of the infection and highlight the delicate balance between both of these T-cell subsets that are required for control of a primary HSV-2 infection [44].

In addition to their importance during a primary HSV-2 infection, T-cells also play a major role in preventing HSV-2 reactivation from latency and controlling recurrent disease. In mouse studies of HSV-1 latency, both $CD4^+$ and $CD8^+$ T-cells are recruited to the ganglion for the life of the animal. Further, the $CD8^+$ cells were found to be in close apposition to the neuronal cell bodies, selectively associating with neurons that harbored latent HSV-1 [58]. Other studies confirmed these findings and further suggested a role for IFNγ secretion by an activated $CD8^+$ effector phenotype in the maintenance of HSV latency [59, 60]. Additionally, analysis of samples collected from human biopsies of genital skin showed that $CD8^+$ T-cells accumulated at the dermal-epidermal junction near sensory nerve endings, suggesting a similar role for T-cells in preventing HSV-2 reactivations [61].

These studies also suggest a potential mechanism for the rapid elimination of virus by the local immune system should a reactivation occur. Likewise, HSV-2 specific $CD4^+$ and $CD8^+$ T-cells accumulate in sites of recurrent lesions during recurrences where they persist for months after wound healing, providing surveillance to quickly contain

reactivated virus and prevent extensive replication [49, 62]. Collectively, these data support an important role for T-cells in the control of HSV-2 reactivations and showed that both $CD4^+$ and $CD8^+$ T-cells are necessary to limiting replication of HSV-2 in the vaginal mucosa and genital skin during recurrent genital herpes disease.

ASSOCIATION BETWEEN GENITAL HERPES AND HIV-1 INFECTION

In addition to the impact on public health caused by genital herpes morbidity, there is an increasing body of epidemiological data that supports a close link between genital HSV-2 infection and human immunodeficiency virus type 1 (HIV-1) infection [63, 64]. In fact, studies have shown that prior infection with HSV-2 increases the risk of HIV-1 infection between 2- to 4-fold [65, 66].

A number of factors are believed to contribute to increased HIV-1 infection in HSV-2 positive individuals. Of these, the primary contributing factor is suggested to be the lytic replication of HSV-2 in the genital epithelium that causes damage leading to breaks in the mucosal barrier, reducing its effectiveness and increasing the potential for HIV-1 infection [67]. Immune cell recruitment to sites of HSV-2 infection also increases the numbers of HIV-1 target cells, including $CD4^+$ T-cells expressing CCR5 (an HIV-1 co-receptor) and/or myeloid dendritic cells (DCs) expressing DC-SIGN or CD123 (both HIV-1 co-receptors) [68, 69]. In HSV-2 infected individuals these cells have been found to be 10-fold higher than at similar sites in non-HSV-2 infected persons [70]. These cells can persist at sites of previous HSV-2 reactivations for months and are not impacted by antiviral therapy [61, 69].

It also bears mentioning that prior HIV-1 infection can similarly impact the susceptibility to HSV-2 acquisition. It has been shown that HIV-1 positive women have an impaired ability to respond to a primary HSV-2 infection, exhibiting diminished anti-HSV activity in cervico-vaginal secretions [71]. Further, women infected with HIV-1 have been shown to shed more HSV-2 during viral reactivations [70, 72]. Thus, there exists a clear

synergistic relationship between HSV-2 and HIV-1 that promotes the acquisition and transmission of these two important human pathogens.

ANIMAL MODELS

Herpes simplex virus has been shown to infect and produce disease in a number of small animal species allowing the development of models that can used for the study of genital herpes disease. These include the mouse, the cotton rat and the guinea pig. Of these models, the mouse and the guinea pig are currently the most commonly used.

One major reason for the use of the mouse to study HSV-2 infection and disease pathogenesis is the availability of specific immune knockout strains, a variety of inbred and transgenic strains, and a well-developed catalog of immunological reagents for this species. The mouse can by inoculated intravaginally (IVAG), but progesterone pre-treatment is required to induce diestrous which thins the vaginal epithelium allowing uniform infection [73]. The mouse model can be used to study primary infection including virus replication in the vaginal mucosa and the onset of genital disease; however, the disease quickly progresses to include CNS involvement with encephalitis and results in high mortality in naïve animals [74]. Despite this high mortality, the mouse is an excellent model for studying the effects of antivirals on acute HSV-2 infection of the genital mucosa and virus replication in the genital tract. Unfortunately, even in animals that survive a primary HSV-2 infection, spontaneous recurrences do not occur making the murine model unsuitable for studying reactivation from latent infection and recurrent disease and evaluating therapeutic interventions that reduce recurrent viral shedding and disease [75]. The hormonal pre-treatment necessary to establish a productive infection may also alter the immune responses to the virus [76, 77]. Additionally, mice do not develop vesicular lesions as seen in humans and are therefore not the most ideal model for studying disease pathogenesis. Even with these limitations, the murine model of genital herpes has been an invaluable resource for studying the immune response to both HSV-1 and HSV-2 primary

infections and remains a widely used model for preclinical studies to evaluate putative HSV-2 vaccines.

In contrast to the mouse, primary genital herpes disease in the cotton rat closely mimics an HSV-2 infection in humans. The cotton rat may be inoculated IVAG without the need for hormonal pre-treatment and the primary infection results in viral spread to multiple organs known to be targets of HSV dissemination in humans[78]. A primary HSV-2 infection in this animal model is self-limiting and leads to the establishment of viral latency in the ganglia. Thus, this animal model may be used to study HSV-2 recurrences and reactivation from latency. Yim *et al*. found that animals undergo spontaneous clinical recurrences with accompanying virus shedding into the genital tract that could be detected by PCR or cell culture methodologies [78]. In addition, HSV-2 reactivations may be induced in this animal model, making it an ideal model for the study of therapeutic interventions for the reduction of recurrent genital herpes disease. A wide variety of reagents, including PCR primers and probes, recombinant proteins, antibodies, and assays to detect cytokine abundance, have been developed and are available for the study of the immune response in this animal [78-80]. However, despite these advantages, the cotton rat has to date failed to become widely used model for the study of genital herpes at this time.

The guinea pig is an excellent model of genital herpes due to its similarity to human disease [81, 82] and is considered a gold standard for evaluating therapeutic vaccine and antiviral candidates [75]. Guinea pigs can be inoculated IVAG without the need for hormonal pre-treatment and develop a self-limiting vesiculo-ulcerative primary disease with many of the clinical and pathologic features observed during human HSV-2 infection [74, 75]. Primary HSV-2 infection results in the development of vesicular lesions similar to humans and these clinical disease manifestations can be evaluated for severity and duration. Additionally, virus replication in the genital mucosa can be quantified by cell culture or PCR methodologies using this animal model. As seen in humans, primary infection results in the virus establishing latency in the dorsal root ganglia (DRG) that

persists for the life of the animal. This latent virus undergoes spontaneous reactivations that can lead to virus being shed into the genital tract and can also lead to the development of recurrent skin lesions [81, 83]. These important similarities to human disease make the guinea pig an ideal animal for studying both prophylactic and therapeutic HSV-2 vaccine candidates, although there are limited reagents currently available to provide a detailed characterization of the immune responses during either a primary or recurrent infection in this animal.

ANTIVIRAL SUPPRESSIVE THERAPY AND VACCINE DEVELOPMENT

Antiviral treatment for genital herpes has been available since the 1980s with the discovery of acyclovir (ACV), a deoxyguanosine analog that competitively inhibits the viral DNA polymerase to prevent elongation of the viral genome during replication [84, 85]. ACV and its derivatives, valacyclovir (VACV) and famciclovir (FCV), all share this common mechanism of action that is highly specific to the viral thymidine kinase. Briefly, these drugs are phosphorylated by the viral thymidine kinase and then undergo additional phosphorylations by host cell kinases to produce a triphosphate that serves as a substrate for the viral DNA polymerase during replication of the viral genome [85]. Incorporation into the replicating viral DNA strand leads to a dead-end complex and prevents further elongation of the DNA molecule. Due to the specificity of this mechanism of action these drugs have a remarkable safety profile and have become the first line antivirals for prophylaxis and treatment of genital and oral herpes [86, 87].

HSV antivirals were initially used episodically to reduce disease symptoms and prevent complications associated with primary HSV-2 infections, however more recently ACV and its derivatives have been employed as a suppressive treatment for genital herpes. These drugs have proven highly effective for reducing the duration of recurrent disease and for preventing reactivations and reducing episodes of clinical (associated with disease) and subclinical (no overt disease symptoms) viral shedding [86, 88]. Additionally, studies using

discordant heterosexual couples have shown that VACV taken once-daily could reduce the transmission of HSV-2 by about 50% [89]. However, suppressive therapy does not clear the latent infection and long-term treatment may lead to the development of drug resistance, particularly in immune-compromised individuals [85]. Further, more recent studies undertaken to investigate the impact of high-dose regimens of ACV and VACV on short duration subclinical shedding contrast the previous findings of Wald *et al.* In these studies, high-doses of ACV and VACV eliminated clinical recurrences and reduced the frequency of high copy number shedding episodes but did not affect the frequency of subclinical breakthrough episodes of viral shedding into the genital tract [90]. These results suggest that daily suppressive therapy, even at high doses, cannot completely abrogate transmission of HSV-2 and further support the need for development of an effective vaccine.

Currently there is no licensed vaccine available for the prevention or treatment of genital herpes. Although there have been many candidate vaccines that have proven efficacious in animal models (reviewed in [91]) and some of these have moved forward into human clinical trials (reviewed in [92]), to date none have successfully progressed to licensure. The most successful vaccine candidate to date, a glycoprotein D (gD) subunit vaccine adjuvanted with AS04, the trade name for a combination of aluminum hydroxide and 3-O-deacylated monophosphoryl lipid A (MPL), showed promise in an initial Phase III trial but subsequently failed to protect from HSV-2 infection in HSV seronegative women in a large follow up phase III clinical trial [93].

Traditionally, the primary goal for development of an HSV-2 vaccine has focused on a prophylactic vaccine capable of inducing a robust mucosal and systemic immunity that could prevent infection [94]. This putative vaccine would rely heavily on neutralizing antibodies for the prevention of infection and targeted two viral glycoproteins, gB and gD, involved in viral entry into the cell [94]. While this strategy proved unsuccessful at preventing infection (sterilizing immunity has been difficult to achieve for HSV-2 [95])

many putative vaccines utilizing glycoproteins have shown promise in preventing or reducing disease severity [91, 92, 96]. Thus, more realistic goals for a prophylactic vaccination may be to prevent or reduce disease associated with a primary infection and reduce or prevent virus shedding into the genital tract and virus transmission [94]. There has also been increasing recognition of the need for development of a therapeutic vaccine that could prevent recurrent symptoms and/or transmission of the virus by infected individuals [91].

Vaccine development for HSV-2 has become a complex field but can be broadly divided into two general categories of vaccines: live or inactive [95]. These groups can be further stratified into 3 sub-categories each as shown in **Table 1.1**. Thus, live vaccines may be attenuated, replication-limited/incompetent, or vectored and inactive vaccines may contain inactivated virus, HSV subunits or components, or plasmids [95].

Table 1.1. Generalized categories of herpes simplex virus vaccines.

Vaccine type, category	Advantage(s)	Disadvantage(s)
Live		
Attenuated	Induces broad, durable immunity	Safety/stability concerns
Replication-limited	May have advantages of live-attenuated w/ better safety profile	May not be as immunogenic as live-attenuated; potential safety concerns
Vectored	May have advantages of live-attenuated w/ better safety profile	Due to limited HSV gene products expressed may be less immunogenic as live-attenuated
Inactive		
Inactivated virus	Easily prepared; cannot cause HSV disease	Not as immunogenic as replicating vaccines; adjuvants necessary to bolster immunogenicity
Subunit	Excellent safety profile; recombinant subunits ensure consistent product	Limited number of epiptopes leads to narrow immunity; adjuvants required to induce cellular responses
Nucleic acid	Induces both humoral and cellular immunity	Limited number of epiptopes leads to narrow immunity; potential safety concerns

Adapted with permission from: Stanberry et al. *Prospects for Control of Herpes Simplex Virus Disease through Immunization*. Clin Infect Dis, 2000. 30: p. 549-66.

Typically, live virus vaccines generate a robust immune response that is durable, however these vaccines also present concerns for the stability and safety of the formulation [94, 95]. A number of approaches to live-attenuated vaccines have been tried, however it has proven difficult to develop candidates that are sufficiently attenuated to be safe for use while retaining the replicative capacity to generate a robust and durable immune response. Attenuated viruses have been genetically engineered, but this approach has resulted in a minimally immunogenic vaccine, although further development is ongoing [95]. Molecular methods have been used to develop a replication-incompetent virus containing single or multiple gene deletions. These defective viruses, known as DISC (disabled infectious single cycle) viruses, are grown in the presence of a complementing cell line that supplies the necessary genes in trans, resulting in a virus that is incapable of infecting a secondary cell [94, 97]. A final strategy that retains the live virus advantages without the safety concerns has also been developed. This involves the use of replicating vectors (viral or bacterial) that contain HSV genes to encode immunogenic proteins that can be produced and stimulate an immune response in the host [95].

Killed-virus vaccines do not offer the immunogenicity observed with live virus preparations but have improved safety profiles [95], although early killed-virus vaccines only showed short-term benefits [97]. Recombinant DNA technology has made possible the development of subunit vaccines. These vaccines offer the advantage of being free from infectious virus or viral DNA and can present a more complex antigen to the host immune system for stimulation of $CD4^+$, $CD8^+$ and B cell responses [95, 97]. Unfortunately, due to the limited number of viral epitopes that can be contained in subunit vaccines, the immune response that is elicited is less broad and may not be long-lasting [95]. The newest strategy for developing HSV-2 vaccines is the use of plasmids. These vaccine formulations contain plasmids that encode one or more viral gene targets (or host cytokines) that are produced *in vivo* by host cells [91, 98], and induce both cell mediated and humoral immunity [95].

Despite these and other advances, HSV-2 vaccine development has proven difficult. Initial strategies revolving around the robust antibody response elicited by HSV-2 infection were unsuccessful and the importance of neutralizing antibodies in the control of disease pathogenesis and virus dissemination remains controversial. Many studies using the murine model of genital herpes have demonstrated the protective effects of anti-HSV antibodies with regards to reduction of viral titers, the frequency of clinical disease and reductions in virus transmission [99-101]. More recent studies supported these earlier findings with similar data indicating that administration of gD-specific antibody reduced viral loads in the vaginal epithelium and protected from disease onset [102]. However, in all of these studies HSV-2 specific antibodies did not prevent infection. Further, studies in B-cell deficient mice showed that resistance to lethal HSV-2 challenge was not B-cell dependent [50] and that the protection from HSV-2 specific antibodies could be compromised by T-cell depletion [103], suggesting the contribution of humoral immunity to HSV-2 control is limited and requires the cellular component of the adaptive response.

Thus, while HSV-2 vaccine formulations have typically included one of the outer membrane glycoproteins necessary for virus entry into host cells, typically gD or gB, as the major antigenic component, there is a need for additional antigenic targets to stimulate both T- and B-cell responses. These targets stimulate a vigorous humoral response but lack a cellular component that has been shown to be crucial for protection [97]. Additionally, the lack of successes with vaccine formulations focusing solely on neutralizing antibody epitopes further suggests a vaccine formulation containing limited HSV antigenic targets does not have the “antigenic breadth” necessary for immunogenic stimulation of both the humoral and cellular immune responses capable of maintaining protection from infection or viral reactivations [104]. It has been argued that for novel vaccine formulations to be effective, they must include multiple antigenic targets for innate immunity and/or stimulation of both a B- and T-cell specific immune response [92] while others proposed

vaccines containing live attenuated or replication-deficient virus to increase the antigenic payload presented to the immune system [105].

This leaves a clear need for a novel HSV-2 vaccine formulation that can elicit both a robust and durable humoral and cellular immune response. Such a vaccine could be used prophylactically to provide protection from primary disease symptoms while limiting the amount of virus capable of establishing latent infection. Additionally, it could be used in a therapeutic setting to reduce recurrent disease manifestations and virus shedding, thereby reducing the potential for transmission of the virus to uninfected individuals.

CHAPTER II[§]

A Vaxfectin®-Adjuvanted HSV-2 Plasmid DNA Vaccine is Effective for Prophylactic and Therapeutic Use in the Guinea Pig Model of Genital Herpes

INTRODUCTION

As discussed in the previous Chapter, genital herpes is a sexually transmitted infection of global importance [3, 106]. Initial infection can be asymptomatic or can result in painful skin or mucosal lesions [22]. In addition, a lifelong latent infection is established [26]. Periodic reactivations of latent virus can result in recurrent lesions or more frequently asymptomatic genital tract shedding that is a major source of virus transmission [39]. Genital herpes infection also increases the risk for HIV acquisition [107] and thus represents a worldwide public health problem with significant attendant economic burden [108].

Unfortunately, there are no licensed vaccines against genital herpes and recent clinical trials with subunit vaccines containing viral glycoproteins B and/or D have proven disappointing, including the recent failure of a Herpes simplex virus type 2 (HSV-2) glycoprotein D (gD2) vaccine developed by GlaxoSmithKline [93]. Evidence suggests that successful vaccines must generate a robust T-cell response in addition to the humoral immunity elicited to viral glycoproteins [106, 108].

Plasmid DNA (pDNA) vaccines can elicit both cellular and humoral immune responses [98, 109-111]. We have previously shown that a non-adjuvanted gD2 pDNA

[§]Chapter II previously published and taken from: Veselenak, R.L., et al., A Vaxfectin®-adjuvanted HSV-2 plasmid DNA vaccine is effective for prophylactic and therapeutic use in the guinea pig model of genital herpes. Vaccine, 2012. 30(49): p. 7046-51. doi: 10.1016/j.vaccine.2012.09.057. Epub 2012 Oct 4. PMID: 23041125. Used with permission.

vaccine provided partial protection against genital herpes in the guinea pig model [112]. The cationic lipid-based adjuvant Vaxfectin® increased the immunity of pDNA vaccines Chapter II taken from: in Phase 1 clinical trials [113-115]. Recently, we showed that Vaxfectin® improved protection provided by a codon-optimized gD2 pDNA vaccine in a murine model of genital herpes [116]. While we were interested in evaluating the protective efficacy of the Vaxfectin®-gD2 pDNA vaccine in the guinea pig model, we also believe that a successful genital herpes vaccine will require additional antigens and will need to elicit cell-mediated as well as humoral immunity. Accordingly, we included pDNAs containing HSV-2 UL46 and UL47 genes encoding the VP11/12 and VP13/14 tegument proteins, respectively, both known to be potent inducers of $CD8^+$ T-cells [8, 9] in our lead vaccine candidate, Vaxfectin®-gD2/UL46/UL47.

Here we describe studies in the guinea pig showing that prophylactic immunization with both Vaxfectin®-gD2 and Vaxfectin®-gD2/UL46/UL47 provided protection against primary and recurrent disease, with Vaxfectin®-gD2/UL46/UL47 being superior in reducing latent viral load. Further, we explored the efficacy of Vaxfectin®-gD2/UL46/UL47 as a therapeutic vaccine in infected animals and showed that it was able to reduce both recurrent disease and viral shedding into the genital tract.

METHODS

Guinea Pigs

Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were housed in AAALAC-approved facilities. All animal studies were approved by the UTMB IACUC.

Plasmid DNAs and Vaccine Formulation

HSV-2 genes were codon-optimized using proprietary algorithms (Vical; San Diego, CA) and DNA synthesized by GeneArt (Regensburg, Germany). Sequences coding

full length gD2 and genes UL46 and UL47 were individually sub-cloned into plasmid VR1012 containing the hCMV immediate early promoter as described previously [9, 117], creating plasmids VR2149, VR2144 and VR2145. pDNAs were formulated with Vaxfectin® adjuvant as described previously [116]. Briefly, on the day of immunization, vials containing Vaxfectin® were reconstituted with 1 mL 0.9% saline. At the same time, pDNA was prepared in 0.9% saline, 20 mM sodium phosphate, pH 7.2. The Vaxfectin® was then streamed into the pDNA at a 1:1 volume dilution yielding 1 mg/mL pDNA and 1.09 mg/mL Vaxfectin® with a final pDNA nucleotide: cationic lipid molar ratio of 4:1 [116].

Guinea Pig Model of Genital HSV-2 Infection

For all studies, guinea pigs were inoculated intravaginally with 6.0 $\log_{10}$ plaque forming units (pfu) HSV-2 strain MS as described previously [96]. The animals were evaluated daily and primary genital skin disease quantified. The resultant cumulative lesion score was used to measure primary genital skin disease severity [96]. Following resolution of primary disease, animals were examined daily for spontaneous recurrent lesions and the number of lesion days was used to determine the frequency of recurrent disease [96].

Prophylactic Vaccination Studies

Forty-five guinea pigs were assigned to three groups (n=15/group). Animals were immunized intramuscularly in each rear leg. Group 1 received 300 µg Vaxfectin®-gD2 in one leg and 150 µg each of Vaxfectin®-UL46 and Vaxfectin®-UL47 in the other. Group 2 received 300 µg Vaxfectin®-gD2 in one leg and 300 µg of Vaxfectin®-VR1012 in the other. Group 3 received saline in both legs. The animals were immunized three times at two week intervals. Serum was collected after the second and third immunizations. Two weeks after the final immunization, animals were virus challenged as described above. Vaginal swabs were collected on days 1, 2, 3 and 5 and stored (-80°C) to determine viral load by

quantitative real-time PCR (qPCR) [118, 119]. After recovering from primary infection, animals were monitored from days 15-63 for recurrent herpetic lesions. Vaginal swab samples were collected from days 21-41 and stored (-80°C) to determine viral shedding into the genital tract by qPCR. At the conclusion of the study, animals were humanely sacrificed, the dorsal root ganglia (DRG) harvested, and the magnitude of latent viral infection determined by qPCR.

Therapeutic Vaccination Studies

Guinea pigs were inoculated with HSV-2 and primary genital skin disease quantified as described above. After recovery from primary infection, animals that had experienced symptomatic disease and with genital skin that could be evaluated for recurrent disease were randomized to groups based on primary genital skin scores. Animals were immunized on days 15, 29 and 43 post-challenge intramuscularly in the rear legs. In Study 1, Group 1 (n=18) received 300 µg Vaxfectin®-gD2 in one leg and 150 µg each of Vaxfectin®-UL46 and Vaxfectin®-UL47 in the other. Group 2 (n=18) received 300 µg Vaxfectin®-VR1012 in one leg and 150 µg each of Vaxfectin®-UL46 and Vaxfectin®-UL47 in the other. Group 3 (n=18) were saline controls. In Study 2, Group 1 (n=17) were immunized as for Study 1 while Group 2 (n=16) were saline controls. In both studies, animals were monitored from days 15-63 for recurrent disease. Daily vaginal swabs were taken and stored (-80°C) to determine viral shedding into the genital tract by qPCR. Over the course of the experimental period for Study 2, several animals developed secondary infections of the perineum and could not be evaluated for recurrent disease. As such, these animals were not included in subsequent viral shedding comparisons. This resulted in final group numbers for Study 2 of: Group 1 (n=14) and Group 2 (n=14).

Neutralization Assays

Neutralizing antibody titers were determined by a modification of our previous methods [119]. Briefly, serum from all animals in each group was pooled and heat inactivated (15 minutes, 56°C). Serial two-fold dilutions were prepared using titration medium (Dulbecco's modified Eagle medium, 2% newborn calf serum, 2% penicillin/streptomycin and 2% amphotericin B) and Low Tox M rabbit complement (CedarLane, Ontario, Canada). A known titer HSV-2 strain MS stock was added to each dilution and the serum/virus mixture incubated (37°C, 60 minutes). Aliquots of each sample dilution were plated in duplicate on Vero cell monolayers and incubated (3 days, 37°C). Plates were stained with crystal violet and viral plaques enumerated. Counts were compared to those from the control group. The neutralizing titer was defined at the log10 of the final serum dilution that reduced plaque number by 50% [119].

HSV-2 DNA Isolation and Quantification

DNA was isolated from swab samples (DNeasy 96 Blood and Tissue Kit; Qiagen, Valencia, CA) and from DRG using an automated sample disruption system (TissueLyser II; Qiagen) followed by spin-column extraction (DNeasy Blood and Tissue Kit; Qiagen) as previously described [119]. All extracted DNA was eluted in carbon-free water and stored at -20°C until analyzed.

qPCR utilized the CFX optical platform (Bio-Rad, Hercules, CA) and associated chemistries. HSV-2 DNA was quantified in 25 µl reactions as described previously [119]. qPCR mixtures for all samples contained 1x iQ Supermix (Bio-Rad), 5 pmol each forward and reverse primers, 2.5 pmol gB HSV-2 specific TaqMan probe, and 5 µl template DNA (2.5% of total DNA sample). A second, parallel qPCR was conducted using guinea pig housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ensure sample integrity and extraction efficiency. This second PCR run controlled for cellular load in each PCR and was used to normalize HSV-2 copies for each sample to more accurately

compare results. Cloned amplimers were included on each plate as a 10-fold dilution series as quantitation standards. Negative template controls and water-only samples were included on each run to ensure integrity. PCR run efficiencies were between 80-120% with correlation coefficients >0.96. Assay sensitivity for these reactions allowed for 100 genome equivalents (GE) per reaction to be detected 100% of the time.

Statistical Analysis

Comparisons between two groups were analyzed by Student's t-test. Analysis of primary viral replication during the prophylactic immunization study was conducted using a mixed model ANOVA on group by day. Comparisons among multiple groups in the therapeutic vaccination studies were made by one-way analysis of variance with Bonferroni correction. Incidence data was compared using Fisher's exact test. All comparisons were two-tailed.

RESULTS

Prophylactic Immunization Study

VACCINE IMMUNOGENICITY

Both Vaxfectin®-gD2 and Vaxfectin®-gD2/UL46/UL47 elicited detectable neutralizing antibodies after the second immunization (titers 2560 and 1280, respectively). Titers increased following the third immunization (Vaxfectin®-gD2 10240; Vaxfectin®-gD2/UL46/UL47 5120) indicating that both vaccines elicited robust and comparable functional humoral immune responses.

GENITAL TRACT INFECTION AND INITIAL VAGINAL HSV-2 REPLICATION

Vaginal HSV-2 DNA was detected in all animals in each group on multiple days after viral challenge, indicating that neither vaccine prevented vaginal virus infection. Virus load in both vaccine groups was significantly lower than controls on all days

(p<0.0001, mixed model ANOVA on group by day; **Figure 2.1**). Although the viral load was consistently lower in animals immunized with Vaxfectin®-gD2/UL46/UL47 than those receiving Vaxfectin®-gD2 on all days the differences did not reach significance.

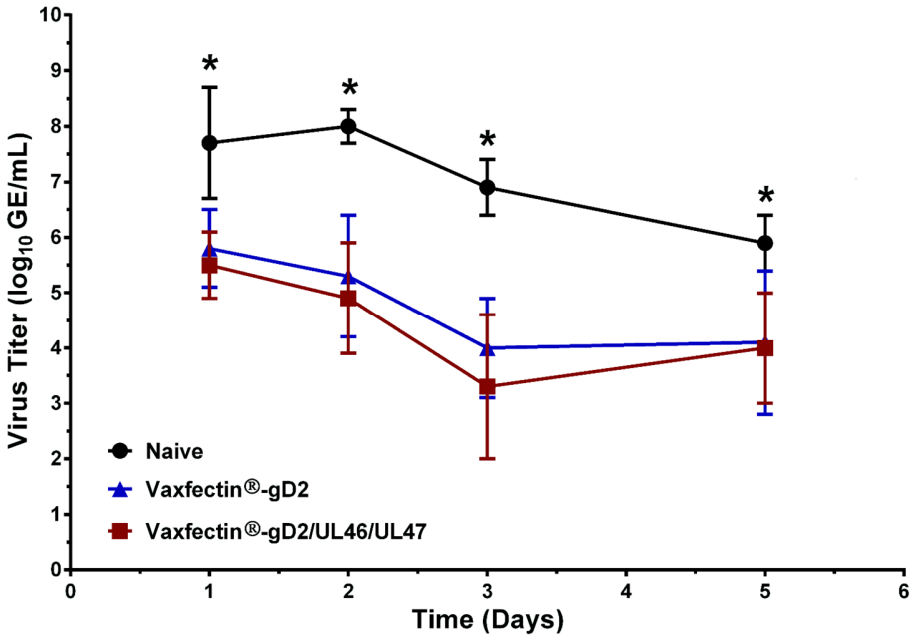

Figure 2.1. Effect of prophylactic vaccination on primary vaginal HSV-2 titers.
Vaginal virus titers were determined by qPCR on days 1-5 after intravaginal HSV-2 challenge in guinea pigs immunized three times with Vaxfectin®-gD2, Vaxfectin®-gD2/UL46/UL47 or saline controls. Titer values are mean ± SD genome equivalents/ml. Effect of vaccination was compared using a mixed model ANOVA on group by day.
* p<0.0001 compared to saline controls for both vaccinated groups.
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GENITAL SKIN DISEASE

Table 2.1 shows that all control animals developed primary vesiculo-ulcerative disease and subsequently 10/11 control animals experienced spontaneous recurrent disease (4 control animals were lost to analysis of recurrent disease due to the severity of primary infection). In contrast, both vaccine formulations provided complete protection against both primary (p<0.001 each, Fisher's exact test) and recurrent (p<0.001 each, Fisher's exact test) genital skin disease.

Table 2.1. Prophylactic vaccination with Vaxfectin® pDNA prevents primary and recurrent disease in the guinea pig model.

Group	Primary disease		Recurrent disease	
	Incidence	Severity[a]	Incidence	Frequency[b]
Naïve	15/15	9.3 ± 4.7	10/11	5.5 ± 3.9
Vaxfectin®-gD2	0/15[c]	0 ± 0	0/15[c]	0 ± 0
Vaxfectin®-gD2/UL46/UL47	0/15[c]	0 ± 0	0/15[c]	0 ± 0

[a] Primary disease severity defined as cumulative daily lesion score, mean ± SD.

[b] Recurrent disease frequency defined as recurrent lesion days between days 15-63 post-inoculation, mean ± SD.

[c] $p<0.001$ compared to control, Fisher's exact test.

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qPCR Determination of Virus Shedding and Latent Viral Load

To further evaluate the protection provided by the two vaccines, vaginal swabs collected from days 21-41 post-challenge were evaluated by qPCR to determine the number of animals shedding HSV-2. Immunization with either Vaxfectin®-gD2 or Vaxfectin®-gD2/UL46/UL47 reduced the number of animals that shed virus, with 6/15 in each vaccine group, compared to 9/11 control animals ($p=0.05$ for both vaccine groups, Fisher's exact test).

At the conclusion of the study, DRG were harvested and latent viral DNA load determined. It should be noted that one animal in the control group died as a result of unrelated complications prior to DRG harvest, resulting in 10 animals for this group. **Figure 2.2** shows that HSV-2 DNA was detected in all control animals (mean 3.3±0.5 $\log_{10}$ GE/mL). In contrast, immunization with Vaxfectin®-gD2 reduced both the number of animals in which viral DNA was detected (5/15 vs 10/10; $p<0.001$, Fisher's exact test) and the amount of virus in those animals (mean 1.9±0.6 $\log_{10}$ GE/mL; $p<0.001$ Student's t-test). The impact of immunization with Vaxfectin®-gD2/UL46/UL47 was even greater with HSV-2 DNA being detected in only one animal ($p<0.0001$, Fisher's exact test; viral load 1.6 $\log_{10}$ GE/mL); the remaining 14 animals were below the limit of detection of our assay.

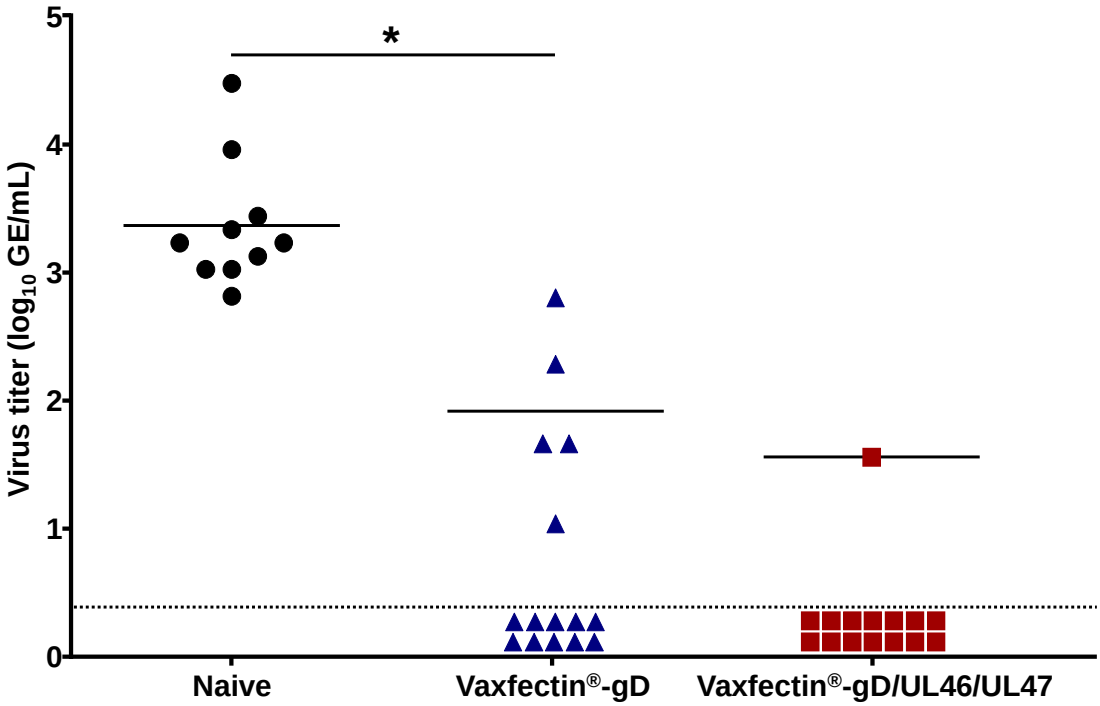

Figure 2.2. Effect of prophylactic vaccination on latent HSV-2 burden in the dorsal root ganglia.

Following determination of the effect of vaccination on recurrent disease, dorsal root ganglia were harvested from guinea pigs vaccinated with Vaxfectin®-gD2, Vaxfectin®-gD2/UL46/UL47 or saline controls. Dorsal root ganglia were collected from surviving animals (10 control and 15 in each vaccine group) on Day 67 and stored -80C until the latent HSV-2 burden was determined by qPCR. Viral load data for individual animals is shown. Horizontal lines indicate group averages. Animals below the dotted line were below the level of detection for our assay and were not included in group means for HSV-2 DNA load. As only one animal in the Vaxfectin®-gD2/UL46/UL47 had a detectable level of HSV-2 DNA in the DRG, no statistical comparison could be made between this group and the Vaxfectin®-gD2 or saline controls. * indicates $p<0.001$ compared to saline controls, Student's t-test. Reproduced with permission.

Therapeutic Vaccine Studies

STUDY 1

We first examined the ability of Vaxfectin®-gD2/UL46/UL47 and Vaxfectin®-UL46/UL47 to impact recurrent disease when administered following resolution of primary disease. **Figure 2.3A** shows that the frequency of recurrent disease in animals immunized with Vaxfectin®-UL46/UL47 was comparable to controls (8.7±4.6 vs 8.1±4.7

mean ± SD recurrent lesion days) between days 15-63 post viral challenge. However, animals immunized with Vaxfectin®-gD2/UL46/UL47 experienced significantly less recurrent disease (5.1±4.6 recurrent lesion days; $p<0.05$, ANOVA with Bonferroni correction).

STUDY 2

A second study was undertaken to confirm the therapeutic efficacy of Vaxfectin®-gD2/UL46/UL47 on recurrent disease. **Figure 2.3B** shows that the vaccine again significantly reduced recurrent disease compared to controls (6.07±4.7 vs 11.4±6.6 recurrent lesion days, $p<0.05$, ANOVA with Bonferroni correction). In addition, we examined the impact of therapeutic immunization on vaginal viral shedding between days 46-59 post challenge by qPCR (**Table 2.2**). As with the impact on recurrent disease, immunization significantly reduced the number of days on which shed virus was detected compared to controls ($p<0.05$, Student's t-test), but not the amount of virus shed per episode.

Table 2.2. Therapeutic vaccination with Vaxfectin®-pDNA vaccine decreases days of viral shedding.

Group	Animals shedding virus	Days of virus shedding [a]	Amount of virus shed (log_{10} GE/mL)[b]
Naïve	12/14	2.29 ± 0.41	2.91 ± 0.19
Vaxfectin®-gD2/UL46/UL47	9/14	1.07 ± 0.27[c]	2.45 ± 0.20

[a] Mean (± SE) number of shedding days/animal over the 14 day period.
[b] Mean (± SE) HSV-2 genome copies per shedding event.
[c] $p<0.05$ compared to controls, Student's t-test.
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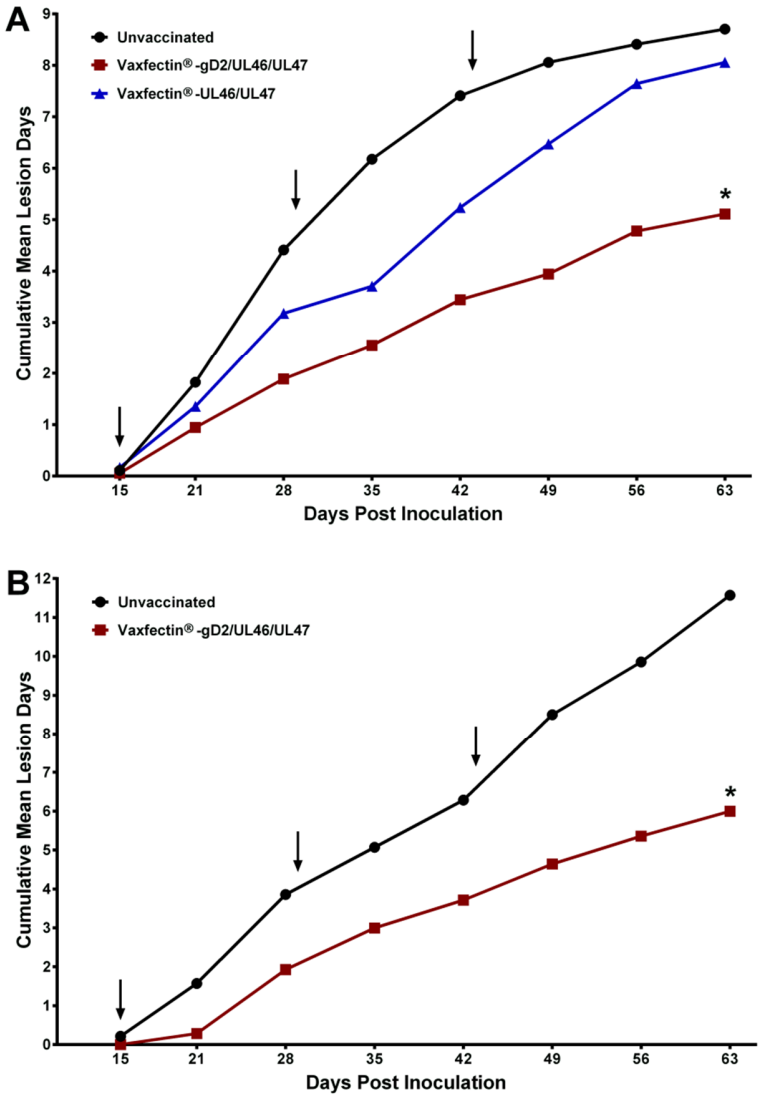

Figure 2.3. Effect of therapeutic vaccination on the frequency of recurrent genital skin disease.

Results are cumulative frequency of recurrent genital skin lesions over time. Arrows indicate days of vaccination relative to intravaginal HSV-2 challenge. **A**) Study 1: animals were vaccinated with Vaxfectin®-gD2/UL46/UL47, Vaxfectin®-UL46/UL47 or were saline controls. Statistical comparisons were made using the cumulative mean lesion day values at Day 63 for each of the three tested groups using ANOVA with Bonferroni correction. **B**) Study 2: animals were vaccinated with Vaxfectin®-gD2/UL46/UL47 or were saline controls. * indicates $p<0.05$ compared to saline controls, Student's t-test. Reproduced with permission.

DISCUSSION

Recent studies showed that addition of Vaxfectin® to a full length codon-optimized gD2 pDNA vaccine (Vaxfectin®-gD2) increased survival in a mouse genital herpes model compared to a gD2 pDNA vaccine alone. Further, Vaxfectin®-gD2 immunized mice had reduced vaginal viral replication and surviving animals had less latent viral DNA in the DRG than animals immunized with gD2 pDNA only [116]. Further, we found that addition of the adjuvant Vaxfectin® to our vaccine formulation (Vaxfectin®-gD2) was sufficient to completely abrogate HSV-2 disease during primary HSV-2 infection in the guinea pig model of genital herpes. This contrasts with a study by Strasser *et al*. where a gD2 pDNA vaccine was not able to completely protect animals from acute HSV-2 disease [120]. It should be noted that while both the gD2 construct and the amount of pDNA used for immunization in the studies of Strasser *et al*. differed from our studies, we believe that our data shows that the adjuvant Vaxfectin® will be an important tool for developing more effective herpes vaccines.

We and others have shown that prophylactic immunization with HSV-2 gD pDNA vaccines without adjuvants provides some protection against genital herpes in the guinea pig model [112, 120]. Given the results in murine studies with Vaxfectin®-adjuvanted pDNA, we examined the protection afforded by prophylactic immunization with Vaxfectin®-gD2 in the guinea pig, which allows the impact of immunization on spontaneous recurrent disease to be evaluated. Because optimal protection against genital herpes will likely require a vaccine containing multiple antigens, we included a Vaxfectin®-gD2 vaccine with plasmid DNAs for genes UL46 and UL47 that encode viral tegument proteins VP11/12 and VP13/14, respectively [8, 9]. Both proteins contain epitopes important in $CD4^+$ and $CD8^+$ T-cell responses in humans [121], making them good candidates for a more broadly immunogenic vaccine. It should also be mentioned that VP11/12 can activate Lck signaling in T-cells [122]. This activation does not globally alter

all Lck-dependent signaling events and the HSV-induced signaling blockade of the T-cell receptor does not require this protein [122], therefore incorporation of the UL46 plasmid should not affect the immunogenicity of this vaccine. In our study, neither vaccine prevented genital infection following a high titer viral challenge. It is worth noting that a number of other prophylactic vaccines evaluated in the guinea pig have also failed to elicit sterilizing immunity [112, 123]. Further, guinea pigs latently infected with HSV-2 and rechallenged intravaginally are not protected against vaginal viral replication [124]. Together, these data indicate that it is extremely difficult to elicit immune responses that provide complete protection of the genital mucosa against large viral inocula.

Although neither vaccine provided sterilizing immunity, both significantly reduced viral replication in the genital tract during primary infection. While the vaginal viral load in animals immunized with the two vaccines did not differ significantly, it was consistently lower in Vaxfectin®-gD2/UL46/UL47 than Vaxfectin®-gD2 animals.

All controls developed primary genital skin disease and subsequently all but one of the animals that could be evaluated developed spontaneous recurrences. In contrast, both vaccines provided complete protection against primary and recurrent genital disease. We also examined the impact of immunization on the number of animals in which vaginal viral shedding was detected. The incidence of shedding was high among controls (9/11) and both vaccines produced a comparable reduction (6/15 each; p=0.05, Fisher's exact test). At the conclusion of the study we examined the latent viral load in the DRG. HSV-2 DNA was detected in all controls. Immunization with Vaxfectin®-gD2 significantly reduced both the number of animals with detectable HSV-2 DNA and the viral load in those animals. Immunization with Vaxfectin®-gD2/UL46/UL47 produced an even greater effect, reducing the burden of latent virus below the limit of detection of the qPCR assay in all but one animal.

While the development of an effective prophylactic vaccine to control genital herpes is important, it will not benefit those already infected with HSV-2. A number of

therapeutic vaccine strategies have been evaluated in the guinea pig model. In early studies, glycoprotein-based vaccines reduced recurrent disease; however, results were variable and highly dependent on both the antigen and adjuvant selected [120, 125]. Interestingly, in one study therapeutic immunization also reduced vaginal HSV-2 shedding measured by the number of days that infectious virus was recovered by culture from vaginal swabs [126]. The efficacy of glycoprotein vaccines used therapeutically in these studies resulted in a number of clinical efficacy trials. In the first of these, two immunizations with a vaccine containing 100 µg gD2 protein with alum reduced recurrent disease [127]. However, a second study using 4 immunizations with a vaccine containing 10 µg each of gD2 and gB2 proteins with MF59 failed to significantly reduce recurrent disease resulting in discontinuation of this candidate [128]. Recently there has been a resurgence of interest in therapeutic immunization for genital herpes due in part to the potential not only to reduce recurrent disease, but also asymptomatic shedding, a major source of virus transmission [8, 129]. Both short-term clinical trials with suppressive antiviral therapy and mathematical modeling for prophylactic vaccines strongly suggest that decreased shedding is critical in reducing genital herpes transmission [89, 130, 131]. One advantage of therapeutic immunization over suppressive antiviral therapy in this regard would be that it would not be dependent on long-term compliance to a daily treatment regimen.

Consequently, we examined the impact of therapeutic immunization with Vaxfectin®-gD2/UL46/UL47 beginning after primary genital skin disease resolution. Because $CD8^+$ T-cell responses are known to be important in the control of recurrent lesions in humans [51], we also included a group of animals immunized with Vaxfectin®-UL46/UL47 alone. Vaxfectin®-gD2/UL46/UL47, but not Vaxfectin®-UL46/UL47, significantly reduced recurrent disease. The different activities of the two vaccine formulations in this therapeutic setting strongly suggests that the efficacy of Vaxfectin®-gD2/UL46/UL47 resulted from the combined antigen formulation rather than non-specific pDNA effects. To confirm the observed therapeutic immunization impact, a second study

was undertaken with Vaxfectin®-gD2/UL46/UL47. Here, immunization again significantly reduced recurrent disease with the impact in both studies being similar (41% and 47%). The reduction in recurrent disease seen with Vaxfectin®-gD2/UL46/UL47 contrasts results reported for bupivacaine-formulated gD2 pDNA which failed to significantly reduce recurrent disease [119]. This strongly suggests that the combination of Vaxfectin® adjuvant and the addition of UL46/UL47 pDNAs to the vaccine are important for its therapeutic efficacy. Further, in the second study we examined the impact of immunization on vaginal virus shedding over 14 days and showed that the number of shedding events in immunized animals was reduced by >53% compared to that in controls ($p<0.05$, Student's t-test). The amount of viral DNA detected during shedding events was comparable in immunized and control animals. To our knowledge this is the first demonstration that a therapeutic vaccine can impact the frequency of shedding into the genital tract as well as the frequency of recurrent disease. Studies to further the development of the therapeutic vaccine are currently in the planning stages. These studies will include additional controls, including irrelevant plasmid formulated with Vaxfectin®.

These studies show that prophylactic immunization with Vaxfectin®-gD2/UL46/UL47 afforded excellent protection against genital herpes disease. Importantly, the vaccine also reduced recurrent disease when used therapeutically. An intervention that could be used periodically to provide an extended period of reduced recurrent disease would be an important addition to treatment options for genital herpes. Further, the observation that therapeutic immunization with Vaxfectin®-gD2/UL46/UL47 significantly reduced the frequency of virus shedding has public health implications given that this is thought to be a major source of transmission. It is important to note that while immunization reduced the potential for transmission of the virus due to a reduction in the frequency of genital tract shedding, the potential for infection by exposure during a shedding event is not decreased. However, these promising results strongly suggest that Vaxfectin®-gD2/UL46/UL47 warrants further development as a therapeutic vaccine and if

the results of our studies can be replicated in clinical trials, this vaccine would provide an excellent alternative to suppressive antiviral therapy.

However, it must be noted that we are currently limited in our ability to evaluate the subtleties of the immune response in the guinea pig due to limitations in available immune reagents for this species. Thus, while our studies would suggest the importance of including the T-cell targets UL46 and UL47 to the efficacy of this vaccine formulation, we do not currently have sufficient means to fully explore this hypothesis. Our future research efforts will therefore be directed at the development and optimization of guinea pig-specific immune assays that will greatly expand our ability to evaluate the immune response in this important research species.

CHAPTER III[§]

Development and utilization of a custom PCR array workflow: analysis of gene expression in *Mycoplasma genitalium* and guinea pig (*Cavia porcellus*)

INTRODUCTION

In the previous Chapter we discussed the lack of guinea pig-specific immune reagents that hinders the characterization of the immune response in this important research species. Our goal was to therefore create a cost-efficient and reproducible method that would allow a more detailed examination of immune events in the guinea pig. This would provide a means to better evaluate the inclusion of the two T-cell antigens in the vaccine initially tested and discussed in Chapter II.

However, for many novel, emerging or under-studied organisms the lack of assays to quantify and characterize proteins and phenotype necessitate the evaluation of nucleic acids. While partial or complete genomic sequence may be available for many of these organisms, the expertise and cost required for *de novo* sequencing or to develop assays for measuring transcription level changes can be a significant deterrent to the development of custom in-house reagents. The lack of demand for niche products to address under-studied or emerging organisms further diminishes the likelihood of commercial production of reagents.

Transcriptional analysis can yield important information about expression patterns for developmental processes, evolving gene regulation, and for highlighting differences

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between healthy and diseased states [132]. For the last few decades, gene expression analysis has been dominated by the use of microarrays. Recently, new approaches using next-generation sequencing (NGS) have led to the development of improved transcriptomic techniques such as RNA-seq [133, 134]. However, transcriptome analysis by either microarray or RNA-seq are currently widely utilized in a discovery role for large-scale gene expression studies [134] and transcript-level analyses made possible by both technologies have revolutionized molecular biology [133, 135]. Microarrays are especially useful to explore gene expression of multiple cellular pathways in parallel and these assays are widely available for a number of model organisms [134]. The newer RNA-seq technologies can also fill this role and additionally offer lower background signal over microarray techniques [134]. Coupled with the detection of transcripts that may be at very high or very low abundance levels [134], RNA-seq is quickly displacing microarrays as the preferential tool for gene expression analysis [136, 137]. While these methods have undoubtedly increased our understanding of transcriptomics, they require specialized equipment and expensive reagents, particularly with respect to RNA-seq, leading to increased costs per sample interrogated. An additional concern surrounding RNA-seq is the introduction of bias at many steps during the process associated with technical inexperience, leading to unwanted variation across samples [132, 136, 138, 139].

Real-time reverse transcription PCR (RT-PCR) has been a widely used methodology for detecting transcriptional changes in mRNA [140, 141] and is the preferred method for validating both microarray and RNA-seq gene expression studies [134, 142]. Recently, this technique has been used to create PCR arrays for use in high-throughput gene profiling. These arrays combine ease of use, reliability, and reproducibility of RT-PCR into a more cost-effective method for screening large numbers of genes simultaneously [134] and so provide an attractive alternative to microarray or NGS methodologies. However, their utility is currently limited due to the availability of arrays for only a few of the more widely used research species. The restricted number of genes

that may be analyzed simultaneously also presents another disadvantage to this methodology [134].

Optimal screening of broad transcriptional changes by RT-PCR requires a design approach that allows for up to 96 targets to be processed through a single set of PCR parameters. PCR primer design has been described previously for single target and multiplex designs [143] but required some enhancement to address the needs created by the transcriptional array. Our goal was to create a highly adaptable and cost-effective workflow with enhanced primer design, a broadly useful PCR program and algorithms for data analysis to facilitate gene expression analysis in under-studied organisms. As a result we present a flexible PCR array development and validation method that creates adaptable screening tools to study changes in the transcriptome of any species with adequate genomic sequence. As proof of concept, we present the successful development and validation of two disparate arrays targeting the bacterial pathogen *Mycoplasma genitalium* (MG) and the commonly utilized guinea pig (*Cavia porcellus*), both of which currently lack commercially available assays for transcriptome analysis.

MATERIALS AND METHODS

Optimized Thermocycling Protocol

To identify a thermocycling protocol central to the functionality of our array platform, we first tested four established protocols using primer pairs from our first-generation designs. These first evaluations targeted RNA species produced by the sexually-transmitted bacterial pathogen MG. Specifically, type strain MG G37 genomic sequence (GenBank Accession: L43967) was used as a template to design 11 sets of forward and reverse primers for highly conserved genes within the bacterium using generic primer selection parameters. Each primer pair was used to amplify three distinct concentrations of purified MG genomic DNA covering a range of 10^5-10^7 copies per reaction. The resulting quantification cycle (C_q) values [143], generated during SYBR green-based real-time PCR

amplification, provided the opportunity to calculate the amplification efficiency of the 11 primer pairs using each of the four PCR thermocycling protocols. These preliminary studies identified a protocol that produced the most consistent PCR efficiencies across the 11 primer pairs. In addition, a melt temperature (T_m) analysis was performed to provide information on the identity of the amplimer created in each reaction.

The optimal protocol identified by this initial evaluation was repeatedly modified by empirical testing of additional primer pairs to refine the thermocycling conditions. This resulted in a stable set of parameters to serve as the fundamental PCR protocol for our array system. The resultant protocol, designated "KS," was established as our standard and subsequently used to evaluate and refine primer design methods to maximize successful targeting of selected genes.

Primer Design and Optimization

Primers were designed using Beacon Designer v7.91 (PREMIER Biosoft; Palo Alto, CA, USA) and regions of each gene were selected by prescreening to identify areas that were highly conserved based on alignments of all available sequences. Initial observations from data obtained during our thermocycling protocol validation studies suggested that primers composed of less than 40% guanine/cytosine (G/C) content were more efficient with the optimized KS protocol, a finding that was confirmed through subsequent primer design and testing studies. Thus, for maximum compatibility with the array platform, the optimal primer should incorporate ≤40% G/C content. Our results also correlated PCR amplimer length with overall primer efficiency success rates. The data from almost 200 primer pairs led us to conclude that optimal amplimer length should be between 70 and 200 base pairs (bp) and led to the primer design specifications described below. All subsequent primer design was undertaken using these parameters.

Forward and reverse oligonucleotide primers were designed with custom settings in Beacon Designer v7.91. Previously reported primer design approaches [143] provided

the basis for the initial parameters that were then empirically optimized. Primer length was 18-24 nucleotides and PCR amplimers were selected to be between 70 – 200 bp with primers designed to contain ≤40% GC where possible. Primers were purchased as desalting-purified lyophilized material from Sigma-Aldrich (St. Louis, MO) and reconstituted with sterile Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA Na2, pH 8.0, Promega, Madison, WI) to a concentration of 500 µM. Aliquots of each forward and reverse primer were made by dilution to a concentration of 5 µM in TE buffer. All primer stocks were stored frozen at -20°C until plated for array assembly. Genomic sequence for the guinea pig (*Cavia porcellus*) was downloaded from the e!Ensembl database (http://useast.ensembl.org/Cavia_porcellus/Info/Index); sequence for *Mycoplasma genitalium* (prototypic laboratory strain G37) was obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Primer Validation

Primer pair candidates were required to pass two validation criteria before being considered for inclusion onto an array assembly. A generalized flow chart of this primer pair validation process is shown in **Figure 3.1**.

Equivalent PCR efficiency is an essential aspect to the effective development of a multi-target system to be used on one sample to produce semi-quantitative data. Based upon extensive experience designing and implementing quantitative PCR (qPCR) assays (13-18), efficiencies between 80-120% with associated correlation coefficients ≥0.95 are required to provide optimal data for comparing transcription levels of selected genes within a sample and to ensure correct normalization across comparator samples. To determine the PCR efficiencies of newly designed primer pairs, a PCR master mix was made (see section "PCR") for each primer pair set and a series of three, 10-fold dilutions of DNA (for *Mycoplasma genitalium* primers) or cDNA (for *Cavia porcellus* primers) was prepared and used as the template for the PCR. This series of dilutions served as a calibration curve that

provided an accurate assessment of efficiency for each putative gene target. Additionally, a negative template control (NTC) was included on all validation runs to identify primer pairs that formed primer-dimers in reactions with little or no target sequence. Amplification of a primer pair that results in a PCR product from primer-dimer formation may still be

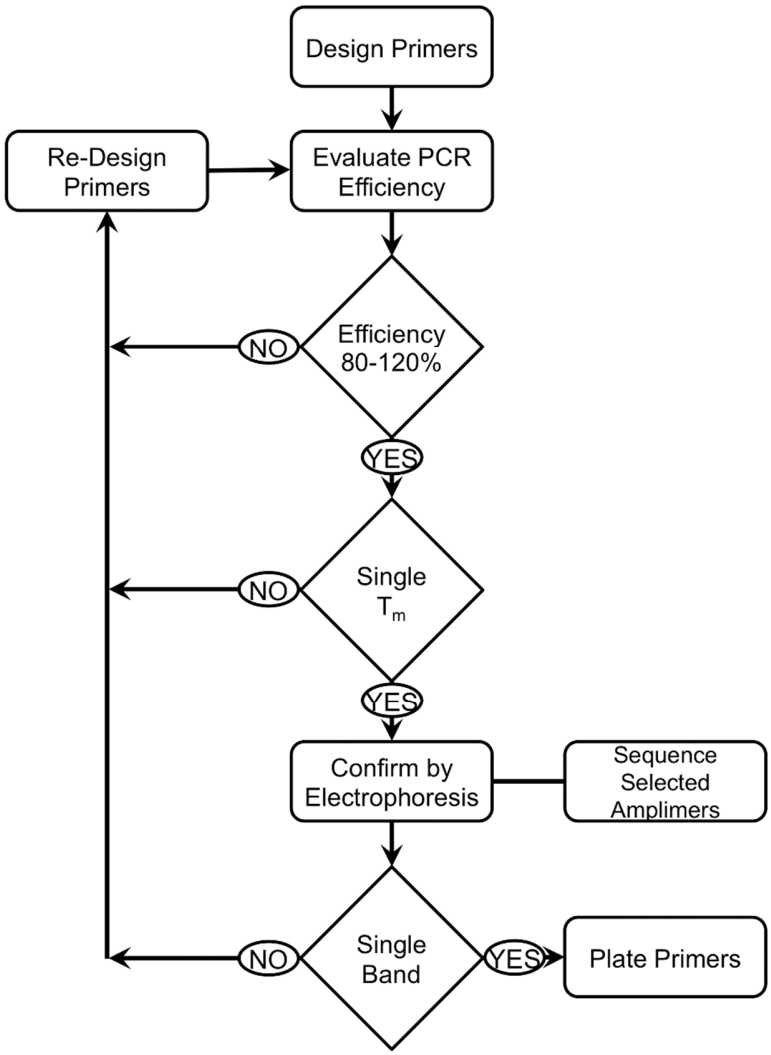

Figure 3.1. Generalized workflow for primer validation.
Primers first met PCR efficiency criteria (80-120%). Secondary evaluation of target specificity used high resolution melt temperature analysis to confirm the presence of a single melt peak. Final confirmation of primer specificity utilized electrophoresis through agarose to ensure only one product was produced, validating the primer pair and establishing the expected T_m for each corresponding amplimer. Approximately 40% of all amplimers were sequenced with 100% confirmation of their identities. Reproduced with permission.

considered for inclusion on an array build provided that the T_m obtained varies by at least 5°C from the T_m of the expected product (**Figure 3.2C**). If a primer pair results in a PCR efficiency of 80-120%, primer pair specificity is next confirmed to ensure the fidelity of the reaction.

Primer specificity was a second and equally important consideration during the validation process. Confirmation of the identity of each amplimer was completed by melt temperature analysis at the end of the amplification cycles to create a specific T_m for each of the amplified products. A single T_m suggested that only one PCR product was produced. In cases of a single T_m (**Figure 3.2A**), agarose gel electrophoresis confirmed the product size. Successful amplification was indicated by a single band that corresponded to the predicted amplimer size. During optimization, if multiple T_m's (**Figure 3.2B**) or a band of an unexpected size was observed, the primer pair was considered sub-optimal and was subjected to redesign and re-validation. Sanger sequencing of randomly selected PCR products (to date approximately 40% of the total targets) showed 100% specificity was achieved using our validation approach. The T_m established for each successful primer pair was used as a subsequent quality control evaluation for experimental runs prior to analyses of gene expression differences. This workflow, using the optimized KS protocol and refined PCR primer pair design parameters, created an overall success rate of 80% for over 400 first round primer pairs tested to date. Second round re-design led to success for the majority of the targets, although 5% required a third round of design, suggesting that a small subset of target genes may fail our array approach.

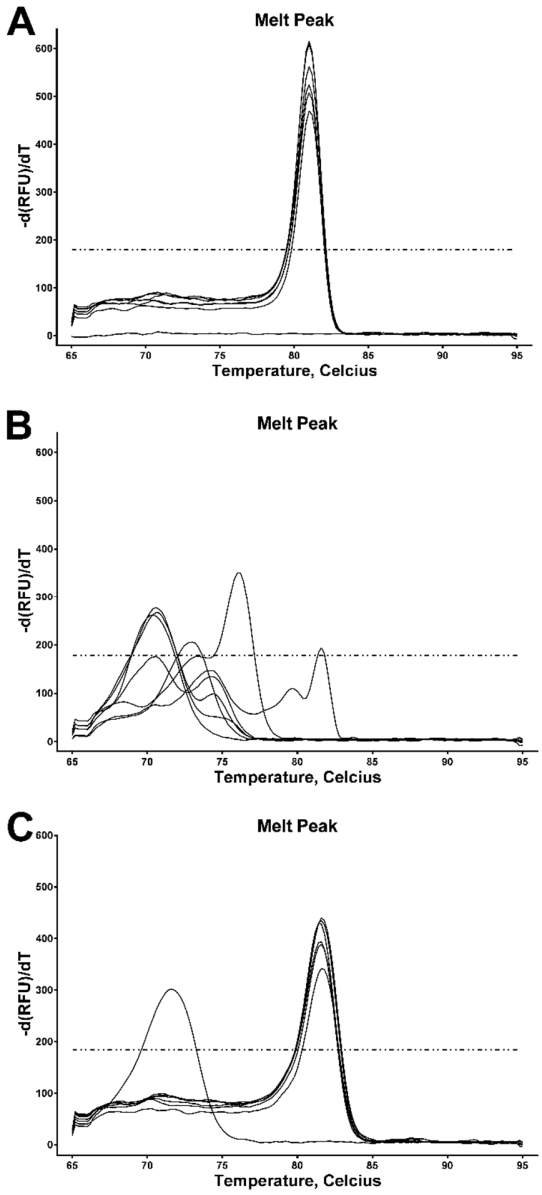

Figure 3.2. Secondary primer pair validation.

Target specificity was confirmed by melt temperature (T_m) analysis for all putative primer pairs of an array. All reactions were carried out as three, 10-fold dilutions analyzed in duplicate. **A)** A single melt peak indicated production of one product and a passed secondary evaluation. **B**) Multiple melt peaks were indicative of off-target primer binding or primer-dimer formation, resulting in multiple amplification products. The lower panel primer pair did not pass validation and was re-designed. **C**) An example of a primer pair that produced a primer-dimer in the no template control well. This primer pair passed validation as the T_m of the primer-dimer was ≥5°C from the expected T_m. All panels: the dotted line indicates the baseline threshold. Reproduced with permission.

Assembly of Array Plates

Validated, optimized primer pairs were robotically plated into 96-well skirted PCR plates. Use of a 12-channel pipet for distribution of primers to array plates was confirmed to be a viable, although laborious, alternative to robotic plating. After array plating, multiple array plates were chosen at random and tested to address quality and contamination concerns. Water only control PCR confirmed that no contamination occurred during the plating process. Assembled array plates were tested using target nucleic acid to confirm primer activity conformed to results obtained during optimization and validation studies.

For the guinea pig host immune response transcription array (gpArray), two additional quality controls were conducted after array plates were produced. Purified Vero and human DNA were tested in the gpArray to determine if the guinea pig primer pairs would recognize homologies in those genomes. These DNAs were specifically selected to identify possible sources of contamination that could be introduced at the time of sampling or during downstream sample manipulation: human DNA from laboratory personnel collecting and preparing the samples and Vero cell DNA to control for extraneous genetic material present in virus stocks grown in Vero cells and introduced during inoculation of test animals. Interestingly, both Vero and human DNA were recognized by the guinea pig primers (data not shown) and resulted in successful amplification of gene targets. Melt temperature analysis of the corresponding PCR amplimers showed that 82% of the resulting Vero T_ms and 85% of the human T_ms were sufficiently different (±0.8°C) to be distinguished by our standard quality assessment prior to data analysis. Of those targets that were ≤0.8°C, only 7 Vero and 7 human DNA amplimers produced T_ms that were indistinguishable from their guinea pig counterparts.

RNA Extraction and cDNA Synthesis

Template RNAs were extracted using the Aurum Total RNA 96 kit (Bio-Rad) following the manufacturer's instructions and included an on-column DNase I digestion to remove genomic DNA contamination. Prior to reverse-transcription of the RNA using the iScript cDNA Synthesis kit (Bio-Rad), lack of DNA contamination was confirmed using a Taqman qPCR targeting the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). cDNA synthesis reactions were carried out in a final volume of 60 µl per sample and contained the following reaction components: 12 µl 5x iScript Reaction Mix, 3 µl iScript Reverse Transcriptase (225 units), and 45 µl template RNA (~600 ng). cDNA synthesis was carried out using a three-step PCR protocol of 25°C for 5 minutes followed by 30 minutes at 42°C and a final 5 minute incubation at 85°C to stop the reaction. Resulting cDNAs were stored frozen at -20°C until assayed.

Production of Arrays

For array plating, a fresh 1 ml primer stock containing both forward and reverse primers for each gene target was prepared in a sterile 1.8 mL cryovial at a final concentration of 434.8 nM in TE. This concentration of primer stock was used to ensure a final 0.2 µM concentration for both forward and reverse primers upon addition of reaction mix (see next section "PCR") to all wells of the array plate. The aliquots were stored on ice until primer mixes were produced for all targets on a given array. The cryotubes were briefly centrifuged (300 RCF, ~30 seconds) before being pipetted into 96-well skirted PCR plates (Thermo Fisher Scientific, Waltham, MA) at a volume of 11.5 µl per well using sterile, RNase/DNase-free filter tips (Mettler-Toledo, Columbus, OH). Initial plating was accomplished using a 12-channel pipet (Mettler-Toledo), however subsequent plating utilized a Tecan Evo 200 robotics platform (Tecan US, Inc., Morrisville, NC) to increase the throughput, efficiency and accuracy of primer distribution. Finished array plates were

covered with sterile foil sealing tape (Thermo Fisher Scientific) and centrifuged briefly (300 RCF, ~30 seconds) to collect the liquid in the bottom of the wells. Array plates were stably archived at -20°C for long-term storage until used for sample screening. Validated gpArray and MG Array plates can be obtained from our lab group under MTA or through collaboration.

PCR

All PCR array reactions were completed in 96-well plates in a total volume of 25 µl. Prior to assembling the reaction mix, template cDNA was diluted 1:5 in sterile DNase/RNase-free water. The final volume for the PCR and dilution of starting material is a best practice suggestion based on our experience with PCR development [144-149]. The final reaction volume and/or cDNA dilution may be adjusted as determined by the end user through experimental in-house validation. The reaction mix was assembled using 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad) and 1 µl of diluted cDNA for each well to be tested. A volume of 13.5 µl of the SYBR-cDNA reaction mix was then added to each well of an assembled array plate containing primers (0.2 µM final primer concentration) using a 12-channel pipet and low-retention, RNase/DNase-free, filtered pipet tips (Mettler-Toledo) . Plates were then sealed with optical tape and briefly centrifuged (6000 RCF, ~30 seconds) to collect the liquid at the bottom of the wells prior to PCR. Reactions were carried out on either CFX or CFX Connect Real-Time PCR detection systems (Bio-Rad) using the optimized KS protocol consisting of an 8 cycle amplification of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds followed by a 40 cycle amplification of 95°C for 15 seconds, 56°C for 20 seconds and 72°C for 20 seconds during which real time data were acquired at the annealing step. C_q analysis was completed by the Bio-Rad CFX Manager software with a constant baseline adjustment to 50 relative fluorescent units for all array runs to provide for accurate comparisons of C_q values across gene targets.

A high resolution melt temperature analysis was included in the thermocycling program following the second amplification step. Starting at 65°C, the temperature was raised incrementally by 0.2°C every 5 seconds, followed by data acquisition to an ending temperature of 95°C. T_m values for all amplicons were evaluated by comparing each corresponding Tm to established historical values with an acceptable range of ± 0.8°C prior to data analysis.

Reference genes were needed to ensure reliable normalization across samples. However, the expression of many widely used reference genes can vary due to the tissue or cell type from which the sample is collected [141]. It has been shown that multiple reference genes should be used for normalization to avoid unintentional biasing of the results [150]. Thus, for all array runs, gene expression levels were normalized using the mean of four reference genes included on each plate using $^{\Delta\Delta}C_q$ [150, 151]. Fold change was calculated by the normalized gene expression of the test sample divided by the normalized gene expression of the control sample. Values greater than one represented an up-regulation and were considered equal to the fold change. Fold change values less than one were down-regulation and were represented by the negative inverse of the fold change.

Cloning of PCR Amplimers

Selected PCR amplimers (~40% of targets) of the proper size created by initial array runs were purified (QIAquick PCR Purification kit; Qiagen, Valencia, CA) and then cloned using the TOPO TA Cloning kit (Life Technologies, Carlsbad, CA). Selected clones that were within 0.2°C of their corresponding control were sequenced using an ABI Prism 3130XL Genetic Analyzer (Carlsbad, CA). Positive clones were also used to confirm T_m values and were archived for use in future single target qPCR assays.

qPCR Validation of Array Results

Each qPCR mix contained 1X iQ SYBR Green Supermix, 0.2 µM of each forward and reverse primer, and 1 µl template cDNA. Cloned amplimers for each gene target were included on each plate as a series of 10-fold dilutions ($10^6 – 10^2$) as quantitation standards to provide a means of calculating the quantities of each target gene. Two additional qPCRs were conducted in parallel using the housekeeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and beta actin to control for cDNA quality and quantity. Negative template controls were included to ensure PCR integrity and indicate potential contamination. All reaction efficiencies were between 80-120% with correlation coefficients of >0.95. Assay sensitivity for these reactions allowed for 100 copies to be detected 100% of the time. T_m analysis was used to confirm primer specificity and amplimer identity as described above. Target gene expression levels were normalized using the geometric mean of the housekeeping genes HPRT1 and beta actin prior to analyses. The mean of normalized test sample values for each target gene were divided by normalized control sample values to indicate the fold change for comparisons to array results. Values greater than one represented an up-regulation and were considered equal to the fold change. Fold change values less than one were down-regulation and were represented by the negative inverse of the fold change.

Guinea Pig Splenocyte Cell Culture

Female Hartley guinea pigs (250-350 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and housed in American Association for the Accreditation of Laboratory Animal Care-certified facilities. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch (UTMB) and all animals were humanely euthanized following UTMB IACUC-approved procedures. Guinea pig spleens were then harvested using sterile

technique and placed into Hank's Balanced Salt Solution (HBSS; Corning Life Sciences-Mediatech, Inc., Manassas, VA) supplemented with 5% (vol/vol) newborn calf serum (Life Technologies Incorporated, Carlsbad, CA) and 1% penicillin/streptomycin (10,000 U/ml penicillin/10,000 µg/ml streptomycin stock; Sigma-Aldrich). All manipulations of the tissues were conducted at room temperature. Spleens were minced and pushed through a metal mesh sieve to produce single-cell suspensions as described previously [152]. Cells were pelleted and washed with HBSS a total of 3 times. Erythrocytes were lysed by exposure to acetic acid prior to splenocyte enumeration. Cells ($1x10^6$ cells/well) were seeded into 24-well plates in medium containing RPMI 1640 (Corning Life Sciences-Mediatech, Inc.), 10% (vol/vol) fetal bovine serum (FBS; Corning Life Sciences-Mediatech, Inc.), 1% penicillin/streptomycin, 1% L-glutamine (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 1% non-essential amino acids (Sigma-Aldrich) and 50 µM β-mercaptoethanol (Sigma-Aldrich). Half of the wells were further supplemented with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 mM ionomycin to stimulate the cells. Cells were incubated (37°C, 5% CO_2) for 24 hours and then re-suspended in 0.2 ml Aurum Total RNA Lysis Solution (Bio-Rad, Hercules, CA) containing 1% BME, vortexed and stored at -80°C until used for RNA isolation and cDNA preparation.

Statistical Analysis

Comparisons to detect significant variations in expression for each target gene were carried out using a two-tailed Student's t-test after $2^{-\Delta Cq}$ conversion of individual C_q data to a linear form [151]. Comparisons between gene expression levels from qPCR assays were made using a two-tailed Student's t-test after log conversion of the qPCR results for each target with Prism software (v6.0; GraphPad, La Jolla, CA). For all comparisons a *P* value of 0.05 was used to designate significance.

RESULTS

Development of the RT-PCR arrays first required the selection and optimization of a permissive thermocycling protocol that would allow multiple, diverse primer pairs of differing annealing temperatures to amplify targets with similar PCR efficiencies. After optimizing a PCR thermocycling protocol, we refined sequence target selection parameters to create an optimal primer design paradigm that increased the success rate for selecting array-compatible primer pairs. Additionally, we established target quality control and validation methodologies to ensure adequate PCR efficiency and to confirm primer specificity. Finally, using optimized methods two disparate example array assemblies were completed and tested to confirm the flexibility and utility of our approach.

Assembled Transcription Arrays

Several genetically distinct target sets have been successfully evaluated using our optimized array design and assembly methods. As proof of concept we offer results for two distinct types of biological target sets with disparate goals and outcomes.

MG ARRAY. Our initial efforts to develop an array platform were driven by our research into the under-studied bacterial pathogen MG [147, 153]. This bacterium is responsible for genital tract infection and can develop an intracellular niche leading to persistent colonization of an infected individual [154]. MG provides a unique opportunity for studying the genes associated with infection and the establishment of persistence as it contains one of the smallest genomes capable of self-replication. To better understand the pathogenesis of this bacterium we designed primers to target 188 conserved genes representing ~40% of the known genes of this organism. Using the optimized workflow, the success rate for first-round primer design was 83% with an 80% success rate for the re-design of suboptimal primer pairs.

The assembled two 96-well plated array (see [155]) was utilized to study transcription profiles of two similarly cultured clinical isolates, MG 2300 and MG 2341

[156], under normal growth conditions. Array profiling of these two cultures showed 13 genes that were significantly up-regulated in MG 2300 compared to MG 2341 under similar conditions (**Figure 3.3**). Additionally, 27 significantly down-regulated genes were identified in the MG 2300 culture compared to that of cultures containing isolate MG 2341.

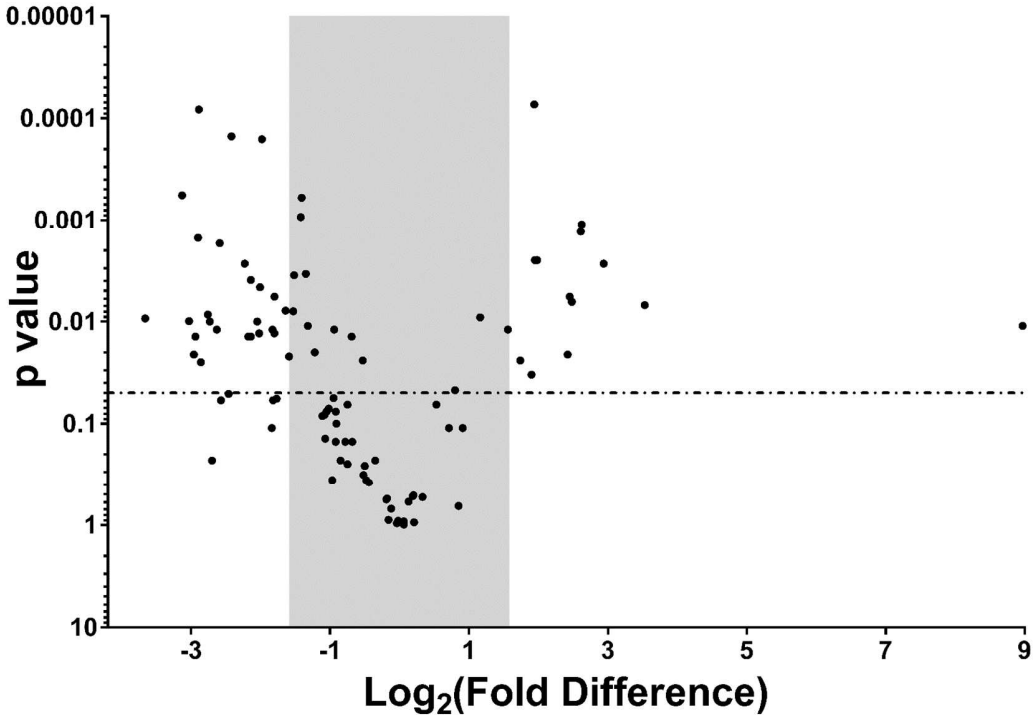

Figure 3.3. MG array comparisons between MG 2300 and MG 234.
Comparisons made between the two MG strains showed significant transcription level changes in 42% of genes evaluated. A plot comparing MG 2300 to MG 2341 showed that significant up-regulation was detected for 13 genes (upper right quadrant). Additionally, 27 genes were significantly down-regulated (upper left quadrant). The grey rectangle indicates a 3-fold up- or down-regulation. Approximately 58% of observed gene transcription levels were found to fall within this range and were considered unchanged. Selected genes were evaluated by qPCR and both their magnitude and direction of change were in agreement with the data obtained from the array. The dotted line indicates a *P* value of 0.05. Reproduced with permission.

GUINEA PIG ARRAY (GPARRAY). Due to its similarity to humans, the guinea pig is often used to study human infection and disease but currently suffers from a dearth of associated immunologic reagents [157, 158]. To increase our understanding of the immune response in these animals, we utilized the flexibility of our platform to develop an array

capable of characterizing transcription changes in the guinea pig. First-round primer design success rates for the gpArray were 73% with an associated 50% success rate for primer re-design, consistent with the greater complexity of this genome compared to that of MG. **Appendix I** provides a summary of primer pair sequences and associated T_ms for the gpArray. Final array assessment was conducted using guinea pig splenic cells cultured with phorbol myristate acetate (PMA) plus ionomycin, an immunostimulatory cocktail known to activate lymphocyte proliferation and cytokine production. These samples were studied for known shifts in transcription levels associated with PMA/ionomycin stimulation.

We first compared gpArray data from 2 sets of duplicate cultures of unstimulated splenocytes ($1x10^6$ cells/sample). A scatterplot (**Figure 3.4A**) of this comparison showed that no genes were up- or down-regulated more than 3-fold, indicating that there were no meaningful differences in transcription between these unstimulated samples. This method also helped to establish the level of biologic and technical noise in the PCR array. Comparison of array data from stimulated splenocytes with the untreated control values identified a number of genes that were differentially expressed (**Figure 3.4B**). When these results were statistically analyzed, transcription levels of 15 genes were significantly up-regulated and the transcription levels of an additional 55 genes were significantly down-regulated (**Figure 3.4C**).

Confirmation of identified differential gene expression

Sampling error and limits of detection can be serious confounders for generalized array approaches. To address these concerns, we utilized subsequent qPCR that included 10-fold dilution series of cloned amplimers from the gpArray as standard curves. Single target qPCR allowed for larger sample size with increased statistical power to confirm array indicated shifts in the transcriptome of the stimulated cell population. To establish this method and effectively define confidence in meaningful array-based differences, we selected qPCR targets representing the highest up- and down-regulated differences as well as differences representing mid- and minimally different fold changes established in the

arrays. Most of these targets were deemed as significantly different in treated guinea pig splenocytes.

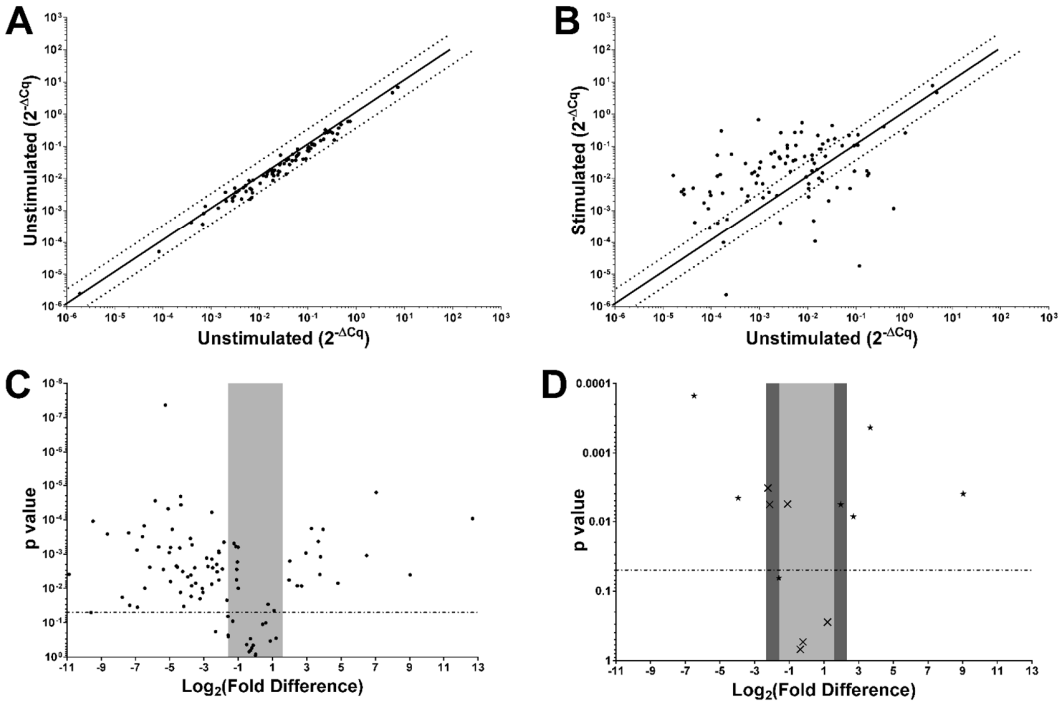

Figure 3.4. Initial validation of the gpArray.
Primary gpArray evaluations were undertaken using guinea pig splenocytes treated with medium only for 24 hr (unstimulated) or PMA/ionomycin (stimulated). Both **A**) and **B**): The solid line indicates baseline expression; the dotted lines indicated 3-fold up or down expression differences. **A**) Replicates of unstimulated splenocytes showed no differences validating the reproducibility of the array and establishing the level of biological and technical noise. **B**) When stimulated splenocytes were compared to unstimulated samples, 16% of genes were found to be up-regulated, 60% were down-regulated and the remaining genes showed no change. Both **C**) and **D**): The dotted line indicates *P* value of 0.05. The light grey rectangle indicates a 3-fold up or down change in expression levels. Gene transcription levels within this range were considered unchanged. **C**) Statistical significance of expression level differences from stimulated splenocytes compared to unstimulated; 15 genes were significantly up-regulated (upper right quadrant) and 55 were significantly down-regulated (upper left quadrant). **D**) qPCR results were concordant for direction of change and P value for 54% of genes evaluated (indicated by ★). Genes that were discordant with respect to direction, *P* value or both were found within a 5-fold up- or down-regulation and are denoted by (x). The dark grey rectangles indicate ±5-fold change in expression levels with all genes that showed >5-fold change corresponding to 100% concordance with array data as confirmed by qPCR. Reproduced with permission.

Initial evaluation of the array results suggested ±3-fold change would likely be meaningful but in pilot studies that were intentionally underpowered we discovered ±5-fold to be more accurate. Comparison of the array and qPCR data (**Table 3.1**) confirmed that all fold changes >5-fold were concordant and significant comparing triplicate stimulated samples to unstimulated controls ($P<0.05$). Of eight targets tested by qPCR with <5-fold difference indicated by the array, 2 matched both direction and P value; 3 matched direction only and the remaining 3 were discordant for both direction and significance (**Figure 3.4D**). gpArray results found to be ≤5-fold change therefore should be confirmed by an alternative method. Importantly, all values of >5-fold change are expected to be solidly concordant.

Table 3.1. qPCR validation of expression level changes from PMA/ionomycin-stimulated guinea pig splenocytes compared to unstimulated cells.

Gene	Array Indicated Fold Change	Array *P* value <0.05	qPCR Fold Change	qPCR *P* value <0.05
IFNγ	522.5 ↑	Y	2.3 ↑	Y
TLR-3	89.9 ↓	Y	1.4 ↓	Y
CD8α	15.4 ↓	Y	1.1 ↓	Y
CXCL10	12.8 ↑	Y	1.6 ↑	Y
OX40L	6.5 ↑	Y	4.7 ↑	Y
CD107b	4.7 ↓	Y	1.0 ↓	N
CD107a	4.4 ↓	Y	1.0 ↓	N
IL-21	3.9 ↑	Y	1.8 ↑	Y
B2M	3.0 ↓	N	1.0 ↓	N
IFNAR1	2.3 ↑	N	1.2 ↑	Y
CXCR3	2.1 ↓	Y	1.3 ↑	Y
TGFβ	1.3 ↓	N	1.2 ↑	Y
CD28	1.2 ↓	N	1.0 ↑	N

Y: Yes, indicates a *P* value <0.05
N: No, indicates a *P* value >0.05
↑: indicates up-regulation
↓: indicates down-regulation
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DISCUSSION

The work described here illustrates the utility of our array platform by providing two new resources for the evaluation of transcriptional profiling for organisms that currently suffer from a lack of commercially available assays. By utilizing the reliability of SYBR green-based chemistries [159], we have developed a cost-efficient, highly specific and reproducible methodology that can be adapted to any target species for which whole or partial annotation of its genome is available. Our enhanced array system builds on previous primer design and PCR parameters [143] to create a system compatible with standard molecular biology laboratories. Our array platform can accommodate any number of genes spanning multiple 96-well plates, making it feasible to screen one sample across many genes or narrowing the focus to evaluate only a few important genes using a "mini array." In this manner, multiple samples may be assayed per plate, reducing costs while increasing throughput. Thus, the platform is an ideal screening tool for detecting transcription changes from individual or biologically pooled samples to identify relevant, differentially-expressed genes for subsequent evaluations. Additionally, the targets included on each array can be converted easily to specific, sensitive qPCR assays using the primer sets already incorporated on the platform. Such assays enable efficient confirmation of the transcriptional changes detected by an array for many individual samples. Finally, our approach allows for the construction of a secondary array using only those genes identified and confirmed by qPCRs, further reducing overall costs and increasing the efficiency of array screens.

There has been intense development in the area of transcriptomics and associated technologies in recent years [136], resulting in several options for gene expression analysis. RNA-seq can provide *de novo* evaluation of gene expression in the absence of a reference genome [136], making this an attractive option for researchers working with an uncommon research species. However, this technology can be cost-prohibitive and requires a multi-

step sample preparation that can introduce unwanted bias into the results [138, 139]. In contrast, PCR arrays are relatively inexpensive on a per sample basis and can provide a more user-friendly approach to transcription analysis. Unfortunately, while many signaling pathways and disease states may be interrogated using commercially available PCR arrays, the limited number of target species prevents study of many important animal models.

Initial development of the array platform was driven by our research into the emergence of MG as a sexually transmitted pathogen [160, 161]. With the availability of commercial reagents lacking for such an under-studied organism, our goal was to provide an important tool for understanding the transcriptome of this bacterium. Thus, the MG array was developed to include individual genes that are crucial to motility [162, 163], important in regulating responses to environmental changes [164] and other potential pathogenicity cues [165].

Utilizing the MG array, we investigated the variability in gene transcription between different clinical isolates to identify genes important to survival or adaptation to the host environment. Screening detected transcriptional changes that could be associated with drug resistance or immune evasion. These observations resulted in a more refined list of gene targets and the flexibility of our platform provided the ability to easily re-configure a more specific, focused version of the MG array. This refined array will be used to identify new targets for therapeutic interventions and vaccine development.

As evidence of success with a more complex genome, we developed an array for the guinea pig because it is an excellent model for the study of infectious diseases [81, 158, 166-168]. The guinea pig boasts immune and pathophysiologic similarities to humans that make it a preferred animal for studying disease pathogenesis (38-40) and for evaluating new vaccines [119, 169]. Despite the value of this small animal model, full utilization of possible outcomes has been hampered by a lack of commercially available immunological assays. Independent researchers have attempted to overcome these limitations, however

the resulting assays are often specific for only a single target [157, 170, 171], restricting their utility to a smaller subset of research applications.

Using the validated gpArray, pilot studies were completed to compare PMA/ionomycin-stimulated splenocytes to unstimulated cells to identify changes in expression levels associated with this non-specific immune activator cocktail. This approach also helped validate the sensitivity of the gpArray providing defined cutoffs for meaningful differences identified by the array. As expected, the expression levels of interferon gamma (IFNγ) increased significantly compared to unstimulated controls. Not surprisingly, additional interferon-stimulated genes (CXCL10 and CXCL11) were also found to be up-regulated along with several interferon-induced lymphocyte activation markers (CD69 and CD223). These results were in agreement with known lymphocyte activation [172-174] and confirmed the gpArray could detect transcriptional changes and could be useful as a screening tool.

The changes in expression levels that were observed were confirmed by subsequent single target qPCR to validate initial array results. The qPCR of selected targets evaluated both large- and small-fold changes in up- and down-regulated genes and showed that transcription level changes of ±3-fold were less meaningful than suggested by initial array evaluations. Importantly, qPCR evaluations showed 100% concordance for expression changes of >5-fold and indicated that changes <5-fold should be confirmed by alternate approaches or increased sample size. Single target qPCR adapted from the existing primers on the array proved an efficient and straightforward means of addressing both statistical confirmation and increased numbers of samples analyzed in cost effective fashion.

Collectively, the presented data showed that our array development platform was a reproducible and highly adaptable resource for examining changes at the transcriptional level in disparate organisms that lack optimal assays for research. Importantly, the system can be used with only partial genomic sequence from pathogens and commensal organisms that are not yet cultured in a laboratory as well as from more complex species currently

lacking a fully annotated genome. To our knowledge, the MG array created by our method represents the first resource for evaluating large-scale expression level changes in this emerging, sexually transmitted pathogen. Additionally, while microarray technologies have been used to study the guinea pig previously [175, 176], restrictions inherent to these technologies (e.g. increased costs, diminished throughput that can reduce statistical power) can be overcome using RT-PCR arrays. With real time PCR instruments increasingly common in research laboratories, we believe RT-PCR arrays to be more approachable and easily adopted by scientists studying emerging organisms or species lacking available assays. In addition, this developmental platform has the potential to create tools to study multiple organisms present in a single sample, i.e. pathogen in the context of host, providing a simple and accurate new screening tool for gene expression analysis.

CHAPTER IV

Transcriptional Analysis of the Guinea Pig Mucosal Immune Response to Intravaginal Infection with Herpes Simplex Virus Type 2

INTRODUCTION

As we have detailed in previous Chapters, Herpes simplex virus type 2 (HSV-2) is the most common cause of genital herpes, one of the most prevalent sexually transmitted infections in the world [2, 3]. Infection is frequently asymptomatic, leaving many individuals unaware of their infection, but it can also be accompanied by painful skin or mucosal lesions. Replication at the mucosal site of infection is followed by virus movement to the ganglia of innervating neurons where a lifelong latent infection is established [22, 32]. Periodic reactivation from this latent virus pool produces recurrent lesions and more frequently virus shedding which is frequently asymptomatic [38, 39] and is regarded as a major source of virus transmission to susceptible partners [39, 177], complicating efforts to control the spread of this disease. As mentioned previously, HSV-2 infection has also been shown to increase the risk of HIV acquisition by over three-fold in both men and women [64] making HSV-2 a significant worldwide public health concern.

While suppressive antiviral therapies exist for the treatment of genital herpes, studies have shown that not even high-dose regimens are completely effective in abrogating HSV-2 transmission [90]. In addition, there are currently no licensed vaccines against genital herpes and recent clinical efficacy trials conducted to evaluate a promising vaccine candidate have proven unsuccessful [93]. A better understanding of viral pathogenesis and virus-host interactions is clearly needed to optimize strategies to control this important disease.

The mouse and guinea pig are the two small animal models most commonly used in the study of genital herpes pathogenesis and for evaluating novel interventions [74]. Although the murine model allows the application of a wide variety of immunologic reagents, it is not the most representative model for studying human disease [75]. Vaginal HSV-2 infection produces high lethality in mice and surviving animals do not experience spontaneous recurrent disease. Consequently, the model is not well suited for the study of latent infection or the recurrent disease and viral shedding associated with HSV-2 transmission in humans. In contrast, the guinea pig provides a more clinically relevant model of human genital herpes, with animals experiencing a self-limiting primary infection that closely resembles HSV-2 disease in humans that results in a latent infection in sensory neurons. This remarkable similarity to human disease has made the guinea pig the "gold standard" for the study of both primary and recurrent genital herpes disease for a number of years [75, 81, 83]. However, as discussed previously in Chapters I-III, the model has suffered from the paucity of immunologic reagents available for the guinea pig [157], hindering disease pathogenesis studies and limiting the characterization of the immune response while evaluating putative vaccines [178]. In an attempt to address this deficiency, as described in Chapter III, we recently developed a guinea pig PCR array (gpArray) that includes gene targets for cytokines, chemokines, and cell-specific surface markers to provide a means of examining the immune response in these important research animals [155].

The primary objective of the studies described here was to utilize this array to characterize the local alterations in immune gene expression levels temporally throughout vaginal primary genital herpes infection in the guinea pig. While there has been limited work using microarray assays to evaluate changes in gene expression during a bacterial infection in guinea pigs [175], to our knowledge this is the first time that a temporal characterization of the immune transcriptome has been undertaken for the guinea pig vaginal mucosa during primary genital herpes infection.

METHODS

Guinea Pig Model of Primary Genital Herpes

Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were housed in AAALAC-approved vivarium and allowed to acclimate prior to use. All animal studies were approved by the UTMB Institutional Animal Care and Use Committee. Animals were inoculated intravaginally with 6.0 $\log_{10}$ plaque forming units (pfu) HSV-2 strain MS as described previously [96]. Following HSV-2 inoculation, animals were evaluated daily through resolution of the primary infection (d10 post inoculation; p.i.) and the severity of genital vesiculo-ulcerative disease quantified as described [74].

Vaginal swabs were collected from each guinea pig immediately prior to HSV-2 inoculation and on days 1, 2, 3, 5, 7, and 10 p.i. Swabs were placed into 1 ml of Dulbecco's Modified Eagle Medium (Corning Life Sciences-Mediatech, Inc., Manassas, VA) supplemented with 2% (vol/vol) newborn calf serum (Life Technologies Incorporated, Carlsbad, CA) and 1% penicillin/streptomycin (10,000 U/ml penicillin/10,000 µg/ml streptomycin stock; Sigma-Aldrich, St. Louis, MO). The samples were vortexed and 100 µl removed and added to 100 µl Total RNA lysis buffer (Bio-Rad, Hercules, CA) containing 1% β-mercaptoethanol (BME; Sigma-Aldrich). Both this sample and the original vaginal swab sample were stored at -80°C until used for RNA isolation and cDNA preparation or virus titration.

For the IFNγ reporter assay, vaginal lavages were collected by washing the vaginal vault three times with 200 µl of Hank's Balanced Salt Solution supplemented with 5% (vol/vol) newborn calf serum and 2% penicillin/streptomycin (20,000 U/ml penicillin/20,000 µg/ml streptomycin stock). Immediately following collection, samples were centrifuged (16000 x g, 5 min) to pellet cellular material and the supernatant transferred to a new, sterile tube. Collected cellular material was preserved by addition of

Total RNA lysis buffer containing 1% BME and stored at -80°C for RNA isolation and cDNA preparation.

RNA Extraction and cDNA Synthesis

RNAs were extracted using the Aurum Total RNA 96 kit (Bio-Rad) following the manufacturer's instructions and reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad) as previously described [155]. Briefly, each reaction contained: 45 µl RNA template, 3 µl iScript Reverse Transcriptase and 12 µl 5x iScript Reaction Mix. A three-step protocol consisting of 25°C for 5 minutes, 30 minutes at 42°C and 5 minute at 85°C to stop the reaction was utilized for cDNA synthesis and the resulting cDNAs were stored at -20°C until assayed.

gpArray Transcription Analysis

cDNAs were assayed using a guinea pig immune response array. Individual cDNA samples were prepared and PCR array reactions completed in 96-well plates in a total volume of 25 µl using previously described methods [155]. PCR array reactions were carried out on CFX or CFX Connect Real-Time PCR detection systems (Bio-Rad) using a protocol that consisted of an 8 cycle amplification of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds followed by a 40 cycle amplification of 95°C for 15 seconds, 56°C for 20 seconds and 72°C for 20 seconds during which real time data were acquired at the annealing step. Bio-Rad CFX Manager software was used to complete C_q analysis with a constant baseline adjustment of 50 relative fluorescent units for all array runs.

For quality control, the identities of each PCR array product were confirmed using a high resolution melt temperature analysis included in the thermocycling protocol following the second amplification step [155]. Melt temperatures (T_m) for each amplicon were compared to historical values with an acceptable range of ±0.8°C prior to data

analysis. For gene targets that had T_ms >0.8°C, corresponding C_q values were considered "40" (the maximum number of amplification cycles included in the PCR) during analysis to indicate the lack of a successful amplification of the target gene in the reaction. Finally, the C_q values obtained from gpArray runs were quantile normalized using the HTqPCR package for the R/Bioconductor framework [179]. Comparisons to identify statistically significant changes in gene expression levels between uninfected controls and samples from each time point post-inoculation were calculated using the R/Bioconductor limma package [180, 181] with a *P* value cutoff of 0.05.

Gene Ontology (GO) Analysis

Genes found by gpArray analysis to have significantly altered expression levels ($P<0.05$, Student's t test) compared to pre-challenge values for the same animal were analyzed using the Enrichr application [182] to rank enriched terms from each day of sampling throughout the primary HSV-2 infection study. GO biological processes were assigned by Enrichr for these individual gene lists identified from gpArray analysis. Calculations to determine the statistical relevance of the results were conducted by the Enrichr application and used Fisher's exact test as described [182].

Additionally, the INTERFEROME (http://www.interferome.org) database [183] was used to identify the members of the gene sets analyzed by GO that had known associations to IFNγ or IFNγ-mediated immunity.

Confirmatory qRT-PCR

PCR primers used for confirmatory qRT-PCR assay have been previously described [155]. Cloned amplimers of selected gene targets were produced and included as a series of duplicate 10-fold dilutions (10^6 - 10^2) as quantitation standards. All reactions were conducted in a total volume of 25 µl and utilized the CFX or CFX Connect optical platforms (Bio-Rad). Reaction mix contained 1X iQ SYBR Green Supermix, 0.2 µM of

each forward and reverse primer, and 1 µl template cDNA. To control for cellular load and normalize IFNγ copies for each sample, parallel qPCRs were conducted using the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin. Negative template controls were included to ensure PCR integrity and all reaction efficiencies were between 80-120% with correlation coefficients of >0.96. The sensitivity of these reactions allowed for 100 copies to be detected 100% of the time. Confirmation of primer specificity and amplimer identity was undertaken using T_m analysis. IFNγ expression levels were normalized using beta actin or the geometric mean of the housekeeping genes GAPDH and beta actin prior to analyses.

Guinea Pig IFNγ Reporter Assay

Vaginal lavage samples were diluted (1:3, 1:12, 1:48) in Hank's Balanced Salt Solution supplemented with 5% (vol/vol) newborn calf serum and 2% penicillin/streptomycin and a 100 µl aliquot added to duplicate wells of a 96-well plate containing guinea pig fibroblast cells (a kind gift of Dr Hubert Schafer, Robert Koch Institute, Berlin, Germany) stably transfected with a luciferase reporter under the control of the interferon stimulated response element (ISRE) [184]. Cells were incubated (37°C, 5 hrs), lysed by addition of ONE-Glo reagent (Promega, Madison, WI) and luciferase activity quantified using a Centro XS3 LB 960 luminometer (Berthold Technologies, Oak Ridge, TN) with reads of 1 sec/well. The luciferase activity in lavage samples was defined as the fold difference compared to that seen in samples from cells treated with medium alone.

Virus Titer

HSV-2 titers were determined from daily vaginal swab or lavage samples by plaque assay [74]. Briefly, 24-well plates with confluent Vero cell monolayers were inoculated with serially diluted samples in duplicate. Plates were incubated for 1 hour at room temperature and then an agarose overlay added. The plates were incubated (37°C, 5% CO_2)

for 2 days. The supernatant was aspirated, monolayers stained with crystal violet solution (Sigma-Aldrich) and viral plaques enumerated to determine the titer. .

Statistical Analysis

For all groups, data were analyzed for mean and standard deviation (SD). Comparisons between multiple groups were made by one-way ANOVA with Bonferroni correction and correlation coefficients were calculated using Spearman nonparametric correlation using Prism software (v6.0; GraphPad, La Jolla, CA). Comparisons of IFNγ gene expression levels were made using a two-tailed ANOVA after log conversion of the qPCR results with Prism software. For all comparisons a *P* value of 0.05 was used to designate significance.

RESULTS

HSV-2 Infection and Overview of Gene Expression Following Virus Challenge

Eighteen outbred Hartley guinea pigs were intravaginally (IVAG) inoculated with HSV-2. Subsequently 15/18 animals developed vesiculo-ulcerative primary genital skin disease. Vaginal swabs were collected from animals on days 1 and 2 p.i. and used to confirm successful inoculation of animals with HSV-2. Replicating virus was recovered from vaginal swabs of all animals confirming that all 18 animals were infected. Vaginal virus titers were highest on day 1 p.i. (5.96 ± 1.2 $\log_{10}$ pfu/ml) and showed a significant reduction by day 2 p.i. (5.05 ± 0.9 $\log_{10}$ pfu/ml; $P<0.05$, Student's t test). Genital skin lesions were detected beginning on day 4 p.i. with peak disease severity on day 6 p.i. and resolution of genital herpes disease by day 10 p.i. (**Figure 4.1**).

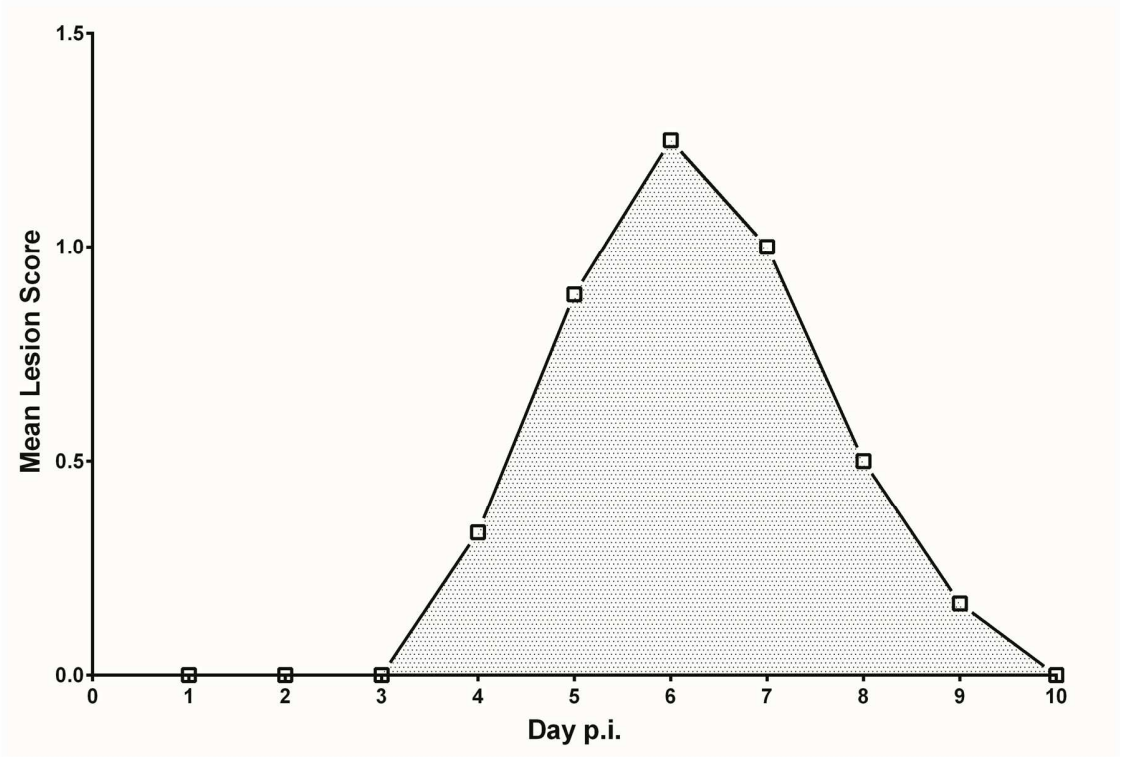

Figure 4.1. Graphical representation of disease scoring for animals inoculated with HSV-2.

Genital lesions appeared between day 3 and 4 p.i. with peak disease severity peaked observed on day 6 p.i. Genital lesions healed and were undetected by day 10 p.i.

To characterize the temporal host immune response to HSV-2 infection at the vaginal epithelium we evaluated samples obtained on days 1, 3, 5, 7, and 10 p.i. using the gpArray and compared them to those obtained from the guinea pigs prior to virus infection with each animal serving as its own control. In these analyses we identified a total of 193 genes with significantly altered expression ($P<0.05$, Student's t test). The highest number of differentially expressed genes were found day 1 p.i. with 45 genes showing significant changes in expression levels. Subsequently, the number of differentially expressed genes decreased on days 3 (39 genes) and 5 p.i. (36 genes) and then increased on day 7 (43 genes) before decreasing again on day 10 (30 genes). **Figure 4.2** provides a summary of the temporal expression of the differentially altered genes grouped by fold change for each day of sampling. Moreover, the individual fold changes for the top 25 differentially expressed

genes (both up- and down-regulated) for each day of sampling are summarized in **Table 4.1**.

For nine genes expression was significantly altered on all of the days. Of these, three (IFNγ, CXCL10 and CXCL11) were up-regulated throughout the course of the infection while six (SOD1, CCL20, TLR-6, IFNAR1, BPI, and IL-16) were down-regulated.

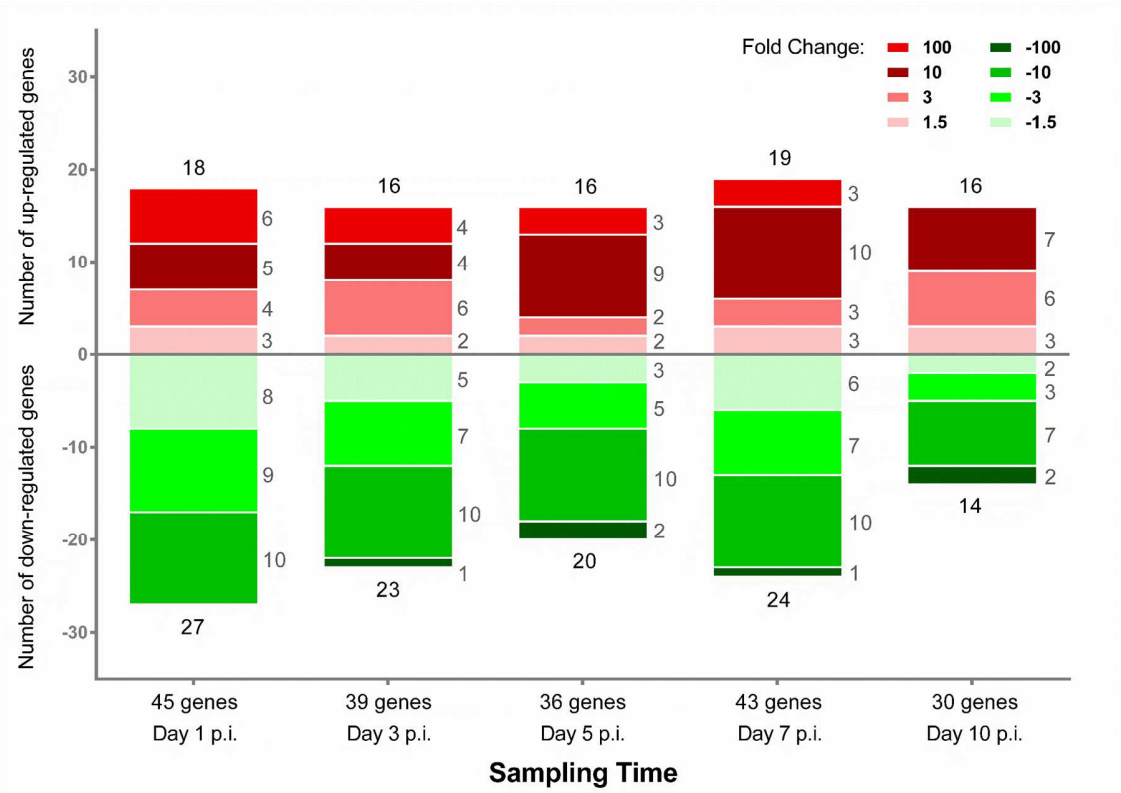

Figure 4.2. Temporal expression of differentially expressed genes by time point following HSV-2 inoculation.
Genes are arranged into groups by fold change and indicate the number of up- and down-regulated genes for each day of sampling. The total of up- and down-regulated genes is indicated at the top and bottom of each column for each day sampled.

Gene Ontology (GO) Analysis

To provide more information about the temporal immune response to HSV-2 infection, GO analysis was undertaken to identify the biological processes associated with

the gene expression patterns elucidated by the gpArray studies. We previously showed that significantly altered genes with a ±3-fold alteration to their expression levels are more likely to indicate significant alterations in transcription levels when measured by more sensitive methodologies such as qPCR [155, 185], thus only genes meeting this criteria were included in the GO analysis studies.

Table 4.1. The top 10 differentially expressed genes for each day of sampling.

Gene	Fold Change Day 1 p.i.	Gene	Fold Change Day 3 p.i.
IFNG	3405.6	CXCL11	997.7
CXCL11	1052.9	IFNG	285.6
CXCL10	422.5	CXCL10	218.4
IL27	203.8	CCL7	172.9
IL12B	147.8	CCL5	36.9
CCL7	118.1	KLRD1	17.2
IL12A	52.0	CD96	13.6
CXCL12	15.7	IL27	10.7
TNFSF4	14.0	CD2	9.8
IL2RA	13.6	IL2RA	8.8
CD69	11.1	LAG3	8.0
TNF	6.9	CD69	6.5
TLR6	**-97.6**	TLR7	4.7
CCR6	**-63.3**	CCL2	4.3
SOD1	**-47.6**	CCL20	**-172.9**
ENTPD1	**-28.6**	CCR4	**-72.1**
CD36	**-26.8**	BPI	**-42.9**
CCL20	**-20.7**	CD19	**-27.7**
LAMP1	**-15.0**	CD93	**-21.5**
IL23R	**-13.6**	TLR6	**-19.4**
IL16	**-13.5**	CCR3	**-12.5**
HPRT1	**-10.3**	SOD1	**-12.3**
BPI	**-9.2**	IL23R	**-11.4**
CD19	**-9.1**	TLR9	**-11.3**
TGFB1	**-7.4**	TGFB1	**-10.2**
Gene	**Fold Change Day 5 p.i.**	**Gene**	**Fold Change Day 7 p.i.**
CXCL11	385.9	CXCL11	613.7
CXCL10	172.5	IFNG	352.6
IFNG	111.9	CXCL10	150.6
KLRD1	23.1	CCL5	46.9
CCL5	22.7	KLRD1	24.3
IL2RA	19.0	CD2	21.2

IL21	17.5	CD96	20.4
CXCR3	17.2	IL21	20.3
CTLA4	15.1	CXCR3	16.8
CD2	12.3	LAG3	16.5
LAG3	11.7	CTLA4	12.7
IL27	11.1	CCL7	12.5
CD96	9.8	IL2RA	12.0
CCL20	**-840.2**	CD28	6.2
BPI	**-197.5**	CCL20	**-469.6**
CD19	**-45.7**	BPI	**-81.7**
SELL	**-33.3**	TGFB1	**-67.7**
FCER2	**-21.5**	CD19	**-39.4**
TNFRSF8	**-20.2**	SOD1	**-16.8**
IL12A	**-18.7**	FCER2	**-15.9**
SOD1	**-18.4**	IL7	**-14.7**
IL16	**-13.0**	IL16	**-14.6**
CSF2	**-12.7**	SELL	**-11.6**
IL15	**-10.3**	CSF2	**-11.3**
CTSG	**-10.1**	IL23R	**-11.3**

Gene	**Fold Change Day 10 p.i.**
CXCL11	33.9
CCL5	30.4
CD79A	20.0
CXCL10	12.9
CD2	11.9
CXCR3	11.2
IFNG	10.2
KLRD1	9.6
CD96	9.1
IL27	7.9
IL27RA	7.3
CD28	5.3
IL1B	4.0
CCL20	**-241.5**
BPI	**-189.4**
CSF2	**-60.1**
SOD1	**-22.8**
IL15	**-22.4**
IL7	**-14.9**
IL23R	**-13.3**
TLR6	**-12.8**
IL16	**-11.4**
FCER2	**-8.7**
TLR3	**-5.0**
IL6ST	**-4.4**

Results from GO analysis showed that on day 1 p.i most of the differentially expressed genes were associated with the broad categories of signal transduction, immune response, defense response and immune system process (**Table 4.2**). These processes involve many functions related to the presence of a foreign body and the response to potential damage due to an invasive threat. Signal transduction was one of the most significant processes identified and was associated with nearly half of the significantly altered genes found at this time point. The processes of regulation of cytokine production and the regulation of lymphocyte activation were also indicated and included several of the most highly up-regulated genes (IFNγ, IL-27, IL-12A, IL-12B) on day 1.

By day 3 p.i. GO analysis showed changes in the categories represented by the altered genes with more genes belonging to the general category response to chemical stimulus. This broad category includes a more specific biological process, chemotaxis, that was indicated for this day of sampling. Five of the most up-regulated genes at this time point (CCL2, CCL5, CCL7, CXCL10, and CXCL11; **Table 4.1**) were chemotaxins that overlap within these related GO processes.

On day 5 p.i. many of the differential altered genes were identified by GO as being associated with positive regulation of metabolic process and the regulation of cell proliferation, and the regulation of lymphocyte and T-cell activation (**Table 4.2**), likely due to up-regulation of CD2, CD28, CTLA4, and IL-21 as these genes are common to both processes.

Day 7 gene expression patterns indicated a more generalized set of processes (**Table 4.2**) such as defense response, immune response, and immune system process. Additionally response to chemical stimulus and chemotaxis were again prominent due to a resurgence in increased expression of the chemotactic genes CCL2, CCL5, CCL7, CXCL10 and CXCL11 (**Table 4.1**). The processes regulation of lymphocyte activation and regulation of T-cell activation were also identified, likely due to increased expression of several markers of T-cell populations (CD2, CD28, CD96, and LAG3) at this time point.

Table 4.2. The top ten GO terms from each time point sorted by increasing *P* value (Fisher's exact test).
The table also lists the number of genes identified from gpArray analysis that are associated with each corresponding GO term.

Day 1 p.i. GO Terms	***P* value**	**Number of Genes**
regulation of cytokine production (GO:0001817)	4.90E-11	11
immune response (GO:0006955)	3.25E-07	13
regulation of immune response (GO:0050776)	3.25E-07	8
regulation of lymphocyte activation (GO:0051249)	3.25E-07	7
immune system process (GO:0002376)	3.34E-07	14
positive regulation of cytokine production (GO:0001819)	4.27E-07	6
regulation of cytokine biosynthetic process (GO:0042035)	1.43E-06	6
defense response (GO:0006952)	7.02E-06	10
chemotaxis (GO:0006935)	8.04E-06	7
signal transduction (GO:0007165)	9.27E-06	20
Day 3 p.i. GO Terms	***P* value**	**Number of Genes**
chemotaxis (GO:0006935)	2.40E-09	10
locomotory behavior (GO:0007626)	2.69E-09	10
regulation of cytokine production (GO:0001817)	1.24E-08	9
defense response (GO:0006952)	4.08E-08	12
behavior (GO:0007610)	5.45E-08	10
response to chemical stimulus (GO:0042221)	1.92E-07	14
immune system process (GO:0002376)	1.34E-06	13
regulation of lymphocyte activation (GO:0051249)	3.89E-06	6
immune response (GO:0006955)	6.21E-06	11
positive regulation of cytokine production (GO:0001819)	8.46E-06	5
Day 5 p.i. GO Terms	***P* value**	**Number of Genes**
regulation of cytokine production (GO:0001817)	1.35E-09	10
regulation of lymphocyte activation (GO:0051249)	1.20E-08	8
regulation of T cell activation (GO:0050863)	7.48E-08	7
immune response (GO:0006955)	1.34E-07	13
immune system process (GO:0002376)	2.46E-07	14
positive regulation of metabolic process (GO:0009893)	1.68E-06	12
regulation of T cell differentiation (GO:0045580)	1.77E-05	4
regulation of cytokine biosynthetic process (GO:0042035)	2.96E-05	5
positive regulation of tyrosine phosphorylation of STAT protein (GO:0042531)	2.96E-05	4
regulation of cell proliferation (GO:0042127)	3.34E-05	10
Day 7 p.i. GO Terms	***P* value**	**Number of Genes**
regulation of cytokine production (GO:0001817)	1.08E-09	10
regulation of lymphocyte activation (GO:0051249)	1.05E-08	8
regulation of T cell activation (GO:0050863)	2.96E-06	6

defense response (GO:0006952)	8.50E-06	10
immune response (GO:0006955)	9.28E-06	11
chemotaxis (GO:0006935)	9.28E-06	7
immune system process (GO:0002376)	1.02E-05	12
locomotory behavior (GO:0007626)	1.02E-05	7
positive regulation of cytokine production (GO:0001819)	1.02E-05	5
response to chemical stimulus (GO:0042221)	1.20E-05	12

Day 10 p.i. GO Terms	***P* value**	**Number of Genes**
regulation of cytokine production (GO:0001817)	1.44E-09	9
regulation of T cell activation (GO:0050863)	8.35E-09	7
regulation of lymphocyte activation (GO:0051249)	2.40E-08	7
regulation of T cell differentiation (GO:0045580)	7.10E-06	4
regulation of cell differentiation (GO:0045595)	7.24E-06	7
regulation of cytokine biosynthetic process (GO:0042035)	7.24E-06	5
immune response (GO:0006955)	3.00E-05	9
immune system process (GO:0002376)	3.00E-05	10
positive regulation of metabolic process (GO:0009893)	3.69E-05	9
regulation of protein amino acid phosphorylation (GO:0001932)	1.09E-04	5

Finally, day 10 expression patterns indicated roles for regulation of cytokine production and the regulation of T-cell activation and differentiation (**Table 4.2**). Sampling at this time point showed the least amount of significantly altered genes of any day throughout primary infection (**Figure 4.2**) and those genes that were significantly altered showed less differential expression compared to naïve controls.

Interferome Analysis

In addition to GO analyses, the significantly altered genes on each day were interrogated against the INTERFEROME database (http://www.interferome.org) to determine genes were associated with IFNγ antiviral host responses. Of the 45 genes identified from 1 day p.i. samples, 51% were associated with IFNγ. Likewise, 17 of the 41 genes (41%) with altered expression on day 3 p.i., 17 of 39 (44%) on day 5 p.i., 19 of 41 (46%) on day 7 p.i., and 15 of 31 (48%) on day 10 had known associations to IFNγ-related immune responses as identified by the database.

Confirmatory Quantitative RT-PCR

Because both gpArray studies and INTERFEROME analysis indicated the importance of IFNγ in the response to HSV-2 infection. We undertook a series of quantitative RT-PCR (qRT-PCR) to provide additional information about IFNγ RNA levels for each animal over the course of the study. The RNA samples used were the same as were assayed on the gpArray. As shown in **Figure 4.3**, qRT-PCR results confirmed that IFNγ RNA was expressed at significantly higher levels on days 1-7 p.i. ($P<0.05$, ANOVA) showing good agreement with the results obtained from gpArray screening. The levels of IFNγ in day 10 p.i. samples were also increased compared to those seen pre-challenge but the increase did not reach significance (1.39 ± 1.0 vs 1.15 ± 0.7 $\log_{10}$ copies).

IFNγ Reporter Assay

Having confirmed that IFNγ mRNA was increased on each day sampled, we next examined whether these increased levels of mRNA were indicative of corresponding increased IFNγ protein levels. In a second series of studies the amount of biologically active IFNγ protein in vaginal lavages collected from guinea pigs prior to and at different times following HSV-2 inoculation and were quantified using an *in vitro* reporter assay [184].

Samples collected from HSV-2 infected animals showed significantly increased luciferase activity, indicating increased IFNγ protein levels on all days tested with the increase being significant on days 2 and 5 p.i. ($P<0.05$ each compared to naïve controls; **Figure 4.4A**). Peak luciferase activity was seen on day 2 p.i., subsequently decreasing significantly by day 5 p.i. with a further reduction between days 5 and 7 p.i. **Figure 4.4B**

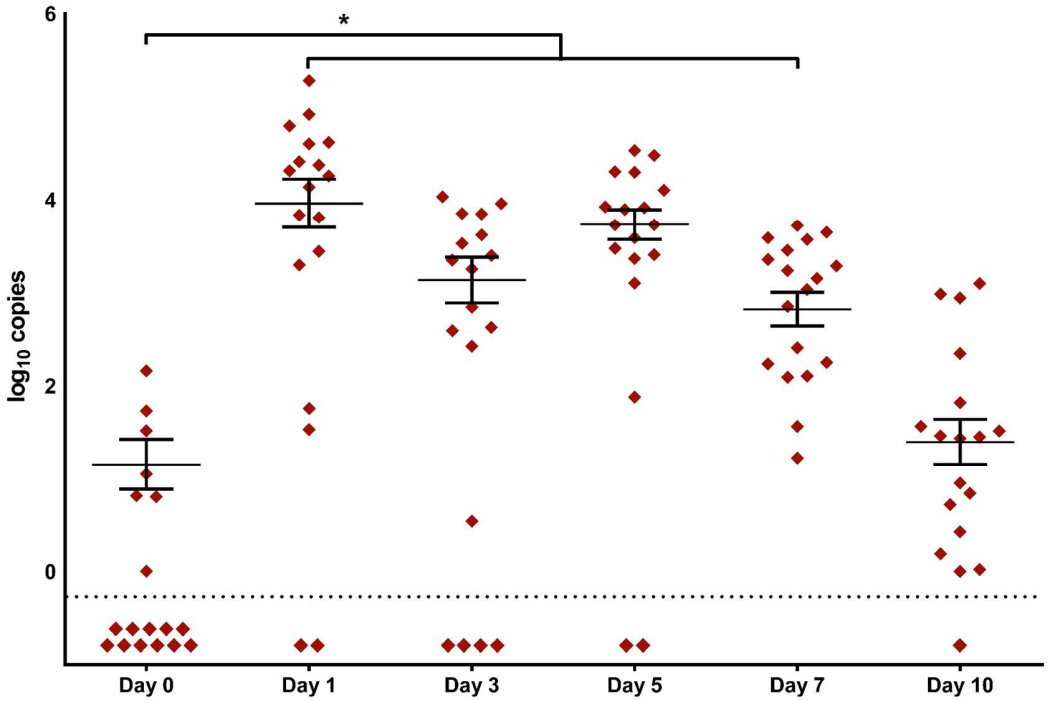

Figure 4.3. Confirmatory IFNγ qPCR results from gpArray samples.
IFNγ mRNA expression was found to be significantly up-regulated compared to naive controls on days 1-7 p.i. correlating well with gpArray screening results. Analysis of day 10 p.i. samples showed increased expression levels of IFNγ mRNA compared to naïve controls but this difference did not reach significance.

shows that IFNγ mRNA isolated from cellular material recovered from the vaginal lavages in this study was significantly increased on all days of sampling compared to controls ($P<0.05$, ANOVA) however there was no significant difference in IFNγ mRNA between days.

A portion of vaginal lavage from this IFNγ protein study was also used to determine the amount of replicating virus in the vagina on each day of sample collection. The highest titers were observed on day 2 (4.93 ± 0.8 $\log_{10}$ pfu/ml) with a significant reduction in the amount of HSV-2 detected by day 5 (2.93 ± 0.8 $\log_{10}$ pfu/ml; $P<0.05$ ANOVA). Virus titers were again reduced on day 7 (2.53 ± 0.8 $\log_{10}$ pfu/ml) compared to day 5 but this reduction was not significant. Additionally, a strong positive correlation was found

between HSV-2 titers and IFNγ protein levels as measured by luciferase activity (r=0.788, *P*<0.01; Spearman correlation).

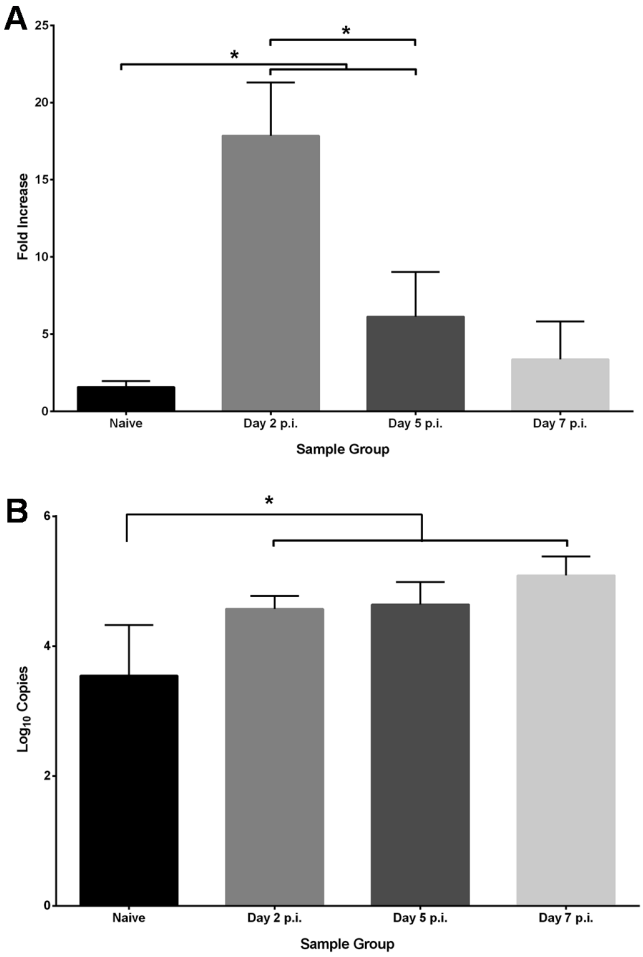

Figure 4.4. Relationship between IFNγ protein abundance and mRNA levels.
Vaginal lavages were collected from animals prior to and after HSV-2 inoculation to determine the relationship between IFNγ mRNA and protein production. **A**) The mean fold increase in luciferase activity indicated significantly increased levels of IFNγ protein on days 2 and 5 compared to naïve controls. In addition, Day 5 expression levels indicated a significant reduction from day 2. Day 7 IFNγ protein levels were similar to day 5. **B**) IFNγ mRNA levels were found to be significantly up-regulated on all days of sample collection compared to naïve controls with no difference in expression levels between individual days of sample collection. * $p<0.05$, ANOVA with Bonferonni correction.

DISCUSSION

Using a custom PCR-based transcription array we undertook a series of studies to evaluate the temporal maturation of the immune response of the vaginal mucosa throughout a primary genital herpes infection in the guinea pig. These studies demonstrated a major role for IFNγ in response to acute HSV-2 infection, a finding that is in good agreement with the importance of IFNγ in the control of genital herpes in mice [55, 73, 186]. Additionally, we showed that approximately 40% of the gene targets included on the array showed significant alterations in expression levels and were linked to IFNγ-mediated antiviral immunity, further highlighting the important role of this inflammatory cytokine in the control of HSV-2 infection.

Using criteria that we had developed in previous studies we undertook GO analysis to provide a biological context to the gpArray screening results. Thus, the biological processes identified using GO analysis provided us with a means to infer the temporal maturation of the mucosal immune response in the guinea pig to an acute genital herpes infection.

Transcriptome analysis showed that the highest number of differentially expressed genes was found 24 hours p.i, corresponding temporally with the peak of viral replication within the vaginal mucosa, the greatest level of IFNγ mRNA expression and most IFNγ-related immune response genes. Unsurprisingly, GO analysis of these altered genes confirmed a general immune defense response with lymphocyte activation and cytokine production indicative of a robust immune response to viral challenge. Further, early up-regulation of CCL2 (+3.4 fold) and CCL7 (**Table 4.1**) at this time suggested monocyte-derived antigen presenting cell recruitment into the infected epithelium [186]. These results are consistent with the initiation of a vigorous Th1 response to HSV-2 infection and, taken together, show good agreement with previous findings regarding the early events in the response to intravaginal HSV-2 infection in mice [73, 186].

The chemotaxins CCL2, CCL5, CCL7, CXCL10 and CXCL11 were all highly up-regulated on day 3 p.i. These chemokines have previously been reported to be critical to host resistance following HSV-2 infection [56, 186-188]. These results are consistent with the recruitment of lymphocytes to the vagina in response to an inflammatory environment, likely stimulated by the Th1 response observed on day 1 p.i. and are supported by GO analysis of day 3 p.i. gene expression in which the top processes identified had shifted to chemotaxis and cellular locomotion. In addition, the INTERFEROME database indicated that these chemokines are all associated with IFNγ-mediated antiviral responses, either directly by IFNγ-stimulated target cell production or through IFNγ-induced up-regulation of cell adhesion molecules. Interestingly, our findings are in good agreement with those reported recently by Cherpes *et al*. who described strong indications of interferon-mediated immunity and increases in the levels of CCL5, CCL7 and CXCL10 3 days following intravaginal HSV-2 challenge in mice using an oligonucleotide microarrray [189].

Transcription data in our studies showed that CXCR3 was one of the most up-regulated genes on day 5 p.i., suggesting vaginal infiltration by a mixed population of activated T-cells [190]. This finding was supported by GO analysis of day 5 gene expression patterns which indicated an increase to the processes of lymphocyte and T-cell activation that would be expected with the arrival of T-cells at the vaginal mucosa. It is also possible that increased expression of CXCR3 is a specific indicator of $CD4^+$-mediated T-cell recruitment of an effector $CD8^+$ population to the mucosal site of infection [57]. Coupled with the effect of IL-21 for the promotion of $CD8^+$ T-cell proliferation and effector functions [191], these results strongly support the appearance and maturation of an adaptive immune response that corresponds with a significant reduction in replicating virus titers compared to those seen on day 2 p.i. Further, the timing of this apparent influx of T-cells into the vagina is similar to the previous reports of the first appearance of IFNγ-secreting lymphocytes into the genital tract of mice on day 5 after HSV-2 challenge [152].

Gene expression data from Day 7 p.i. showed continued up-regulation of a number of potent chemotaxins, including CXCL10, CXCL11, CCL5, CCL7, as well as increased expression of T-cell activation markers (CD2, CD28, CTLA4). Taken together with the results of GO analysis, these results suggested continued lymphocyte and T-cell activation and would be compatible with continued influx and expansion of a $CD8^+$ population at the vaginal epithelium. As IFNγ mRNA expression was also found to be up-regulated with a concomitant increase in the number of IFNγ-associated immune genes, it is likely that day 7 is the peak of $CD8^+$ activity associated with mucosal clearance of replicating virus [43, 54] in the guinea pig. Vaginal virus titers on day 7 p.i. were lower than those in day 5 samples and further support this hypothesis. Additionally, a recent study by Xia *et al*. [192] reported the recovery of large numbers of HSV-specific IFNγ-secreting T-cells from the vaginal mucosa of guinea pigs on day 7 p.i., further supporting our interpretation of the gpArray results.

Analysis of gene expression levels day 10 p.i. samples was similar to day 7, with a further increase in the number of genes associated with IFNγ antiviral immunity. Interestingly, at this time although the amount of IFNγ mRNA detected by qRT-PCR was higher than in uninfected animals this increase did not reach significance. However, the amount of IFNγ mRNA on day 10 p.i. was significantly reduced compared to that seen on day 1 p.i. Coupled with the reduction in the total number of genes with significantly altered expression levels, this data suggests that the vaginal transcriptome was returning to its normal non-perturbed state as the initial virus infection at the genital epithelium was cleared.

Of the six genes that were significantly down-regulated on all days, BPI functions as an innate anti-infective defense molecule recently found to be expressed on the surface of epithelial cells [193] and IL-16 is a $CD4^+$ chemotactic cytokine linked to IFNγ stimulation. To our knowledge, down-regulation of these genes has not previously been associated with HSV-2 infection and consequently these genes may represent novel targets

for future studies. IFNAR1 is the alpha chain of the type I interferon receptor and production of this protein has been reported to be down-regulated during both HCV and HSV infections [194, 195], however no studies have previously shown transcriptional alterations for this gene during viral or bacterial infections. TLR-6, is a pattern recognition receptor that detects bacterial lipoproteins at the cell surface, it is expressed at high levels on the epithelial cells of the lower genital tract [145]. Transcriptional down-regulation of TLR-6 has been reported in lung epithelial cells treated with TLR-2 agonists [196]. These studies also showed that increased levels of IFNγ and TNFα had a suppressive effect on TLR-6 mRNA expression [196]. HSV-2 is known to be recognized by TLR-2 [43, 197] and the levels of TLR-6 mRNA in our studies were lowest when IFNγ and TNFα mRNA expression were increased suggesting that the decreased transcription of TLR-6 may have been due to a similar mechanism.

SOD1 mRNA expression has also been reported to be reduced during viral infections with a direct correlation to viral gene expression [198]. In addition, TNFα has been shown to reduce SOD1 transcription [199, 200] and in our studies the highest level of down-regulation of SOD1 was coincident with the greatest up-regulation of TNFα transcription.

Finally, CCL20, a highly potent chemokine constitutively expressed by vaginal epithelial cells [201], has been shown to be up-regulated during periods of inflammatory stimulation [201] and results in a rapid recruitment of lymphocytes to the site of perturbation [202]. In contrast, our analysis of CCL20 transcription levels showed significant reductions during the primary HSV-2 infection. While this was unexpected, Sperling *et al*. recently showed that human papillomavirus type 8, another DNA virus that can establish a persistent infection, encodes a novel viral protein that directly interferes with CCL20 promoter activation, thereby reducing transcription and expression and potent suppression in the ability of the cells to attract Langerhans cells to the site of infection

[203]. Our results could therefore be suggestive that HSV-2 employs a similar mechanism of transcriptional suppression of this molecule.

Of the genes found to be significantly up-regulated across the entire sampling period, IFNγ is known to be an important effector molecule against genital herpes in mice, including functions for the recruitment of lymphocytes to the vaginal epithelium [73] and resolution of a HSV-2 infection [55]. CXCL10 and CXCL11 are potent chemotactic interferon-stimulated genes [204, 205] and it is not surprising that the expression of these three genes was found to be highly up-regulated throughout the course of the HSV-2 infection. In addition, evaluation of alterations in gene expression levels following HSV-2 infection showed that over 40% of the genes with significantly altered expression had a connection to IFNγ based on INTERFEROME database analysis for each day sampled. IFNγ is known to be important in the resolution of genital herpes in mice and our results suggested similar importance in the guinea pig. Utilizing qRT-PCR, we confirmed increased expression of IFNγ throughout primary HSV-2 infection. These results suggest that IFNγ is also crucial to the control of a vaginal HSV-2 infection in the guinea pig.

mRNA expression levels do not always correlate well with protein levels [206, 207]. Thus, to evaluate the relationship between IFNγ transcription and protein levels, a second series of experiments was undertaken to quantify the amount of biologically active IFNγ protein present in the vagina during primary HSV-2 infection. These studies showed that on days 2 and 3 after HSV-2 infection there was increased IFNγ protein production. The results were in good agreement with the IFNγ mRNA levels detected by the gpArray and confirmed by qRT-PCR. Further, levels of IFNγ mRNA recovered from cellular material collected from vaginal lavages also correlated well with IFNγ protein levels in the lavage supernatant. Concordant with our previous study, we observed a significant increase in luciferase activity suggestive of high levels of IFNγ protein on day 2 p.i. and found that IFNγ mRNA expression was also significantly increased at this time point. Interestingly, IFNγ protein levels subsequently decreased over the course of the study, however the

expression levels of IFNγ mRNA remained up-regulated compared to uninfected on all days of sample collection. This phenomenon has been reported previously for enriched populations of T-cells and represents a general uncoupling of transcription from translational processing to prepare and maintain potent cytokines for memory responses while protecting from an improper release of inflammatory molecules [208, 209]. These findings proved that increased mRNA expression for IFNγ was indicative of a greater abundance of IFNγ protein, however they also highlighted potential differences in post-transcriptional regulation or post-translational modifications that prevent a direct linear relationship for correlating mRNA expression levels to protein production under certain conditions [210].

Our comparative analysis studies utilized a PCR-based array to characterize changes in the host immune transcriptome in the guinea pig model of genital herpes. To our knowledge this is the first evaluation of its kind in this animal model. While we acknowledge that limitations exist in our analyses due to the limited number of gene targets available for interrogation using the gpArray, GO analysis of the genes with significantly altered expression patterns identified by this system were still successful in providing a temporal examination of the biological processes associated with the host immune response to HSV-2 infection. These results were in line with the known vaginal immune response observed in mice and have allowed a more detailed examination of the host response to genital herpes in an animal model that more closely resembles human disease. The gpArray also expands the capabilities for studying the vaginal immune response during recurrent disease for which there is no good murine model. Additionally, a key finding of our array studies was the crucial role for IFNγ in the resolution of an HSV-2 infection and these results were confirmed using an in vitro reporter assay to measure the abundance of biologically active IFNγ protein at the vaginal mucosa. These findings indicated the importance of IFNγ in stimulating an exuberant antiviral immunity against HSV-2 and

show good agreement with studies conducted in the murine model of genital herpes [55, 73, 189].

The gpArray has proven to be a useful new tool for understanding immune responses in the guinea pig model of genital herpes. Importantly, it has extended our understanding of the host response to a primary HSV-2 infection by allowing us to characterize the immune response throughout the entire acute infection, a feat that is not possible in mice due to the mortality associated with genital herpes infection. However, further exploration of the immune response, including the contribution of different lymphocyte cell populations, will be necessary to fully delineate host immunity to this important sexually transmitted infection, especially with regard to recurrent disease and virus transmission.

CHAPTER V[§]

Use of a Guinea Pig-Specific Transcriptome Array for Evaluation of Protective Immunity against Genital Chlamydial Infection following Intranasal Vaccination in Guinea Pigs

INTRODUCTION

Because we could not evaluate the HSV-2 vaccine originally tested in follow-up studies using the gpArray we instead undertook a series of studies to characterize the effects of a genital infection with the bacterial pathogen *Chlamydia caviae* on the immune response in the guinea pig. We further compared the protective effects of intranasal *C. caviae* vaccination on differences in the mucosal immune responses between naïve and vaccinated animals.

Urogenital infection with *Chlamydia trachomatis* is one of the most common bacterial sexually transmitted infections (STIs) worldwide [211]. Recent surveys have shown that over 105 million new cases were reported in 2008 and that more than two-thirds of these occurred in developing countries [211, 212]. The prevalence of chlamydial infections have been found to be similar in both women and men [213], however it is a greater public health concern for women due to the major clinical sequelae that can result from infection, including ectopic pregnancy, infertility and pelvic inflammatory disease (PID) [214, 215]. Infection is asymptomatic (i.e. subclinical) in 70-90% of women but these individuals are capable of transmitting the infection to others [214, 216]. Additionally, despite many decades of sexual health programs [217] incidence rates are

[§]Chapter V previously published and taken from: Wali, S., et al., Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

still increasing in many countries including the United States and Canada [216].

C. trachomatis is an obligate intracellular bacterium that normally infects the cervical (women) or urethral (men) epithelia [218]. In untreated women, the bacteria can produce an ascending infection that spreads along the endometrial epithelium to the fallopian tubes [216]. Here, the bacteria can establish a persistent infection that can result in a chronic host inflammatory immune response and the development of PID [216].

Chlamydia exists in two distinct forms: a small, infectious, nonreplicating, extracellular elementary body (EB) and a larger, intracellular, noninfectious, replicating reticulate body (RB). EBs are internalized by epithelial cells into an endocytic vesicle wherein they subvert lysosomal processing by the cell [219]. The EBs then modify the endosomal compartment and undergo transformation into the metabolically active RBs [218, 219]. As RBs undergo cellular division, the endosome expands to accommodate the increased numbers of progeny and becomes an inclusion [219]. After continued logarithmic growth, the RBs' depletion of host cell nutrients triggers a return to the infectious EB form. The new EBs are released to infect neighboring cells through extrusion of the inclusion or lysis of the host cell [219].

The immune response to chlamydial infection in humans results from a myriad of host responses that include both innate and adaptive components [220]. TLR recognition of the bacteria results in the production of interferon gamma (IFNγ) and other inflammatory cytokines that stimulate dendritic and natural killer cell activation [221]. The resulting cytokine milieu favors the development of a T helper 1 (Th1) response necessary for effective bacterial clearance. Specifically, it is the $CD4^+$ component of the Th1 T cell response that is the critical element in resolution of a chlamydial infection [221-223]. $CD8^+$ T-cells play a more controversial role in resolution of infection, but can contribute as a secondary source of IFNγ production or through killing of infected cells [218, 221-223]. Additionally, while antibodies are important contributors to a successful immune response in both the human [224-227] and mouse [228, 229], an antibody response by itself is not

capable of resolving the infection. Rather, the major role of antibodies in clearance of a chlamydial infection is to further enhance Th1 activation, providing increased levels of IFNγ secretion by $CD4^+$ effector T-cells [218, 220].

A number of animal models have been reported for the study of chlamydial genital infection [230-234]. While there is currently no ideal animal model, the guinea pig model offers distinct advantages over the others for evaluating chlamydial infection of the genital tract [231, 234]. The causative agent of guinea pig inclusion conjunctivitis (GPIC or *C. caviae*) produces a genital tract infection remarkably similar to human *C. trachomatis* genital infection with regard to pathogenesis, immunity, and the ability to be transmitted sexually [231, 235]. In addition, *C. caviae* is a natural pathogen of the guinea pig that can infect superficial epithelial cells of the ectocervix and endocervix [236], producing an ascending infection to the endometrium and oviducts [235]. Further, the female reproductive system of the guinea pig is also closely related to that of the human with regard to histological features and physiology including an estrous cycle with active hormone secretion from a corpus luteum, thus eliminating the need for hormonal pre-conditioning necessary for infection, colonization and pathogen ascension in other animal models [231, 235]. However, as described for herpes simplex virus (HSV) vaccine studies conducted in the guinea pig (see Chapter II; [169]), the lack of guinea pig-specific reagents has limited the use of this animal model for evaluating the efficacy of putative chlamydial vaccine candidates.

In the current studies we determined protective immunity against intravaginal (IVAG) infection in guinea pigs vaccinated with chlamydial EBs that are known to provide robust protection against genital challenge(s) [237, 238]. Here we immunized female guinea pigs intranasally (IN) with *C. caviae* EBs or delivered PBS to controls (mock vaccinated) and then challenged the animals IVAG with *C. caviae*. Further, as the major sequelae from chlamydial infections result from the ascending nature of the infection that can lead to chronic inflammation [220, 221], we examined the tissues from both the lower

and upper genital tracts early (3 days post-challenge) and later in the course of the infection (9 days post-challenge). These studies provided an excellent opportunity to utilize the guinea pig array (gpArray) described in Chapter III [155] to analyze differences in the regulation of genes that may contribute to innate responses, Th1-cellular/inflammatory, and Th2-humoral immunity in vaccinated animals compared to mock controls. gpArray data revealed increased Th1- and Th2-associated gene expression in vaccinated guinea pigs by day 3 post challenge. Further, vaccinated guinea pigs cleared IVAG infection by day 3 post challenge and displayed significantly less upper genital pathological damage compared to mock vaccinated controls.

MATERIALS AND METHODS

Bacteria

Chlamydial stocks (obtained from Dr. Harlan Caldwell at the Rocky Mountain Laboratory, NIAID/NIH) were prepared as described previously [239]. EB (infectious form) of *C. caviae* were harvested from infected HeLa cell monolayers and stored at -80°C in sucrose-phosphate-glutamine (SPG) buffer. *C. caviae* stock titers were determined as previously described [240] and diluted appropriately in PBS for both IN immunization and IVAG challenge.

Guinea Pigs

Dunkin Hartley strain guinea pigs (350-450g) were purchased from Charles River Laboratories (Massachusetts, USA) and housed, infected and processed by our collaborators in AAALAC-accredited vivarium at the University of Texas at San Antonio. Food and water were supplied *ad libitum* and all experimental studies were completed humanely and followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved and

overseen by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at San Antonio.

Immunization and Challenge

Guinea pigs were immunized IN with 1 x 10^5 *C. caviae* EB. Immunized guinea pigs were rested for one month and then challenged IVAG with 1 x 10^5 EB of *C. caviae*. Following challenge, vaginal swabs were collected every 3 days and used to inoculate HeLa cell monolayers to determine infection status. Chlamydial inclusions were detected in the infected cells after 30h incubation using an anti-Chlamydia genus specific rabbit monoclonal primary antibody and goat anti-rabbit IgG secondary antibody conjugated to FITC plus Hoescht nuclear stain.

Guinea Pig Transcriptome Analysis

To prepare guinea pig nucleic acids for gpArray analysis, RNA was extracted from genital tract tissues harvested from three humanely euthanized guinea pigs from each group at the indicated times post challenge using the Aurum RNA extraction system (Bio-Rad; Hercules CA). Briefly, small (<3mm3) tissue pieces collected from the vagina and cervix representing the lower genital tract (LGT) and uterine horns and oviducts representing the upper genital tract (UGT)s of individual guinea pigs were homogenized in kit-provided lysis solution supplemented with 1% beta-mercaptoethanol. Following the kit instructions, total RNA (~2ug per sample) was collected in a 96 well format and then immediately converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was analyzed by PCR array (~2ng of RNA per well) immediately or stored at -20°C until analysis [155]. Gene expression data were normalized using quantile transformation to provide a more uniform distribution of intensities as described by Bolstad *et al*. [241]. This approach normalized each gene expression level and each sample to the others to account for differences in RNA quality and quantity. Comparisons of the transcription profiles

among the lower and upper genital tracts of naïve, mock vaccinated challenged, and vaccinated challenged animals at days 3 and 9 post-challenge were performed using delta delta C_q analyses [151] within the HTqPCR package for the R/Bioconductor framework [179-181]. This allowed us to establish fold change (FC) values for subsequent comparisons of individual genes between test groups. The FC values were then evaluated by Student's t-test (Prism v6.0; GraphPad) to identify significantly differentially regulated genes ($p<0.05$). In addition, the HTqPCR package provided us with a means to conduct hierarchical clustering analysis on selected genes for comparisons between groups and time points.

Selected genes that were expressed differently between groups were subsequently analyzed by qRT-PCR with single target assessment under optimal conditions established for each specific target using the same RNA analyzed in the array. Single target expression data were normalized against the averaged housekeeper expression levels for HPRT1 and eEF1a1. Expression profiles for these two housekeepers were indistinguishable across all the samples in the study (correlation coefficient $R^2=1.0$, n=36). This approach confirmed the data from the quantile normalized C_q values generated by the array and provided accurate quantified outcomes to compliment FC calculations. For each qPCR run, a 10-fold dilution series of known copy number was processed in parallel as described previously [149] to provide a means of extrapolation of C_q value to actual copy number in a given sample. All PCR analyses were completed in CFX real time instruments (Bio-Rad) using optimized thermocycling conditions.

Determination of *C. caviae* Loads in Infected Genital Tracts by qPCR

DNA was collected from each tissue [147] and subjected to qPCR assays. Primers targeting the tryptophan synthase gene beta subunit of *C. caviae* were used to quantify bacterial load in the lower and upper genital tract of infected guinea pigs. These evaluations utilized forward (5' - AGAGGATCTTCTACATACAG - 3') and

reverse (5'- CCATGAAAATCACACATTC-3') primers with a corresponding melting temperature analysis to identify and confirm all qPCR products with a resulting 84°C T_m for the *C. caviae* product. Guinea pig GAPDH served as a DNA quality and quantity indicator and was used to normalize the *C. caviae* loads in individual samples. Guinea pig GAPDH qPCR utilized forward (5'-AAT GGG AAG CTC ACA GGT ATG G-3') and reverse (5'-ATG TCA TCG TAT TTG GCC GGT-3') primers and a TET-labeled TaqMan probe (5'-TET-TCC AGG CGG CAG GTC AGA TCC ACA-BHQ1-3'). The lower limit of detection for the assays was 50 copies. Comparisons between bacterial loads in tissues at day 3 and day 9 post-inoculation were made using Student's t-test with a *P* value of 0.05.

Genital Tract Pathology

Genital tract tissues of all surviving animals were harvested on day 80 post-challenge as previous extensive analyses demonstrated the suitability of this time period to evaluate upper genital tract sequelae following IVAG Chlamydia challenge [242-244]. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E). Histological images were recorded at ×200 or ×400 magnifications under an Olympus AX80 light microscope (Olympus, Center Valley, PA) and evaluated in a blinded manner for pathological damage. The microscopic findings were either graded as none/minimal (0), slight (1), moderate (2), or severe (3) histological alteration. Histological scores were obtained by examining 5 consecutive sections (2mm-interval) of cervix, oviducts, and uterus from every animal. Scores assigned to individual guinea pigs were used to calculate the pathology scores for each group of animals and presented as mean ± standard deviation.

RESULTS

Intranasally Vaccinated Guinea Pigs are Protected Against Intravaginal *C. caviae* Infection

IN vaccination of mice with *C. muridarum* EBs has been shown to provide robust protection against genital *C. muridarum* infection [240, 243]. To evaluate a similar vaccination regimen against IVAG *C. caviae* infection in guinea pigs, we immunized guinea pigs IN with 1 x 10^5 *C. caviae* EBs. Guinea pigs administrated PBS IN were used as a mock vaccination control, similar to previously reported studies in mice that were found to be comparable to an adjuvant-alone control group [242]. As shown in **Figure 5.1**, *C. caviae* EB-vaccinated animals cleared subsequent IVAG *C. caviae* challenge at day 3 whereas mock vaccinated guinea pigs shed *C. caviae* ($1x10^6$ inclusion forming units; IFU) for 6 days post challenge, followed by reduced bacterial loads from days 9-18, and no recoverable bacteria by day 21 post challenge.

Evaluation of *C. caviae* Bacterial Burden by Genomic Analysis

In order to increase the sensitivity of detection and measure *C. caviae* that was no longer infectious because of host inactivation, bacterial genomic burdens in lower and upper genital tract of guinea pigs at days 3 and 9 post challenge were estimated using qRT-PCR. Although *C. caviae* loads were comparable at day 3 post challenge in the lower genital tract (LGT) and upper genital tract (UGT) of vaccinated and mock vaccinated guinea pigs (**Figure 5.2**), *C. caviae* EB-vaccinated guinea pigs displayed 4-6 logs fewer bacterial genomes in lower and upper genital tracts by day 9 post challenge. Additionally, of the twelve tissues from *C. caviae* EB-vaccinated animals, three had no detectable bacterial genomes.

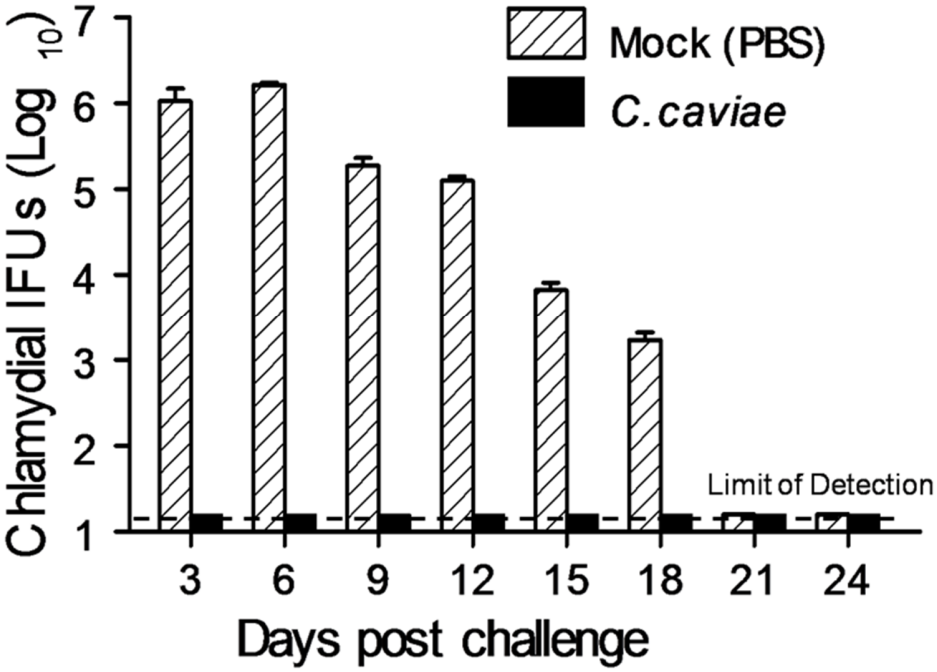

Figure 5.1. Vaccination of guinea pigs with *C. caviae* protects against genital chlamydial infection.

Groups (5 per group) of guinea pigs were immunized IN with 1 x 105 IFU *C. caviae* or treated with PBS as mock vaccination controls. All animals were rested for 30d and challenged IVAG with 1 x 10^5 IFU *C. caviae*. Chlamydial shedding in vaginal swab samples was monitored every third day after challenge for a month. Data are presented as mean ± SD for each group at each time point. Adapted from: Wali, S., *et al*., *Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs*. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

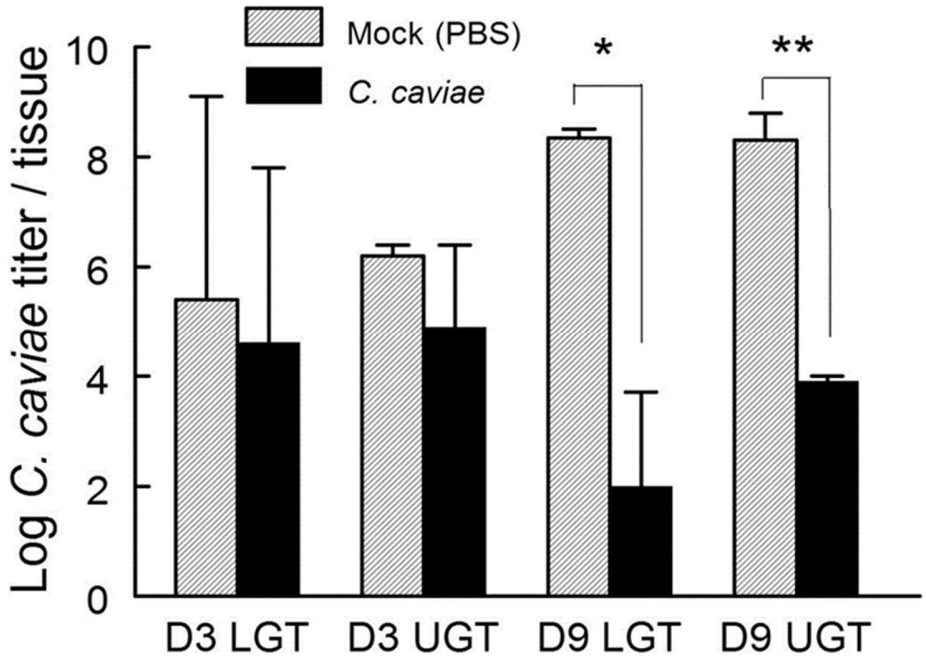

Figure 5.2. Quantitative PCR assessment of bacterial genomic burdens in lower and upper genital tracts from *C. caviae* mock (PBS) or vaccinated guinea pigs.
Groups of three animals were euthanized on days 3 and 9 after *C. caviae* IVAG challenge and tissues representing the lower or upper genital tract were aseptically collected. Extracted DNA was subjected to qPCR for single copy guinea pig GAPDH (used for normalization) and the single copy *C. caviae* tryptophan synthase gene (quantification of bacterial genomic load). The average bacterial burdens for each tissue are depicted as grey (mock-vaccinated) or black (vaccinated) bars for each tissue and time point. LGT: lower genital tract. UGT: upper genital tract. * $p < 0.05$, ** $p < 0.01$, Student's t test. Adapted from: Wali, S., *et al*., *Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs*. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

C. caviae EB-vaccinated Guinea Pigs are Protected Against Development of Reproductive Tract Pathology Following Intravaginal Challenge

To evaluate the effect of *C. caviae* EB vaccination on the development of pathological lesions in the genital tract, tissue sections were obtained from challenged guinea pigs at day 80 after challenge [242-244]. Histological analysis of the uterus of mock-vaccinated animals revealed pathological damage that was characterized by the presence of a severe inflammatory cell infiltration (the majority of the inflammatory cells were lymphocytes and macrophages) with moderate superficial layer exfoliation and hemorrhage [185]. In contrast, *C. caviae* EB-vaccinated animals had an intact endometrial epithelium that exhibited reduced inflammation and hemorrhage in the uterus [185]. The mean histopathology severity scores for the uterus demonstrated significantly ($p<0.05$) reduced inflammatory cell infiltration, superficial layer exfoliation and hemorrhage upon *C. caviae* EB vaccination compared to controls [185]. In addition, congestion and edema were also found to be reduced in vaccinated animals but the associated scores were not statistically different from the mock-vaccinated guinea pigs.

Host Responses are Increased Following *C. caviae* EB Vaccination in Guinea Pigs

We next utilized the guinea pig-specific PCR array [155] to elucidate the impact of vaccination with *C. caviae* EB on gene expression in both the upper and lower genital tract following intravaginal challenge. As expected, comparisons of gpArray results between vaccinated and mock-vaccinated groups revealed modulation of a number of genes. From these, 19 genes were selected for more extensive qRT-PCR analyses based on their involvement in either innate, Th1 or Th2-humoral immune pathways. In addition, the gpArray results for these genes were subjected to hierarchical clustering analyses for probable co-regulation of immune components in *C. caviae* EB-vaccinated or mock vaccinated guinea pigs. As shown in Figure 3, hierarchical clustering of the data indicated 3 major groups including innate (NK), Th2-humoral (including CD93, CD39, IL-4R, β2-

microglobulin) and Th1-cellular/inflammatory responses that was in agreement with the known immune functions of the selected genes, further validating the gpArray results.

Interestingly, when the gpArray screening results of tissues from *C. caviae* EB-vaccinated animals were compared to those of mock vaccinated controls at day 3 after chlamydial challenge (**Figure 5.3**), we observed decreased innate gene transcription and a

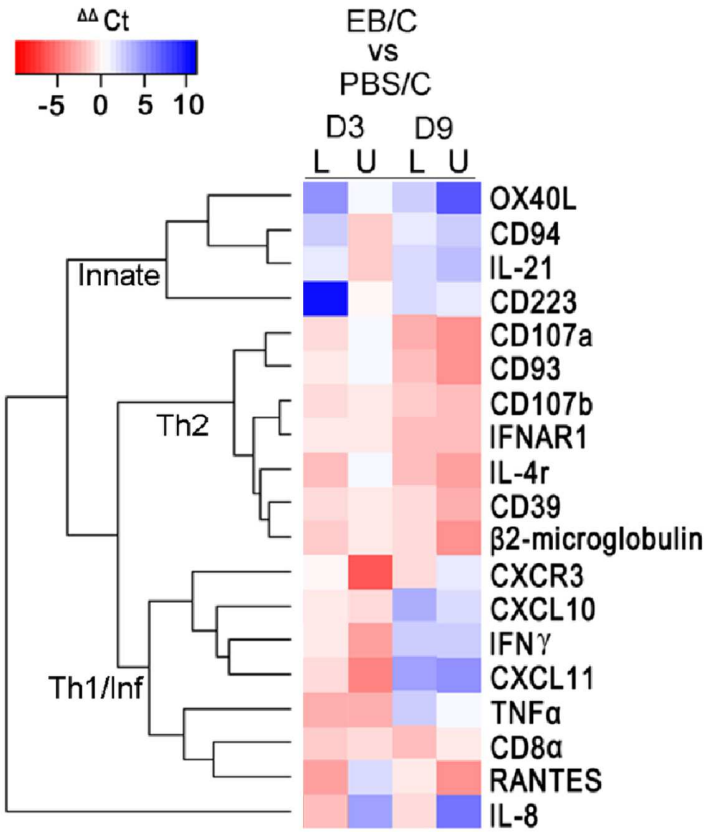

Figure 5.3. Comparative heatmap depiction of differential gene expression using RT-PCR array screening.

Groups of mock vaccinated and challenged (PBS/C) were compared to *C. caviae* EB-vaccinated and challenged (EB/C) animals to evaluate differences in gene transcription. Each group contained three animals. Tissues from both upper and lower genital tracts (U and L, respectively) from the respective groups of animals were collected at days 3 and 9 after challenge. Red and blue shading indicate increased or decreased expression of the indicated genes, respectively. Lighter shades, including white, indicate similar levels of expression. Functional gene clustering is indicated by the brackets on the left that show 3 major groups consisting of innate, Th2 and Th1/inflammatory related genes. Adapted from Wali, S., *et al.*, *Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs*. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

concomitant increase in the transcription of genes associated with both Th1 and Th2 immune responses in the LGT of the vaccinated animals. For these selected genes, transcription in the UGT of vaccinated animals was generally up-regulated compared to the controls. By day 9 the gpArray indicated that innate gene transcription was still reduced in both the LGT and UGT of vaccinated animals. But Th2-associated gene transcription was up-regulated in both the LGT and UGT of vaccinated animals. Finally day 9 Th1-associated gene transcription had waned in both tissues of vaccinated animals, although CD8α and RANTES were found to be highly transcribed at this time.

Next, to confirm the results of the gpArray screening, single qPCR assays were performed for the selected genes associated with both innate and acquired immunity. The data are summarized in **Tables 5.1** (LGT) and **5.2** (UGT) and provided an overall confirmation of the array results depicted in **Figure 5.3**.

Table 5.1. Fold change between groups from qPCR assays of selected immune response genes from the guinea pig lower genital tract.

	[a]Comparison Between Groups	CXCL10	CXCL11	CXCR3	CD8α	CD107a	CD107b	IL-21	IFNAR1	RANTES	β2-μglobulin	OX40L	IFNγ
D3	PBS/C vs Naive	6.3↑	3.3*↑	1.4↑	1.6↑	2.3↑	1.6↑	1.3↓	1.6↑	1.7↑	2.9*↑	1.2↑	1.4↑
	EB/C vs Naive	9.5*↑	6.7**↑	2.5↓	1.1↑	5.6*↑	3.0*↑	>10↓	6.1*↑	5.6↑	6.8**↑	>10↓	5.0↓
	EB/C vs PBS/C	1.4↑	2.0**↑	3.5↓	1.4↓	2.5↑	2.0↑	6.0↓	3.3↑	3.3↑	2.5**↑	>10↓	6.7↓
D9	PBS/C vs Naive	>10*↑	>10↑	1.0↑	1.6↑	1.7↓	1.4↓	1.1↓	2.5↓	7.4*↑	1.3↑	1.0↓	2.8↑
	EB/C vs Naive	1.7↑	1.0↑	5.0↓	1.4↓	1.2↑	1.7↑	5.0↓	3.1**↑	1.7↑	2.4**↑	>10↓	>10↓
	EB/C vs PBS/C	9.5*↓	>10↓	4.2*↓	2.3*↓	2.0**↑	2.5**↑	4.8*↓	>10**↑	4.5*↓	1.7**↑	>10↓	>10↓

Arrows indicate direction of fold change.

* indicates $p<0.05$; ** indicates $p<0.01$

[a]PBS/C: mock vaccinated and challenged; EB/C: *C. caviae* vaccinated and challenged; Naïve: non-vaccinated and not challenged.

Adapted from: Wali, S., *et al*., *Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs*. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

The qPCR data from day 3 post-challenge LGT samples confirmed increased expression of CXCL10, CD107a, CD107b, and IFNAR1 in *C. caviae* EB-vaccinated animals compared to mock controls with significantly higher transcription levels for CXCL11 and β2-microglobulin ($p<0.01$, Student's t test). CXCL10, CXCL11, CXCR3, CD8α, IL-21, and IFNγ were similarly found to be up-regulated in EB-vaccinated animals for samples collected from UGT tissues at day 3 post-challenge although the expression of these genes was not significantly increased from the mock controls.

Table 5.2. Fold change between groups from qPCR assays of selected immune response genes from the guinea pig upper genital tract.

	[a]Comparison Between Groups	CXCL10	CXCL11	CXCR3	CD8α	CD107a	CD107b	IL-21	IFNAR1	RANTES	β2-μglobulin	OX40L	IFNγ
D3	PBS/C vs Naive	1.1↓	3.3↓	3.3↓	1.4↓	1.2↑	2.3↑	1.4↓	2.1↑	1.4↑	2.3↑	1.3↓	1.1↓
	EB/C vs Naive	1.2↑	1.1↓	2.0↓	1.3↑	3.3**↓	1.4↓	1.7↑	1.5↑	3.3↓	1.3↓	2.0↓	1.9↑
	EB/C vs PBS/C	1.3↑	3.3↑	1.7↑	2.0↑	4.5**↓	3.2↓	2.5↑	1.4↓	4.4*↓	3.0*↓	1.5↓	2.0↑
D9	PBS/C vs Naive	>10↑	>10↑	2.0↑	2.8↑	>10*↓	>10*↓	5.2↑	>10**↓	1.2↑	5.0↓	3.9*	>10↑
	EB/C vs Naive	4.2↑	7.0↑	3.4↑	5.4↑	1.9*↑	1.9*↑	5.4↑	5.9**↑	5.9*↑	3.8**↑	4.4↑	3.9↑
	EB/C vs PBS/C	2.5↓	7.1↓	1.7↑	2.0↑	>10**↑	>10**↑	1.0↑	>10**↑	5.0↑	>10**↑	1.1↑	2.8↓

Arrows indicate direction of fold change.

* indicates $p<0.05$; ** indicates $p<0.01$

[a]PBS/C: mock vaccinated and challenged; EB/C: *C. caviae* vaccinated and challenged; Naïve: non-vaccinated and not challenged.

Adapted from: Wali, S., *et al*., *Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs*. PLoS One, 2014. 9(12): p. e114261. Reproduced with permission.

In addition, analysis of day 9 post-challenge qPCR data confirmed a decrease in transcription of CXCL11 with significantly reduced expression levels of IL-21, OX40L, CXCL10, and IFNγ ($p<0.05$) in LGT tissues. There was also agreement between gpArray screening results and qPCR data for the UGT samples. Here, qPCR confirmed increased transcription of CD8α and RANTES with associated down-regulation of CXCL10, CXCL11 and IFNγ. Further, the Th2-associated genes CD107a, CD107b, IFNAR1 and β2-

microglobulin were shown to be significantly increased in the UGT at this time point (p<0.01; Student's t test).

DISCUSSION

This study showed that intranasal immunization with *C. caviae* EB provides robust protection against IVAG *C. caviae* challenge through the induction mucosal Th1 and Th2 humoral immune responses within the genital compartment in the guinea pig model. Biologically, immunization reduced reproductive tract pathological sequelae in the guinea pig that are similar to those in human vaginal infections associated with reduced fertility and increased susceptibility to other infections including HIV. Importantly, our study is the first to carefully interrogate the gene expression patterns in immunized animals after challenge with this important bacterial STI.

The utilization of guinea pigs as an alternate and complimentary animal model to mice is important because (1), despite a number of vaccine studies in murine models no anti-chlamydial vaccine as yet been licensed [240, 243, 245-249]; (2), the use of the guinea pig to provide additional data is cost effective compared to non-human primates and bovine models [230, 233, 249, 250]; (3), the guinea pig model is remarkably similar to human *C. trachomatis* infection with regards to bacterial ascension, colonization and related pathogenesis [236, 251, 252]; and, (4), the ability to study transmission dynamics of chlamydial infections following sex between males and females provides the potential to evaluate the ability of a vaccine to interrupt sexual transmission [253]. However, in spite of being one of the oldest animal models used for immunological studies [166] and in research related to several pathogens (*Chlamydia sp*. [231, 234], *Mycobacterium sp*. [254, 255], *Legionella sp*. [256], *Francisella sp*. [257] and, *Neisseria sp*. [258]) the limited availability of guinea pig-specific reagents [166] has led to it being an animal model with limited or constrained utility. The application of a novel guinea pig gene expression qRT-

PCR array both advances the utility of the animal model and helps to increase our understanding of the immune outcomes of IN vaccination against Chlamydia.

Screening with the gpArray revealed significant modulation in several immunity markers associated with NK cells and Th1/Th2-specific cytokines and chemokines in immunized guinea pigs. Increased NK cell activation markers, such as CD94, CD233 and IL-21, with concomitant down-regulation of Th2/humoral responses and increased Th1 responses, were observed and are consistent with a previous report that described an important role for NK cells early following genital Chlamydia infection [259]. These studies showed that the production of IFNγ by NK cells was essential in the development of Th1 CD4+ T cell responses and facilitated the subsequent clearance of infection.

Array screening also demonstrated that *C. caviae* EB-vaccinated animals exhibited increased Th1- and Th2-related gene transcription at day 3 compared to mock vaccinated guinea pigs and suggested that the vaccinated animals mounted a quicker response to the infection following challenge. Specifically, gene expression data suggested an early, robust cellular response as indicated by increased expression of three potent T-cell chemokines: RANTES, CXCL10 and CXCL11. Up-regulation of these genes in vaccinated animals correlated with the abrogation of bacterial shedding by day 3 post challenge (**Figure 5.1**) and significantly reduced numbers of bacterial genomes in both lower and upper genital tract tissues by day 9 post chlamydial challenge (**Figure 5.2**). Taken together, diminished bacterial burden, undetectable levels of bacterial shedding (**Figure 5.1**) and the analyses of immune responses (**Figure 5.3**, **Tables 5.1** and **5.2**) strongly support the generation effective Chlamydia-specific immune responses in *C. caviae* EB-vaccinated guinea pigs compared to mock vaccinated controls.

In contrast, mock vaccinated animals did not exhibit marked transcription of essential Th1 or Th2/humoral genes until 9 days post challenge. Specifically, the expression pattern of the T-cell chemokine RANTES was consistent with the kinetics of bacterial clearance from the infected genital tract. Further, this pattern of RANTES gene

regulation has been reported in a male guinea pig genital chlamydial infection model where elevated levels of this chemokine were associated with T-cell influx into the urethra following *C. caviae* challenge [260]. Additionally, as reported by Sakthivel *et al*. [261], inhibition of RANTES in mice led to reduced antigen-specific activation (IL-12 and IFNγ production) of $CD4^+$ T cells isolated from lymphoid tissues and genital tract and was associated with prolonged *C. muridarum* shedding. These previous studies support the data obtained from gpArray screening and further suggest an important role for priming T-cells in early immune responses and control of a genital chlamydia infection and that RANTES may serve as a marker for optimal vaccine selection.

Additionally, the diminished need for elevated cellular response(s) at day 9 post challenge in *C. caviae* EB-vaccinated animals (clearance of bacterial shedding was observed by day 3; **Figure 5.1**) was consistent with significantly lower expression of inflammatory- and T-cell-associated genes, including CXCL10, CXCR3, CD8α, IL-21, RANTES, OX40L and IFNγ, relative to mock vaccination (**Table 5.1**).Importantly, these data strongly suggested that IN vaccination reduced the expression of these inflammatory immune genes at day 9 post challenge in the UGT with this reduction corresponding to significantly decreased pathology in the genital tract of vaccinated animals compared to mock controls. Histopathology results showed less inflammatory cell infiltrate and reduced damage in UGT tissue samples and were in good agreement with the array screening results. In contrast, mock vaccinated guinea pigs exhibited high levels of inflammatory gene expression at this time point, including IFNγ and its associated chemokines (e.g. CXCL10, CXCL11), compared to their *C. caviae* EB vaccinated counterparts. This expression correlated with an active/ongoing infection that can exacerbate tissue damage and is believed to be a major factor in the sequelae associated with chronic infection [220, 221].

Other differentially expressed immune genes revealed by the array, including IFNγ, CXCL10, IFNAR1 and OX40L, have previously been established to be associated with

genital chlamydial infection in the murine model [262-264] but have not previously been examined in guinea pigs. As such, the prior data sets help to validate our findings and the gpArray approach to vaccine evaluations. The array also identified modulation of genes which have not been previously reported in chlamydial infections. CD36 was up-regulated in both the LGT and UGT on day 9. CD81 and CD130 were found to be up-regulated, and IL-21 and CD96 were down-regulated, in the UGT on day 9. CD36 is expressed on monocytes/macrophages and has been shown to be critical in atherosclerotic lesions [265] although its contribution to the resolution of a genital chlamydial infection has not been previously reported. CD81 is expressed on B-cells, T-cells and dendritic cells and has been shown to co-stimulate T-cell activation and is required for induction of Th2 biased immune responses [266, 267]. In contrast, the role of IL-21 (an NK and T-cell activator; upregulated in non-protected guinea pigs, **Table 5.1**) is believed to be important in HIV-1 induced $CD8^+$ T cell activation and poorer disease outcomes [268] but has not been previously investigated in Chlamydia-induced pathology. These data provide additional support for the idea that the guinea pig may be a more useful model to study chlamydial pathogenesis than previously utilized mouse models.

Although our immune gene-specific qRT-PCR array provided additional insights into the regulation of selected immune pathways following vaccination and *C. caviae* genital infection in the guinea pig model, we analyzed whole genital tract tissue and thus the identification of the specific cell types responsible for particular gene expression could not be identified. Subsequent studies using cell sorting techniques would be required to better appreciate the sources and locations of the differently expressed genes. Similarly, the multi-cell type nature of the tissues led to an averaging effect of the gene expression differences common to all methods of whole tissue analysis. Here, such averaging likely masked cell type-specific responses in the minor cell populations within the tissue(s). Despite such limitations, these novel analyses provided an extensive view of immune gene expression within the genital compartment of guinea pigs following IN vaccination and *C.*

caviae challenge. These results extend our current understanding of the immune responses in this animal model of chlamydial infection of the genital tract and extend the utility of the guinea pig model for the study of chlamydial pathogenesis.

CHAPTER VI

Summary and Future Directions

Genital herpes is a significant worldwide public health concern. Infection with HSV-2, the virus that causes most genital herpes disease, is accompanied by lifelong latency in the neuronal tissues where the virus lays dormant undergoing periodic reactivations that may be accompanied by recurrent disease symptomology on external genitalia or the mucous membranes or may be asymptomatic with shedding of virus into the genital tract. Both outcomes of reactivation can lead to virus transmission to susceptible partners. Current estimates show that over 550 million people worldwide are infected with this virus and that as many as 27 million new infections occur each year, most of which are likely due to asymptomatic transmission from individuals that may not even know they are infected. Additionally, the virus can be transmitted to neonates during delivery where it can cause neonatal herpes, a devastating disease that can produce severe lifelong complications or even death. Current antiviral treatments can reduce disease symptoms and, when used suppressively, have shown the ability to reduce transmission, at least in the short-term, but such treatments do not clear the virus. Additionally, many people who are infected and can transmit the virus are asymptomatic and so unlikely to take suppressive antiviral therapy. Thus a prophylactic vaccine that could prevent infection or interrupt the establishment of latency or a therapeutic vaccine capable of reducing viral reactivations and/or viral shedding into the genital tract would appear to be urgently needed to reduce the spread of this global STI. Unfortunately, despite multiple clinical trials to date no such vaccine has reached licensure.

Following promising results obtained in murine models of genital herpes [116] we used an outbred guinea pig genital herpes model to evaluate the efficacy of an HSV-2 pDNA vaccine that included plasmids encoding antigenic targets to prime both humoral

(gD2) and T-cell (UL46 and UL47) adaptive responses formulated with the novel adjuvant Vaxfectin® [169]. Prophylactic vaccination with this vaccine was successful at completely abrogating primary genital skin disease and eliminating the appearance of recurrent genital herpes disease in all vaccinated animals. The vaccine also reduced the latent HSV-2 load in the neuronal ganglia compared to mock-vaccinated controls or animals receiving adjuvant and gD2 alone. Importantly, therapeutic usage of this vaccine formulation reduced the frequency of recurrent genital herpes disease and, notably, this was the first time that a therapeutic vaccine was shown to reduce the number of days on which virus was shed into the genital tract. Thus, this vaccine could provide a means to prophylactically reduce disease in uninfected persons and additionally reduce the risk of transmission when used as a therapeutic measure.

Overall, these vaccine efficacy studies suggested that the inclusion of the T-cell targets UL46 and UL47 provided enhanced protection compared to guinea pigs that were vaccinated with the same formulation containing only gD2 (Chapter II). We therefore hypothesized that the improved efficacy was due to increased cell-mediated responses elicited by inclusion of the T-cell targets UL46 and UL47. However testing this hypothesis was hindered by the lack of available immune reagents for the guinea pig. The dearth of such important tools hampered our ability to accurately and efficiently characterize the contribution of these antigens and assess their individual impacts on the protection observed with this vaccine. Consequently, we focused our efforts on the development and optimization of a platform that would allow a more detailed characterization of the guinea pig immune response to both vaccination and disease (Chapter IV). Our goal was to provide a means both to better evaluate vaccine candidates for HSV-2 and more generally to allow more detailed evaluation of immune responses in the guinea pig, a commonly used animal model for a number of medically important diseases.

Concomitant with our studies to evaluate the Vaxfectin®-adjuvanted HSV-2 vaccine, we developed a PCR-based platform to characterize gene expression in

understudied pathogens. Incorporating newly published genome sequence for the guinea pig, we adapted these successful methodologies to develop a PCR array that would provide a reproducible and inexpensive assay to characterize changes in gene transcription in this important research animal [155].

Following preliminary evaluations of ~120 individual primer pairs targeting various immune-specific genes, we undertook confirmatory studies using identical populations of guinea pig splenocytes cultured with or without PMA/ionomycin to determine the most reproducible primers for inclusion in our array. From these secondary studies we identified 92 target and 4 reference genes that were used to populate our array assembly. Additionally, these studies allowed us to establish a level of both biological and technical noise associated with gpArray screening results. Finally, using cloned amplimers from the gpArray, we developed single-target qPCR assays that allowed quantitative confirmation of the indicated changes in the transcriptome of the stimulated cell population. These additional validation assays allowed us to better define the meaningful limits of transcriptional changes suggested by gpArray evaluations and provided a metric for future gpArray-based comparisons between experimental groups.

Next, we utilized the newly developed gpArray to undertake studies that would for the first time provide a temporal evaluation of the mucosal immune response to primary vaginal HSV-2 infection in the guinea pig model of genital herpes. These studies allowed a comparison of the response to genital herpes in the guinea pig with those seen in the extensively used murine model of genital herpes and also a more direct comparison to immune responses known to be associated with genital herpes infection in humans.

To better establish a biological context for the changes in gene expression identified by the gpArray, we took advantage of the growing number of available gene ontology databases to assist in the determination of the specific immune pathways involved in the host immune response to HSV-2 infection indicated by our screening assays. Analyses showed that the highest number of differentially expressed genes were found 24 hours p.i.

and included IFNγ and the IFNγ-stimulated chemokines CXCL10 and CXCL11. Additionally, up-regulation of the chemokines CCL2 and CCL7 suggested early monocyte-derived antigen presenting cell recruitment to the epithelium [186]. Together these results indicated a robust Th1 response to the viral infection at the vaginal mucosal surface and were in good agreement with results reported previously in murine studies. Further analyses of early transcription changes showed a continued up-regulation of chemotaxins that was in good agreement with known recruitment of lymphocytes in response to an inflammatory environment [56, 186-188] and was comparable to a recent transcriptome study undertaken with an oligonucleotide array in mice [189]. As the primary vaginal infection progressed, evaluations from day 5 p.i. strongly suggested a shift to adaptive immunity with increased expression of CXCR3 (indicating infiltration of activated T-cells to the vagina) that were in good agreement with T-cell influx described by others at this time point for the murine model [152]. Because the genital herpes infection in mice is accompanied by rapid progressive neurologic disease and death by encephalitis, data about the temporal immune response in the vaginal epithelium at later times are difficult to obtain. Our analyses of transcription changes in the guinea pig from days 7-10 p.i. showed continued increases of T-cell activation markers (CD2, CD28 and CTLA4) that indicated a likely peak for $CD8^+$ activity on day 7 in the guinea pig based on decreasing viral titers that correlated to the increases observed for T-cell marker transcription. Further supporting these findings, Xia *et al*. found increased numbers of HSV-specific T-cells in the vaginal mucosa at day 7 p.i. in guinea pigs inoculated with HSV-2 [192].

The collective findings from the temporal gpArray screening studies showed good agreement with previously reported data from the mouse model of genital herpes and, most importantly, are the first evaluation of its kind in the guinea pig, providing a detailed description of the host response to genital herpes in a model where the primary vaginal infection is self-limiting and so more closely resembles human disease. Specifically, our evaluation of gene expression levels showed IFNγ mRNA transcription was significantly

up-regulated following IVAG HSV-2 inoculation and that over 40% of the genes with significantly altered expression levels on each day sampled were associated with IFNγ signaling. IFNγ has been shown to be important against genital herpes in mice [55, 73] and is suggested to be important in humans [43, 269], however we were aware that the increased IFNγ mRNA expression observed in our screening studies might not correlate with similar increases in protein expression levels [206, 207]. Thus, we utilized a stably transfected guinea pig fibroblast cell line with a reporter under the control of an IFNγ response element to determine the relationship between IFNγ transcription and protein levels during an acute genital herpes infection.

In these studies we used vaginal lavages collected following IVAG HSV-2 infection to determine both IFNγ protein levels in addition to IFNγ mRNA expression. Our results showed both increased IFNγ protein in the day 2 and day 3 p.i. samples and similarly increased expression of IFNγ mRNA that correlated well with the original gpArray screening results. Further samples collected on days 5 and 7 p.i. indicated levels of IFNγ protein that were elevated compared to naïve controls, however IFNγ protein levels decreased steadily from day 2 to day 5 and from day 5 to day 7. Of interest, we found that while IFNγ protein levels experienced a general decline from the apparent peak at day 2 p.i. the corresponding IFNγ mRNA expression levels remained significantly elevated compared to those of uninfected animals. While this phenomenon was not altogether unexpected [208, 209], it did highlight the importance of post-transcriptional or post-translational modifications that prevent a direct linear relationship between mRNA expression and protein production that must be taken into account when evaluating gpArray screening results.

While both gpArray screening and the IFNγ protein/mRNA studies allowed us to conduct more detailed analysis of the host immune responses to genital herpes infection in the guinea pig than had previously been possible, there were limiting factors that similarly affected each study. Chiefly, both the vaginal swab samples used for the gpArray screening

and the vaginal lavages collected to evaluate IFNγ protein and mRNA levels produced samples that contained mixed cellular populations. The analyses of these studies were therefore resultant of an averaging effect of the gene expression differences that such multi-cell samples inherently produce making it difficult to define the role of individual cell types responsible for particular gene or gene pattern expressions. Additionally, sample collection was undertaken every 24h during the temporal gpArray HSV-2 study. This provided an overview of the day-to-day changes in the immune response but limited our ability to more accurately dissect the kinetics that define the immune response with respect to not only cell types but also to individual immune pathway activations. Finally, as these studies utilized either vaginal lavages or swab samples there is a concern that the samples were limited to cellular material on the luminal side of the vaginal mucosa and may not have accurately or fully represented the immune cell populations within the epithelial layer. Thus, additional studies will therefore be necessary to fill in the gaps left by these preliminary studies.

As more reagents become available for the guinea pig, particularly antibodies that either feature cross-reactivity with this species or, of greater value, are guinea pig-specific, it would be very useful to conduct a series of experiments that examine the gene expression of discrete cellular populations over the course of an acute HSV-2 infection. For these studies groups of animals would need to be euthanized and the vaginal epithelium of both the lower and upper genital tracts as well as the inguinal and iliac lymph nodes harvested. From these tissues, cellular populations, with emphasis on early responders such as NK cells, macrophages and DCs and later with $CD4^+$ and $CD8^+$ T-cells, could be quantified and then screened using the gpArray to determine the associated gene expression patterns for each cell type. Such studies would not only produce data with regard to the types of cells present during each time point but also the timing of immune cell migrations to the vaginal mucosa throughout the course of the infection. This would more accurately reflect the immune response occurring within the epithelial tissue and provide a more detailed accounting of the host response to genital herpes.

To better examine the contribution of the epithelial cell layer to the immune response, particularly with respect to early innate antiviral and chemotactic immune cell signaling, a more controllable environment would be required. Producing an immortalized cell line using guinea pig vaginal epithelial cells would be a major advantage in this regard. Our group has had success with immortalizing vaginal epithelial cells from human donors and also at developing a transwell culture system that produces an *in vitro* multilayer cell culture closely mimicking the natural vaginal epithelium [270]. If a similar culture system could be developed for the guinea pig, it would allow a more efficient and inexpensive means to observe and define the kinetics of the immune response by providing an opportunity to sample more often than can be achieved with live animals. Vaginal swab samples collected from animals once every 24h do not by themselves elicit an immune response, however to obtain a more detailed overview of immune events samples would ideally need to be collected with much higher frequency, possibly every 5 to 10 minutes. Not only would the logistics of such sampling prove to be problematic but over the course of a primary genital herpes infection this would most certainly influence the immune response in animals thus sampled, possibly even increasing the severity of disease. With a cell culture system, the samples could be much more efficiently collected with far less effort. Additionally, as more reagents become available for the sorting of guinea pig immune cell populations, studies could be conducted with the addition of these cells to better simulate the normal epithelial environment. Taken further, such studies could potentially identify target cell types or specific immune pathways that are more pertinent to controlling or resolving the infection and may provide new targets for vaccine development.

During the planning of this dissertation project we envisioned that after the development and optimization of the gpArray we would return “full circle” and re-evaluate the vaccine that initially spurred my interest in the production of new guinea pig-specific reagents. Unfortunately, there were unforeseen complications that precluded additional

studies with this vaccine formulation. Had it been possible, our plan was to conduct a series of experiments to evaluate the immune response in animals with and without vaccination using gpArray screening to determine the effects of both prophylactic and then therapeutic vaccination on activation of immune pathways. Additionally, as we discussed in Chapter II and more recently in this Summary, this vaccine contained two antigenic targets that were included specifically to boost the T-cell response to HSV-2. Using the methods perfected by Xia *et al*. [192] we had also intended on harvesting vaginal epithelium from both vaccinated and mock vaccinated animals 30d following the completion of the vaccine dosing regimen to determine whether there was a vaccine-induced increase in the number of resident T-cells present in these tissues. A second group of animals would be similarly evaluated to determine whether T-cells migrated to the tissues quicker and/or in greater numbers in vaccinated animals following HSV-2 challenge. Finally, we planned to culture these T-cells in the presence of feeder B-cells that were infected with an adenovirus producing UL46 and UL47 to confirm that vaccination with these antigens induced an immune-specific response.

Because we could not evaluate the HSV-2 vaccine originally tested we instead evaluated the protective effects of intranasal (IN) *Chlamydia caviae* vaccination on the mucosal immune response to genital *C. caviae* inoculation. These studies showed that the IN vaccination successfully primed the immune response in animals receiving vaccination as those animals exhibited enhanced Th1 inflammatory and Th humoral responses sooner following challenge than mock-vaccinated controls. Further, vaccinated animals were shown to clear the bacteria earlier and, most importantly, experienced less genital tract inflammation and had reduced reproductive tract sequelae than mock-vaccinated controls. This is particularly important due to the often chronic nature of a Chlamydial infection that can lead to severe complications such as ectopic pregnancy or pelvic inflammatory disease due to the inflammatory environment stimulated by the chronic infection. As with our HSV-2 studies, the samples evaluated here represented mixed cellular populations and

therefore it is difficult to accurately determine the types of immune cells responsible for particular gene expression patterns. Additionally, the number of animals used per experimental group was limited to reduce costs for these preliminary studies. It would therefore be interesting to undertake subsequent studies with an increased number of animals per group to better define the effects produced by IN vaccination and to determine the cellular populations responsible for the enhanced protection observed in our initial studies.

Our work has produced a valuable research tool for characterizing the host immune response in the guinea pig, a widely used research animal. Through a variety of studies we have proven its utility, particularly with respect to sexually transmitted infections, and believe that this reagent will be of great use to not only infectious disease research but to all areas of study that utilize the guinea pig model, particularly allergy and high-containment disease research. While the number of genes represented on our gpArray is currently limited to 96 individual genes, the array was designed to be flexible and may easily be tailored to include more or less targets as desired by the end user. Until such time as a reliable and cost-efficient commercial replacement becomes available we believe that this array will provide invaluable information to researchers using the guinea pig animal model.

Appendix I

List of Primers on the gpArray

Gene		Sequence (5' – 3')	Length (bp)	Amplimer T_m (°C)
TLR-2	F	GTGCATATTCCAAACTTCTA	83	75.6
	R	TCCAGTGTGATTCGTTTA		
CD4	F	AGACCTTGAACCTGATTG	174	84.8
	R	ACTGGAGATACTTCTTGTC		
CD126	F	CCAACATCATTGTCACTG	92	81.8
	R	CCTGTAGAAGTCTGAGTTC		
CD94	F	TGGAGGATAATTTCTGGAA	97	75.8
	R	GACTCATCAAGAGACACTTA		
CD115	F	GGAAGATCATTGAGAGCTA	80	80.6
	R	CTCCCACTTCTCATTGTA		
KLRG1	F	GTGCCAGTGACATTTATTTC	73	78.2
	R	TGCTGTGGTCTGTAGTTA		
NFκB1	F	CTCCGAAGTATAAAGACATCA	82	79.8
	R	CTCGTTTCCAAGTCTGAC		
TNFSF4	F	TTGGCTTACAAAGACAAA	96	79.6
	R	CTGGATGAGATACAGTTC		
SOD1	F	GAGCAAATTCCATCATTG	89	80.5
	R	CGTCTTTGTACTTTCTTCA		
IL-23 receptor	F	GACACCAACTTCACATAC	74	79.4
	R	CGCACTTGAAATACGTAA		
CCR3	F	CCAACATTTACCTGCTCAA	82	79.7
	R	CAGTGAACATAGTAAGTCCA		
CXCR1	F	TGGTCAAGTTCATCTGTA	115	81.4
	R	GACTCATAGCAGACAAAG		
TLR-3	F	GGTCCTGTTCATTTTCTAA	126	78.6
	R	CCCTAAATTGATGCTCTTAA		

CD8α	F	GGAACAGAAGCGATTCAG	100	82.6
	R	GACCGAGCAGAAGTAGTA		
CD130	F	CCACCTCGTAATTTATCAG	96	75.8
	R	CAGTCTCATAACATTCATAATAC		
CD23	F	CCCTGAAGAACGTTAAAC	90	81.0
	R	TCTGGTTACTGTGATGTC		
CD2	F	AGACCTGAAATTGTACTTCA	83	78.6
	R	TGCAGATGTAGCAAATGA		
β_2 microglobulin	F	AGTCGAATTGCTGAAGAA	71	77.0
	R	AGTCCTTGCTGAAAGAAA		
CCL20	F	GACTGTTGTCTCAGATACA	129	81.8
	R	CACAGATAGTCTCTTCTTTG		
CCR6	F	CTTGCCAGATACCTCATAA	176	84.6
	R	GAACCTTTGACCGATAAAC		
IFNAR2	F	GAAGGTATGAAGATTGTGAAG	72	76.8
	R	TTGCCACACATCTGTTAG		
IL-27	F	CTGCTCTACACCTATCAG	81	82.3
	R	CTTGGACAGCAGTAGTAA		
IL-8	F	CCCAAATTTATCAAAGAACTG	77	76.9
	R	CTGAGTTTCACAATGATTTC		
CXCR3	F	TCTCCTTACGACTATGGA	84	81.8
	R	GTCGAAGTTTAGGCTGAA		
TLR-4	F	CCTTCACTACAGAGACTTTA	146	82.3
	R	GAGCAATCTCATATTCAAAGA		
CD62 ligand	F	GTACCAAAACCACAACAA	121	86.4
	R	CACAGTGACGTAGTAAAC		
CD19	F	CCACTGAGATACACATGA	110	83.8
	R	GCAGAAGATCAGATAAACC		

CD25	F	CCCATAATGCAAGAGAAC	87	77.9
	R	TAGGCTGTATCTGACTTTG		
IFNγ	F	CCATCAAGGAACAAATTATTAC	90	77.7
	R	TGACCGAAATTTGAATCAG		
MHC-II	F	GGATCATGTGTCAACATTTG	77	78.7
	R	CCTCATCAAGCTCAAACATA		
IL-15	F	GGCAAATAGCAGTTTAAATTC	70	74.9
	R	CTCCAGTTCTTCACATTC		
CD36	F	GGATGTTTACAGACAGTTC	89	79.2
	R	CTCTCTGCTTAACCTTTATG		
CD96	F	GCACTCAGGAAACTATTATC	91	78.4
	R	AGTCGATAGCTTGTATCTAG		
IL-27 receptor α	F	GTGGACTTCTCAGAGGAC	100	85.6
	R	TCTGGTAGTAGAACTGACAG		
SOD2	F	GGAACAACAGGTCTTATTC	98	77.7
	R	ATGGCTTTTAGATAATCAGG		
CXCR2	F	CTGAGCCAAATTTCATCA	70	77.1
	R	CTACGACATAAGTATTGATTTC		
TLR-6	F	CTCACTTGAACCTAAATTAC	85	75.0
	R	GTCAGAATTTGAAGATTCTC		
CD107a	F	CAGAGTGGTCAACATCAA	91	83.4
	R	GCTCTCTTCACTCTTCAG		
CD22	F	CTCCTACCTTCAGAAATAAAG	82	78.4
	R	GGATGTCATACTCAAAACA		
CD39	F	TGCTCTCAAAATATCCTG	98	80.2
	R	TCAGGTAATGGTTTGTTC		
IFNAR1	F	CTGAGATGGATAATTGGATAA	150	77.4
	R	CAACGTAATACCATGAAGA		

CIITA	F	AGCGAAATCAAGGACAAG	82	75.8
	R	CTTCCATCCAGTTGTCATA		
CXCL10	F	GCCACAATGAAAATGAATG	76	70.6
	R	CTGCTTTCAGTAAATTCTTAATG		
CD180	F	CTACCAAACACAACAGAA	72	73.6
	R	GCTGAAGGTTATATCTTGAA		
IL-12p40	F	TCCAGGTCAAAGAGTTTG	114	81.2
	R	CAGTGGACCAAATTTCATC		
CTSG	F	CCTCTGGTATGTAACAATG	82	78.7
	R	TCCTGGTAAAGACTTCTG		
TGFβ	F	CACAGTATATATATGTTCTTCAAC	110	83.9
	R	TCCACATTTAACTTGAGTC		
IL-5 receptor β-chain	F	ATGGGGAGAAATTCATAAG	128	83.3
	R	CTCGATGTGGGAATAGTA		
TLR-7	F	GCTGAAATACTTAGACTACTC	123	79.0
	R	CCTTCTGATTGAAAATAATGG		
CD107b	F	GCTTCAGTTATTAACATCAAC	90	79.0
	R	GTCACTGTTATTCAGCTTAA		
CD79a	F	ATGGCAACAACTCCAAAG	126	83.8
	R	GACTCTTGTTCACATTGGA		
CD69	F	CTAGCTTCCGTTTTGAAA	106	79.2
	R	GCGATGACAGTAATGAATA		
IFNGR1	F	CCGAAATGGTTCCGATAG	101	80.2
	R	CCTGTCCTTCTGTCTTTA		
RANTES	F	CAAGGAATATTTCTACACCA	128	81.6
	R	TCTCCAAAGAGTTGATGTA		
CXCL11	F	GCTTCCCTATGTTCAAAA	100	79.1
	R	CTTGGGTAAATTATAGAGGC		

CD28	F	ATGGGAATTTGGACAATG	88	79.2
	R	ACCTCAATTTTGCAGAAG		
IL-4 receptor	F	CAACCTGACCTACAAGGA	123	85.6
	R	CTCCACTCACTCCAGATG		
CD14	F	CCTGTCCTTGAAAGGAAA	82	81.0
	R	CACGTTAGACTCAGAGTTC		
TNFα	F	GGAAGAGCAGTTCTCCAG	89	83.3
	R	GCTTGTCATTATCGTTTTGAG		
GAPDH	F	CTCGTCATCAATGGAAAG	98	83.6
	R	GTGGATTCCACTACATAC		
TLR-8	F	GTCTGGGATTTCTTGAAA	87	75.4
	R	GCTCATTTTCCTCTGTTA		
CD134	F	GCTGGTTTCAGAGAGAAG	100	83.4
	R	AGGAGAAAGAAGGTCACA		
CD79b	F	GCGGAATACACTGAAAGA	72	77.6
	R	ATGGGCACAATGATGAAA		
CD72	F	ACGCTGCTTTTACTTTTC	75	76.2
	R	GTGGATGACAGAGATGTA		
GM-CSF	F	ATGCCACCATCAATGAAG	90	81.5
	R	GGTCATAGACAACTTCTACTG		
MCP-1	F	AGGGTTATGAAAGAATCAC	78	79.2
	R	AGACCTCCTTGTTCTTTA		
IL-12p35	F	CACTGGAATTAGTCAAGAA	132	81.8
	R	AGTCCTCATAGATACTGTTA		
CD40	F	TACGGCTACTTCTGATAC	84	79.5
	R	GGGTGACACTTTTCAAAA		
IL-7	F	GATCCTTGTTCTGTTACC	91	75.2
	R	GATGCTGACCAGTATAAC		

IL-7 receptor	F	GACGCAATGTATGAGATTA	102	79.7
	R	CTCTGGAGTTTTGAAGTG		
BPI	F	TCCTGAGTTTCCATCTTC	95	82.0
	R	CCCATTCATAACGACATTG		
HPRT1	F	GACCTAGATTTATTTTGTATTCC	81	74.2
	R	GTCCATAATTAGTCCATGAG		
TLR-9	F	CACTCAAGTATAACAATCTTAC	99	82.4
	R	CCGATTCCAGTTTAATAATG		
CD152	F	CCAGATTCTGACTTCCTC	76	79.2
	R	CTGTGATGAGGAAACTGTA		
CD20	F	TCCCGTGACATATATTAACA	94	77.8
	R	GGACTGTATGGTGTAACA		
CD92	F	TCCTGATAATGCTGGTTA	102	81.5
	R	GTTGGAAGACTAACAGTG		
LTA	F	CCTCAATGGCTTCTCTTTG	71	80.6
	R	GCGAGTAGACAAAGTACAG		
MCP-3	F	AGACCAAATTTAACAGAGAAA	91	77.9
	R	GGAGTTTTGGATTTCTTATCTA		
GNCP-1b	F	GCTGTATTTGCACAACAA	73	79.6
	R	ACTCGATTCTGGAAGATG		
CD44	F	AGGAGAATACAGAACACA	115	81.4
	R	GCCATAAGTGCTTCTTTC		
IL-17α	F	AAGGCAGGAATACCAATC	111	80.6
	R	TTGGGTAAGAGGATTGAAG		
IL-18	F	CCTCCTGATAATATCAATGAC	107	76.2
	R	TAGCCTTTATACAATGAAGAC		
CXCL12	F	CAGCCTGAGTTACAGATG	86	82.0
	R	GAGTGTTGAGGATTTTGAG		

β-actin	F	CTACCTTCAACTCCATCA	166	84.6
	R	GGAGCAATGATCTTGATC		
TLR-10	F	TGGGTAAAGAATGAATTGG	117	78.8
	R	GCAGTTAATGATGTTTTCAG		
CD223	F	CAGGAGTCCTCACATCAC	104	82.2
	R	CCATCTCTGTAAGTAAGAATGC		
CD30	F	CACAGTCCTGTCCTCAG	79	82.2
	R	TAGCCGCTCTCATCTAC		
CD93	F	TGCTGCTGTTCTACATC	120	86.6
	R	GTGGCTTCTTCTCCTTTA		
Lysozyme	F	TGGGAGAGTGATTATAATACA	86	76.8
	R	CGACTATTGATCTGGAATATC		
F_c γ1/γ2 receptor	F	CGCTAAATCTGAGGTTGA	95	79.4
	R	TGTGGTTCTGATAGTCATG		
IL-1β	F	CACAGTGGAATTTGAATCC	129	80.8
	R	GACACTAGTTCTAACTTGAAG		
CD81	F	GCTCCAACACATTGATTG	89	81.8
	R	CTTCCATAAGGTATTTATGAAGG		
IL-16	F	GGCTGAAGAAGTCTTTTG	86	80.4
	R	GTCGCTTTTGAAGTATTTTC		
IL-21	F	AAGGCTCAACTCAAGTTA	76	76.2
	R	TCCTCTTCAACTGCTTAA		
CCR4	F	TGAGGACCTTTACGTATG	187	84.8
	R	CCTAGAATGTTGATCTCCA		
eEF1a1	F	TGGGTGTGAAACAACTAA	99	79.4
	R	TGCTGACTTCCTTAACAA		

Shaded boxes indicate primers that may form primer-dimers in reactions with little to no template.

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VITA

Ronald Lee Veselenak II was born in Cambridge, Ohio on November 23, 1973 to Ronald L and Patty Jo Veselenak. Ronald earned a double B.S. in Marine Biology and Marine Fisheries from Texas A&M University in 1997 after which he became employed by the University of Texas Medical Branch at Galveston. Here he acquired new research skills until accepting an offer to take part in an HCV antiviral screening project under the direction of Dr Nigel Bourne. Finally, Ronald worked with Drs Bourne and Pyles to establish the Assay Development Service Division (ADSD) within the Galveston National Laboratory. It was during his tenure in the ADSD that Ronald became interested in vaccine studies and designing and optimizing new assays to aid the research community.

During Ronald's graduate career he was honored with the McLaughlin Travel Award, the Arthur V Simmang Scholarship award and multiple travel awards from the Sealy Center for Vaccine Development.

Ronald had been successfully employed as a Field Application Specialist with Tecan U.S. since October 2014.

HONORS and AWARDS

Arthur V. Simmang Scholarship Fund 2013 Award Recipient

Sealy Center for Vaccine Development Graduate Student Award 2013 Recipient

Sealy Center for Vaccine Development Travel Award Recipient. 2013

McLaughlin Travel Award Recipient. 2012

Sealy Center for Vaccine Development Travel Award Recipient. 2012

Sealy Center for Vaccine Development Travel Award Recipient. 2011

Hosted University of Texas Medical Branch President Dr David Callendar during an "In Your Shoes" program visit. 2010

Hosted Scientific Advisory Board tour of the Assay Development Service Division, Galveston National Laboratory. 2010

Hosted Community Advisory Board tour of the Assay Development Service Division, Galveston National Laboratory. 2010

ARTICLES IN PEER REVIEWED JOURNALS

Wali S, Gupta R, **Veselenak RL**, Li Y, Yu JJ, Murthy AK, Cap AP, Guentzel MN, Chambers JP, Zhong G, Rank RG, Pyles RB, Arulanandam BP. Use of a Guinea pig-specific transcriptome array for evaluation of protective immunity against genital chlamydial infection following intranasal vaccination in Guinea pigs. PLoS One. 2014 Dec 11;9(12):e114261. doi: 10.1371/journal.pone.0114261. eCollection 2014. PMID: 25502875.

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MANUSCRIPTS IN PREPARATION

Veselenak RL, Milligan GN, Pyles RB, Bourne N. Characterization of the Guinea Pig Mucosal Immune Response to Intravaginal Infection with Herpes Simplex Virus Type 2.

ABSTRACTS

RL Veselenak, AL Miller, GN Milligan, RB Pyles, N Bourne. Temporal Characterization of the Mucosal Immune Response to Primary Genital Herpes Infection in the Guinea Pig. 2014 IHII/McLaughlin Colloquium on Infectious Diseases & Immunity. Galveston, TX. April 2014.

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