Genital herpes meets its match: A live HSV-2 *ICP0*$^{-}$ virus vaccine that succeeds where subunit vaccines have failed

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ABSTRACT

Millions of people suffer with recurrent genital herpes. Antiviral drugs and subunit vaccines have been thrown at the problem for decades, but the pandemic spread of genital herpes has not abated. In 2007, I proposed a live HSV-2 vaccine was the most viable solution. A decade and a clinical trial later, it is clear that live HSV-2 *ICP0*$^{-}$ virus vaccines are safe and elicit complete protection against genital herpes, whereas herpes subunit vaccines elicit a fraction of this response. After decades of failed subunit vaccines, live HSV-2 *ICP0*$^{-}$ virus vaccines represent a new opportunity to solve the world's herpes problem. It would be a violation of the Hippocratic Oath to further ignore or delay exploration of a live HSV-2 vaccine's capacity to prevent genital herpes.

KEYWORDS

- herpes simplex virus (HSV)
- live HSV-2 *ICP0*$^{-}$ virus vaccine
- recurrent herpetic disease
- antigenic breadth
- preventative HSV-2 vaccine
- therapeutic HSV-2 vaccine
- live-attenuated virus (LAV) vaccine
- rationally-engineered live virus (RELV) vaccine
- subunit vaccine
- acyclovir-based antiviral drugs

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BODY OF ARTICLE

Introduction

In this introduction, I briefly recount how an aspiring marine biologist came to discover a herpes simplex virus (HSV) vaccine. My interest in marine biology was piqued when I attended an oceanography course at Occidental College in 1985. This led me back to the University of California as an undergraduate in 1986, and from there I pursued research opportunities in Santa Barbara, Australia, and Antarctica. Due to a double-major in marine and microbiology, my studies cut across systems that ranged in scale from biochemistry to ecology, and everything in between. Thus, I came to appreciate that, in a wide variety of biological systems, true understanding always transformed initially "complex" problems into simple and elegant solutions.

In October 1991, I returned home and faced the reality that it was time to pursue a research career that included a salary. I started as a technician in the laboratory of Dr. James Hill studying herpes simplex virus (HSV) biology. Unlike the problems I had studied as an undergraduate, HSV latency and reactivation were problems whose solutions had yet to be discovered. I was enthralled by HSV's biology, which challenged me to think both in terms of the micro-scale of molecular virology as well as the macro-scale of the immune and nervous systems that dictate much of HSV's biology. HSV established infections in the epithelium and nervous systems of animal models, and thus closely mimicked the human condition. HSV was an ideal model for studying the biology of persistent viral infections, which straddle the fields of immunology and virology. In 1992, I commenced my graduate studies with Drs. Daniel Carr and Bryan Gebhardt and an 18-year journey towards understanding how the immune system delivers signals that trigger HSV to establish a latent infection rather than face destruction by host T-cells (reviewed in Ref. [1]).

As a broadly trained biologist, I learned long ago that the most "complex" problems in biology were those described in the least accurate terms. Natural systems always seem more complex when a group of experts settle on a flawed explanation (hypothesis) of how the system "should work." Subsequent generations often accept the flawed hypotheses they inherit as truths, and tend to ignore all evidence to the contrary. So, we arrive at the central tenet of this article.

To this student of HSV biology, the reasons we lack an effective herpes vaccine could not be clearer. Most herpes vaccine concepts originated 25 years before scientists understood HSV's *in vivo* biology. In the 1970s, scientists proposed that any enveloped virus could be defeated with a glycoprotein subunit vaccine [2]. If that were true, then HSV-2 would have become vaccine-preventable in the 1990s.

In 1976, it was proposed that a herpes glycoprotein subunit vaccine would be required to circumvent HSV-2's propensity to caused cancer [2-4]. This was wrong; human papillomaviruses (not HSV-2) proved to be the STD that promoted genital cancers [5]. In the 1970s, scientists also speculated that glycoproteins were the dominant antigens of HSV-2 and would thus yield an effective vaccine [6, 7]. On both counts, they were wrong; ***1.*** HSV-2's most dominant antigens are infected cell proteins rather than glycoproteins [8]; and ***2.*** HSV-2 glycoprotein D (gD-2)-based vaccines have been failing to prevent genital herpes in clinical trials since the 1980s [9-14].

Vaccine scientists have long assumed that HSV was another three-letter antigen that could be defeated with a subunit vaccine. In warfare, there is no better recipe for defeat than to underestimate one's enemy. Vaccine scientists continue to ignore the nuances of HSV-2's *in vivo* biology, which suggest a live HSV-2 vaccine is the only solution that may conquer this foe. In my experience, most vaccine scientists are loathe to acknowledge ***1.*** the underlying principles that explain why HSV-2 subunit vaccines keep failing (Fig. 1), and ***2.*** the value of what we might learn

from clinical trials of a live HSV-2 vaccine (Fig. 4 – 8). In science, it is exceedingly difficult to make new discoveries in areas that one systematically avoids exploring.

A live varicella-zoster virus (VZV) vaccine was proposed in 1974 [15], and has since curbed the incidence of VZV-induced disease [16, 17]. VZV and HSV-2 are related alpha-herpesviruses that infect human neurons and share more than 60 genes in common [18]. If a live VZV vaccine is safe and effective, then a live HSV-2 vaccine should be equally feasible.

In 2007, I proposed that a live HSV-2 *ICP0*$^{-}$ virus would yield a safe and effective herpes vaccine [19]. Below, I provide an update in light of a decade of pre-clinical and human studies of a live HSV-2 *ICP0*$^{-}$ virus vaccine. The available evidence suggests a live HSV-2 *ICP0*$^{-}$ virus vaccines represent a viable treatment option for ***1.*** protecting vulnerable partners from the risk of contracting genital herpes (Fig. 4-6), and ***2.*** therapeutically reducing the symptoms of recurrent genital herpes (Fig. 7-8). Applying Occam's Razor, I would suggest that it is time to embrace the most likely possibilities that ***1.*** genital herpes is a vaccine-preventable disease, and ***2.*** genital herpes will begin to be eradicated the day a live HSV-2 vaccine is made available to patients.

Current herpes treatment and prevention options

i. A 21st Century view of HSV-1 versus HSV-2 infections. Herpes is a disease manifestation that may occur at a variety of anatomic locations (e.g., mouth, eye, brain, cuticles, genitalia, buttocks, peripheral ganglia, intestines, or liver) [20-24]. The causative agents of herpetic disease are two serotypes of herpes simplex virus, HSV-1 and HSV-2, which are nearly identical viruses that encode ~75 equivalent proteins from co-linear genomes. The ~75 proteins encoded by HSV-1 and HSV-2 differ enough in their amino acid usage (epitope signatures) that the adaptive immune response to HSV-1 only partially cross-protects against HSV-2.

HSV-2 spreads faster than HSV-1 and differs enough in amino acid sequence to allow HSV-2 to superinfect HSV-1-positive individuals. HSV-2 is predominantly sexually transmitted, and thus primarily causes genital herpes. In contrast, HSV-1 is more adept at establishing infections anywhere in the body and causes a wider variety of diseases (e.g., ocular herpes, whitlow, encephalitis). Unlike past generations, young adults today are often HSV-1 seronegative when they reach adulthood; hence, HSV-1 now causes >50% of cases of genital herpes in young women [25, 26]. Doctors have long been trained to believe HSV-1 infections predominantly occur "above the belt," but recent epidemiological data tells a different story.

ii. HSV seroprevalence versus recurrent herpetic disease. About 4 billion people worldwide are infected with HSV-1 and/or HSV-2 [27, 28]. Every week, more than 1 million people are newly infected with HSV-1 or HSV-2 during their interactions with 4 billion carriers who often shed infectious HSV-1 or HSV-2 in the absence of symptoms [29-32].

Like many viral diseases [33], only a subset of HSV infections progress to high-level disease. About 80% of HSV infections produce no visible symptoms, and about 10% of HSV infections resolve within the first month of primary infection. The 2 - 3% of HSV-infected persons

who progress to a lifetime of herpetic disease represent about ~100 million people who suffer with a chronic disease and the fear of transmitting their infection to others. Recurrent herpetic disease afflicts more people than reside in Germany, the most populous country in Europe.

iii. <u>Antiviral drugs and synthetic herpes vaccines: Still center-stage</u>. Herpetic disease represents a major challenge that is too often underestimated in terms of its human toll. An essential step towards a viable herpes solution is to first understand why past solutions have failed to slow the pandemic spread of herpes. Only three strategies have been proposed that have an established track record of clinical success in preventing viral disease, and these are ***1.*** antiviral drugs [34]; ***2.*** viral protein subunit vaccines [35]; and ***3.*** live-attenuated virus (LAV) vaccines [15, 36-42].

Acyclovir-based drugs are effective at preventing HSV replication in cultured cells, but have limited bioavailability *in vivo*. Famciclovir and valacyclovir partially address this limitation [43, 44], but have not slowed the pandemic spread of herpes [45, 46]. Herpes glycoprotein subunit vaccines have been investigated in six U.S. clinical trials, but have not curbed the symptoms of recurrent genital herpes [12, 13] nor protected naïve recipients from contracting genital herpes [9-11, 14]. *Bona fide* live-attenuated HSV-2 virus vaccines have the potential to stop the spread of herpes, but have not been tested in a single U.S. clinical trial (reviewed in Ref. [47]).

Bona fide live-attenuated HSV-2 virus vaccines should not be confused with replication-defective viruses that deliver a cargo of foreign antigens to a recipient [48-50]. Such replication-defective, antigen-delivery vehicles have yet to yield a single viable vaccine, and one such HIV vaccine candidate made recipients more susceptible to HIV infection [51]. In the current article, the term "live HSV-2 vaccine" is reserved for live viruses that undergo limited replication and spread in vaccine recipients, as is true of all LAV vaccines that have succeeded in clinical practice.

Live versus subunit HSV-2 vaccines: Why does it matter?

i. ***General concepts***. Synthetic virus vaccines expose recipients to a subset of virus-encoded peptides with the goal of eliciting protective immunity against a viral pathogen. Glycoprotein subunit vaccines are one iteration of this concept. Hundreds of synthetic virus vaccine concepts have been proposed over the past 30 years for a variety of diseases, but few have yielded viable vaccines. Only the hepatitis B and human papillomavirus subunit vaccines have been effective in reducing the incidence of human disease caused by a viral pathogen [35, 52].

There are several reasons that a live HSV-2 virus vaccine should succeed where subunit vaccines have failed. HSV-2 is a 154-kb dsDNA genetic element that is 50 times larger than hepatitis B virus [47], and which encodes 30 different viral proteins that are targeted by host B- and T-cells [8, 53, 54]. It would be difficult to recapitulate this level of antigenic complexity with a synthetic HSV-2 vaccine. Live virus vaccines offer a simple approach to develop a superior HSV-2 vaccine because we do not need to define the combination of HSV-2 epitopes that elicit a protective immune response. Rather, the only question that need be addressed is, "*Which mutation(s) in the HSV-2 genome yield a live-and-appropriately attenuated virus vaccine?*" An appropriate level of attenuation simply requires ***1.*** eliminating HSV-2's disease-causing potential whilst ***2.*** retaining HSV-2's capacity to elicit a protective immune response.

ii. ***Underlying principles***. At least three principles should be considered in designing an effective HSV-2 vaccine, and these are *i.* *antigenic breadth*; *ii.* *antigenic context*; and *iii.* *antigen load*. *Antigenic breadth* refers to the percentage of an infectious agent's proteome represented in a vaccine. Most gD-2 subunit vaccines introduce recipients to 1% of HSV-2's proteome (Fig. 1A). The 99% of HSV-2's proteome that is missing from subunit vaccines limits their efficacy [47]. In contrast, live HSV-2 *ICP0*$^{-}$ virus vaccines retain 99% of HSV-2's antigenic breadth (Fig. 1A).

Antigenic context refers to the 3-dimensional array of epitopes that drive a protective immune response. Glycoprotein D (gD) is but 1 of 10 glycoproteins that appear on the surface of HSV virions (Fig. 1B) [55]. The 3-dimensional array of B- and T-cell epitopes on HSV-infected epithelial cells and neurons is at least an additional two orders of magnitude more complex (Fig. 1B). Only a live HSV vaccine may recapitulate the full array of virus epitopes to which B-cells, $CD4^+$ T-cells, and $CD8^+$ T-cells cooperatively respond to generate a protective immune response.

Regarding *antigen load*, a live HSV virus will undergo amplification and limited viral spread in human vaccine recipients, which favors a larger peak antigen load than a synthetic HSV vaccine (Fig. 1C). Moreover, a live HSV virus may be capable of low-level, episodic synthesis of viral antigen over time (Fig. 1C). The potential of a live α-herpesvirus vaccine to establish a life-long infection that sub-clinically reactivates may be required to elicit durable protective immunity against genital herpes, as appears to be the case with the live VZV Oka vaccine [56, 57]. Provided that the latent α-herpesvirus vaccine is *appropriately attenuated*, such small-scale (molecular) reactivations of viral antigen synthesis will be insufficient to spread and cause disease. Despite initial concerns surrounding the live VZV Oka vaccine's capacity to establish latent infections, it now has a strong safety record spanning 40 years of clinical use [58, 59].

iii. Live HSV-2 ICP0⁻ virus vaccines elicit superior protection. The evidence that HSV-2 viral vaccines elicit superior protection relative to glycoprotein subunit vaccines is briefly reviewed. In the study of Halford, et al, 2013 [60], guinea pigs were immunized on Days 0 and 30 with ***1.*** culture medium (naïve); ***2.*** gD-2 + alum/MPL adjuvant; or ***3.*** a live HSV-2 *ICP0⁻* virus, HSV-2 0ΔNLS. Following a lethal HSV-2 challenge, naïve guinea pigs shed high titers of HSV-2 from their vaginas and developed florid perivaginal disease that required their sacrifice (Fig. 2). Guinea pigs immunized with gD-2 + alum/MPL shed 4-fold less HSV-2 per vagina, but still developed disease

(Fig. 2). In contrast, guinea pigs immunized with HSV-2 0ΔNLS shed an average 200-fold less HSV-2 per vagina at Days 1 – 3 post-challenge, and were completely protected from perivaginal disease and death (Fig. 2). Halford, et al., (2011) reported similar side-by-side test results in mice [61]. Other research groups have also concluded that whole viral vaccines are superior to gD-2 subunit vaccines [48, 62-64]. Thus, adjuvanted gD-2 subunit vaccines are not only ineffective in human clinical trials [9-14], but are equally ineffective in protecting mice and guinea pigs against HSV-2 genital herpes [48, 60-64].

iv. Live HSV-2 ICP0⁻ virus vaccines elicit a superior antibody response. Adjuvanted gD-2 vaccines elicit a high titer of antibody against the 55 kDa gD-2 protein, but elicit poor protection against HSV-2 [8, 61]. The pan-HSV-2 IgG antibody response elicited by a variety of HSV-2 vaccine candidates correlates with protection against HSV-2 challenge [60]. This observation began to shed light on why gD-2 subunit vaccines were ineffective; HSV-2 0ΔNLS elicited 40-fold higher levels of pan-HSV-2 IgG antibody relative to a gD-2 subunit vaccine [60, 61]. Likewise, the live HSV-2 0ΔNLS vaccine elicited a polyclonal IgG antibody response that targeted 9 to 19 different HSV-2 proteins, and >90% of those antibodies targeted viral proteins other than gD-2 (Fig. 3) [8, 47]. If HSV-2's most dominant antigens were infected-cell proteins, rather than glycoproteins as postulated in the 1970s and 1980s [2, 7, 65], then it would stand to reason that gD-2 subunit vaccines should be insufficient to prevent HSV-2-induced herpetic disease.

Live HSV-2 ICP0⁻ virus vaccines: Why are they safe?

i. Why knock out ICP0 to make a live HSV vaccine? In 1997, I joined Dr. Priscilla Schaffer's laboratory as a postdoctoral fellow and there demonstrated that infected cell protein 0 (ICP0) was necessary and sufficient to trigger HSV-1 to reactivate in neurons [66, 67]. In 1997, I first observed

that HSV-1 *ICP0*$^{-}$ viruses established self-limited infections in the corneas of mice with severe-combined immunodeficiency (SCID) (Fig. 1 in [68]). By 2005, it was evident that HSV-1 *ICP0*$^{-}$ viruses were avirulent in SCID mice because the host interferon response was adequate to suppress their replication *in vivo* [68 , 69, 70] At the very end of this SCID mouse study, I inoculated some immunocompetent mice with an avirulent HSV *ICP0*$^{-}$ virus and observed they acquired robust immunity to HSV-1. Hence, after nine years, it finally dawned on me that HSV *ICP0*$^{-}$ viruses might serve as an exceptionally safe class of live HSV vaccines [68].

ii. Live HSV-2 ICP0^{-} viruses establish self-limited infections in animal models. In 2010, my laboratory demonstrated that live HSV-2 *ICP0*$^{-}$ viruses were, as predicted, ***1.*** interferon-sensitive; ***2.*** attenuated in SCID mice; and ***3.*** capable of eliciting a robust protective immune response [71]. HSV-2 *ICP0*$^{-}$ viruses, such as HSV-2 0ΔNLS, were attenuated because they established self-limited infections that failed to spread adequately to cause disease *in vivo* [71]. The live HSV-2 0ΔNLS virus was later proven to be avirulent in guinea pigs [60] and humans (Fig. 4-8).

iii. HSV-2 0ΔNLS: A rationally-engineered live virus (RELV) vaccine. Eight *live-attenuated virus* (LAV) vaccines have been advanced to clinical usage over the past 200 years, and these are vaccinia [36], yellow fever strain 17D [37], polio Sabin strains 1, 2, and 3 [38], mumps Jeryl Lynn strain [39], rubella Cendehill strain [40], measles Schwarz strain [41], varicella-zoster Oka strain [15], and human rotavirus 89-12 strain [42]. These LAV vaccines have a record of acceptable safety and superior efficacy, but are accidents of nature whose attenuated phenotype is documented but not well explained [72]. Such LAV vaccines are far too similar to their disease-causing counterparts, and this well-documented fact underlies legitimate concerns about their safety.

In the 21st century, it is possible via genetic engineering [73] to create more stable *rationally-engineered live virus* (RELV) vaccines by introducing large deletions into viral genes.

In the case of live HSV-2 *ICP0*$^{-}$ viruses, my laboratory engineered nine different HSV-2 *ICP0*$^{-}$ mutant viruses to find three that were appropriately attenuated: 0ΔNLS, 0ΔRING, and 0ΔMD [71]. If a live HSV-2 *ICP0*$^{-}$ virus vaccine, such as HSV-2 0ΔNLS, is advanced to clinical usage, then it will be the first example of a RELV vaccine where ***1.*** the attenuating mutation removes >2,000 bp of a virus's genome and ***2.*** the basis of viral attenuation is well defined. Given that live HSV *ICP0*$^{-}$ viruses are attenuated in SCID mice, it is unlikely that they would pose a risk to immuno-compromised patients [68, 71]. Such considerations suggest that a RELV vaccine, like HSV-2 0ΔNLS, would be far safer than the LAV vaccines that are administered to children today [17, 74].

Human Recipient #1: Self-testing of a live HSV-2 ICP0^{-} *virus in WPH*

i. How I came to be the first person inoculated with a live HSV-2 ICP0*$^{-}$ *virus. In February 2011, I was diagnosed with sinonasal undiffererentiated carcinoma. A golf ball-sized tumor was breaking through the cribiform plate that separated my sinuses from my cranium. Given the 5-year-survival rate for such patients [75], it was unlikely that I would survive long enough to advance a live HSV-2 *ICP0*$^{-}$ virus vaccine to clinical trials by the traditional route. My cancer responded to chemotherapy and radiation by August 2011, but by then I had come to appreciate that time was of the essence; *carpe diem!* Under this mindset, I, William P. Halford (WPH), wrote a protocol of how I would conduct experiments in a single trial participant, WPH, and I then proceeded to administer an intradermal injection of 3 x 10^6 plaque-forming units (pfu) of the live HSV-2 0ΔNLS vaccine into the dermis of my left forearm (Fig. 4A). Unlike the 5- to 10-year timeline required to initiate an FDA-sanctioned Phase I clinical trial in the United States, all of this happened on September 14, 2011.

Some readers may find this course of action reckless, as it deviates from how HSV-2 vaccines have traditionally advanced to U.S. clinical trials. However, from my perspective, I would suggest

the opposite. The protracted process by which HSV-2 vaccines currently advance to U.S. clinical trials is well meaning, but violates the Hippocratic Oath; do not harm! There is an ongoing herpes pandemic that demands the scientific community's attention today, not tomorrow. The *risk* I accepted by self-injecting the live HSV-2 0ΔNLS vaccine pales in comparison to the morbidity that *actually occurred* in the ~1.5 billion people who were newly infected with HSV-1 or HSV-2 whilst FDA-sanctioned herpes subunit vaccine trials have failed for three decades [9-14, 58, 76].

There are too many variables in the dozens of immunizations I have self-administered for the results to define an ideal formulation for a HSV-2 preventative vaccination series. However, the results do suggest that the key properties of the live HSV-2 0ΔNLS vaccine, defined in animal models [8, 60, 61, 71, 77], do in fact translate to humans. I review the evidence that the live HSV-2 0ΔNLS vaccine is, in at least n=1 human subject, well tolerated, immunogenic, and elicits a protective humoral immune response that may be transferred to naïve μMT mice (Figs. 4-6).

ii. Safety of a live HSV-2 ICP0$^-$ virus in Recipient #1. I have given myself more than two dozen immunizations with the live HSV-2 0ΔNLS vaccine. I have found the live HSV-2 0ΔNLS virus to be well tolerated, despite my being ***1.*** HSV-seronegative (naïve) at the time of my first vaccination and ***2.*** repeatedly immunosuppressed with chemotherapy over the past five years. Following my first intradermal vaccination with a dose of 3 x 10^6 pfu in my left forearm, I observed an outward spread of HSV-2 0ΔNLS infection in the skin between Days 2 and 6 post-vaccination, which produced an area of inflammation surrounding the injection site that peaked in size at ~6 cm in diameter (Fig. 4A).

Following vaccination of my forearm, I opted for an injection site that placed the immunization site as far away from the brain as practical, the inner calf, in order to minimize any potential concern that the live HSV-2 0ΔNLS vaccine might cause encephalitis (Fig. 4B). Secondary HSV-2 0ΔNLS boosters of the inner calf produced an inflammatory response in the skin that was virus dose-

dependent, and which peaked at 48 hours post-immunization (Fig. 4B). Secondary boosters that contained doses less than 10^6 pfu failed to elicit an inflammatory reaction in the skin (Fig. 4B) and failed to elicit an increase in serum levels of pan-HSV-2 IgG antibody. In contrast, secondary boosters with $>10^7$ pfu HSV-2 0ΔNLS consistently elicited an inflammatory response in the skin and a corresponding increase in serum levels of pan-HSV-2 IgG antibody (data not shown).

One of the concerns with a live HSV-2 vaccine is that the attenuated virus may, in principle, establish a latent infection in the recipient that later produces recurrent lesions at the injection site. Despite receiving more than two dozen self-immunizations, I have never observed a secondary recurrence of inflammation at a prior HSV-2 0ΔNLS vaccination site. This is hardly surprising, as HSV *ICP0*$^-$ viruses are grossly impaired in their capacity to reactivate from latency [78].

iii. Immunogenicity of a live HSV-2 ICP0*$^-$ *virus in Recipient #1. My laboratory has developed a flow-cytometry-based test that measures antibody-binding to virus-infected cells (ABVIC) [79]. The ABVIC test accurately quantifies pan-HSV-2-specific IgG antibody levels in mice [8, 61, 77], guinea pigs [60], and humans (unpublished data). Pan-HSV-2 IgG levels correlate with HSV-2 virion-neutralizing antibody titers, but are far more precise than the 2-fold increments in antibody titer returned by virion-neutralization assays [61, 77].

When serum from a HSV-seronegative individual is added to fixed and permeabilized test cells (i.e., a mixture of uninfected [UI] cells, HSV-1^+ cells, and HSV-2^+ cells), the ABVIC test demonstrates that virus-infected test cells remain at the same low vertical position on the y-axis of flow cytometry plots as UI cells devoid of HSV antigen (Fig. 5A, left panel). In contrast, when serum from patients with recurrent HSV-2 genital herpes is added to the same test cells, the HSV-1^+ and HSV-2^+ cells shift up the y-axis of flow cytometry plots relative to UI cells (Fig. 5A, middle and right panels). Using patient serum samples as controls, it is possible to define the range of serum levels of pan-HSV-2-IgG

that naturally occur in the human population (Δ2 in Fig. 5A; Fig. 5C). Relative to these controls, Recipient #1 of the live HSV-1 0ΔNLS vaccine, WPH, was seronegative for HSV-2 antibodies at multiple times prior to vaccination (Fig. 5B, 5D). In contrast, 29 days after Vaccination #1, WPH exhibited a significant increase in pan-HSV-2 IgG antibody levels (Figs. 5B, 5D). At all times post-vaccination (with the benefit of dozens of boosters), WPH remained HSV-2 seropositive, although antibody levels did wane in between boosters (Fig. 5D). WPH's highest serum levels of pan-HSV-2 IgG were observed on Day 1,556 (October 2015), which occurred one month after receiving two intradermal immunizations of $2x10^8$ pfu HSV-2 0ΔNLS in August and September 2015 (Fig. 5B, 5D). Importantly, peak levels of pan-HSV-2 IgG in WPH approached the levels of pan-HSV-2 IgG antibody that normally occur in patients with HSV-2-driven genital herpes (Fig. 5C vs 5D).

iv. Mouse model to evaluate the protective efficacy of HSV-2 vaccines in humans. B-cell-deficient μMT mice produce functional T-cells, but lack the complementary functions of antibody required for complete vaccine-induced protection against HSV-2 [77]. Reciprocally, adoptive transfer of HSV-2 0ΔNLS antiserum from *vaccinated C57BL/6 mice* protects naïve μMT recipient mice from HSV-2 challenge [77]. Therefore, I wished to determine if adoptive transfer of HSV-2 0ΔNLS antiserum from *a vaccinated human (WPH)* might similarly protect naïve μMT recipient mice from HSV-2 challenge. If so, this might provide a useful tool for gauging the protective efficacy of preventative HSV-2 vaccine candidates in human clinical trials.

To explore this possibility, two groups of n=20 naïve B-cell-deficient μMT mice were treated with either ***1.*** seronegative WPH pre-serum or ***2.*** WPH post-0ΔNLS-vaccination serum, and were then ocularly challenged with wild-type HSV-2 MS-GFP (Fig. 6). Naïve μMT mice that received WPH post-0ΔNLS antiserum (d1556) exhibited a transient, but significant, 5-fold decrease in ocular HSV-2 MS-GFP shedding at 24 hours post-challenge relative to recipients of WPH pre-serum (Fig. 6A).

Profound changes were noted in the extent of HSV-2 challenge virus-induced pathogenesis that developed in naïve μMT mice that received pre- versus post-0ΔNLS-vaccination serum (Fig. 6B, 6C, 6D). Only 9 of 20 recipients of WPH pre-serum survived ocular HSV-2 MS-GFP challenge (Fig. 6B), and all survivors exhibited overt disease (survivor image in Fig. 6C). All non-survivors exhibited typical symptoms of herpes encephalitis prior to death, and dissection revealed large areas of GFP expression in the brain, confirming that HSV-2 MS-GFP challenge virus had invaded the CNS (non-survivor image in Fig. 6C). In contrast, all 20 naïve mice that received WPH post-0ΔNLS vaccination serum survived ocular HSV-2 MS-GFP challenge with little to no disease (Fig. 6B, 6D).

Human Recipients #2 - 21: Phase I trial of a therapeutic live HSV-2 ICP0⁻ vaccine

i. Why should a live HSV-2 ICP0⁻ virus vaccine help genital herpes sufferers? In March 2015, I co-founded Rational Vaccines, Inc. (http://rationalvaccines.com/) with my business partner, Agustin Fernandez III, as a means to fund a Phase I clinical trial to investigate the potential of the live HSV-2 0ΔNLS vaccine to therapeutically reduce the symptoms of recurrent genital herpes. Most therapeutic HSV-2 vaccine candidates today are based on ~2% of HSV-2's antigenic breadth, and are thus similar to HSV-2 subunit vaccines that have been failing for decades [9-14, 76]. These synthetic HSV-2 vaccine candidates include Genocea's GEN-003 (gD + ICP4 [80]); Vical's Vaxfectin (gD + UL46 [81]); Admedus' COR-1 (gD [82]); and Agenus's HerpV (32 T-cell peptides; [83]). A recent Phase I trial of a therapeutic HSV-2 0ΔNLS vaccine was initiated in March 2016. Herein, I briefly highlight the findings as a means to convey that HSV-2 0ΔNLS may offer a superior vaccine, as it offers 50-fold greater antigenic breadth than GEN-003, Vaxfectin, COR-1, or HerpV [80-83] (Fig. 1A).

Prior studies have reported that when inactivated or live HSV-2 vaccines are administered to genital herpes sufferers, recipients often report a reduction in their herpes symptoms [84-87]. These

studies have gone largely unnoticed because the findings are both phenomenological and paradoxical in nature. Moreover, a dearth of evidence has been offered to explain why a therapeutic HSV-2 vaccine should alter the course of recurrent genital herpes in a patient who contracted HSV-2 years to decades earlier [84-87]. The preliminary findings of the recent HSV-2 0ΔNLS vaccine trial may offer a surprisingly simple explanation to this paradox; *reversal of immunological tolerance to HSV-2*.

Clonal exhaustion of lymphocytes explains how the vertebrate immune system becomes tolerant, or unresponsive, to many chronic viral infections [88-90]. If immunological tolerance to HSV-2 is common in patients who are "high reactivators," then treatment with a therapeutic HSV-2 vaccine might reduce their herpes symptoms by re-awakening their HSV-2-specific B- and T-cells out of a state of anergy (tolerance), and eliciting a more protective immune response (Fig. 8). I briefly review the findings of a live HSV-2 0ΔNLS vaccine trial that support this interpretation.

ii. Tolerability of the live HSV-2 0ΔNLS vaccine in genital herpes sufferers. More than 100 individuals were interviewed to review their case histories and/or test results that led them to conclude they had contracted genital herpes. A subset of 47 individuals who self-reported high levels of recurrent herpes symptoms were screened by the HSV ABVIC test to verify they possessed antibodies to HSV-1 and/or HSV-2 (data not shown). Twenty citizens of the U.S. or United Kingdom who were HSV-1 and/or HSV-2 seropositive were enrolled in the trial. On average, these individuals had contracted genital herpes 11 years prior to receiving the HSV-2 0ΔNLS vaccine.

Trial participants were flown to the Federation of St Kitts and Nevis on three occasions to receive three intradermal immunizations with 2 x 10^8 pfu of the live HSV-2 0ΔNLS vaccine in the inner calf of the left, right, and left legs (i.e., Shots 1, 2, and 3, respectively). The first group of n=10 participants received a live HSV-2 0ΔNLS vaccination series between March and June, 2016. Having established tolerability in Group 1, another n=10 participants received the same HSV-2 0ΔNLS

vaccination series between June and August, 2016. When questioned about the discomfort caused by intradermal immunization with HSV-2 0ΔNLS, participants rated the discomfort as a 2 ± 2 on a 0- to 10-point pain scale (Fig. 7A). When asked how HSV-2 0ΔNLS vaccine-induced discomfort compared to their genital herpes symptoms, 19 of 20 indicated the vaccine was less painful (Fig. 7B).

iii. Therapeutic effect of live HSV-2 0ΔNLS vaccine in genital herpes sufferers. All but one trial participant had attempted to use, or were actively using, prophylactic antiviral drugs to manage their herpes symptoms prior to receiving the HSV-2 0ΔNLS vaccine. Of the n=17 who completed the HSV-2 0ΔNLS vaccination series, participants self-reported an average 3.1-fold reduction in their frequency of herpes-symptomatic days during the first 4 to 6 months post-vaccination relative to their symptoms prior to vaccination (black versus hatched bars in Fig. 8A). All trial participants discontinued the use of antiviral drugs at least three days prior to Shot 1, and most discontinued the use of antiviral drugs between Shot 1 and the end of the initial evaluation period (i.e., Sept 30, 2016).

Participants self-reported a significant reduction in their frequency of herpes-symptomatic days after receiving the live HSV-2 0ΔNLS vaccination series (Fig. 8A; $p<10^{-5}$ by a paired t-test). Taken alone, these results should be as unconvincing as the phenomenological observations of Skinner, et al. [84-86] and Casanova, et al. [87]. However, the results of the HSV-2 0ΔNLS vaccine trial differ in that objective evidence was collected that the vaccine elicited significant changes in the immune response to HSV-2 (Fig. 8B, 8C). To illustrate the point, I focus on the analysis of vaccine-induced changes in the immune response of a single participant, CE-27 (white arrow in Fig. 8A).

CE-27 contracted HSV-2 genital herpes in 1992, and had been on daily antiviral therapy since 1995. It should be noted that individuals who use daily antiviral drugs for >6 months often exhibit a significant contraction in their antibody response to HSV-2 [91, 92]. Despite daily valacyclovir, CE-27 experienced an average of 5 outbreaks per year that lasted 6 to 8 days per outbreak (Fig. 8A). Two

days after receiving Shot 1 containing 2 x 10^8 pfu of HSV-2 0ΔNLS, CE-27 returned for his post-vaccination follow-up and exhibited a 60 cm^2 area of inflamed epithelium at the injection site (Fig. 8B). When CE-27 received equivalent HSV-2 0ΔNLS vaccinations on Days 40 and 70, the inflamed area surrounding the injection site decreased in size to 40 and 9 cm^2, respectively (Fig. 8B). This would be expected in a HSV-2-seronegative vaccine recipient (Fig. 4). However, CE-27's improved capacity to contain the live HSV-2 0ΔNLS virus injected in the skin between Shots 1 and 3 (Fig. 8B) suggested that CE-27's first 24 years of experience with wild-type HSV-2 had not elicited an optimal (protective) immune response to HSV-2.

Western blot analysis demonstrated that CE-27's HSV-2 antibody response was detectable, but marginal, prior to receiving Shot 1 of HSV-2 0ΔNLS (Days -42 and 0 in Fig. 8C). In contrast, CE-27's serum collected on Day 40 exhibited a 3-fold increase in pan-HSV-2-specific IgG by ABVIC test (data not shown). Western blot analysis indicated that CE-27's increased levels of HSV-2-specific antibodies were directed against >10 HSV-2 proteins (Day +40 in Fig. 8C). A therapeutic HSV-2 vaccine based on gD or isolated HSV-2 peptides would be unlikely to elicit such a polyclonal B-cell response [80-83]. CE-27's self-reported cessation of genital herpes outbreaks over the first 6 months post-vaccination represents "soft science," and should be viewed with skepticism (white arrow in Fig. 8A). However, it is unlikely that the demonstrable changes in CE-27's immune response to HSV-2 are psychosomatic in nature (Fig. 8B, 8C). Hence, a live HSV-2 *ICP0*$^-$ virus, which retains 99% of HSV-2's antigenic breadth (Fig. 1A), should offer long-time herpes sufferers some hope that it may be possible to better manage their genital herpes symptoms via therapeutic vaccination (Fig. 8A). Further clinical trials will be required to fully vet this clinically important hypothesis.

FUTURE PERSPECTIVE

The failures of HSV-2 subunit vaccines have a human toll; every week that the world lacks a preventative herpes vaccine means that >1 million people will be newly infected with HSV-1 or HSV-2. VZV and HSV are closely related α-herpesviruses [18]. If the live VZV Oka vaccine is safe and effective, then live HSV vaccines should be equally feasible. Not only is this true in principle (Fig. 1), but my research of the past decade suggests that it is true in practice (Fig. 2-8).

Since the 1970s, vaccine scientists have rejected the possibility of human testing of a live HSV-2 vaccine in a U.S. clinical trial based on unfounded fears [2]. I believe that it is time to move forward into the 21st century, and embrace the evidence that ***1.*** herpes gD subunit vaccines have been failing for decades [9-14]; ***2.*** live HSV-2 *ICP0*$^{-}$ virus vaccines are safe and well tolerated (Fig. 4, 7); and ***3.*** live HSV-2 *ICP0*$^{-}$ virus vaccines are 50- to 100- times more effective than herpes gD subunit vaccines [8, 60-62].

The available evidence indicates the world has little to lose, and much to gain, by advancing live HSV *ICP0*$^{-}$ virus vaccines into Phase I/II clinical trials (Fig. 4-8). Applying Occam's Razor, I would suggest that it is time to embrace the most likely possibilities that ***1.*** genital herpes is a vaccine-preventable disease, and ***2.*** genital herpes will begin to be eradicated the day a live HSV vaccine is made available to patients.

EXECUTIVE SUMMARY

- Four billion people are infected with HSV-1 and/or HSV-2, and the life-long condition of recurrent herpetic disease afflicts ~100 million people.
- In 2015, the World Health Organization called for increased efforts to develop a HSV-2 vaccine.
- HSV-2 glycoprotein D (gD-2) subunit vaccines have been the primary herpes vaccine candidate for three decades, but have failed in clinical trials spanning three decades.
- In animal models, live HSV-2 *ICP0*$^{-}$ viruses, such as HSV-2 0ΔNLS, elicit 50- to 100-fold greater protection against genital herpes than a gD-2 vaccine.
- The live HSV-2 0ΔNLS vaccine elicits 40-fold higher levels of HSV-2-specific antibody than a gD-2 vaccine, and elicits an antibody response against 9 to 19 HSV-2 proteins.
- HSV-2 0ΔNLS belongs to a new class of rationally-engineered live virus (RELV) vaccines that are safer than the live-attenuated virus (LAV) vaccines administered to children today.
- In one seronegative person (the author), the live HSV-2 0ΔNLS vaccine elicited high levels of HSV-2-specific antibody that, upon transfer, protected naïve μMT mice from HSV-2 challenge.
- In a recent Phase I trial (n=20 persons), the live HSV-2 0ΔNLS vaccine was well tolerated by genital herpes sufferers and they reported reduced herpes symptoms after vaccination.
- Genital herpes is almost certainly a vaccine-preventable disease, but a live HSV-2 vaccine will be required to achieve this goal.

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FINANCIAL DISCLOSURE

William Halford is a co-author on United States Patent Application 8,802,109, which describes the uses of HSV-2 *ICP0*$^{-}$ viruses in the design of a live-attenuated HSV-2 vaccine. William Halford is a co-founder of Rational Vaccines, Inc. that was established in March 2015 for the purpose of advancing live HSV-2 *ICP0*$^{-}$ viruses as a new class of therapeutic and preventative vaccines. Rational Vaccines, Inc. holds the exclusive licenses to U.S. Patents 7,785,605 and 8,802,109, and International PCT/US2014/070915, which collectively describe the use of HSV-1 or HSV-2 *ICP0*$^{-}$ mutants in the design of live-attenuated vaccines and an improved herpes serological testing procedure, ABVIC. The decade of research summarized herein was supported by (1) Central Research, Excellence in Academic Medicine, and Concept Development Awards from the Southern Illinois University School of Medicine; (2) a grant from the National Institutes of Health to study the Development of an Effective Genital Herpes Vaccine (R21 AI81072); (3) private donations to the Halford Vaccine Research Fund administered by the Southern Illinois University Foundation; and (4) private investment into Rational Vaccines, Inc. which supported the Phase I clinical trial described herein. None of these funders, donors, nor investors influenced the timing nor content of this manuscript. The financial interests of Rational Vaccines, Inc. have not altered nor influenced the interpretation of the evidence presented herein. William Halford is an Associate Professor at the Southern Illinois University School of Medicine. The viewpoints expressed herein are solely that of the author, and should not be construed as an official statement made on behalf of the Southern Illinois University, the School of Medicine, nor any of the academic departments contained therein.

FIGURE LEGENDS

Figure 1. Advantages of a live HSV-2 *ICP0*$^{-}$ virus vaccine versus a gD subunit vaccine. (**A**) The HSV-2 *US6* gene encodes the 393 amino-acid glycoprotein D (gD-2), and the N-terminal 302 amino acids of mature gD-2 were the basis of the Herpevac vaccine trial [9]; this corresponds to 0.8% of HSV-2's antigenic breadth. In contrast, the live HSV-2 0ΔNLS vaccine retains 99.3% of HSV-2's antigenic breadth. **(B)** Glycoprotein D's 3-dimensional structure is depicted on the left [93], and glycoprotein D is shown in the context of an infectious HSV virion, which contains >30 HSV-encoded proteins [55]. The right panel shows the *full antigenic context* of an HSV-infected cell, which serves as virion-factories that propagate HSV infection *in vivo* and which are likely the most relevant target of a protective B- and T-cell response to HSV. **(C)** A glycoprotein D subunit vaccine should provide a one-time bolus of 1 of HSV's 75 proteins, whereas an optimal live-attenuated HSV vaccine would initially express ~75 HSV proteins and would establish a latent infection that supports the episodic, low-level (asymptomatic) expression of HSV antigens, hence providing 'endogenous HSV boosters' and promoting life-long protection against exogenous exposures to wild-type HSV. *The glycoprotein D image shown in Panel B is reprinted from Di Giovine, et al., 2011, PLoS Pathog 7:e1002277 [93] with the permission of the corresponding author, Claude Krummenacher and is reprinted in accordance with PLoS's Open Access agreement (https://creativecommons.org/licenses/by/4.0/legalcode). The HSV virion image in Panel B was purchased from https://www.dreamstime.com/ as a Royalty Free Stock Image.*

Figure 2. Comparison of vaccine-induced protection against genital herpes in guinea pigs immunized with adjuvanted gD-2 or live HSV-2 0ΔNLS. Guinea pigs were immunized in their rear footpads on Days 0 and 30 with culture medium (n=5), 5 μg gD-2+ alum/MPL (n = 4), or 2 x

10^6 pfu live HSV-2 0ΔNLS (n = 5). On Day 75, blood was harvested, and on Day 90, guinea pigs were challenged with 2×10^6 pfu per vagina of wild-type HSV-2 MS. (**A**) Mean ± sem pfu of HSV-2 shed per vagina between Days 1 and 8 post-challenge in guinea pigs that were naïve or were immunized with adjuvanted gD-2 or live HSV-2 0ΔNLS. A single asterisk (*) denotes $p<0.05$ and a double asterisk (**) denotes $p<0.0001$ that HSV-2 MS vaginal shedding was equivalent to naïve guinea pigs on that day, as determined by one-way ANOVA and Tukey's post hoc t-test. (**B**) The worst case of perivaginal disease in each group of naïve or immunized guinea pigs on Day 7 post-challenge. Survival frequency refers to the frequency with which animals in each immunization group survived until Day 30 post-challenge. *Reprinted from Halford, et al., 2013, PLoS ONE 8:e65523 under an Open Access agreement [60].*

Figure 3. Western blot comparison of the HSV-2-specific antibody response elicited by adjuvanted gD-2 versus live HSV-2 0ΔNLS . Western blots of (UI) uninfected Vero cells or cells inoculated with 5 pfu/cell of HSV-1 KOS, a HSV-1 ΔgD virus, HSV-2 MS, or a HSV-2 ΔgD virus incubated with 1:20,000 dilutions of serum from mice immunized with **(A)** gD-2 + alum/MPL adjuvant or **(B)** HSV-2 0ΔNLS. Red diamonds (1–9) denote the positions of viral proteins commonly targeted by mouse IgG antibodies. *Panels A and B reprinted from Geltz, et al., 2015, PLoS ONE 10:e116091 under an Open Access agreement [8].*

Figure 4. WPH self-testing of live HSV-2 0ΔNLS in an initially seronegative recipient: Immunization site reactions after intradermal vaccination. (A) Left forearm of WPH on Days 2 - 9 after primary intradermal immunization with 3 x 10^6 pfu of HSV-2 0ΔNLS (Sept 2011). **(B)**

Inside of left calf of WPH on Day 2 after secondary intradermal immunizations with, from bottom to top, 10^4, 10^5, 10^6, or 3 x 10^6 pfu of HSV-2 0ΔNLS (Jan 2012). *This is an Original Figure.*

Figure 5. HSV-2 0ΔNLS vaccine-induced antibody response (WPH) versus natural antibody response of herpes sufferers to wild-type HSV-2. Three-population flow cytometric analysis comparing human IgG antibody-binding to a mixture of Vero cells that were uninfected (UI) or were inoculated with 5 pfu per cell of HSV-1 KOS or HSV-2 MS twelve hours prior to harvest. Each cell population was dispersed, differentially labeled with 0, 0.1, or 2 μM CFSE, fixed, permeabilized, and combined for antibody staining and flow cytometry. **(A and B)** Test cells were incubated with 1:5,000 dilutions of serum from **(A)** control patients who were HSV-seronegative, HSV-2-seropositive, or double seropositive for HSV-1 and HSV-2, or **(B)** WPH before or after immunization with HSV-2 0ΔNLS. Pan-HSV-2 IgG binding (y-axes) was detected using APC-labeled goat anti-human IgG, and was enumerated based on the increase in mean fluorescent intensity (ΔMFI) of HSV-2 MS^+ cells relative to UI cells (Δ2). **(C)** Graphical representation of mean ± sem of pan-HSV-2 IgG levels in n=4 HSV-seronegative persons vs n=6 genital herpes sufferers who were HSV-2^+ (n-=4) or HSV-1^+2^+ (n=2). **(D)** Graphical representation of the levels of pan-HSV-2 IgG observed in WPH at times before and after immunization with the live HSV-2 0ΔNLS vaccine. The positioning of downward arrows in Panel D is not a precise representation of the number, or timing, of >24 booster shots administered to WPH. The dashed black line in panels C and D represents the lower-limit of detection of the ABVIC test, and corresponds to a threshold above which the probability of a serum sample being "HSV-2-seronegative" is $p < 0.0001$ relative to the n=4 human HSV-seronegative controls in Panel C. The dashed red line in panels C and D represents the average level of pan-HSV-2 IgG observed in genital herpes sufferers who are

strongly HSV-2 seropositive. Serum from human seronegative and HSV-2 seropositive controls was obtained in collaboration with the Westover Heights Clinic, as described under IRB Protocol #14-152 entitled "*Evaluation of a novel antibody test for HSV-2*," which was approved by the Springfield Committee for Research Involving Human Subjects. *This is an Original Figure.*

Figure 6. Adoptive transfer of human post-0ΔNLS vaccine serum protects naïve μMT mice from lethal HSV-2 challenge. Groups of n=20 naïve μMT mice received three adoptive transfers of ***i.*** WPH pre-serum or ***ii.*** WPH post-vaccination serum on Days -1, +1, and +3 relative to the timing of HSV-2 MS-GFP challenge; at each transfer, 0.25 ml serum was delivered to mice by i.p. injection. On Day 0, mice were bilaterally challenged with 400,000 pfu per eye of HSV-2 MS-GFP, which expresses a GFP reporter protein [71]. WPH pre-serum was harvested 7 days prior to the first immunization with HSV-2 0ΔNLS. WPH post-vaccination serum was harvested on Day 1,556 after primary immunization, and one month after receiving the second of two booster shots of $2x10^8$ pfu HSV-2 0ΔNLS. **(A)** Mean ± sem HSV-2 MS-GFP shedding from mouse eyes at 24 hours post-challenge (n = 20 per group). The dashed line denotes the lower limit of detection of the plaque assay. **(B)** Mean ± sem of disease scores in mice on Day 15 post-challenge. Disease scores were assigned, as follows: 0 = indistinguishable from uninfected control; 1= change in corneal opacity noted; 2 = frank clouding of cornea and/or periocular fur loss; 3 = constitutional disease symptoms such as hunched posture or compromised balance; 4 = dead. (**C)** Representative disease observed in mice that received WPH pre-serum, and which either ***i.*** survived until Day 15 (left panel; 9 of 20) or ***ii.*** succumbed to herpes encephalitis and exhibited GFP expression in the cerebrum and/or cerebellum upon dissection of the brain (right panel; 11 of 20). **(D)** Representative disease observed in mice that received WPH post 0ΔNLS-vaccination serum (n=20). In Panels A

and B, ** and *** denote $p < 10^{-6}$ and $< 10^{-14}$ by a two-sided t-test, respectively, that the measured parameter was equivalent in mice that received pre- vs post-0ΔNLS vaccination serum. Mice were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the AVMA Guidelines for Euthanasia. These experiments were conducted as described in protocol 205-13-010 approved by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee. *This is an Original Figure.*

Figure 7. The HSV-2 0ΔNLS vaccine is well tolerated in long-time genital herpes sufferers. Self-reported pain and tolerability metrics provided by n=20 participants in a Phase I trial of a therapeutic HSV-2 0ΔNLS vaccine. These metrics were reported 7 to 10 days after receiving Shots 1, 2, or 3 of HSV-2 0ΔNLS (2×10^8 pfu per shot) delivered into the dermis of the left or right calf. All n=20 participants suffered from HSV-1 or HSV-2-driven genital herpes, and received at least two shots of the HSV-2 0ΔNLS vaccine; n=17 participants received all three shots. Attrition at Shot 3 occurred for a variety of reasons; one participant expressed concerns over the potential for an adverse event and was withdrawn; one participant was withdrawn for behaviors that violated the conditions set forth in the Informed Consent; one participant's son experienced a medical emergency at the time of Shot 3. **(A)** Self-reported pain scores as provided by Groups 1 and 2 (n=10 per) after receiving Shots 1, 2, or 3 of an identical HSV-2 0ΔNLS vaccination series. Participants reported the discomfort of vaccination between Days 7 and 10 post-vaccination both in terms of **(A)** the discomfort they had experienced on a 10-point pain scale (0 = no pain; 10 = intolerable pain), and **(B)** whether the discomfort of the HSV-2 0ΔNLS vaccination series was less, similar, or more painful than their genital herpes symptoms. This Phase I clinical trial was conducted in compliance with the principles outlined in the Declaration of Helsinki. Prior to

enrollment, trial participants were interviewed by telephone on at least three occasions to assess their eligibility and to review a 14-page Informed Consent Form that described all aspects of the trial including the potential risks of participation. Participants were only enrolled in the trial after the Informed Consent Form was signed and countersigned. At every vaccination appointment, trial participants were required to re-affirm their consent to participate in the trial, in both verbal and signatory form, before the Administering Physician was allowed to proceeded with the interview, blood draw, and vaccination. *This is an Original Figure.*

Figure 8. Therapeutic effects of live HSV-2 0ΔNLS vaccine in genital herpes sufferers. (A) Participants enrolled in a Phase I trial of a therapeutic HSV-2 0ΔNLS vaccine were queried via telephone and Pre-Vaccination Questionnaires as to the frequency and duration of their genital herpes-associated symptoms in the three years prior to receiving the HSV-2 0ΔNLS vaccine. Each pair of black and hatched bars represents a single trial participant. The black bars represent each individual's self-reported frequency of herpes symptoms prior to the first vaccination; 16 of 17 participants had used antiviral drugs episodically or daily prior to their first vaccination (pre-vax + antivirals). The hatched bars represent each participant's self-reported frequency of herpes symptoms in the 4 to 6 months after receiving the first vaccination (post HSV-2 0ΔNLS). The difference in participants' self-reported herpes symptoms was significant, as determined by a paired t-test ($p=0.000005$). Participants are segregated into four categories based on their genital herpes symptoms, and whether these symptoms were driven by HSV-1 or HSV-2. Two of 17 HSV-2-seropositive individuals reported "herpes neuralgia" that not only accompanied visible herpes outbreaks, but also occurred daily in between visible HSV-2-driven herpes outbreaks. Similar to VZV-induced neuropathic pain, this neuralgia presented as a stabbing or burning pain.

Other participants reported similar sensations preceding or accompanying their herpes outbreaks, but this first group of 2 of 17 participants in the "herpes neurlalgia" category suffered with daily neuropathic pain for the three years leading up to the HSV-2 0ΔNLS vaccine trial. A second group of "Menstural HSV-2 OB" participants were women who reported 12 – 24 herpes outbreaks on their labia, thighs or buttocks, and in which one herpes outbreak per month coincided with the onset of their menstrual cycle. The latter two groups of participants experienced more classic genital herpes outbreaks driven by HSV-2 or HSV-1, which were associated with a variety of stimuli including stress, fatigue, sun exposure, excessive alcohol consumption, or trigger foods such as peanuts, coffee, or chocolate. The white arrow and blue box highlight Participant CE-27 whose vaccination site reactions and Western Blots are shown in Panels B and C. **(B)** Inside of calf of Participant CE-27 two days after receiving Shot 1 (left panel), Shot 2 (middle panel), or Shot 3 (right panel). A ruler was included in each photograph to allow calculation of the area of erythema and edema surrounding the injection site 48 hours after Shots 1, 2, and 3. **(C)** Western blots analysis of CE-27's pre- and post-0ΔNLS vaccination serum. Pre-serum (Day -42) was collected for the purpose of verifying that CE-27 was HSV-seropositive (a requisite for trial enrollment). Serum collected on Days 0, 40, and 70 just prior to Shots 1, 2, and 3, respectively. Each Western blot contained protein lysates harvested from, left to right, Vero cells that were uninfected (UI) or were inoculated with 2.5 pfu/cell of HSV-1 KOS or HSV-2 MS, and which were incubated with 1:5,000 dilutions of serum from CE-27's serum collected on Days -42, 0, +40, or +70 relative to Shot 1 of the HSV-2 0ΔNLS vaccine. Red diamonds on the Day +40 blot denote the positions of HSV-2 proteins that appeared to be targeted by increased levels of human IgG antibodies relative to blots incubated with CE-27's pre-vaccination sera (Days -42 and 0). This Phase I clinical trial was conducted in compliance with the principles outlined in the Declaration

of Helsinki, as detailed in the Legend to Figure 7. The alphanumeric identifiers used for trial participants in Panels A, B, and C are arbitrary, and have no relationship to these individuals' names, birthdays, or federal/state identification numbers. *This is an Original Figure.*

Figure 1 – Halford

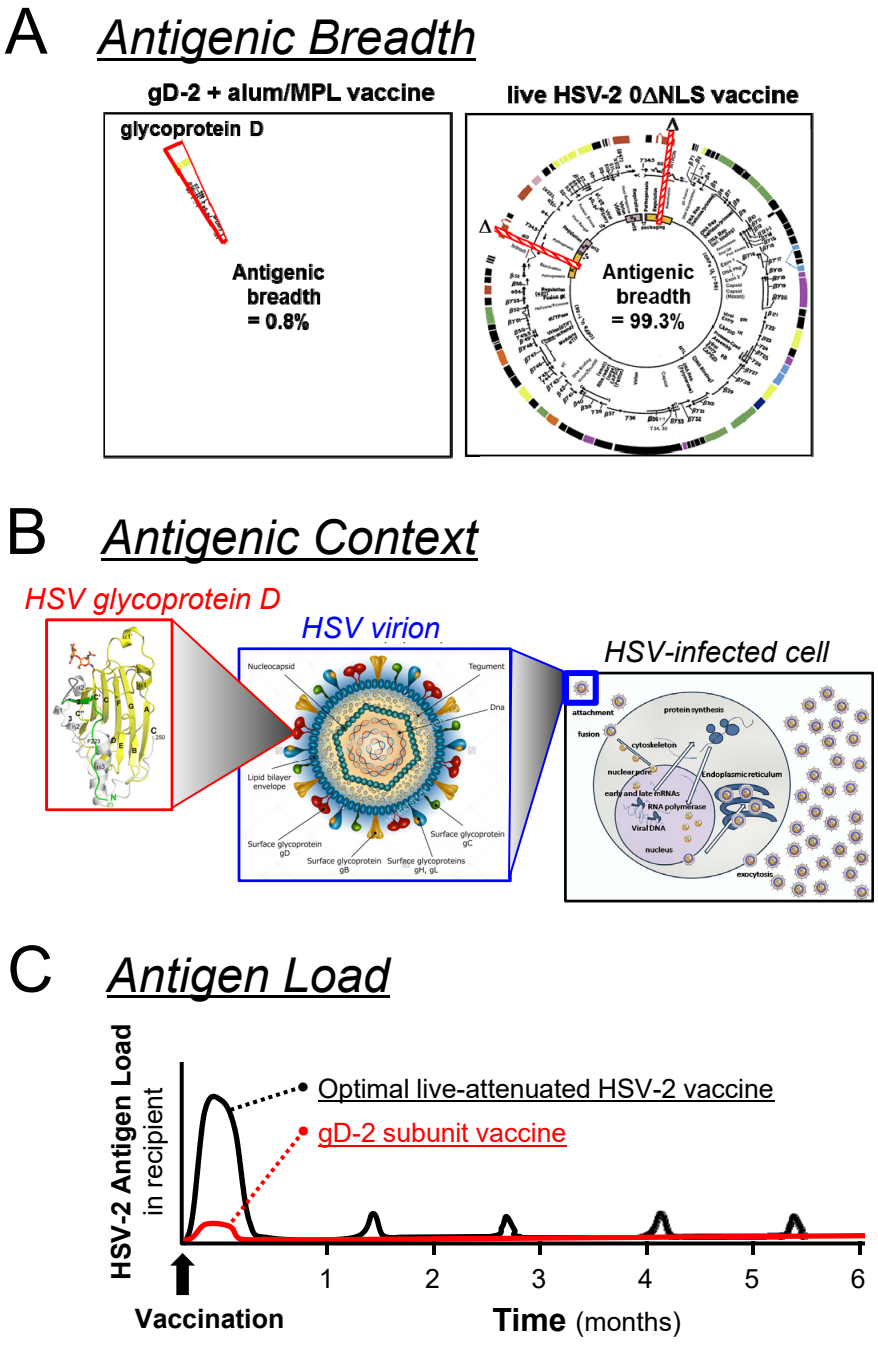
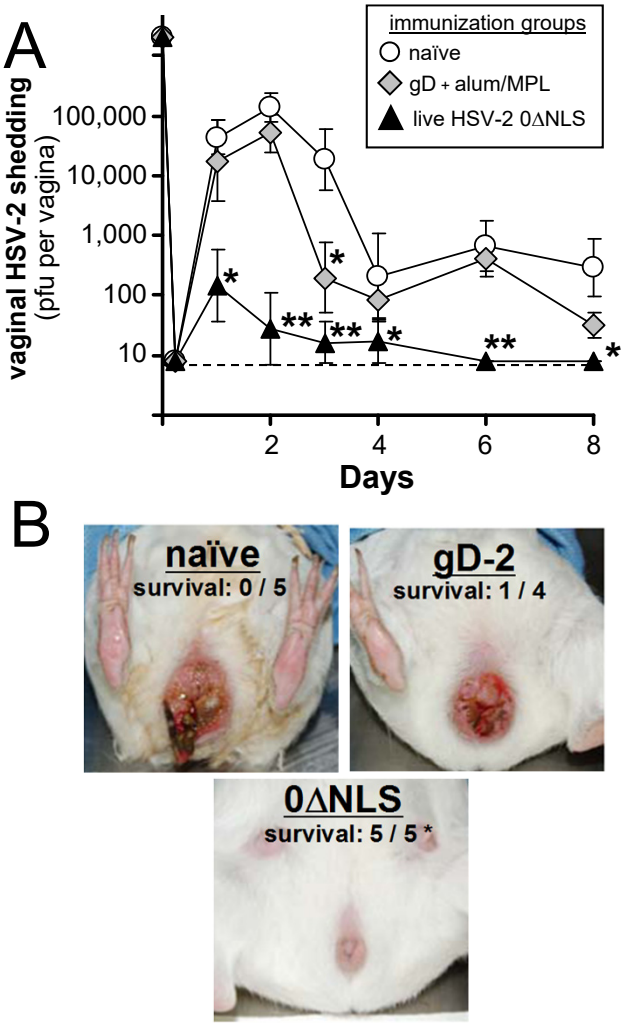


Figure 2 – Halford



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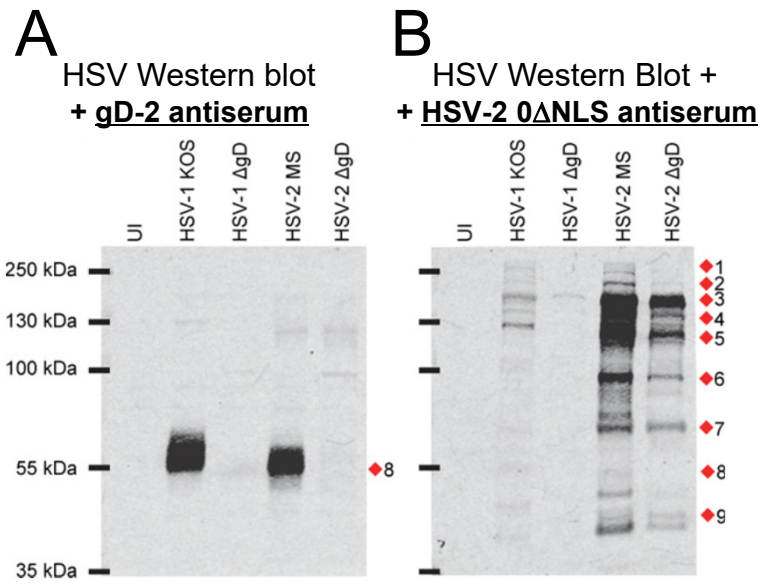
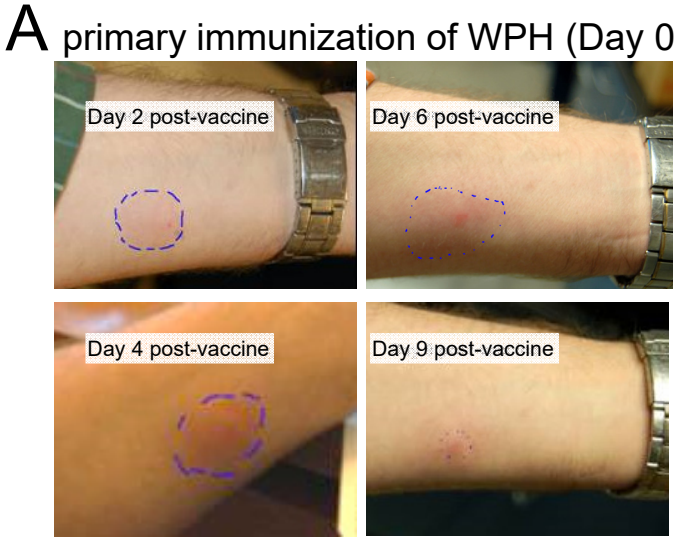
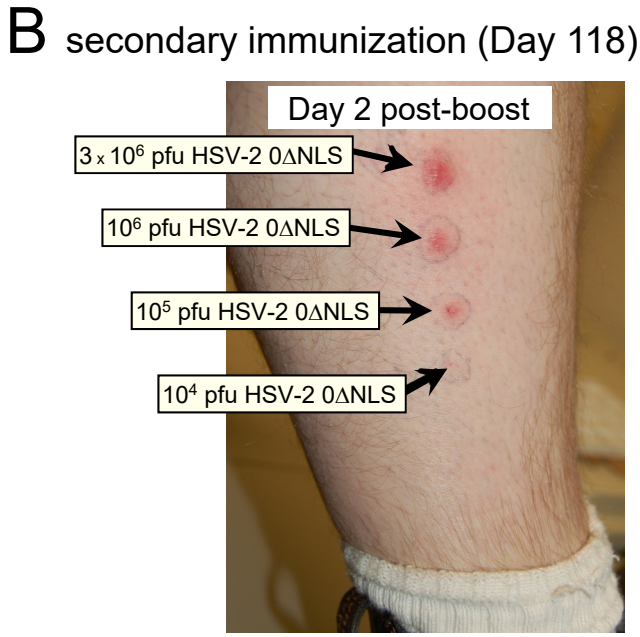


Figure 4 – Halford





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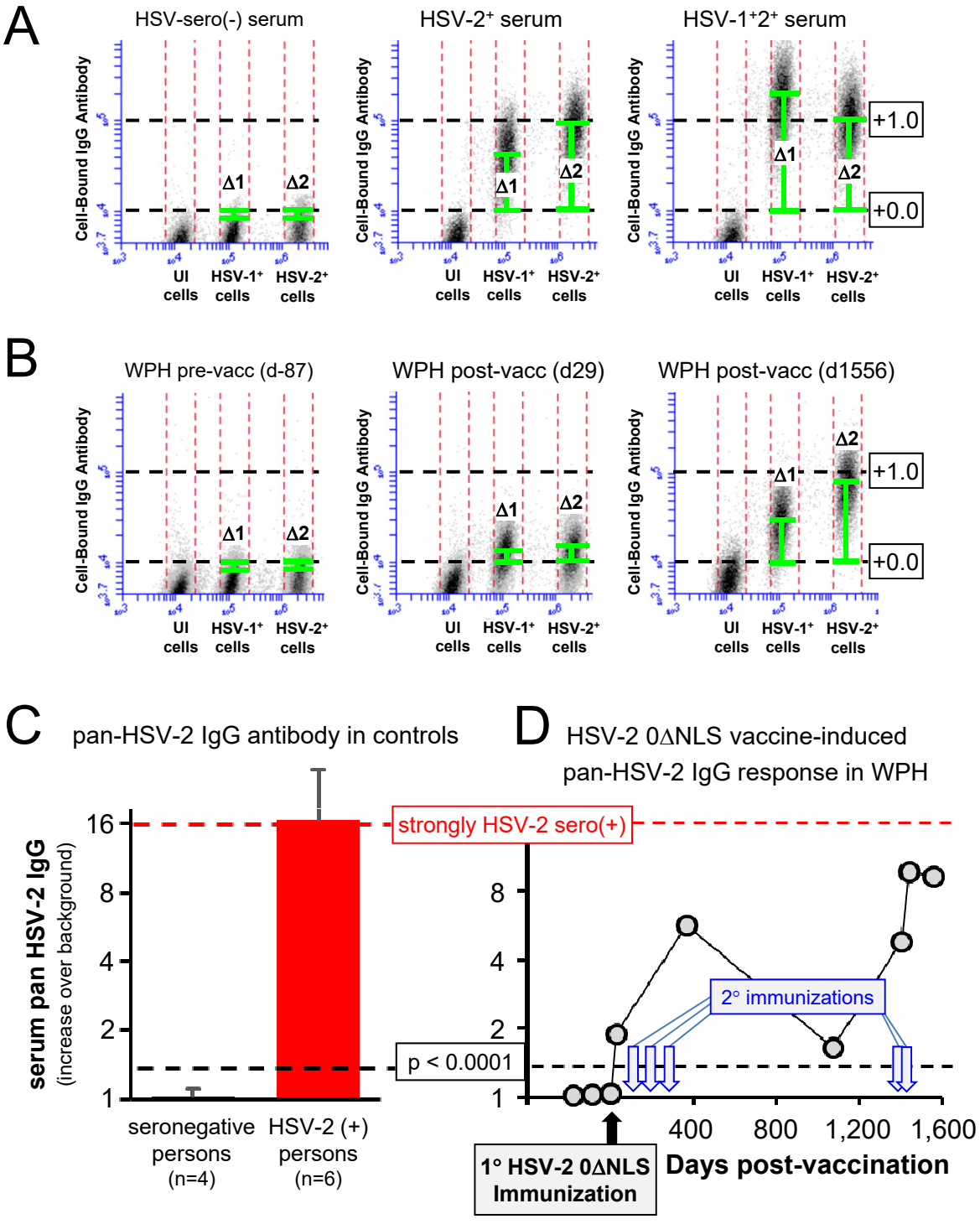

Figure 6 – Halford

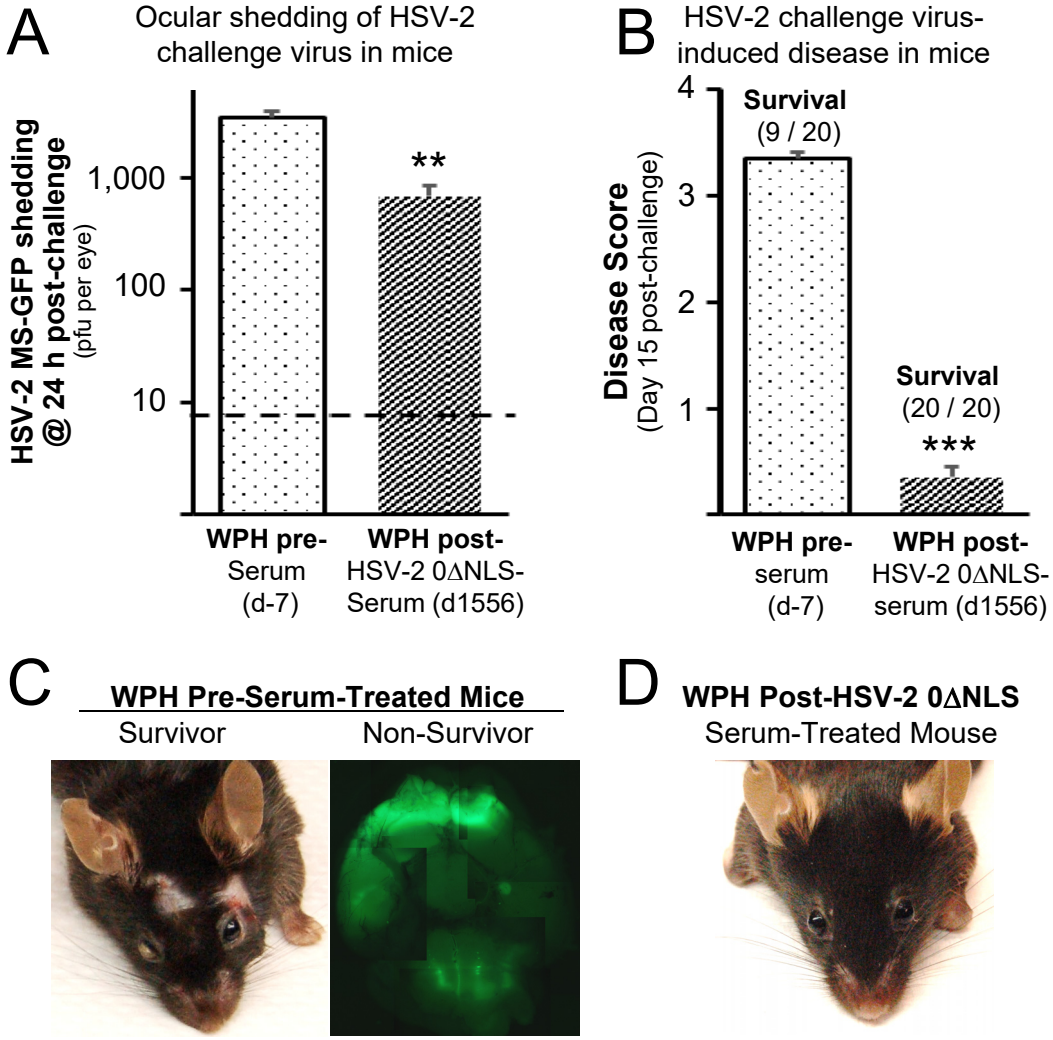

Figure 7 – Halford

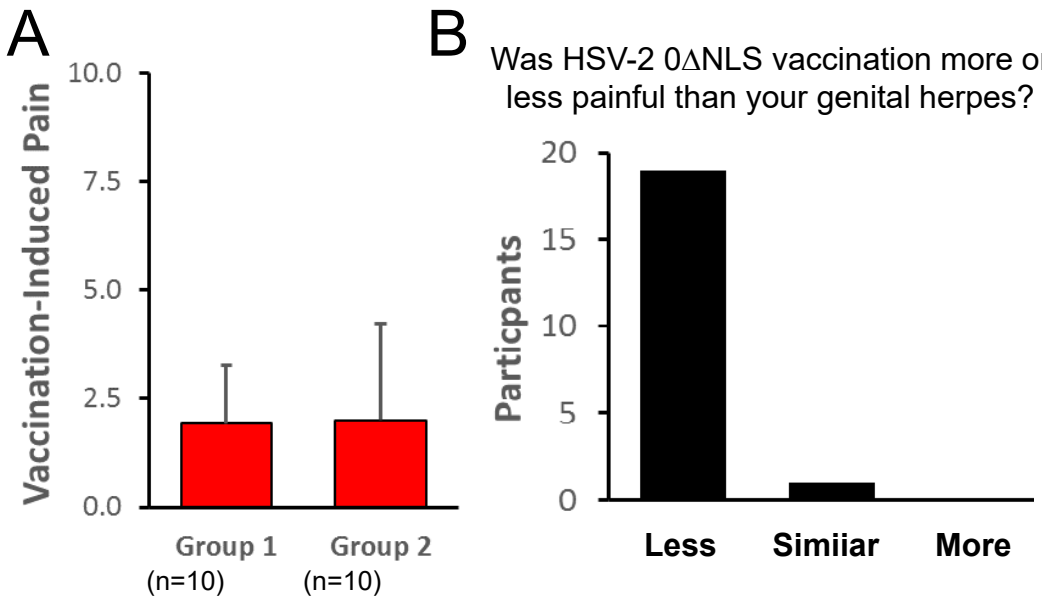

Figure 8 – Halford