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Liposomal gD Ectodomain (gD₁₋₃₀₆) Vaccine Protects Against HSV2 Genital or Rectal Infection of Female and Male Mice

K. Olson^a, P. Macias^a, S. Hutton^a, W. A. Ernst^b, G. Fujii^b, and J. P. Adler-Moore^{a,*}

^aCalifornia State Polytechnic University Pomona, 3801 West Temple Ave., Pomona, CA 91768, USA

^bMolecular Express, Inc. 2011 E. University Drive, Rancho Dominguez, CA 90220, USA

Abstract

Herpes simplex virus type 2 (HSV2) is the most common causative agent of genital herpes, with infection rates as high as 1 in 6 adults. The present studies were done to evaluate the efficacy of a liposomal HSV2 gD₁₋₃₀₆ vaccine (L-gD₁₋₃₀₆-HD) in an acute murine HSV2 infection model of intravaginal (female) or intrarectal (male or female) challenge. Two doses of L-gD₁₋₃₀₆-HD containing 60µg gD₁₋₃₀₆-HD and 15µg monophosphoryl lipid A (MPL) per dose provided protection against HSV2 intravaginal challenge (86-100% survival, $P \leq 0.0003$ vs control liposomes; $P = 0.06$ vs L-gD₁₋₃₀₆-HD without MPL). Both male and female mice (BALB/c and C57BL/6) immunized with L-gD₁₋₃₀₆-HD/MPL were significantly protected against HSV2 intrarectal challenge, with higher survival rates compared to controls (71-100%, $P \leq 0.007$). L-gD₁₋₃₀₆-HD/MPL also provided increased survival when compared to a liposomal peptide vaccine, L-gD₂₆₄₋₂₈₅-HD/MPL (male BALB/c, $P \leq 0.001$; female BALB/c and male C57BL/6, $P = 0.06$). Mice given L-gD₁₋₃₀₆-HD/MPL also had minimal disease signs, reduced viral burden in their spinal cords and elevated neutralizing antibody titers in the females. The vaccine also stimulated gD₁₋₃₀₆-HD specific splenocytes of both male and female mice with significantly elevated levels of IFN- γ compared to IL-4 ($P \leq 0.01$) indicating that there was an enhanced Th1 response. These results provide the first evidence that the L-gD₁₋₃₀₆-HD vaccine can protect both male and female mice against intrarectal HSV2 challenge.

Keywords

liposomal gD vaccine; HSV2 infection; male and female mice

Introduction

Herpes genitalis caused by Herpes simplex virus type 2 (HSV2) is one of the most common, sexually transmitted diseases in humans [1], [2], [3], [4]. Epidemiological studies show that as many as 1 out of 6 Americans have been infected by HSV2 [3]. The disease affects both normal and immunosuppressed adults, and is associated with increased susceptibility to the human immunodeficiency virus [5], [6]. Serious clinical disease can occur in neonates following transmission of virus from their infected mothers [7] who are also more likely to

*Corresponding Author: Jill Adler-Moore, jpadler@csupomona.edu, 909-869-4047.

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develop cervical cancer than non-infected women [4]. Thus, there is a critical need to develop an effective vaccine against this disease.

The most promising HSV2 vaccine targets have been the viral envelope glycoproteins [8], especially the 393 amino acid gD protein [9], [10], [11]. The gD envelope protein (59kDa) (ectodomain 36.2kDa) has been the focus of much attention because it has been shown to elicit neutralizing antibodies, and serves as a target for cellular immune responses in animal models [11], [12], [13] and more importantly, in humans [14]. The cellular immune response is believed to be a critical component for providing protection against both primary and recurrent disease [15], [16], [17], [18], [19]. In guinea pigs, immunization with gD has shown some success when given prophylactically, but is less effective in preventing the recurrence of lesions in already infected animals [20], [21].

To date, the most successful vaccine candidate in clinical trials is composed of the gD protein and an adjuvant system (AS04), consisting of aluminum hydroxide and 3-*O*-deacylated-monophosphoryl lipid A (Simplirix[®], manufactured by GlaxoSmithKline). This vaccine demonstrated significant efficacy against disease in women who were seronegative for HSV. However, it was not effective in men [22]. This suggests that there were gender differences in the immune responses to Simplirix[®] and underscores the need for preclinical testing of potential HSV2 vaccine candidates in both female and male animal models.

In previous studies [23], we observed significant protective effects in female mice challenged intravaginally with HSV2 following immunization with vaccines consisting of single epitopes derived from the HSV2 gD envelope protein and presented to the immune system by a highly immunogenic liposomal delivery vehicle. Within the gD envelope protein, a number of continuous small epitopes have been identified that are of potential vaccine interest because they are postulated to be located on protein surfaces, especially in regions where highly hydrophilic residues are present in beta turns [24], [25], [26], [27], and they mediate antibody-dependent cellular cytotoxicity [28]. We hypothesized that the same type of liposomal delivery system that had been used for the single gD epitope vaccines could also be employed in the design of a vaccine incorporating the entire gD ectodomain (gD₁₋₃₀₆) and that the larger, potentially more immunogenic protein would stimulate a protective immune response in both female and male mice. In this study, we evaluated the efficacy of a liposomal formulation of gD₁₋₃₀₆ in an intravaginal HSV2 murine model using different adjuvant and antigen doses and boosting schedules, compared its effectiveness in different mouse strains following HSV2 intrarectal challenge of male and female mice, and characterized the immune response of both sexes to the vaccine.

Materials and Methods

Cloning and purification of HSV2 gD-HD protein

The nucleotide sequence was derived using optimized *Escherichia coli* codon preferences for the gD amino acid sequence. To prepare the gD₂₆₄₋₂₈₅ gene, two overlapping oligonucleotides (sense: 5' CATATGACCCAGCCGGAAGTGGTTCCGGAAGACCCGGAAGACTCCGCT and antisense: 5' GTCGACGGTGCCAGCCGGGTCTTCCAGCAGAGCGGAGTCTTCCGGGTC) resulting

in the gene product gD₂₆₄₋₂₈₅ (TQPELVPEDPEDSALLEDPAGT) were synthesized and flanked by convenient restriction sites (Nde I and Sal I underlined). Polymerase chain reaction (PCR) was used to amplify the overlapping oligonucleotides. One microgram of sense and antisense pair was incubated with 1× Pfx buffer, 2.5mM dNTPs, 1µl of Pfx polymerase (Invitrogen, San Diego, CA). The reaction mix was incubated using the following cycle 30 times: 94°C for 1 minute, ramp to 50°C in 15 minutes hold at 50°C for 1 minute, 72°C for 1 minute. Following PCR, the product was cleaved with Nde I and Sal I, and isolated by gel

electrophoresis. The gD₂₆₄₋₂₈₅ gene was ligated into a similarly cleaved expression plasmid containing the proprietary hydrophobic domain (HD) gene (*i.e.*, pET28a). The gD₁₋₃₀₆ gene was created using the *E. coli* optimized sequence (BlueHeron Biotechnologies, Bothel, WA). The gD₁₋₃₀₆ was subcloned into the pET28a vector using the Nde I and Sal I sites. The genes encoding gD₂₆₄₋₂₈₅ and gD₁₋₃₀₆ were cloned upstream of the gene encoding the HD. The final genes were verified by sequencing the DNA. The gD₁₋₃₀₆-HD and the gD₂₆₄₋₂₈₅-HD containing plasmids were transformed into BL21 *E. coli* for expression. The bacteria were incubated in terrific broth medium with kanamycin (30 µg/ml) at 37°C until mid-log phase and induced with 0.75mM IPTG (isopropyl-beta-D-thiogalactopyranoside). The bacteria were harvested by centrifugation and the cell pellets lysed with 8M urea/50mM Tris-HCl pH 7.8 and centrifuged at 30K × g to remove cellular debris. The gD-HD proteins were purified by selective nickel affinity (Qiagen, Chatsworth, CA). Endotoxin was removed using detoxi-gel (Pierce, Rockville, IL). Endotoxin levels were determined by the Limulus Amebocyte Lysate assay (Cambrex Bioscience, Walkersville, MD). Protein purity and concentration were evaluated by Coomassie stain and the bicinchoninic acid (BCA) assay, respectively (Pierce, Rockville, IL).

Liposome preparation

The liposomes were prepared by dissolving the phospholipids, cholesterol, and monophosphoryl lipid A (MPL; Sigma, St Louis MO) with recombinant gD₁₋₃₀₆-HD, recombinant gD₂₆₄₋₂₈₅-HD protein or without protein (control liposomes) in an organic solvent mixture of chloroform/methanol (1:1 v/v). The recombinant proteins with the HD were designed to stably integrate into the lipid bilayer of the liposomes. Thin lipid films were created by evaporating the solvent at 65°C under a stream of nitrogen gas. The dried films were placed under vacuum for at least 24 hours to remove residual organic solvent. Preparation of the liposomes was accomplished by hydrating the lipid films with buffer and incubating the suspension at 65°C for 5-10 minutes before subjecting them to probe sonication. The liposomes were sterile filtered post-production through a 0.22 µm filter and sized by dynamic light scattering using a UPA-150 (Microtrac, North Largo, FL). To verify that the protein was incorporated in the liposomes, liposomes were passed over a size exclusion column (DG-10, BioRad, Hercules, CA). Samples of the liposomes pre- and post-filtration were dissolved in 2.5% CHAPS detergent (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined by the BCA assay (Pierce, Rockford, IL). The total encapsulated protein was greater than 95%.

Mice

BALB/c and C57BL/6 male and female mice (5–6 weeks) were purchased from Harlan Labs (Indianapolis, IN). All mice were housed and maintained in a pathogen-free environment, in accordance with the Animal Care and Use protocols at the California State Polytechnic University, Pomona vivarium.

Vaccination of mice

Six week old BALB/c or C57BL/6 mice (*n*= 7/group) were vaccinated subcutaneously (*s.c.*) on day 0 and day 56 (unless specified otherwise) with liposomes containing MPL and HSV2 gD amino acids 1-306 (L-gD₁₋₃₀₆-HD/MPL) or HSV2 gD amino acids 264-285 (L-gD₂₆₄₋₂₈₅-HD/MPL) or with liposomes containing only MPL (L-control/MPL) provided by Molecular Express, Inc. The mice were monitored for adverse reactions to vaccination including redness, swelling, or the formation of granulomas at the injection site. Mice were weighed daily for one week post-vaccination and weekly between the prime and boost.

Vaginal HSV2 infection of mice

At the time of boost (day -7 prior to challenge) and day -1 prior to challenge, mice were given a s.c. injection of Depo-Provera® (Pharmacia Corporation, MI) (2.67mg medroxyprogesterone acetate/mouse). On the day of HSV2 challenge, mice were anesthetized intraperitoneally (i.p.) with ketamine (80 mg/kg) and xylazine (16 mg/kg) and the vagina of each mouse was swabbed with a Calcium Alginate Fiber Tipped Ultrafine Aluminum Applicator Swab (Fisher Scientific, PA) followed by intravaginal challenge with 50LD₅₀ of HSV2 (strain G). Mice were weighed and observed for morbidity on a daily basis for 28 days. Vaginal scores were as follows: 0= no lesion; 1= erythema; 2= mild inflammation; 3= severe inflammation, fecal impaction or urinary incontinence; 4= severe inflammation, epithelial tissue damage, fecal impaction and urinary incontinence. Neurological scores: 0= no neurological signs; 1= tail paralysis; 2= tail and one hind leg paralyzed; 3= tail and both hind legs paralyzed/hind quarter atrophy; 4= total paralysis.

Rectal HSV2 infection of mice

Twelve hours prior to HSV2 challenge, food was withheld from the mice. On the day of challenge, mice were anesthetized i.p. with ketamine (80 mg/kg) and xylazine (16 mg/kg). The rectum of each mouse was washed 4× with 10µL sterile PBS followed by 20µL 2% nonoxynol-9 (Tergitol, Type NP-9, Sigma-Aldrich, MO) delivered into the rectum of each mouse and withdrawn after 5 minutes. Mice were intrarectally challenged with 10LD₅₀ of HSV2 (strain G). The challenge dose for the intrarectal infection was lower than that for the intravaginal challenge because the intrarectal infection is more severe and we tried to match the severity of the intravaginal and intrarectal infections by adjusting the challenge dose. If we had used the same viral dose for the intrarectal challenge as we did for the intravaginal infection (50LD₅₀ of HSV2 (strain G)), deaths would have occurred within the first three days following intrarectal challenge. Mice were weighed and observed for morbidity on a daily basis for 28 days. Rectal scoring was the same as in the vaginal infection model except the rectum was observed for infection instead of the vagina. Neurological scoring was the same as in the vaginal infection model.

Tissue and peritoneal fluid collection from viral-challenged mice

For the viral localization study, spinal cords, brain, adrenal glands, bladder, heart, kidneys, liver, lungs, spleen, and peritoneal fluid were collected from non-vaccinated, intrarectal or intravaginal HSV-2 challenged female BALB/c mice at the height of their disease signs (i.e. days 8-12), weighed and assayed for viral burden by the plaque forming assay. In the immunization studies, BALB/c or C57BL/6 mice were vaccinated with L-gD₁₋₃₀₆-HD/MPL or L-gD₂₆₄₋₂₈₅-HD/MPL or L-control/MPL, challenged intravaginally or intrarectally with HSV2 one week post-boost, and then spinal cords collected at the height of the disease signs for viral burden determination by the plaque forming assay.

Neutralizing antibody assay

BALB/c or C57BL/6 mice were vaccinated with L-gD₁₋₃₀₆-HD/MPL or L-gD₂₆₄₋₂₈₅-HD/MPL or L-control/MPL. One week post-boost, blood was collected from vaccinated mice (n=3/group) by cardiac puncture and the serum from each mouse in each group assayed for neutralizing antibody titer. Serial serum dilutions were made in a 96-well round bottom Nunclon™ Surface plate (Nalge Nunc International, NY) with a final volume of 120µL per well. To each well containing a different serum dilution, 120µL of 1:2000 HSV2 stock (strain G), diluted in CMEM was added. This volume of viral stock contained ~200 plaque forming units (PFU)/mL. The plates were incubated for one hour at 34°C in a CO₂ incubator and the contents of each well then transferred from the 96-well plates to Multiwell™ 24 well flat bottom tissue culture plates (Becton Dickinson and Company, NJ); 240µL of Vero cells (1.1×10^6

cells/mL) were added to each well. Plates were incubated at 34°C for 5 hours and 500µL of 3% methylcellulose in CMEM was then added to each well. Plates were incubated for an additional 23 hours, cells fixed with 4% formaldehyde and stained with 0.1% crystal violet. Plaques were counted microscopically and the PFU/mL was determined in the positive control wells (no serum). The neutralizing antibody titer for each mouse was the serum dilution in which the PFU were equal to 50% of the PFU in the positive viral control wells.

Viral burden assay

To each tared tissue, 500µL of chilled PBS was added and the tissue was sonicated in an icy water bath sonicator (Branson Ultrasonics, CT) for 2 or 3, one-minute interval bursts until a homogeneous slurry was formed. Two fold dilutions of each tissue homogenate or peritoneal fluid were dispensed into a 24 well tissue culture plate (240µL/well) and an aliquot of 240µL of Vero cells (1.1×10^6 cells/mL) added to each well. Plates were incubated at 34°C for 5 hours, and 500µL of 3% methylcellulose in CMEM was then added to each well; the plates were incubated for an additional 23 hours. Cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet. Plaques were counted microscopically to determine PFU/g tissue for each mouse.

ELISPOT assay for IFN-γ and IL-4

L-gD₁₋₃₀₆-HD/MPL or L-control/MPL vaccinated BALB/c male and female mice ($n = 24$ /group) were euthanized by carbon dioxide inhalation on day 3 post-vaccine boost. The spleens were removed, minced and pressed through a 70µm Sterile Cell Strainer (Fisher Scientific, PA) while remaining in contact with CMEM and pooled for each group of mice. Each pooled splenocyte suspension was passed through a second 70µm strainer and five 100µL aliquots (2×10^7 splenocytes/mL) from each group were added to wells of an ELISPOT plate (BD Biosciences Pharmingen, San Diego, CA) containing 2.5µg of gD₁₋₃₀₆-HD in 100µL CMEM or 100µL CMEM without antigen. ELISPOT assays were run for both IFN-γ and IL-4 according to the manufacturer's directions with the IFN-γ plates being incubated for 24 hours with the antigen (gD₁₋₃₀₆-HD) and the IL-4 plates incubated for 48 hours with the antigen.

Cytokine Singleplex bead assay for IFN-γ and IL-4

Aliquots of the same pooled splenocyte samples used in the ELISPOT assay were tested for production of cytokines by Cytokine Singleplex bead assay for IFN-γ and IL-4. Five aliquots (100µL/well) of a given splenocyte suspension were added to wells of a sterile 96 well round bottom plate and incubated for 48 hours with 2.5µg of gD₁₋₃₀₆-HD in 100µL CMEM or 100µL CMEM without antigen. After incubation, plates were centrifuged for 10 minutes at $575 \times g$. Supernates were stored at -80°C and then analyzed using cytokine bead Singleplex assay kits for mouse IFN-γ and for mouse IL-4 (Bio-Rad Laboratories, CA). Five aliquots (50µL/well) of a given supernate were added to 1.2µm Millipore MultiScreen_{HTS} 96-Well Filter Plates (Millipore, MA) and the cytokine bead assay was done as described in the Bio-Plex Cytokine Assay Instruction Manual. Plates were read in the Luminex[®] 100 IS[™] System (Luminex Corporation, TX).

Results

Liposomal gD-HD Vaccines

Two batches of L-gD₁₋₃₀₆-HD and two batches of L-gD₂₆₄₋₂₈₅-HD were sized post-filtration by dynamic light scattering. The L-gD₁₋₃₀₆-HD had mean diameters of 147nm and 55.8nm, and the L-gD₂₆₄₋₂₈₅-HD had mean diameters of 45.1nm and 75.1nm (Figure 1). Samples stored at 4°C for over 40 days remained stable as judged by visual inspection. (left out endotoxin levels!!)

Dosing of gD₁₋₃₀₆-HD

Initial studies were focused on determining the protective dose of gD₁₋₃₀₆-HD to be delivered as L-gD₁₋₃₀₆-HD/MPL. Vaccination of female BALB/c mice with L-gD₁₋₃₀₆-HD containing 15µg MPL provided significant protection compared to the L-control/MPL vaccinated mice against intravaginal HSV2 challenge at all doses of gD₁₋₃₀₆-HD (15µg, $P=0.0230$; 30µg, $P=0.0006$; 60µg, $P=0.0002$). The 60µg L-gD₁₋₃₀₆-HD/MPL provided better protection (86% survival) than the lower doses which produced 43% survival, although the difference did not reach significance (60µg vs 15µg L-gD₁₋₃₀₆-HD/MPL $P=0.0729$; 60µg vs 30µg L-gD₁₋₃₀₆-HD/MPL $P=0.1110$) (Figure 2A). Vaccination with 60µg L-gD₁₋₃₀₆-HD/MPL resulted in lower vaginal scores, with a mean peak score of ≤ 1.3 throughout the study, compared to the 15µg and 30µg doses which had mean peak scores of 2.9 (day 8) and 2.6 (day 9), respectively (Figure 2B). The 60µg dose also completely protected mice from developing neurological disease signs whereas mild neurological disease signs were observed with the 15µg and 30µg doses (mean peak score of 0.9 (day 9) and 0.7 (day 9), respectively)(Figure 2C). L-control/MPL vaccinated mice developed severe vaginal (mean peak = 4.0) and neurological (mean peak = 3.0) disease signs. (should have mentioned that there were no granulomas, reddening or other side effects at the site of immunization)

Dosing of MPL

Having established that the optimal antigen dose in these studies was 60µg gD₁₋₃₀₆-HD, other groups of female BALB/c mice were vaccinated with L-gD₁₋₃₀₆-HD containing different amounts of the adjuvant MPL. These results demonstrated that all doses of MPL (10µg, 15µg or 25µg) incorporated into the L-gD₁₋₃₀₆-HD provided significant protection against intravaginal HSV2 challenge compared to the L-control/MPL mice ($P=0.0003$; survival =100% for 10µg or 15µg MPL/dose, 86% for 25µg MPL/dose, 0% for L-control/MPL). In comparison, the L-gD₁₋₃₀₆-HD without MPL group had only 57% survival which trended strongly towards significance compared to the 10µg or 15µg MPL/dose groups ($P=0.0597$) (Figure 3A). The vaginal scores were lowest in the 15µg and 25µg MPL/dose groups (mean peak score of 0.8 (day 10 and 11, respectively) while mice given 10µg MPL/dose or no MPL had scores that were twice as high as the 15µg and 25µg MPL groups (mean peak score of 1.4 (day 10) and 1.7 (day 9), respectively) (Figure 3B). The mice which received 15µg MPL/dose were completely protected from developing neurological disease signs, whereas mice receiving either 10µg or 25µg MPL/dose had low neurological disease signs (mean score of ≤ 0.2 throughout the study). Mice without MPL had higher neurological scores (mean peak score of 0.4 (day 9)) (Figure 3C). L-control/MPL vaccinated mice developed severe vaginal (mean peak = 4.0) and neurological (mean peak =3.0) disease signs. Based on survival (100%), vaginal (≤ 0.8) and neurological (0) scores, the optimal dose of MPL in the L-gD₁₋₃₀₆-HD vaccine was found to be 15µg/dose.

Boosting schedule

Using a dose of 60µg gD₁₋₃₀₆-HD and 15µg MPL, we wanted to determine whether or not the boosting schedule would affect the vaccine efficacy. Vaccination of female BALB/c mice with L-gD₁₋₃₀₆-HD/MPL provided significant protection against intravaginal HSV2 challenge compared to the L-control/MPL mice for all boosting regimens with the same degree of protection (57% survival), (4 week, $P=0.0002$; 6 week, $P=0.0019$; 8 week, $P=0.0003$) (Figure 4A). Additionally, all boosting regimens resulted in similar mean peak vaginal scores (4 week=2.6 on day 10; 6 week=2.3 on day 9; 8 week=2.4 on day 11) (Figure 4B) and neurological scores (4 week=0.6 on day 10; 6 week=0.5 on day 11; 8 week=0.6 on day 11) (Figure 4C). The lower survival rate (57%) for the mice given L-gD₁₋₃₀₆-HD/MPL was probably a result of these mice being given a challenge dose greater than 50LD₅₀ based on the severity of the neurological scores on day 7 for all the liposome controls.since the higher neurological scores

are indicative of increased infection. In comparison the neurological score on day 7 for the mice in the liposome control group in the previous experiment (Figure 3) was much lower and the survival of the L-gD1-306-HD/MPL vaccinated mice in that case was 100%.

Establishment of intrarectal HSV2 infection model

Given the difference reported between male and female immune responses to vaccines [22], [29], the L-gD₁₋₃₀₆-HD vaccine had to be tested in comparable male and female infection models. The intrarectal infection model was developed given its clinical relevance and reproducibility [30]. To determine the spectrum of protection that could be generated by the vaccine, we also developed the infection model in mice with different genetic backgrounds (*i.e.*, BALB/c and C57BL/6). The intrarectal infection was comparable in BALB/c male and female mice, with all the male mice becoming moribund by day 10 and all female mice becoming moribund by day 11 (Figure 5A). The intrarectal infected BALB/c male and female mice also showed the same disease progression as seen in the graphs of the rectal (Figure 5B) and neurological (Figure 5C) signs of disease. Unlike the BALB/c mice however, the infection in C57BL/6 mice was not the same between the sexes as the females were very resistant to the infection (86% survival; $P=0.0003$ male survival vs female survival) (Figure 5A). The female C57BL/6 did show rectal signs of infection (Figure 5B), but neurological disease signs remained very low throughout the study (mean peak score =0.2) (Figure 5C). These results emphasize the differences in susceptibility between the strains of mice.

HSV2 infection localization

Before testing the vaccine in the intrarectal model, we wanted to be sure that the infection localized in the same tissues as it did in the intravaginal model and that the tissue we harvested for evaluating viral burden was appropriate. Our data (Table 1) showed that viral presence in the intrarectal infection model ($n=5$ female BALB/c mice/group) was restricted to only the brain and spinal cord. The mean value for the spinal cords was 52,200 PFU/g (intrarectal) and 37,400 (intravaginal); for the brain the mean value was 3,338 PFU/g (intrarectal) and 2,160 (intravaginal). The spinal cords consistently had higher levels of virus than the brains, although the difference was not significant in the intravaginal model ($P=0.0269$ intrarectal and $P=0.3063$ intravaginal), and thus, the spinal cords were chosen for tissue analysis of viral burden.

Liposomal ectodomain protein (gD₁₋₃₀₆) vs peptide (gD₂₆₄₋₂₈₅) vaccines

Once the intrarectal infection model was established in male and female mice and the L-gD₁₋₃₀₆-HD/MPL vaccine was optimized for antigen and MPL dose, the efficacy of the gD₁₋₃₀₆ antigen was compared with the immunogenic epitope, gD₂₆₄₋₂₈₅ [31],[32] incorporated into the same type of liposome. The L-gD₂₆₄₋₂₈₅-HD/MPL epitope vaccine had been previously tested in our laboratory using the intravaginal HSV2 infection model in BALB/c mice, and showed similar protection to the L-gD₁₋₃₀₆-HD/MPL vaccine [33].

In the present study, male and female BALB/c mice, immunized with L-gD₁₋₃₀₆-HD/MPL were significantly protected against intrarectal HSV2 challenge, with 71% survival ($P=0.0002$ males, $P=0.0072$ females compared to the respective male or female L-control/MPL mice) (Figure 6A). In comparison, vaccination with L-gD₂₆₄₋₂₈₅-HD/MPL provided no significant protection (29% survival) of female mice against intrarectal HSV2 challenge ($P=0.1237$) compared to the L-control/MPL female mice although the difference in survival between the L-gD₁₋₃₀₆-HD/MPL antigen and the L-gD₂₆₄₋₂₈₅-HD/MPL epitope vaccine did not reach significance ($P=0.0618$). L-gD₂₆₄₋₂₈₅-HD/MPL did not protect any of the male BALB/c mice as all these mice were moribund by day 15. Unlike the females, the difference in survival between L-gD₁₋₃₀₆-HD/MPL and L-gD₂₆₄₋₂₈₅-HD/MPL in male BALB/c mice was statistically significant ($P=0.0012$).

Vaccination of C57BL/6 male and female mice with L-gD₁₋₃₀₆-HD/MPL paralleled the results obtained with the BALB/c mice (100% survival for both sexes) (Figure 7A). Although survival of the L-gD₁₋₃₀₆-HD/MPL male mice was significantly better than the L-control/MPL vaccinated male mice ($P=0.0003$) this was not the case for the C57BL/6 female mice since the latter were innately resistant to the intrarectal HSV2 infection ($P=0.3173$). When the C57BL/6 male mice were vaccinated with L-gD₂₆₄₋₂₈₅-HD/MPL, there was significant protection (57% survival) compared to male C57BL/6 L-control/MPL mice ($P=0.0003$). In comparison, L-gD₁₋₃₀₆-HD/MPL vaccination of male C57BL/6 mice provided even better protection than vaccination with the L-gD₂₆₄₋₂₈₅-HD/MPL but the difference did not quite reach significance ($P=0.0597$). Unlike the male C57BL/6 mice, the efficacy of L-gD₂₆₄₋₂₈₅-HD/MPL in female C57BL/6 mice was not examined because the female C57BL/6 mice were very resistant to the intrarectal HSV-2 infection, and it was unlikely that we would be able to discern any potential difference in efficacy between the peptide vaccine and the ectodomain vaccine using this model. The L-control/MPL vaccines did not protect any of the male mice, with all mice becoming moribund by day 13.

The disease signs following vaccination corresponded with the survival data. L-gD₁₋₃₀₆-HD/MPL vaccination reduced the severity of rectal lesions (mean peak score ≤ 1) for all mice (Figures 6B and 7B), while the L-gD₂₆₄₋₂₈₅-HD/MPL vaccination did not (mean peak score male BALB/c=4.0, male C57BL/6=1.1, female BALB/c=2.8). No neurological signs were observed in the L-gD₁₋₃₀₆-HD/MPL mice while both L-gD₂₆₄₋₂₈₅-HD/MPL and the L-control/MPL mice had some neurological signs (Figures 6C and 7C). The median PFU/g spinal tissue of the L-gD₁₋₃₀₆-HD/MPL and the L-gD₂₆₄₋₂₈₅-HD/MPL mice was 0 although the L-control/MPL male and female BALB/c and male C57BL/6 mice had high levels of virus in their spinal cords (median values of 162,500 PFU/g, 162,000 PFU/g, and 2000 PFU/g, respectively; $P\leq 0.0066$ vs L-gD₁₋₃₀₆-HD/MPL mice) (Table 2). In comparison, the C57BL/6 female L-control/MPL mice had a median PFU/g value of 0. The lack of virus in the spinal tissue of these mice was probably a result of the female's resistance to HSV2 infection (*i.e.*, 86% survival in the L-control/MPL female group).

Neutralizing antibody titers

To compare the humoral immune response of mice vaccinated with the L-gD₁₋₃₀₆-HD/MPL or L-gD₂₆₅₋₂₈₄-HD/MPL vaccines, we assayed the serum of vaccinated mice for neutralizing antibody titers. The L-gD₁₋₃₀₆-HD/MPL female BALB/c and female C57BL/6 mice produced the highest titers (mean=75 for BALB/c and 96 for C57BL/6) (Table 3). In contrast, the males had much lower neutralizing antibody titers (mean=3.3 BALB/c and 0.67 for C57BL/6 mice). The neutralizing antibody titers from L-gD₂₆₅₋₂₈₄-HD/MPL BALB/c male and female mice were also very low (mean=1.0 males and 1.3 females; the titer for L-gD₂₆₅₋₂₈₄-HD/MPL C57BL/6 mice was not determined). Notably, although the male L-gD₁₋₃₀₆-HD/MPL vaccinated mice had low neutralizing antibody titers, they showed the same degree of protection as the female L-gD₁₋₃₀₆-HD/MPL vaccinated mice against intrarectal HSV2 challenge (100% survival C57BL/6 and 71% survival BALB/c).

Cytokine Singleplex Bead assay for IFN- γ and IL-4 and ELISPOT results for IL-4 and IFN- γ

The results of the neutralizing antibody assays suggested that the Th1 response might play a more critical role than the Th2 response in providing protection against infection. To assess this, we examined cellular cytokine production of male and female L-gD₁₋₃₀₆-HD/MPL BALB/c mice using both the ELISPOT and Cytokine Singleplex bead assays. Male and female BALB/c mice vaccinated with L-gD₁₋₃₀₆-HD/MPL had significantly higher numbers of gD₁₋₃₀₆ IFN- γ -secreting splenocytes than their respective L-control/MPL mice ($P=0.0218$ males; $P<0.0001$ females) (Figure 8A). In addition, the total amount of IFN- γ produced by these splenocytes was also significantly higher for the L-gD₁₋₃₀₆-HD/MPL mice than the L-

control/MPL mice ($P<0.0001$ males and females)(Figure 8B). Compared to the male mice, females produced significantly more IFN- γ -secreting splenocytes ($P=0.0091$) although a greater total amount of IFN- γ was secreted by the male vs the female splenocytes ($P=0.0586$).

Similar results were observed for IL-4. L-gD₁₋₃₀₆-HD/MPL vaccinated male and female BALB/c mice had significantly higher numbers of IL-4-secreting splenocytes than the respective L-control/MPL mice ($P=0.0166$ males; $P=0.0001$ females) (Figure 9A) and the total amount of IL-4 produced by the splenocytes was significantly higher for the L-gD₁₋₃₀₆-HD/MPL vaccinated mice than the L-control/MPL mice ($P=0.0001$ males; $P<0.0001$ females) (Figure 9B). Male and female BALB/c mice did not have significantly different numbers of IL-4-secreting splenocytes and the total amount of IL-4 secreted by the splenocytes was also not significantly different between the sexes.

While the number of splenocytes which secreted IL-4 was significantly higher than the IFN- γ -secreting splenocytes in both the male and female mice ($P=0.0136$ males; $P<0.0001$ females), the total amount of IFN- γ (pg/mL) secreted by the splenocytes was significantly higher than the IL-4 ($P=0.0002$ males; $P=0.01$ females). This data suggests that both the Th1 (IFN- γ) and Th2 (IL-4) responses are upregulated by the L-gD₁₋₃₀₆-HD/MPL in both male and female mice, but that the Th1 response appears to stimulate greater cytokine production.

Discussion

In the present study, the L-gD₁₋₃₀₆-HD/MPL vaccine provided significantly better protection than the L-gD₂₆₄₋₂₈₅-HD/MPL vaccine in both male and female BALB/c mice challenged intrarectally with HSV2. We had previously observed similar results using an intravaginal HSV2 model in immunized female BALB/c mice [33]. The better protection provided by the gD₁₋₃₀₆ antigen is probably a result of the ectodomain containing the gD₂₆₄₋₂₈₅ epitope as well as other known immunogenic regions of the HSV2 gD protein reported to stimulate cell-mediated responses [26],[34],[31], as well as humoral responses [35]. By using the gD₁₋₃₀₆ ectodomain, the host APCs (antigen-presenting cells) can process the entire antigen and present different peptides to the various T cell populations via either MHC class I or class II [36]. The larger size of the gD₁₋₃₀₆ ectodomain antigen in comparison to the much smaller gD₂₆₄₋₂₈₅ epitope segment may also contribute to the enhanced protection that we observed because it would be a larger target for the immune system [23] and this might have enhanced the phagocytic uptake of L-gD₁₋₃₀₆-HD/MPL compared to L-gD₂₆₄₋₂₈₅-HD/MPL.

The L-gD₁₋₃₀₆-HD/MPL vaccine was also found to be more protective than the L-gD₂₆₄₋₂₈₅-HD/MPL vaccine when tested in another strain of inbred mice, *i.e.*, C57BL/6. It has been reported that C57BL/6 mice elicit a stronger Th1 response than BALB/c mice, making them more resistant to infections by pathogens such as *Leishmania major* [37], *Francisella tularensis* [38], and *Mycobacterium tuberculosis* [39]. When Stulting *et al.* [40] compared susceptibility to ocular HSV1 (Herpes simplex type 1) infection in three strains of inbred mice, *i.e.*, A/J, BALB/c, and C57BL/6, they found that only the C57BL/6 mice were resistant to the HSV1 infection. The Th1 response also plays a critical role in protection against HSV2 infection [41],[42],[43],[44],[45] and in the present studies, the L-gD₁₋₃₀₆-HD/MPL vaccine protected both strains of mice, although it produced better survival in the intrarectal HSV2 challenged C57BL/6 male and female mice (100%) than in the BALB/c male and female mice (71%). There was also a marked decrease in severity of the intrarectal HSV2 infection in the non-vaccinated, female C57BL/6 mice. Testing of this vaccine for its efficacy in outbred mouse strains will help determine if the L-gD₁₋₃₀₆-HD/MPL vaccine has the potential to be protective for the genetically heterogeneous human population. Preliminary studies in Swiss Webster male and female mice given this vaccine and challenged intrarectally or intravaginally with HSV2 indicate that the vaccine is protective in outbred mice [46].

There are important differences in the immune responses of males and females which can lead to differences in vaccine efficacy, as evidenced by the HSV2 gD vaccine Simplirix® presently in clinical development. This vaccine is efficacious in HSV-seronegative women, but not in men [22]. Thus, an improved HSV2 vaccine should provide protection to both men and women. We developed the HSV2 intrarectal model of infection in mice to compare male and female vaccine responses. For BALB/c mice, the infection was very similar in males and females, but for C57BL/6 mice, the females were more resistant to the infection. This resistance in the C57BL/6 female mice was probably a result both of the differences between the mouse strains, as well as the protective effects of the estrogen since higher estrogen levels are associated with higher survival following HSV2 challenge in mice [47]. The sex hormones, estrogen and progesterone, lead to changes in the numbers and activity of immune cells in the uterus, cervix and vagina in humans and animals [29]. When women are pregnant and estrogen levels are lower with progesterone dominating, there is a shift toward a Th2 response [48] which may be more similar to the immune response seen in men. The need for Depo-Provera® to produce consistent intravaginal infection in our female mice underscores the susceptibility of animals to HSV2 with high levels of progesterone.

The L-gD₁₋₃₀₆-HD/MPL vaccine tested in this study was able to protect both male and female mice, with elevated IFN- γ levels compared to IL-4 levels, indicating an upregulation in both sexes of the Th1 response to the HSV2 gD antigen protein. Although the results of the ELISPOT assay showed that there were fewer splenocytes producing IFN- γ than IL-4, the total production of secreted IFN- γ was significantly higher than the total production of secreted IL-4. Other investigators have observed that IFN- γ is important in HSV2 genital infections for resolution of lesions and clearance of infection [42], and mice given HSV2 gD DNA as a vaccine along with Th1 cytokine genes had much better survival and fewer lesions, than those given Th2 cytokines when challenged with HSV2 intravaginally [43]. However, along with the enhanced Th1 response reported in our studies, there was also an increased production of IL-4 in the L-gD₁₋₃₀₆-HD/MPL vaccinated mice. This is probably beneficial since both the cell-mediated and the humoral immune responses have been reported to protect mice against HSV2 infection [49],[41]. Our results from the *in vitro* neutralizing antibody assays indicated that the L-gD₁₋₃₀₆-HD/MPL immunized female mice produced higher titers than L-gD₂₆₄₋₂₈₅-HD/MPL immunized female mice and this paralleled the greater protection seen with the L-gD₁₋₃₀₆-HD/MPL vaccine. Female mice had significantly higher neutralizing antibody titers than the males although both male and female mice in our studies were similarly protected by the L-gD₁₋₃₀₆-HD/MPL vaccine, suggesting that the Th1 response was primarily responsible for the protection generated by the vaccine in males, and possibly even in the females.

To optimize the L-gD₁₋₃₀₆-HD vaccine, several variables important for vaccine development [50],[51],[52] were examined including the dose of antigen, the boosting schedule and the amount of MPL adjuvant. As reported with other vaccines [53], there was a clear dose dependent response as the amount of gD was increased, with the highest gD dose eliciting the best protection. We also observed that the time following the priming dose could be reduced from 8 weeks to 4 weeks without loss of protection indicating that one month provided sufficient time to achieve maturation of the memory T cell response to the vaccine [54].

MPL was added to the liposome composition because it is known to be a potent stimulator of the Th1 response [55]. The HSV2 Simplirix® vaccine tested in human clinical trials that was shown to be effective in females, but not males, contains 3-O-deacylated-monophosphoryl lipid A, as well as alum which has been reported to stimulate a Th2 response [56]. The L-gD₁₋₃₀₆-HD/MPL vaccine used in our study does not contain alum and was found to be protective for both male and female mice. The L-gD₁₋₃₀₆-HD without MPL used in the present study was also protective (P=0.0003 vs L-control), but incorporation of MPL in the liposomes further enhanced the protective effects. In preliminary studies, we subcutaneously immunized

mice with a mixture of alum and the L-gD₁₋₃₀₆-HD/MPL vaccine. Although this combination showed comparable efficacy to the L-gD₁₋₃₀₆-HD/MPL vaccine, the addition of alum produced large granulomas and thus, was not further investigated. We also tested non-liposomal gD₁₋₃₀₆-HD mixed with MPL and Alum and it provided no protection against HSV2 challenge (unpublished data). Taken together, this data demonstrates that the liposome carrier itself contributes to the immunogenicity of the vaccine.

In summary, the present studies focused on demonstrating the protective efficacy of the L-gD₁₋₃₀₆-HD/MPL vaccine in acute murine HSV2 infection following intravaginal or intrarectal challenge. The results showed that the L-gD₁₋₃₀₆-HD vaccine provided significant protection of both male and female mice in different inbred mouse strains. The vaccine also stimulated antigen-specific splenocytes to produce elevated levels of IFN- γ compared to IL-4 indicating that there was an enhanced Th1 response in both males and females. To examine whether the vaccine can minimize the recurrence of HSV2 infection, studies have been initiated to test the vaccine's efficacy in a recurrent HSV2 infection in both male and female guinea pigs. Further optimization of the L-gD₁₋₃₀₆-HD vaccine to determine the effect of additional boosts, further increases in gD dosing, and testing of the duration of vaccine protection in different animal models is underway.

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References

1. Breinig MK, Kingsley LA, Armstrong JA, Freeman DJ, Ho M. Epidemiology of genital herpes in Pittsburgh: serologic, sexual, and racial correlates of apparent and inapparent herpes simplex infections. *J Infect Dis* 1990;162(2):299–305. [PubMed: 2165102]
2. Cusini M, Cusan M, Parolin C, et al. Seroprevalence of herpes simplex virus type 2 infection among attendees of a sexually transmitted disease clinic in Italy. *Italian Herpes Forum. Sex Transm Dis* 2000;27(5):292–5. [PubMed: 10821604]
3. Xu F, Sternberg MR, Kottiri BJ, McQuillan G, Lee FK, Nahmias AJ, Berman SM, Markowitz LE. Trends in Herpes Simplex Virus Type 1 and Type 2 seroprevalence in the United States. *JAMA* 2006; (8):964–973. [PubMed: 16926356]
4. Munoz N, Kato I, Bosch FX, et al. Cervical cancer and herpes simplex virus type 2: case-control studies in Spain and Colombia, with special reference to immunoglobulin-G sub-classes. *Int J Cancer* 1995;60 (4):438–42. [PubMed: 7829255]
5. Perez G, Skurnick JH, Denny TN, et al. Herpes simplex type II and Mycoplasma genitalium as risk factors for heterosexual HIV transmission: report from the heterosexual HIV transmission study. *Int J Infect Dis* 1998;3(1):5–11. [PubMed: 9831669]
6. Chen CY, Ballard RC, Beck-Sague CM, et al. Human immunodeficiency virus infection and genital ulcer disease in South Africa: the herpetic connection. *Sex Transm Dis* 2000;27(1):21–9. [PubMed: 10654864]
7. Brown ZA, Selke S, Zeh J, et al. The acquisition of herpes simplex virus during pregnancy. *N Engl J Med* 1997;337(8):509–15. [PubMed: 9262493]
8. Welling-Wester S, Scheffer AJ, Welling GW. B and T cell epitopes of glycoprotein D of herpes simplex virus type 1. *Microbiol Immunol* 1991;76:59–68.
9. Berman PW, Gregory T, Crase D, Lasky LA. Protection from genital herpes simplex virus type 2 infection by vaccination with cloned type 1 glycoprotein D. *Science* 1985;227(4693):1490–2. [PubMed: 2983428]
10. Eisenberg RJ, Cerini CP, Heilman CJ, et al. Synthetic glycoprotein D-related peptides protect mice against herpes simplex virus challenge. *J Virol* 1985;56(3):1014–7. [PubMed: 2999419]

11. Long D, Madara TJ, DeLeon MP, Cohen GH, Montgomery PC, Eisenberg RJ. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. *Infect Immun* 1984;37(2):761–4. [PubMed: 6319291]
12. Landolfi V, Zarley CD, Abramovitz AS, et al. Baculovirus-expressed herpes simplex virus type 2 glycoprotein D is immunogenic and protective against lethal HSV challenge. *Vaccine* 1993;11(4):407–14. [PubMed: 8385842]
13. Geerligs HJ, Kocken CHM, Drijfhout JW, et al. Virus neutralizing activity induced by synthetic peptides of glycoprotein D of herpes simplex virus type 1, selected by their reactivity with hyperimmune sera from mice. *J Gen Virol* 1990;71:1767–74. [PubMed: 2167930]
14. Torseth JW, Cohen GH, Eisenberg RJ, et al. Native and recombinant herpes simplex virus type 1 envelope proteins induce human immune T-Lymphocyte responses. *J Virol* 1987;61(5):1532–9. [PubMed: 3033279]
15. Mitchell BM, Stevens JG. Neuroinvasive properties of herpes simplex virus type 1 glycoprotein variants are controlled by the immune response. *J Immunol* 1996;156(1):246–55. [PubMed: 8598469]
16. Posavad CM, Koelle DM, Corey L. High frequency of CD8⁺ cytotoxic T-lymphocyte precursors specific for herpes simplex viruses in persons with genital herpes. *J Virol* 1996;70(11):8165–8. [PubMed: 8892947]
17. Sethi KK, Omata Y, Schneweis KE. Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. *J Gen Virol* 1983;64(Pt 2):443–7. [PubMed: 6601180]
18. Gytoku T, Ono F, Aurelian L. Development of HSV-specific CD4⁺ Th1 responses and CD8⁺ cytotoxic T lymphocytes with antiviral activity by vaccination with the HSV-2 mutant ICPI0DeltaPK. *Vaccine* 2002;20(2122):2796–807. [PubMed: 12034107]
19. Smith PM, Wolcott RM, Chervenak R, Jennings SR. Control of acute cutaneous herpes simplex virus infection: T cell-mediated viral clearance is dependent upon interferon-gamma (IFN-gamma). *Virolog* 1994;202(1):76–88.
20. Berman PW, Vogt PE, Gregory T, Lasky LA, Kern ER. Efficacy of recombinant glycoprotein D subunit vaccines on the development of primary, recurrent, and latent genital infections with herpes simplex virus type 2 in guinea pigs. *J Infect Dis* 1988;157(5):897–902. [PubMed: 2834467]
21. Stanberry LR, Myers MG, Stephanopoulos DE, Burke RL. Preinfection prophylaxis with herpes simplex virus glycoprotein immunogens: Factors influencing efficacy. *J Gen Virol* 1989;70:3177–85. [PubMed: 2558156]
22. Stanberry LR, Spruance SL, Cunningham AL, et al. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. *N Engl J Med* 2002;347(21):1652–61. [PubMed: 12444179]
23. Fujii G, Ernst W, Adler-Moore J. The VesiVax system: a method for rapid vaccine development. *Frontiers in Bioscience* 2008;13:1968–1980. [PubMed: 17981684]
24. Isola VJ, Eisenberg RJ, Siebert GR, Heilman CJ, Wilcox WC, Cohen GH. Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. *J Virol* 1989;63(5):2325–34. [PubMed: 2467994]
25. Mehra V, Sweetser D, Young RA. Efficient mapping of protein antigenic determinants. *Proc Natl Acad Sci USA* 1986;83(18):7013–7. [PubMed: 2428046]
26. DeFreitas EC, Dietzschold B, Koprowski H. Human T-lymphocyte response *in vitro* to synthetic peptides of herpes simplex virus glycoprotein D. *Proc Natl Acad Sci USA* 1985;82:3425–9. [PubMed: 2582411]
27. Watari E, Dietzschold B, Szokan G, Heber-Katz E. A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. *J Exp Med* 1987;165:459–70. [PubMed: 3029270]
28. Kohl S, Strynadka NC, Hodges RS, Pereira L. Analysis of the role of antibody-dependent cellular cytotoxic antibody activity in murine neonatal herpes simplex virus infection with antibodies to synthetic peptides of glycoprotein D and monoclonal antibodies to glycoprotein B. *Journal of Clinical Investigation* 1990;86:273–278. [PubMed: 2164044]
29. Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunology and Medical Microbiology* 2003;38:13–22. [PubMed: 12900050]

30. Phillips D, Zacharopoulos V. Nonoxynol-9 Enhances Rectal Infection by Herpes Simplex Virus in Mice. *Contraception* 1998;57:341–348. [PubMed: 9673842]
31. Wyckoff JH III, Osmand AP, Eisenberg RJ, Cohen GH, Rouse BT. Functional T cell recognition of synthetic peptides corresponding to continuous antibody epitopes of herpes simplex virus type 1 glycoprotein D. *Immunobiology* 1988;177(2):134–48. [PubMed: 2456985]
32. Chentoufi AA, Zhang X, Lamberth K, Dasgupta G, Bettahi I, Nguyen A, Wu M, Zhu X, Mohebbi A, Buus S, Wechsler SL, Nesburn AB, Benmohamed L. HLA-A*0201-restricted CD8+ cytotoxic T lymphocyte epitopes identified from herpes simplex virus glycoprotein D. *Journal of Immunology* 2008;180(1):426–437.
33. Olson, K.; Avalos, S.; Alvarado, A.; Ernst, W.; Fujii, G.; Adler-Moore, J. Comparative Efficacy of Liposomal gD1-327 and gD264-285 Vaccines in a Lethal Intravaginal Murine HSV-2 Infection Model. 25th Annual Meeting American Society for Virology; 2006. Abstract 16-15
34. Heber-Katz E, Hollosi M, Dietzschold B, Hudecz F, Fasman GD. The T cell response to the glycoprotein D of the herpes simplex virus: The significance of antigen conformation. *The Journal of Immunology* 1985;135(2):1385–1390. [PubMed: 2409148]
35. Cohen GH, Dietzschold B, Ponce deLeon M, Long D, Golub E, Varrichio A, Pereira L, Eisenberg RJ. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulated the production of neutralizing antibody. *Journal of Virology* 1984;49(1):102–108. [PubMed: 6197535]
36. DeBruijn MLH, Schuurhuis DH, Vierboom MPM, Vermeulen H, deCock KAJ, Ooms ME, Rensing ME, Toebes M, Franken KLMC, Drijfhout JW, Ottenhoff THM, Offringa R, Melief CJM. Immunization with Human Papillomavirus Type 16 (HPV16) Oncoprotein-loaded Dendritic Cells as well as Protein in Adjuvant Induces MHC Class I-restricted Protection to HPV16-induced Tumor Cells. *Cancer Research* 1998;58:724–731. [PubMed: 9485027]
37. Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. *Annual Review of Immunology* 1995;13:151–77.
38. Chen W, Shen H, Webb A, KuoLee R, Conlan JW. Tularemia in BALB/c and C57BL/6 mice vaccinated with *Francisella tularensis* LVS and challenged intradermally, or by aerosol with virulent isolates of the pathogen: protection varies depending on pathogen virulence, route of exposure, and host genetic background. *Vaccine* 2003;21:3690–3700. [PubMed: 12922099]
39. Su SH, Chen H, Jen CJ. C57BL/6 and BALB/c Bronchoalveolar Macrophages Respond Differently to Exercise. *The Journal of Immunology* 2001;167:5084–5091. [PubMed: 11673518]
40. Stulting RD, Kindle JC, Nahmias AJ. Patterns of Herpes Simplex Keratitis in Inbred Mice. *Investigative Ophthalmology and Visual Science* 1985;26(10):1360–1367. [PubMed: 4044164]
41. Rager-Zisman B, Allison A. Mechanism of Immunologic Resistance to Herpes Simplex Virus 1 (HSV-1) Infection. *The Journal of Immunology* 1976;116(1):35–40. [PubMed: 173758]
42. Parr MB, Parr EL. The Role of Gamma Interferon in Immune Resistance to Vaginal Infection by Herpes Simplex Virus Type 2 in Mice. *Virology* 1999;258:282–294. [PubMed: 10366565]
43. Sin JJ, Kim JJ, Boyer JD, Ciccarelli RB, Higgins TJ, Weiner DB. In Vivo Modulation of Vaccine-Induced Immune Responses toward a Th1 Phenotype Increases Potency and Vaccine Effectiveness in a Herpes Simplex Virus Type 2 Mouse Model. *Journal of Virology* 1999;73(1):501–509. [PubMed: 9847356]
44. Lekstrom-Himes J, LeBlanc RA, Pesnicak L, Godleski M, Straus SE. Gamma Interferon Impedes the Establishment of Herpes Simplex Virus Type 1 Latent Infection but Has No Impact on Its Maintenance or Reactivation in Mice. *Journal of Virology* 2000;74(14):6680–6683. [PubMed: 10864685]
45. Thapa M, Welner RS, Pelayo R, Carr DJJ. CXCL9 and CXCL10 Expression Are Critical for Control of Genital Herpes Simplex Virus Type 2 Infection through Mobilization of HSV-Specific CTL and NK Cells to the Nervous System. *The Journal of Immunology* 2008;180:1098–1106. [PubMed: 18178850]
46. Macias, P.; Tan, BJ.; Ernst, WA.; Adler-Moore, JP. Multiple dosing with a liposomal HSV-2 vaccine protected outbred Swiss Webster female and male mice challenged intrarectally with HSV-2. 25th Annual Meeting American Society for Virology; 2009. Abstract 715

47. Gillgrass A, Fernandez SA, Rosenthal KL, Kaushic C. Estradiol Regulates Susceptibility following Primary Exposure to Genital Herpes Simplex Virus Type 2, while Progesterone Induces Inflammation. *Journal of Virology* 2005;79(5):3107–3116. [PubMed: 15709030]
48. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunology Today* 1993;14(7):353–356. [PubMed: 8363725]
49. Sin JJ, Kim JJ, Ugen CE, Ciccarelli RB, Higgins TJ, Weiner DB. Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocytemacrophage colony-stimulating factor expression cassettes. *European Journal of Immunology* 1998;28:3530–3540. [PubMed: 9842896]
50. Elhay M, Anderson P. Immunological requirements for a subunit vaccine against tuberculosis. *Immunology and Cell Biology* 1997;75:595–603. [PubMed: 9492199]
51. Cassidy WM, Watson B, Ioli VA, Williams K, Bird S, West DJ. A Randomized Trial of Alternative Two- and Three-Dose Hepatitis B Vaccination Regimens in Adolescents: Antibody Responses, Safety, and Immunologic Memory. *Pediatrics* 2001;107(4):626–631. [PubMed: 11335734]
52. Jansen T, Hofmans MPM, Theelen MJG, Manders FGA, Schijns VEJC. Dose and timing requirements for immunogenicity of viral poultry vaccine antigen: investigations of emulsion-based depot function. *Avian Pathology* 2007;36(5):361–365. [PubMed: 17899459]
53. Malkin E, Diemert DJ, McArthur JH, Perreault JR, Miles AP, Giersing BK, Mullen GE, Orcutt A, Muratova O, Awkal M, Zhou H, Wang J, Stowers A, Long CA, Mahanty S, Miller LH, Saul A, Durbin AP. Phase I Clinical Trial of Apical Membrane Antigen 1: an Asexual Blood-Stage Vaccine for *Plasmodium falciparum* Malaria. *Infection and Immunity* 2005;73(6):3677–3685. [PubMed: 15908397]
54. Wherry E, Ahmed R. Memory CD8 T cell differentiation during viral infection. *Journal of Virology* 2004;78(11):5535–5545. [PubMed: 15140950]
55. DeBecker G, Moulin V, Pajak B, Bruck C, Francotte M, Thiriart C, Urbain J, Moser M. The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells. *International Immunology* 2000;12(6):807–815. [PubMed: 10837408]
56. Pashine A, Valiante NM, Ulmer JB. Targeting the innate response with improved vaccine adjuvants. *Nature Medicine* 2005;11(4):S63–S68.

Fig 1. Liposome sizing. The sizes of two separate batches of L-gD₂₆₄₋₂₈₅-HD/MPL and two separate batches of L-gD₁₋₃₀₆-HD/MPL were determined using dynamic light scattering.

Fig. 2. BALB/c female mice ($n=7$ /group), vaccinated subcutaneously with L-gD₁₋₃₀₆-HD/15µgMPL (15, 30 or 60µg gD₁₋₃₀₆-HD/dose) or L-control/15µgMPL on day 0 and day 56, and challenged intravaginally with 50LD₅₀ HSV2 on day 63. Mice were monitored for morbidity (A), vaginal disease signs (0-4) (B) and neurological disease signs (0-4) (C) for up to 28 days. L-gD₁₋₃₀₆-HD/MPL vs L-control/MPL ($P<0.05$; Kaplan Meier Log Rank test).

Fig. 3. BALB/c female mice ($n=7$ /group), vaccinated subcutaneously with L-gD₁₋₃₀₆-HD/MPL (60µg gD₁₋₃₀₆-HD/dose with no MPL or 60µg gD₁₋₃₀₆-HD/dose with 10, 15 or 25µgMPL/dose) or L-control (15µgMPL/dose prime; 25µgMPL/dose boost) on day 0 and day 56, and challenged intravaginally with 50LD₅₀ HSV2 on day 63. Mice were monitored for morbidity (A), vaginal disease signs (0-4) (B) and neurological disease signs (0-4) (C) for up to 28 days. L-gD₁₋₃₀₆-HD/MPL vs L-control/MPL ($P<0.05$; Kaplan Meier Log Rank test).

Fig. 4. BALB/c female mice ($n=7/\text{group}$), vaccinated subcutaneously with L-gD₁₋₃₀₆-HD/15 μg MPL or L-control/15 μg MPL or with on day 0 and boosted day 28, 42 or 56. Day 63 mice were challenged intravaginally with 50LD₅₀ HSV2. Mice were evaluated for morbidity (A) and vaginal (B) and neurological (C) signs of disease. All boosting regimens were equally protective for L-gD₁₋₃₀₆-HD/MPL vaccinated mice and significantly more effective than L-control/MPL vaccinated mice ($P<0.05$; Kaplan Meier Log Rank test).

Fig. 5.

Male and female non-vaccinated BALB/c and C57BL/6 mice ($n=7$ /group) were challenged intrarectally with HSV2. The infection was comparable for male and female BALB/c mice as well as male C57BL/6 mice based on morbidity (A), rectal (B) and neurological (C) disease signs. C57BL/6 female mice were resistant to the challenge, with most mice surviving (A) despite moderate rectal disease signs (B).

Fig. 6. BALB/c male and female mice ($n=7$ /group), vaccinated subcutaneously with L-gD₁₋₃₀₆-HD/25 μ gMPL (60 μ g gD₁₋₃₀₆-HD/dose), L-gD₂₆₄₋₂₈₅-HD/25 μ gMPL (60 μ g gD₂₆₄₋₂₈₅-HD/dose), or L-control/25 μ gMPL liposomes on day 0 and day 56, and challenged intrarectally with 10LD₅₀ HSV2 on day 63. Mice were monitored for up to 28 days for morbidity (A), rectal disease signs (0-4) (B), and neurological disease signs (0-4) (C). L-gD₁₋₃₀₆-HD/MPL vs L-control/MPL ($P<0.05$; Kaplan Meier Log Rank test); L-gD₁₋₃₀₆-HD/MPL male BALB/c vs L-gD₂₆₄₋₂₈₅-HD/MPL male BALB/c ($P<0.05$; Kaplan Meier Log Rank test).

Fig. 7. C57BL/6 male and female mice ($n=7/\text{group}$), vaccinated subcutaneously with L-gD₁₋₃₀₆-HD/25 μg MPL (60 μg gD₁₋₃₀₆-HD/dose), L-gD₂₆₄₋₂₈₅-HD/25 μg MPL (60 μg gD₂₆₄₋₂₈₅-HD/dose), or L-control/25 μg MPL liposomes on day 0 and day 56, and challenged intrarectally with 10LD₅₀ HSV2 on day 63. Mice were monitored for up to 28 days for morbidity (A), rectal disease signs (0-4) (B), and neurological disease signs (0-4) (C). L-gD₁₋₃₀₆-HD/MPL vs L-control/MPL ($P<0.05$; Kaplan Meier Log Rank test)

A. ELISPOT: Number of IFN- γ Secreting Cells **B. Cytokine Singleplex Bead Assay: Total IFN- γ Secreted (pg/mL)** **Fig. 8.**

IFN- γ secretion by splenocytes from L-gD₁₋₃₀₆-HD/15 μ gMPL or L-control/15 μ gMPL vaccinated male and female BALB/c mice. Splenocytes were pooled for each test group ($n=24$ mice/group); 5 aliquots (2×10^6 cells/aliquot) of each pooled sample were analyzed by ELISPOT (A) and Cytokine Singleplex bead (B) assays. L-gD₁₋₃₀₆-HD/MPL vaccination resulted in significantly higher IFN- γ production by splenocytes from both male and female mice ($P<0.05$; Unpaired t test).

A. ELISPOT: Number of IL-4 Secreting Cells

B. Cytokine Singleplex Bead Assay: Total IL-4 Secreted (pg/mL)

Fig. 9.

IL-4 secretion by splenocytes from L-gD₁₋₃₀₆-HD/15μgMPL or L-control/15μgMPL vaccinated male and female BALB/c mice. Splenocytes were pooled for each test group ($n=24$ mice/group); 5 aliquots (2×10^6 cells/aliquot) of each pooled sample were analyzed by ELISPOT (A) and Cytokine Singleplex bead (B) assays. L-gD₁₋₃₀₆-HD/MPL vaccination resulted in significantly higher IL-4 production by splenocytes from both male and female mice ($P<0.05$; Unpaired t test).

Table 1

HSV2 infection localization in Intravaginally or Intrarectally challenged BALB/c mice

ORGAN^a	Intravaginal female Mean PFU/g (range)^b # infected/# total	Intrarectal female Mean PFU/g (range)^b # infected/# total
Brain	2,160 (0-5100) 3/5	3,338 (0-9300) 4/5
Spinal cord	37,400 (0-120000) 3/5	52,200 (8000-130000) 5/5

^a No recovery of virus from adrenal glands, bladder, heart, kidneys, liver, lungs, peritoneal fluid or spleen (*n*=5 mice/group)^b Assay limit of detection = 320 PFU/g tissue

Table 2

Spinal cord viral burden in immunized mice (n=5/group) challenged intrarectally with HSV2

Vaccine	Median PFU/g spinal tissue (range) ^a			
	BALB/c female	BALB/c male	C57BL/6 female	C57BL/6 male ^b
L-gD ₁₋₃₀₆ -HD/MPL	0	0 ^c (0-340)	0	0
L-gD ₂₆₄₋₂₈₅ -HD/MPL	0	0 ^d (0-2.5×10 ⁴)	ND ^e	ND ^e
L-control/MPL	1.6 ×10 ⁵ ^f (0.33-4.8×10 ⁵)	1.6 ×10 ⁵ (0.66-6.8×10 ⁵)	0	2.0×10 ³ ^g (0-6.2×10 ³)

^a Assay limit of detection = 320 PFU/g tissue^b n= 4 mice^c 4 out of 5 animals had no detectable PFU; one animal had 340 PFU/g^d 3 out of 5 animals had no detectable PFU; one animal had 709 PFU/g and the other animal had 2.5 × 10⁴ PFU/g^e Not Determined^f One mouse died prior to collection of tissues^g 2 out of 5 animals had no detectable PFU

Table 3Neutralizing antibody titer in immunized mice (n=3/group)^a

Vaccine	Mean Neutralizing Antibody Titer (range) ^b			
	BALB/c female	BALB/c male	C57BL/6 female	C57BL/6 male
L-gD ₁₋₃₀₆ -HD/MPL	75 (32-128)	3.3 (2-4)	96 (16-256)	0.67 (0-2)
L-gD ₂₆₄₋₂₈₅ -HD/MPL	1.3 ^c (0-2)	1.0 ^c (0-2)	ND ^d	ND ^d

^a Serum collected 6 days post-boost^b Titer is the reciprocal of the serum dilution in which the PFU were equal to 50% of the PFU in the positive viral control wells^c n=2 mice^d Not Determined