Abstract

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted virus that is highly prevalent worldwide, causing a range of symptoms that result in significant healthcare costs and human suffering. ACAM529 is a replication-defective vaccine candidate prepared by growing the previously described dl5-29 on a cell line appropriate for GMP manufacturing. This vaccine, when administered subcutaneously, was previously shown to protect mice from a lethal vaginal HSV-2 challenge and to afford better protection than adjuvanted glycoprotein D (gD) in guinea pigs. Here we show that ACAM529 given via the intramuscular route affords significantly greater immunogenicity and protection in comparison with subcutaneous administration in the mouse vaginal HSV-2 challenge model. Further, we describe a side-by-side comparison of intramuscular ACAM529 with a gD vaccine across a range of challenge virus doses. While differences in protection against death are not significant, ACAM529 protects significantly better against mucosal infection, reducing peak challenge virus shedding at the highest challenge dose by over 500-fold versus 5-fold for gD. Over 27% (11/40) of ACAM529-immunized animals were protected from viral shedding while 2.5% (1/40) were protected by the gD vaccine. Similarly, 35% (7/20) of mice vaccinated with ACAM529 were protected from infection of their dorsal root ganglia while none of the gD-vaccinated mice were protected. These results indicate that measuring infection of the vaginal mucosa and of dorsal root ganglia over a range of challenge doses is more sensitive than evaluating survival at a single challenge dose as a means of directly comparing vaccine efficacy in the mouse vaginal challenge model. The data also support further investigation of ACAM529 for prophylaxis in human subjects.

Introduction

HSV-2 is a global health problem, causing clinical manifestations ranging from mild skin or mucosal ulcers to lethal disseminated infections in newborns [1,2]. According to a meta-analysis of seroprevalence surveys, over 530 million of 15 to 49-year-olds are infected, and more than 24 million new infections occur each year worldwide [3]. The health economic costs of HSV-2 infections have been projected to reach over $2.5 billion annually in the US alone by 2015 [4]. In an effort to address the medical and societal burden caused by HSV-2, a number of vaccines have been evaluated in the clinic over the past decades [5]. Notably, a subunit vaccine made using recombinant glycoprotein D of HSV-2 (gD-2) strain G was shown to be well tolerated and immunogenic [6], and to achieve a disease reduction of 73% (P=0.01) and a trend for reduction in infections of about 43% (P=0.08) in women who were HSV-1 seronegative [7]; however, a larger clinical trial with a different target population failed to demonstrate efficacy against HSV-2 and significantly increased shedding frequency in vaccine recipients who became infected with HSV-2 [8].

New vaccines are constantly being investigated for HSV prophylaxis [9], including live attenuated [10–12], DNA [13], subunit [14], peptides [15,16], and prime-boost [17]. An alternative approach is the replication-defective virus dl5-29 which was constructed by deleting the UL5 and UL29 genes of herpes simplex virus type 2 (HSV-2) [18]. When administered to mice or guinea pigs subcutaneously, dl5-29 induces robust protective immune responses in vivo, and yet does not replicate or establish latency [19–21]. These data suggest that dl5-29 could be an effective vaccine for human use to prevent HSV-2 infections and genital herpes disease. In preparation for evaluation in a clinical setting, a new production cell line and virus master seed were prepared and the resulting vaccine was renamed ACAM529.

The replication-defective vaccine dl5-29 was previously shown in guinea pigs to be more immunogenic than purified subunit antigen gD combined with Freund’s adjuvant, and, after vaginal challenge, to offer similar protection against disease while also...
significantly reducing shedding [20]. The vaccine was further shown to protect guinea pigs against latent infection regardless of whether they were previously infected with HSV-1 [21]. However, evaluation of the immunogenicity and efficacy of ACAM529, which is prepared using cells and virus seed intended for production of clinical trial material, had not been possible until recently. In addition, prior studies of efficacy were limited to a single challenge dose rather than a range of doses. Under these conditions, it was not possible to estimate by how much the vaccines increased the challenge dose required to infect a certain proportion of animals. This information may be useful in predicting clinical efficacy, particularly in light of the recent observation that gD did not protect against HSV-2 infection [8], predicting clinical efficacy, particularly in light of the recent observation that gD did not protect against HSV-2 infection [8], which is consistent with past observations in guinea pigs [22].

Selection of immunization routes for human vaccines is highly empirical, however it is thought that immunization with live viral vaccines is less sensitive to route due to their ability to spread, while inactivated vaccines are generally most immunogenic when delivered intramuscularly or intradermally [23]. For instance, clinical studies have shown that delivery of live-attenuated polio vaccine is oral but the inactivated vaccine is given subcutaneously or intramuscularly. While the adjuvanted gD vaccine against HSV was given by intramuscular injection [7], the TA-HSV DISC vaccine, which was only tested for treatment of recurrent genital herpes but not prophylaxis, was administered subcutaneously [25]. The immunogenicity of dl29 (5BLacZ) delivered by the intramuscular route was investigated previously [26], but its potency in a prophylactic challenge model has not been described, nor have the relative immunogenicity and protective efficacy of dl29 or dl5-29 delivered by the intradermal, subcutaneous and intramuscular routes. Mucosal delivery of dl5-29 via the intranasal route was reported, but only to show that the vaccine does not establish latency in trigeminal ganglia [19], and, interestingly, vaginal delivery of the vaccine in the guinea pig prophylactic model significantly reduced recurrent disease but the effect was inferior to that afforded by intramuscular gD, and intravaginal dl5-29 failed to reduce challenge virus loads in sacral ganglia, in contrast with gD [20].

In the present studies, we first investigate the optimal route for ACAM529 immunization against a vaginal challenge in mice. Then we compare the vaccine’s efficacy with a glycoprotein D vaccine using four different challenge doses and several measures of immunogenicity and protection against viral infection as well as morbidity and mortality. These studies show that ACAM529 is most immunogenic and protective when given intramuscularly, and that it protects significantly better than gD against infection of the vaginal mucosa and dorsal root ganglia. Implications for preclinical assessment of vaccine candidates based on these observations are discussed.

**Results**

**Immunogenicity and prophylactic efficacy of ACAM529 administered to mice via different routes**

The effect of the route of administration of ACAM529 on its immunogenicity and protective efficacy was first investigated. BALB/c mice were immunized intradermally (ID), intramuscularly (IM), or subcutaneously (SC) and their blood sampled three weeks after the last vaccine dose. Serum prepared from this blood was tested for IgG responses against a commercial HSV-2 viral lysate (Fig. 1A), and assayed for neutralizing antibody responses (Fig. 1B). While all three immunization routes yielded significantly higher IgG responses compared to negative control animals, the IM group showed a significantly higher titer of $1.5 \times 10^6$, nearly 10-fold higher than that shown by either the SC or ID groups. Neutralizing antibody responses observed in IM vaccinated mice were significantly higher than all other groups in the experiment, while the titers of SC and ID vaccinated mice were not significantly higher than the negative control group.

The mice were challenged intravaginally with 50 LD$_{50}$ of HSV-2 strain 333 and their disease symptoms were monitored daily for two weeks. Animals that received the vaccine IM were 100% protected from challenge, which is significantly better than mice immunized SC (50% protection; $P<0.02$; Fig. 2A). ID immunization offered an intermediate degree of protection from death, and all three routes gave significant protection compared to mock immunization.

![Image](https://example.com/image1.png)

**Figure 1. Characterization of antibody responses elicited by ACAM529 delivered via three different parenteral routes.** Groups of ten BALB/c mice were immunized with two doses of $10^6$ pfu of ACAM529 given three weeks apart by one of three routes (SC, subcutaneous; IM, intramuscular; ID, intradermal), or with PBS (negative control given SC). Serum samples were taken 3 weeks after the second immunization. (A) ELISA endpoint IgG titers measured against whole HSV-2 lysate. Each symbol represents one animal and the horizontal bars indicate geometric mean titers (GMT). All differences except SC vs. ID are significant (* $P<0.05$; one way ANOVA, Kruskal-Wallis, Dunn’s multiple comparison test). (B) Neutralizing antibody responses, reported as 50% plaque reduction neutralization titer (50% PRNT), are significantly higher in the IM group than in the SC or ID groups (**P<0.0001; one way ANOVA). The dotted line indicates the lower limit of detection of the assay. doi:10.1371/journal.pone.0046714.g001
Comparison of the immunogenicity of ACAM529 and adjuvanted gD in mice

Having established the optimal delivery route for ACAM529, we wished to compare the immunogenicity and efficacy of ACAM529 and HSV-2 gD adjuvanted with CpG and alum [14,27]. Two doses of 1x10⁶ plaque forming units (pfu) of ACAM529 were administered three weeks apart in the upper thigh, while three doses of 2 μg of adjuvanted gD were given two weeks apart in the gastrocnemius muscle as described previously [14]. Control animals received PBS SC, and all animals received the last vaccine dose on the same day. The animals were bled 10 days after the last dose and sera were assayed for neutralizing antibody titers (Fig. 3A), IgG against HSV-2 lyase (Fig. 3B), as well as IgG against purified recombinant gD (Fig. 3C). Both ACAM529 and gD elicited similar neutralizing antibody titers against HSV-2, and while ACAM529 gave a 16-fold higher IgG titer against HSV-2 lyase than gD, gD-immunized mice showed a 14-fold greater titer against purified gD than ACAM529-immunized mice (both differences statistically significant).

An interferon-γ ELISPOT was carried out on a subset of immunized animals whose spleens were harvested 9 days after the last vaccine dose. Splenocytes were cultured in the presence of an immunodominant I-Aβ-restricted gD peptide and interferon-γ secreting cells were counted. The CD4+ T cell response of ACAM529-immunized animals is significantly greater than that seen in gD-immunized mice or negative control mice (Fig. 3D). Similar results were obtained using UV-inactivated ACAM529 for stimulation of splenocytes in the ELISPOT (data not shown).

Comparison of the prophylactic efficacy of ACAM529 and adjuvanted gD in mice

The immunized animals were challenged with wildtype HSV-2 to compare the efficacy of the two vaccines, however, in order to increase the probability of finding conditions which would reveal any differences, the animals were given one of four different challenge doses. Eleven days after the last vaccine dose, the immunized mice were given 2 mg of medroxyprogesterone and challenged intravaginally 7 days later with one of four challenge doses of HSV-2 strain 333: 15 LD₅₀ (2.4x10⁵ pfu), 50 LD₅₀ (8x10⁵ pfu), 150 LD₅₀ (2.4x10⁶ pfu), or 450 LD₅₀ (7.2x10⁶ pfu). For two weeks following challenge, the animals were observed for morbidity and mortality.

The effect of immunization on disease severity is shown in Fig. 4A. Control animals experienced significantly more severe symptoms than immunized mice at all challenge doses. ACAM529 protected mice significantly better than gD at all challenge doses tested. Animals reaching a disease score of 3 or higher were euthanized in accordance with approved institutional animal care protocols. All mock-immunized mice died or were euthanized by day 9 post-challenge (Fig. 4B and not shown). While four of forty mice were euthanized in the gD-immunized group, none of the ACAM529-immunized animals died or required euthanasia, although this was not a statistically significant difference (Fig. 4C; Fisher's exact test, P = 0.12).

Mucosal replication of challenge virus was measured by taking vaginal swabs of the mice during peak shedding (day 2 post-challenge) and measuring swab viral titers by plaque assay. The observed shedding for animals challenged with the highest dose is shown in Fig. 5A. ACAM529 significantly reduced shedding from 1.9x10⁵ pfu (geometric mean) for mock-immunized mice to 3.2x10⁵ pfu, which is a statistically significant decrease of over 500-fold. Furthermore, two of ten ACAM529-immunized animals

Figure 2. Effect of ACAM529 route of administration on prophylactic efficacy against a lethal challenge. Mice immunized as described in Fig. 1 were treated with medroxyprogesterone three weeks after the second immunization and challenged i.vag. one week later with 50 LD₅₀ (8x10⁵ pfu) of HSV-2 strain 333. (A) The percentage of animals surviving the challenge is plotted as a function of days post-challenge. Survival of IM-immunized animals is significantly better than SC (* P<0.02). All three groups of immunized animals are significantly protected compared to mock-immunized (PBS) animals. (B) Two days after challenge, vaginal swabs were taken from all challenged animals and viral titers determined by plaque assay. Shed virus is significantly reduced in all groups compared to mock-immunized (PBS) animals, and IM-immunized animals shed significantly less compared to SC and ID (* P<0.05; one way ANOVA). One animal in the IM group died during a bleed, prior to challenge. The dotted line indicates the lower detection limit of the plaque assay. doi:10.1371/journal.pone.0046714.g002

Because survival from a lethal challenge is not considered to be a stringent measure of protection in the HSV-2 vaginal challenge model, we evaluated other metrics of infection. Shedding of challenge virus at the site of infection within two days of challenge is thought to be an indication of the extent of mucosal infection [22]. Using vaginal swabs, it is possible to measure the amount of virus shed into the vaginal cavity. Based on previous experiments (data not shown) it was established that the peak time of shedding is 48 hours (day 2) post-challenge. Therefore, the challenged animals were swabbed on the 2nd day after challenge and the harvested virus titer was determined by plaque assay (Fig. 2B). All immunization routes afforded significant reductions in virus shedding compared to negative control animals, but IM immunization gave over 10-fold greater reduction in shed virus relative to SC or ID immunization (P<0.05). Interestingly, three of the IM mice and one of the ID mice had no virus detectable by plaque assay in their swabs, suggesting complete protection from mucosal infection.
had non-detectable shedding. Immunization with gD afforded a non-significant 5-fold decrease in shedding at this challenge dose.

Figure 5B shows the amount of virus shed as a function of challenge dose for mock-immunized, ACAM529-immunized, or gD-immunized animals. Mock-immunized mice shed increasing amounts of virus with increasing challenge dose and reached a plateau at the 2.4 × 10^6 pfu dose. There was a similar trend observed for gD-immunized mice, except that there was significant reduction in shedding observed at the lowest challenge dose. ACAM529 afforded significant reductions in shedding compared to control mice at all challenge doses.

The proportion of mice protected from challenge, i.e., with plaque titers below the detection limit, was also analyzed (Fig. 5C). At all four challenge doses, ACAM529 protected 20% to 40% of animals from shedding, while protection was only observed in one animal (10%) immunized with gD at the lowest challenge dose. Overall, combining all challenge dose groups, 27.5% (11/40) of ACAM529-immunized animals were protected from viral shedding while 2.5% (1/40) were protected by the gD vaccine (P = 0.0033; Fisher’s exact test).

Protection against infection of the dorsal root ganglia (DRG) was done in a subset of animals which was challenged 58 days after the last vaccine dose. Four days after challenge, five pairs of DRG
were extracted from each mouse and their DNA extracted in order to carry out quantitative PCR to measure viral genome copy number. The number of viral genome copies was divided by copy number of the host gene adipin and this ratio is plotted in Fig. 6A for all four challenge doses. Both the ACAM529 and gD vaccines significantly reduce DRG viral DNA loads compared to negative control mice. The proportion of animals with undetectable viral genomes, i.e., protected from DRG infection, is shown in Fig. 6B. Overall, 7 of 20 mice (35%) are protected by ACAM529, while none of the gD-immunized mice were protected (P = 0.0083; Fisher’s exact test).
ACAM529 consistently protects the detection limit on day 4 is plotted as a function of challenge dose. The percentage of mice for which viral genome copies in DRG were below the mean.) (B) Protection from infection of dorsal root ganglia. The copies were increased from 0 to 0.1. Error bars indicate standard error of one way ANOVA, Tukey’s comparison. Twenty animals in each group were divided in to 4 groups of 5 animals for each challenge dose. To discuss the protective effect of ACAM529, it is unlikely to be due to the anatomical proximity of immunization and challenge sites. The same logic applies to the ID injections in the flank of the mice, which were no more immunogenic or efficacious than SC. Recently, Awasthi et al. also reported a trend for intramuscular administration of their HSV-2 gD-deletion mutant vaccine to be more effective against shedding and disease than subcutaneous immunization in mice [28].

Past studies of gD adjuvanted with MPL and alum [22] have shown that protection against mucosal infection in guinea pigs is not achieved with this vaccine despite nearly complete prevention of morbidity and mortality. Therefore it is important to consider more than symptoms of disease when assessing vaccine efficacy. In addition, it is currently very difficult to conclusively compare vaccines using published data due to the variety of challenge strains being used that have different passage histories and apparently different virulence, as well as other differences in infection protocols, animal model used, immunological protocols, virological assays, etc. These considerations militate in favor of side-by-side comparisons of vaccines against HSV-2 with a benchmark immunogen such as adjuvanted gD.

In this study, ACAM529 was compared with gD combined with alum and CpG adjuvant as described by Awasthi et al. [14]. A similar vaccine was recently evaluated by Khodai et al. [29] and found to be more immunogenic than gD adjuvanted with MPL and alum. Because we were interested in finding conditions that optimally differentiate ACAM529 and gD, we challenged groups of immunized animals with different doses of wildtype HSV-2, spanning a 30-fold range from 15 to 450 LD_{50} (2.4×10^4 to 7.2×10^5 pfu). Based on our past experience we doubted that even a severe challenge would be enough to differentiate the two vaccines and this was borne out by the lack of significant differences in mortality, although disease symptoms were significantly lower in ACAM529-immunized mice as compared to gD.

Among mice immunized with gD, there was a trend for mean disease scores to increase with challenge dose until the highest dose, at which point the mean disease score decreased slightly, but these differences were not significant. Also in the gD recipients, more deaths were seen in the 50 LD_{50} group than the 150 or 450 LD_{50} groups, however these differences are not statistically significant and are consistent with the expectation that gD achieves a protection against morbidity and mortality that is robust enough to render these metrics unhelpful in comparing different vaccines.

Mucosal infection can be assessed by measuring challenge virus shedding using vaginal swabs within the first two days post-challenge [22]. Similarly, measuring viral genome copy number in DRG indicates the extent to which the infection has spread to sensory ganglia. ACAM529 achieved significantly greater reductions in mucosal replication (Fig. 5A and B). In addition, ACAM529 achieved significant protection against both mucosal and DRG infection while gD did not (Figs. 5C and 6B). A further benefit of challenging with a range of doses is that the effect of immunization on the dose of challenge virus leading to 90% infection (ID_{90}, or Infectious Dose 90%) can be estimated. While 90% of gD-vaccinated mice could be infected with the lowest challenge dose, the dose required to infect the same proportion of
ACAM529-vaccinated mice was not reached in this study. Therefore, ACAM529 vaccination increases the ID90 by more than 30-fold over gD vaccination. The shift in ID90 relative to mock immunization cannot be estimated because 100% of mice were infected even at the lowest challenge dose.

These differences in efficacy cannot be explained by differences in the neutralizing antibody responses since the titers induced by either vaccine were not significantly different (Fig. 3A). Also, antibody titers against gD are significantly higher in gD-immunized animals compared to those that received ACAM529, as might be expected given the compositions of the vaccines (Fig. 3C). These two observations, along with the observations mentioned earlier of significant protection against morbidity and mortality, are also consistent with a successful immunization with gD. In contrast, antibody titers measured by ELISA against an HSV-2 lysate are significantly higher in the group of animals which received the vaccine that comprises multiple HSV-2 antigens, namely ACAM529 (Fig. 3B). Moreover, interferon-γ ELISPOT data acquired by stimulation of splenocytes with a gD peptide known to stimulate CD4+ T cells indicate a significantly greater response in mice immunized with ACAM529 (Fig. 3D). Therefore, both total anti-HSV-2 IgG responses and CD4+ interferon-γ+ T cell counts correlate with protection in this study. The latter observation is consistent with past reports of the importance of CD4+ T cell counts correlate with protection in this study.

Conclusion

The observations reported here support the proposition that intramuscular immunization with ACAM529 should be investigated in clinical studies. Moreover, we propose that preclinical evaluation of future vaccine candidates should include a benchmark vaccine such as adjuvanted gD, and should measure protection against mucosal and DRG infection in addition to morbidity and mortality, preferably with a publicly available reference challenge strain. Other methods of standardization may further accelerate the search for a potent prophylactic HSV-2 vaccine and should be considered by researchers in the field. Finally, new ex vivo human models of immunogenicity should be considered as a potentially rich new source of information to compare and optimize human HSV vaccines [32].

Materials and Methods

Complementing cells

Complementing cell line AV529-19 was obtained by combining the Vero cell line CCL-81.2 (ATCC, Manassas, VA) with plasmids pCIaUL5, pcDNA1UL29, and pSV2neo, which were provided by Dr. David Knipe (Harvard Medical School). To minimize the chance of homologous recombination between the transgenes and the vaccine genome during manufacturing, all flanking sequences of the HSV-1 U5 and U3 genic was removed before cloning except for a 109 bp segment in the 3’ untranslated region of U5 sharing 72% identity with the vaccine genome. The transfected cells were cloned and screened for their ability to complement d55-29. Despite repeated passaging of ACAM529 in vitro, replication-competent virus has never been observed (data not shown). The cells were maintained in OptiPro (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT) and 4 mM glutamine (Invitrogen) at 37°C in a 5% CO2 atmosphere.

Viruses

Viral genomic DNA encoding vaccine virus d55-29 was provided by Dr. David Knipe (Harvard Medical School). To produce the pre-master seed, this DNA was transfected into AV529-19 and the resulting virus was passaged once to amplify titers. Viral genomic DNA was extracted from this virus passage and used to transfect AV529-19 again under GLP conditions. The resulting virus was harvested, plaque-purified four times, and banked under GMP. The selected clone is identified as ACAM529 clone 4a. Vaccine utilized for in vivo studies was purified in a process to be described elsewhere (Mundle et al., in preparation).

HSV-2 challenge strain 333 was a generous gift of Dr. Jeffrey Cohen (NIAID, Medical Virology Section). Challenge virus was cultivated on Vero cells seeded one day before infection at 1.2×106 cells in T175 flasks. Virus was inoculated at a multiplicity of infection of 0.01 pfu/cell. Flasks were incubated at 37°C, 5% CO2 for 1 h with agitation every 15 minutes. After addition of 20 mL of fresh media (DMEM containing 1% FBS), cultures were allowed to incubate under the same conditions until 100% cell death was reached (about 2 days). Infected cells were harvested and lysed to extract the virus.

gD vaccine

Recombinant HSV-2 glycoprotein D comprising residues 1–306 was produced in baculovirus and provided by Dr. Gary H. Cohen (Department of Microbiology, School of Dental Medicine, University of Pennsylvania) [14,27]. The antigen (gD, 2 μg/mouse) was mixed with CpG oligonucleotide ODN1826 (50 μg/mouse; Invivogen, CA) and with alum (25 μg/mouse; Alhydrogel, Accurate Chemicals and Scientific Corporation, MO) and combined using a vortex mixer for 2 hours at room temperature before injection.

Plaque assays

Samples were serially diluted and plated onto 12-well plates seeded one day prior to inoculation with 4×106 AV529 cells per well. Plates were incubated at 37°C, 5% CO2 for 1 hour with gentle rocking of plates every 15 min. Overlay medium (1 mL) consisting of methyl cellulose in DMEM supplemented with heat-inactivated FBS, L-glutamine and antibiotics was then added to each well. Plates were incubated at 37°C, 5% CO2 for about 48 hours. Following incubation, plates were stained with 1% crystal violet in 70% methanol. Plaques were then counted and titers calculated in pfu/mL.

Ethics statement

All animal experiments were performed according to Animal Research Protocol number 2011-05-01 approved by Sanofi Pasteur’s Institutional Animal Care and Use Committee, Acambis Cambridge Campus.

Mouse challenge model

Female BALB/c mice 6–7 weeks old were purchased from Charles River (Wilmington, MA). Animals were vaccinated with 1×106 pfu ACAM529 in 100 μL of sterile PBS. In the first route study, control animals were inoculated subcutaneously with sterile PBS, while in the gD comparison study, all control animals were immunized intramuscularly in the heavy musculature of the upper thigh. Subcutaneous immunization was administered in the scruff of the neck. Intramuscular immunization of 100 μL of ACAM529 was given in the upper thigh using a 27G needle. Intramuscular immunization with gD was in the gastrocnemius. Intradermal administration was done by first wiping the animal with 70%
ethanol, then the skin of the back was pulled taut with one hand and the 27G needle was injected bevel up at a shallow angle and two injections of 50 μl were given per mouse. Serum samples for serology assays were obtained from mandibular bleeds.

Seven days prior to intravaginal (i.vag.) challenge, mice were injected subcutaneously with 2 μg of medroxyprogesterone acetate injectable suspension diluted in PBS (SICOR Pharmaceuticals Inc., Irvine, CA). On the day of challenge, mice were given, in the route comparison experiment, 50 LD₅₀ (8 x 10⁴ pfu), and in the gD comparison experiment 15, 50, 150 or 450 LD₅₀ of HSV-2 strain 333 i.vag. in 20 μl sterile PBS with a positive displacement pipette. Pathology was scored on a 4 point scale as follows: 0 = no signs of disease, 1 = slight genital erythema and edema; 2 = moderate genital lesion and/or loss of fur; 3 = purulent genital lesion; 4 = hind-limb paralysis. Mice were euthanized upon reaching stage 3 or 4. Animals were observed and disease scores were recorded daily for 14 days after challenge.

Vaginal swabs

Vaginal swabs were taken on day two after challenge, and in some cases on days one, four and/or six, using swabs (CleanTips Swab, Micro CleanFoam Head, ITW Textwise). Swabs were collected in 1 mL stabilization buffer and stored at −80°C until challenge virus titers were determined by plaque assay.

ELISAs

ELISA against HSV-2 lysate was performed using Maxisorp plates (Nunc) which were coated with 100 μl/well of a solution of 2 μg/ml of HSV-2 purified viral lysate in PBS (Advanced Biotechnologies). Serum IgG was detected with biotin-anti-mouse IgG (Fc) (Sigma) diluted 1:2000 in 1% BSA/0.05% Tween 20 in Biotechnologies). Serum IgG was detected with biotin-anti-mouse IgG (Fc) (Sigma) diluted 1:2000 in 1% BSA/0.05% Tween 20 in Biotechnologies). Serum IgG was detected with biotin-anti-mouse IgG (Fc) (Sigma) diluted 1:2000 in 1% BSA/0.05% Tween 20 in Biotechnologies). Serum IgG was detected with biotin-anti-mouse IgG (Fc) (Sigma) diluted 1:2000 in 1% BSA/0.05% Tween 20 in Biotechnologies).

ELISPOT

ELISPOTs were coated with 0.5 μg/ml of HSV-2 using the highest dilution of the serum to reduce the average pfu/well by ≥50% from the average pfu number in the negative controls. In the route comparison experiment, complement was not added to the assay, while in the gD comparison guinea pig serum (Calbiochem) was added to a final concentration of 5%.

Virus neutralization assay

Serum neutralizing antibodies were measured by pre-incubating serum dilutions with HSV-2 strain 333 and plating the mixture over AV529-19 cells for an hour. The resulting assay plates were incubated 2 days at 37°C, 5% CO₂, and fixed and stained using a crystal violet methanol solution. Neutralizing titer was defined as the highest dilution of the serum to reduce the average pfu/well by ≥50% from the average pfu number in the negative controls. In the route comparison experiment, complement was not added to the assay, while in the gD comparison guinea pig serum (Calbiochem) was added to a final concentration of 5%.

Quantification of viral load in DRG

Dorsal root ganglia (DRG) were dissected according to Malin et al. [33], frozen at −80°C in DMEM containing 5% FBS as well as antibiotics, and sent to Dr. Harvey Friedman (University of Pennsylvania) for analysis. DNA was extracted from mouse DRG samples and analyzed using duplex real-time qPCR to quantify the HSV-2 Us9 gene, with a previously reported limit of quantitation of 5 copies, and the mouse adipsin gene as described previously [14].

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Author Contributions

Conceived and designed the experiments: SD SFA PLH MP JA HK. Analyzed the data: SD SFA PLH MP HH JMD. Wrote the paper: SD MP HH JMD.

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