Vaxfectin-adjuvanted plasmid DNA vaccine improves protection and immunogenicity in a murine model of genital herpes infection

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INTRODUCTION

Genital infections caused by herpes simplex type 2 (HSV-2) continue to present serious public-health problems throughout the world (Koelle & Corey, 2008; Xu et al., 2006). HSV-2 initially infects genital epithelial cells. Although the primary infection resolves, the virus undergoes retrograde transport into the dorsal root ganglia (DRG) and establishes a permanent latent neuronal infection (Corey et al., 2004). Periodic reactivation produces HSV-2 viral shedding, often without symptoms or lesions, when the virus can be transmitted (Koelle et al., 1992; Wald et al., 2002). HSV-2 has serious medical consequences, including neonatal infection, and is associated with increased susceptibility to human immunodeficiency virus type 1 infection (Chen et al., 2000; Perez et al., 1998; Wald & Corey, 2003). HSV-2 is thus an important vaccine target.

In an attempt to make an effective HSV-2 vaccine, various strategies have been investigated, including live-attenuated virus, inactivated virus and adjuvanted subunit vaccines (Kemble & Spaete, 2007; Koelle & Corey, 2008). The most thoroughly investigated subunit HSV-2 vaccine candidates have been viral envelope glycoproteins (Stanberry et al., 2002). Immunization with adjuvanted recombinant envelope glycoprotein D (gD) elicits neutralizing antibodies (Balachandran et al., 1982; Long et al., 1984) and CD4+ T-cell responses (Flo, 2003). HSV-2 vaccine clinical trials using gD2 have focused on the extracellular domain, lacking the C-terminal transmembrane and cytoplasmic...
domains of gD2 (Cattamanchi et al., 2008; Koelle & Corey, 2008). To date, adjuvanted gD2 subunit vaccines have shown limited clinical benefits (Belshe et al., 2012; Cohen, 2010; Stanberry et al., 2002). Therefore, there is a need to explore other approaches in vaccine development. Several reports have documented that there are CD4+ and CD8+ T-cell epitopes throughout the gD2 protein (Kim et al., 2008; Koelle et al., 2003), suggesting that full-length (FL) gD2 could potentially improve gD2-based vaccines. Immune response in HSV-2-infected humans and animals showed that T-cell responses are critical for providing protection against both primary and recurrent disease (Koelle & Corey, 2008). DNA vaccination is an attractive approach for developing HSV vaccines because of their potential for producing broad immune responses, including specific CD8+ and CD4+ responses and antibody production (Rajčáni et al., 2005). Plasmid DNA (pDNA) vaccines have been shown to elicit significant cellular immune responses to a variety of bacterial, viral and tumour antigens (reviewed by Donnelly et al., 1997; Sin et al., 1999) and have stimulated protective and therapeutic immune responses in animal HSV models (Bourne et al., 1996; McClements et al., 1996).

The adjuvant activity of Vaxfectin, a cationic lipid-based adjuvant, has been documented extensively for pDNA-based vaccines extending from rodent to non-human primate models of infectious disease (Hartikka et al., 2001; Hermanson et al., 2004; Jimenez et al., 2007; Pan et al., 2008; Reyes et al., 2001). These studies showed that Vaxfectin increased immunogenicity of antigens expressed from pDNA and that animals were protected against infection. Vaxfectin-formulated pDNA-based vaccines have produced protective neutralizing antibody titres and T-cell-specific immune responses in Phase I clinical trials of healthy subjects (Smith et al., 2010). In addition, the vaccines were shown to be safe and well tolerated.

In the present study we evaluated the efficacy of Vaxfectin-formulated gD2 vaccines in an intravaginal HSV-2 mouse model using both FL and secreted (S) gD2. End points included quantification of vaginal and DRG viral loads, survival and antibody responses. Vaxfectin-formulated gD2 pDNA showed a higher degree of sterilizing immunity and reduction in latency compared with pDNA alone.

RESULTS

Vaxfectin-formulated gD2 pDNA produces higher antibody titres than gD2 pDNA alone

We compared the immunogenicity of FL gD2 and S gD2 at high (100 μg) and low (0.1 μg) doses with and without Vaxfectin. Previous experiments using 0.1 μg, 3 μg and 100 μg doses showed that only the 0.1 μg dose provided <100% protection against lethal HSV-2 challenge (unpublished results). Therefore, the 0.1 μg dose was tested to distinguish between treatment conditions for survival and the 100 μg dose was tested for reduction in vaginal and latent HSV-2 copies. IgG serum ELISA titres were measured using gD121–339 protein, which is very similar in sequence to the corresponding region of gD2 (Dolan et al., 1998; Watson, 1983). Results showed that four out of 10 mice seroconverted after three immunizations with 0.1 μg of Vaxfectin-formulated pDNA for both the FL and the S forms of gD2, whereas specific IgG was not observed for non-adjuvanted gD2 pDNA (Fig. 1a). At the 100 μg dose, all animals seroconverted. However, the Vaxfectin-formulated pDNA groups showed significantly higher IgG titres using both forms of gD2 (P=0.034 for S and P=0.026 for FL) compared with the non-adjuvanted gD2 pDNA groups (Fig. 1b). Comparison of the FL and S forms of gD2 showed no significant difference in antibody titres.

Fig. 1. Serum IgG titres from vaccination of BALB/c mice with FL and S gD2 pDNA vaccines, formulated with or without Vaxfectin. Antibodies were measured by ELISA 2 weeks after the third injection. The bars represent geometric mean titres (n=10 mice per group). Antibody titres were determined from A405 values. Titres <1:100 are plotted as 100. (a) Antibody titres at 0.1 μg per dose. (b) Antibody titres at 100 μg per dose. P values as shown.
between Vaxfectin-formulated pDNAs at either the 0.1 µg or 100 µg pDNA doses at any time point (Fig. 1).

**Vaxfectin-formulated gD2 pDNA protects against lethal HSV-2 challenge better than gD2 pDNA alone**

HSV-2 gD2 pDNA alone or formulated with Vaxfectin was tested at the 0.1 µg and 100 µg doses for protection against lethal intravaginal HSV-2 challenge at 50 LD₅₀. Animals in the negative-control group succumbed to infection by day 8, whereas all animals in the thymidine kinase-negative strain of HSV-2 (TK⁻ HSV-2) positive-control group survived (results not shown). At the 100 µg dose of gD2 pDNA, both Vaxfectin-formulated gD2 pDNA and gD2 pDNA alone (FL and S forms of gD2) induced complete protection against this HSV-2 challenge (results not shown). However, at the 0.1 µg pDNA dose, all animals vaccinated with gD2 pDNA alone died by day 8, whereas Vaxfectin-formulated gD2 pDNA, both FL and S forms, provided 80 and 60 % survival, respectively (P<0.0001 for both groups compared with gD2 pDNA alone; Fig. 2). All surviving animals seroconverted against HSV-2, confirming infection for all treatment groups (results not shown).

**Vaxfectin-formulated gD2 pDNA reduces vaginal viral load compared with gD2 pDNA alone**

To determine the effect of vaccination on HSV-2 vaginal replication, quantitative PCR (qPCR) for HSV-2 DNA was performed on swabs obtained after inoculation. At the 0.1 µg dose, no significant differences were observed between Vaxfectin-formulated gD2 pDNA, gD2 pDNA alone and the negative-control group (results not shown). However, vaccination with the 100 µg dose of Vaxfectin-formulated gD2 pDNA resulted in lower HSV-2 DNA copy numbers at days 1 and 3 (Fig. 3a, b) and a significantly lower HSV-2 DNA copy number at day 5 compared with gD2 pDNA alone (Fig. 3c; P=0.024 for S gD2 and P=0.019 for FL). FL gD2 pDNA alone and Vaxfectin-formulated FL gD2 pDNA produced significantly lower HSV-2 copy numbers at day 3 (P=0.05 and P=0.002, respectively; Fig. 3b) and day 5 (P=0.013 and P=0.020, respectively; Fig. 3c) than S gD2 pDNA alone and Vaxfectin-formulated S gD2 pDNA.

**Vaxfectin-formulated FL gD2 pDNA reduces viral latency in DRG compared with gD2 pDNA alone**

The level of HSV-2 viral latency was determined by measuring the HSV-2 DNA copy number in the DRG, normalized per host mouse cell. Lumbar and sacral DRG from surviving animals were pooled from each group, at 90 days after viral challenge. Vaccination with the 100 µg pDNA dose of Vaxfectin-formulated FL gD2 pDNA produced significantly lower DRG HSV-2 latency compared with survivors vaccinated with the same dose of gD2 pDNA alone (P=0.007; Fig. 4). Six of the 10 animals vaccinated with Vaxfectin-formulated FL gD2 pDNA had no detectable HSV-2 DNA in their DRG; compared with only one of nine survivors vaccinated with FL gD2 pDNA alone. When pDNA encoding the S form of gD2 was used, no significant difference was observed with or without Vaxfectin (Fig. 4). The FL form of Vaxfectin-formulated gD2 pDNA produced significantly lower levels of latent DRG HSV-2 DNA than the S form (P=0.028; Fig. 4). There was no difference between the two forms of non-adjuvanted gD2 (P=0.227).

**Vaxfectin-formulated FL gD2 pDNA produces high IgG titres irrespective of dose and protects better against HSV-2 lethal challenge than gD2 pDNA alone**

Vaxfectin-formulated FL gD2 pDNA was selected for further evaluation, as both acute vaginal and latent DRG HSV-2 DNA copy numbers were lower for the FL vaccine compared with Vaxfectin-formulated S gD2 pDNA. Vaxfectin-formulated FL gD2 pDNA again produced higher IgG titres compared with gD2 pDNA alone at both the 0.1 µg and the 100 µg doses (P<0.001 for both comparisons). A dose–response relationship was observed, such that all animals seroconverted at the high (100 µg) but not at the low (0.1 µg) dose (results not shown). To determine the strength of protection, mice were challenged with both 50 and 500 LD₅₀ HSV-2. At the 0.1 µg dose of gD2 pDNA, Vaxfectin-formulated FL gD2 pDNA provided significantly better survival compared with FL gD2 pDNA at both 50 and 500 LD₅₀ of HSV-2 (P<0.0001 and P=0.006, respectively; Fig. 5a, b). After challenge of vaccinated mice with 50 LD₅₀ of HSV-2, all mice vaccinated with Vaxfectin-formulated FL gD2 pDNA were protected, whereas only one
of 10 mice vaccinated with gD2 pDNA alone was protected. Moreover, when the viral challenge dose was increased 10-fold to 500 LD$_{50}$, 80% of mice vaccinated with the Vaxfectin-formulated FL gD2 pDNA still survived, compared with 0% of mice vaccinated with gD2 pDNA (Fig. 5b). At the 100 µg pDNA dose, all mice survived both viral challenge doses regardless of the inclusion of Vaxfectin (results not shown).

**Vaccination with Vaxfectin-formulated FL gD2 pDNA reduces vaginal HSV-2 DNA copies compared with gD2 pDNA alone**

The amount of vaginal HSV-2 DNA at days 1, 3 and 5 was measured by qPCR. Vaccination with 100 µg Vaxfectin-formulated FL gD2 pDNA reduced HSV-2 DNA copy number significantly at both 50 LD$_{50}$ or 500 LD$_{50}$ HSV-2 challenge doses at each time point compared with vaccination with the same dose of FL gD2 DNA administered without adjuvant (Fig. 6). As noted above, all animals survived viral challenge at this vaccine dose, but the viral load data clearly differentiate the efficacy of Vaxfectin-formulated vs non-adjuvanted vaccine. By day 5, three mice challenged with 50 LD$_{50}$ and one mouse challenged with 500 LD$_{50}$ had no detectable vaginal HSV-2 DNA (Fig. 6c). The vaginal HSV-2 copy number detected on day 5 for mice vaccinated with Vaxfectin-formulated FL gD2 pDNA did not differ statistically between the 50 and 500 LD$_{50}$ HSV-2 challenge doses, showing the effectiveness of the vaccine at the highest viral challenge dose.

**Vaxfectin-formulated FL gD2 pDNA significantly reduces latent DRG HSV-2 DNA copy number compared with gD2 pDNA alone**

Animals vaccinated with 100 µg gD2 pDNA dose and surviving HSV-2 vaginal challenge were examined for HSV-2 DNA copies in DRG. Mice vaccinated with Vaxfectin-formulated FL gD2 pDNA had significantly lower DRG HSV-2 DNA copy number at both the 50 and 500 LD$_{50}$ HSV-2 challenge doses, compared with gD2 alone ($P=0.002$ and $P=0.005$, respectively; Fig. 7). Seven out of 10 mice vaccinated with Vaxfectin-formulated FL gD2 pDNA showed no detectable HSV-2 DNA copy number at 50 LD$_{50}$, compared with one of 10 mice vaccinated with gD2 pDNA alone (Fig. 7a). Notably, at 500 LD$_{50}$ of HSV-2, four out of 10 animals had no detectable HSV-2 DNA copy number, whereas all mice vaccinated with gD2 pDNA alone had detectable HSV-2 DNA in their lumbosacral DRG (Fig. 7b). These results are consistent with vaginal HSV-2 DNA levels (Fig. 6), showing efficacy of the Vaxfectin-formulated FL gD2 vaccine in preventing HSV-2 viral latency, even at a viral challenge of 500 LD$_{50}$.

**DISCUSSION**

Subunit vaccines consisting of recombinant envelope glycoproteins gB2 and gD2, or gD2 alone with different
Two different forms of gD2 were first compared: FL gD21–393 and vaccines seroconvert to HSV-2. The use of a pDNA vaccine provides the advantage of allowing the evaluation of a full-length form, FL gD21–393, which includes CD4 and CD8 epitopes distributed throughout the protein (BenMohamed et al., 2003; Chentoufi et al., 2008; Kim et al., 2008; Koelle et al., 2003). There was no difference in the antibody response or survival (Figs 1 and 2) between these two forms of gD2. While there could be a difference in neutralizing antibody titres between the two forms of gD, this was not explored further due to previous studies which showed that antibodies are not required for protection from lethal challenge by either HSV-2 pDNA vaccines or by the live-attenuated virus used as a positive control vaccine in the HSV-2 vaginal challenge model (Iijima et al., 2008; Nass et al., 2001). There was a clear difference in HSV-2 replication in the vagina (Fig. 3) and in DRG (Fig. 4). The host mechanisms controlling virus replication after FL gD2 pDNA vaccination remain undefined. It is possible that quantitative functional differences in antigen-specific CD4+ or CD8+ T-cell responses correlate with the improvements observed with Vaxfectin and such studies are planned for the future.

The vaccine potency can be increased by formulating an adjuvant with an antigen to boost the immune system. Adjuvants have undergone efficacy testing in clinical trials (Belshe et al., 2012; Corey et al., 1999; Stanberry et al., 2002). While strong neutralizing antibodies were induced, efficacy end points were not met (Belshe et al., 2012; Cohen, 2010; Corey et al., 1999). An effective prophylactic HSV-2 vaccine is the most likely option for the successful control of the spread of genital herpes. End points in Phase 3 trials have shifted from prevention of infection in early studies (Corey et al., 1999) to prevention of clinical disease in more recent studies (Stanberry et al., 2002). At the population level, control will be assisted by a reduction in symptomatic and asymptomatic HSV-2 shedding, which is responsible for HSV-2 transmission. Therefore the induction of immune responses which could reduce reactivation and shedding is arguably a valuable end point for a preventative vaccine, even if the vaccine is not sterilizing and vaccines seroconvert to HSV-2.

Two different forms of gD2 were first compared: FL gD21–393 and S gD2, a secreted form of gD2 comprising aa 1–340. The S form lacks the transmembrane and cytoplasmic domains (Higgins et al., 2000), similar to the recombinant gD protein adjuvanted with MF59 (Langenberg et al., 1995) or alum and monophosphorylated lipid A (MPL) (Bernstein et al., 2005).

The vaccine potency can be increased by formulating an adjuvant with an antigen to boost the immune system.
Adjuvants, such as MF59 and alum with MPL, have been used to adjuvant split vaccines containing recombinant truncated envelope glycoproteins. Both of these adjuvants increase the humoral immune response, but have not been shown to increase cell-mediated immunity (Shiver et al., 2002; Ulmer et al., 1999). In this study, Vaxfectin, a cationic lipid adjuvant, was added to improve immunogenicity of the HSV-2 gD pDNA. Vaxfectin was initially developed to enhance immune responses to pDNA-based vaccines (Sullivan et al., 2010). Numerous mouse, rabbit, dog and non-human primate studies have demonstrated the ability of Vaxfectin to boost antibody titres and cell-mediated immunity, resulting in durable responses. (Hartikka et al., 2001; Hermanson et al., 2004; Jimenez et al., 2007; Margalith & Vilalta, 2006; Reyes et al., 2001). As an example, a study in infant and juvenile macaques showed protection against a measles virus challenge 1 year after vaccination (Pan et al., 2008). Moreover, Vaxfectin-formulated pDNA encoding H5 haemagglutinin was able to protect mice and ferrets against a lethal viral challenge. This same Vaxfectin-formulated vaccine candidate was tested in two phase 1 clinical trials producing durable immune responses that resulted in protective neutralizing antibody titres and induction of T-cell-specific immune response (Jimenez et al., 2007; Smith et al., 2010). Unlike the alum/MPL HSV-2 split vaccine, which did not prevent infection in a recently completed phase 3 clinical trial (Aumakhan et al., 2010; Belshe et al., 2012), expression of the FL gD will provide the complete repertoire of T-cell epitopes (Hosken et al., 2006) and Vaxfectin should further boost the cell-mediated immune response against these epitopes.

In the present study, we have shown that both FL and S forms of gD2 formulated with Vaxfectin showed significantly higher antibody titres, compared with gD2 alone (Fig. 1). Previous studies demonstrated the importance of antibodies in the protection of neuronal tissues against HSV (Bourne et al., 2002; Klein, 1980; Simmons & Nash, 1985). However, review of our results shows that some animals that failed to seroconvert were nonetheless protected from death at the 0.1 µg dose of Vaxfectin-formulated FL gD2 (Figs 1 and 2). This apparent
discrepancy may be related to limited sensitivity of our IgG assay or to a protective role for CD4+ T-cells, as demonstrated previously in some pDNA-HSV-2 challenge studies (Manickan et al., 1995; Nass et al., 2001; Sin et al., 2000, 2001).

Our results also showed that vaginal and DRG levels of HSV-2 DNA were reduced in mice vaccinated with Vaxfectin-formulated gD2 pDNA compared with gD2 pDNA alone (Figs 3 and 4). Notably, in our initial experiment, at the low dose of gD2 (0.1 μg), no animals vaccinated with either form of gD2 seroconverted after three injections, whereas in the Vaxfectin-formulated gD2 pDNA groups, seroconversion occurred in 50% of the animals (Fig. 1a). Interestingly, survival for the 0.1 μg dose was extremely dependent on the inclusion of Vaxfectin (Fig. 2), regardless of the form of gD2 pDNA used. Vaxfectin-formulated FL gD2 pDNA reduced vaginal and DRG HSV2 DNA copies significantly, compared with Vaxfectin-formulated S gD2 pDNA (Figs 3 and 4). Some end points were clearly dependent on the length of the gD2 construct used, with FL gD2 performing better than S gD2 either with or without Vaxfectin as measured by vaginal HSV-2 DNA loads on day 5 (Fig. 3).

Mouse and guinea pig infection models (BenMohamed et al., 2003; Bourne et al., 2005; Sawtell, 1998; Sawtell et al., 1998, 2001) suggest that the intensity of viral reactivation corresponds to the number of HSV-2-infected neurons in the DRG and the intensity of infection per neuron, such that non-sterilizing vaccines that perturb natural infection to reduce ganglionic latency could rationally be expected to have a beneficial effect on prevention of latency. Formulation of gD pDNA with Vaxfectin resulted in lower DRG latent HSV-2 loads, correlating with lower vaginal replication; this is consistent with a model of pathogenesis in which intense local replication precedes and predicts access of virus to the ganglia. These data suggest that an optimized HSV-2 vaccine could improve the natural history of HSV-2 shedding, and reduce the transmission of HSV-2 in the population.

Based on comparison of FL and S gD2, and data indicating that Vaxfectin increased immune responses and protection, the stringency of the experimental system was enhanced by increasing the challenge dose of HSV-2 10-fold (500 LD50). Notably, at a 0.1 μg pDNA dose of FL gD2, Vaxfectin-formulated gD2 pDNA still provided a high level of protection against both standard and high-dose challenge in mice (Fig. 5). The results showed that the 100 μg dose of FL gD2 pDNA formulated with Vaxfectin significantly reduced vaginal and DRG HSV-2 DNA copy number equally well after either 50 or 500 LD50 HSV-2 challenge doses (Figs 6 and 7). This indicates that the Vaxfectin-formulated vaccine generates a strong effect that can control primary infection and limit subsequent latent HSV-2 infection in mice. These results suggest that a non-sterilizing vaccine can still have a beneficial influence on both acute vaginal replication, which can be expected to correlate with the intensity of genital symptoms in humans undergoing primary genital herpes infections, as well as the level of DRG latency, which arguably is correlated directly with HSV reactivation.

Overall, the Vaxfectin-formulated vaccine expressing FL gD2 in a mouse HSV-2 model demonstrated excellent immunogenicity, increased protection with low doses of gD2 pDNA vaccine, and led to significant reduction in the vaginal and ganglion HSV-2 DNA copies. While truncated gD2 protein with MPL and alum (MPL-alum) has been disappointing in a recent clinical trial, the benefits seen with expression of FL gD2 formulated with Vaxfectin adjuvant in this report indicate that gD2, combined with other HSV-2 subunits targeting T-cell responses (Muller et al., 2009), may be a viable preventative vaccine candidate. Future studies will evaluate such multivalent vaccines to determine whether a Vaxfectin-adjuvanted DNA vaccine
has prophylactic and therapeutic activity in the guinea pig model of genital HSV-2.

**METHODS**

**Mice.** Female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used at 5–6 weeks of age. Mice were housed in pathogen-free conditions. Protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

**Plasmid DNA (pDNA), formulations and vaccination.** The HSV-2 gD2 sequence was used as either the full-length (FL) gene or a secreted (S) form (aa 1–340) (Dolan et al., 1998). HSV-2 genes were codon-optimized using proprietary algorithms developed at Vical. DNA was transthesized by GeneArt (Regensburg) and sequenced to threefold redundancy. Sequences encoding the FL or S form were inserted into expression plasmid VR1012 (Hartikka et al., 1996) that contains a constitutive hCMV (human cytomegalovirus) immediate-early enhancer/promoter. pDNA was manufactured as previously described (Vilalta et al., 2009). The expression of FL gD2 and S gD2 was confirmed (results not shown) by flow cytometry (Kask et al., 2010). Briefly, COS-7 cells were transfected with FL gD2, S gD2, or empty vector VR1012. At 48 h, trypsin-harvested cells were permeabilized for S gD2, but not for FL gD2, as described previously (Jing et al., 2009). Cells were stained with mouse mAb anti-gD 2C10 (Santa Cruz Biotechnology) or isotype control at 1 gD2, or empty vector VR1012. At 48 h, trypsin-harvested cells were permeabilized for S gD2, but not for FL gD2, as described previously (Jing et al., 2009). Cells were stained with mouse mAb anti-gD 2C10 (Santa Cruz Biotechnology) or isotype control at 1 μg ml−1 and washed. Bound IgG was detected with allop hyococcin-conjugated goat anti-mouse IgG (Biolegend). After washing and fixation, cells were analysed on a FacsCanto II cytometer (Becton Dickinson).

pDNA was formulated with Vaxfectin as first described by Hartikka et al. (2001). Briefly, both GAP-DMORIE ((+)-N-(3-amino prop) N,N-dimethyl-2,3-bis(cis-9-tetradecenyl)-1-propanaminium bromide) and DPyPE (aminopropyl-dimethyl-styreneoxy-propanaminium bromide-diphtyanolphosphatidyl-ethanolamine) were resuspended in chloroform, mixed in 1:1 molar ratio, aliquoted into vials and dried to create Vaxfectin dry lipid film. On the day of injection, the lipid film vials were resuspended in 1 ml 0.9% saline. pDNA was prepared in 0.9% saline, 20 mM sodium phosphate, pH 7.2. The pDNA was formulated with Vaxfectin by gently stirring the lipid into pDNA at a 1:1 equal volume dilution, yielding 1 mg pDNA ml−1 and 1.09 mg Vaxfectin ml−1. The final pDNA nucleotide:cationic lipid molar ratio was 4:1. For the 1 mg ml−1 high gD2 pDNA dose, the formulation was injected undiluted. The formulation was diluted with PBS to yield the 1 μg ml−1 low gD2 pDNA dose.

Mice were injected bilaterally with 50 μl intramuscular injection yielding a total volume of 100 μl per mouse. Mice were vaccinated three times on days 0, 14 and 28. Negative-control animals received no vaccine. Positive-control animals were immunized by intravaginal infection at day 14 with 0.5 × 105 p.f.u. TK − HSV-2 strain 333 after Depo-provera (Pharmacia) pre-treatment (Coen et al., 1989).

**Anti-gD121–339 and anti-HSV-2 antibody measurement.** Recombinant HSV-1 gD protein, expressing aa 21–339, was purchased from Meridian Life Sciences. This protein has a primary sequence that is 89% identical to that of gD2 (Dolan et al., 1998; Watson, 1983) and was used because long polypeptide versions of gD121–339 were not commercially available. ELISAs were performed as described previously (Muller et al., 2009). Briefly, high-binding 96-well flat-bottom ELISA plates (Corning Costar) were coated with 250 ng gD121–339 ml−1. 0.1 M Na2CO3 (pH 9.6) buffer overnight at 4 °C. Subsequently, serial threefold dilutions of sera starting at 1:100 were added overnight at 4 °C. Total IgG was detected by addition of biotinylated goat anti-mouse Fc (Biodesign), followed by streptavi-
genomes were measured with a qPCR primer/probe cocktail (part 4308313) amplifying the GAPDH gene (AB1). Results are presented as HSV-2 DNA copies per million mouse cells.

**Statistical analysis.** Wilcoxon rank sum test or Student’s r-test was used to compare log_{10} IgG titer, and to compare log_{10} vaginal HSV-2 DNA copy numbers between groups. Kaplan–Meier curves were generated to compare survival in days between groups. Survival distributions were compared between groups using the log-rank test. All statistical analysis was performed using sas 9.2 for PC. A two-sided p-value <0.05 was considered statistically significant. No multiple comparison adjustments were made.

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