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

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The herpes simplex type 2 (HSV-2) envelope glycoprotein (gD2) was evaluated as a potential antigen candidate for a plasmid DNA (pDNA)-based HSV-2 vaccine. The pDNA was formulated with Vaxfectin, a cationic lipid-based adjuvant, and tested in a murine HSV-2 lethal challenge model. gD2 was expressed as full-length (FL) and secreted (S) gD2 forms. A 0.1 μg pDNA dose was tested to distinguish treatment conditions for survival and a 100 μg pDNA dose was tested to distinguish treatment conditions for reduction in vaginal and latent HSV-2 copies. Vaxfectin-formulated gD2 pDNA significantly increased serum IgG titres and survival for both FL gD2 and S gD2 compared with gD2 pDNA alone. Mice immunized with FL gD2 formulated with Vaxfectin showed reduction in vaginal and dorsal root ganglia (DRG) HSV-2 copies. The stringency of this protection was further evaluated by testing Vaxfectin-formulated FL gD2 pDNA at a high 500 $LD_{50}$ inoculum. At this high viral challenge, the 0.1 μg dose of FL gD2 Vaxfectin-formulated pDNA yielded 80 % survival compared with no survival for FL gD2 pDNA alone. Vaxfectin-formulated FL gD2 pDNA, administered at a 100 μg pDNA dose, significantly reduced HSV-2 DNA copy number, compared with FL gD2 DNA alone. In addition, 40 % of mice vaccinated with adjuvanted FL pDNA had no detectable HSV-2 viral genomes in the DRG, whereas all mice vaccinated with gD2 pDNA alone were positive for HSV-2 viral genomes. These results show the potential contribution of Vaxfectin-gD2 pDNA to a future multivalent HSV-2 vaccine.

## 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 (Fló, 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 (Rajcáni *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 1 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 $gD1_{21-339}$ 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
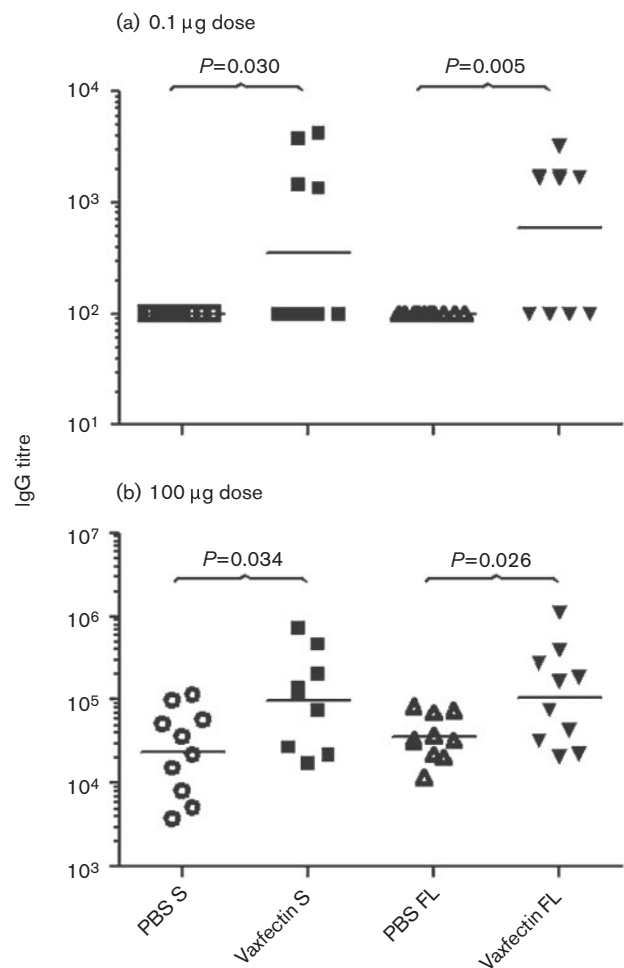


**Fig. 1.** Serum IgG titres from vaccination of BALB/c mice with FL and S gD2 pDNA vaccines, formulated with or without Vaxfectin. Antibodies were measured by ELISA 2 weeks after the third injection. The bars represent geometric mean titres ($n=10$ mice per group). Antibody titres were determined from $A_{450}$ 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 $LD_{50}$. 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
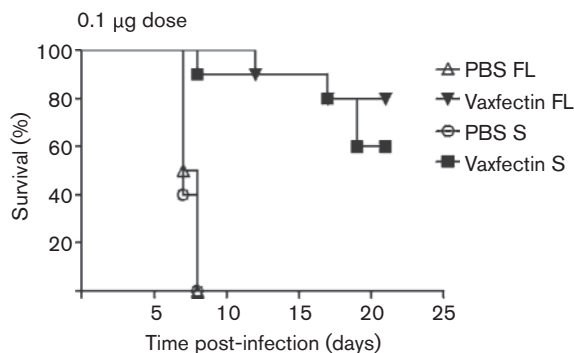


**Fig. 2.** Relationship between adjuvant and survival after HSV-2 challenge with low-dose gD2 pDNA vaccine. BALB/c mice (10 per group) were vaccinated three times intramuscularly with 0.1 µg gD2 pDNA (S and FL). Six days prior to the challenge, 10 animals in each treatment group and five animals in the negative and TK$^-$ HSV-2 control groups were treated subcutaneously with 2 mg medroxyprogesterone (Depo-provera). Two weeks after the last vaccination, mice were inoculated vaginally with 50 $LD_{50}$ of HSV-2. $P<0.0001$ when Vaxfectin FL is compared with PBS FL; $P<0.0001$ when Vaxfectin S is compared with PBS S.

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 $LD_{50}$ 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 $LD_{50}$ of HSV-2 ($P<0.0001$ and $P=0.006$, respectively; Fig. 5a, b). After challenge of vaccinated mice with 50 $LD_{50}$ of HSV-2, all mice vaccinated with Vaxfectin-formulated FL gD2 pDNA were protected, whereas only one
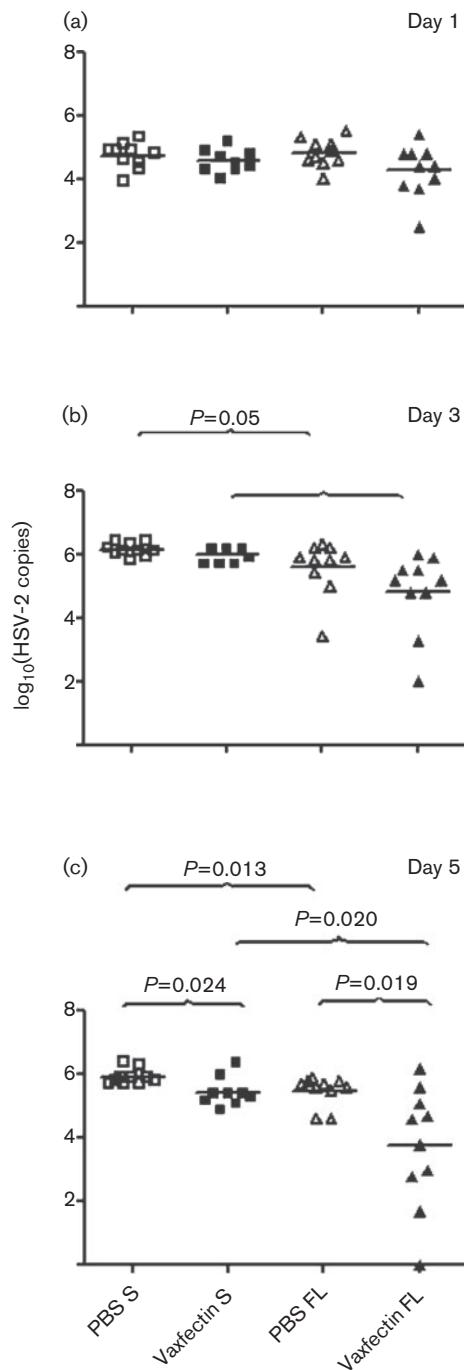
**Fig. 3.** Vaginal HSV-2 DNA copy number on day 1 (a), day 3 (b) and day 5 (c) after vaccination with high-dose gD2 pDNA vaccine followed by lethal challenge with HSV-2. BALB/c mice (10 mice per group) were vaccinated three times intramuscularly with 100 μg gD2 pDNA. At day 14 after the last vaccination, mice were inoculated vaginally with 50 LD$_{50}$ HSV-2. Vaginal swabs were collected and viral genomes were measured by qPCR for gB HSV-2 DNA. Each symbol represents an individual animal. The mean value for each group is shown by a black horizontal line. Values below the lower limit of detection are indicated as zero. *P* values as shown.

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 gD$_2$ 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 the 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
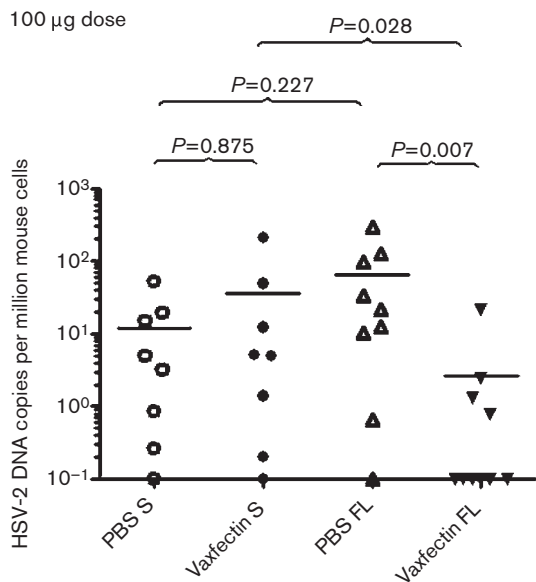
**Fig. 4.** HSV-2 DNA copy number in DRG from mice surviving HSV-2 challenge following completion of pDNA vaccinations. BALB/c mice (10 per group) were vaccinated three times intramuscularly with 100 μg gD$_2$ pDNA (S and FL) per dose. At day 14 after vaccination, mice were inoculated vaginally with 50 LD$_{50}$ HSV-2. DRGs were collected 90 days after challenge and HSV-2 genomes were measured by qPCR assay. Each symbol represents an individual animal. The mean value for each group is shown by a black horizontal line. Viral genome numbers were normalized using mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA. Values below the lower limit of detection are indicated as 0.1. P values as shown.



**Fig. 5.** Inclusion of Vaxfectin improves survival after low-dose FL gD2 vaccination and standard- or high-dose HSV-2 challenge. BALB/c mice (10 per group) were vaccinated three times intramuscularly with 0.1 μg FL gD2 pDNA. Six days prior to the challenge, 10 animals in each treatment group and five animals in the negative and TK$^-$ HSV-2 control groups were treated subcutaneously with 2 mg medroxyprogesterone. Two weeks after the last vaccination, mice were inoculated vaginally with 50 LD$_{50}$ (a) or 500 LD$_{50}$ (b) HSV-2. $P < 0.0001$ when Vaxfectin FL is compared with PBS FL at 50 LD$_{50}$; $P = 0.006$ when Vaxfectin FL is compared with PBS FL at 500 LD$_{50}$.

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 gD2$_{1-393}$ 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).
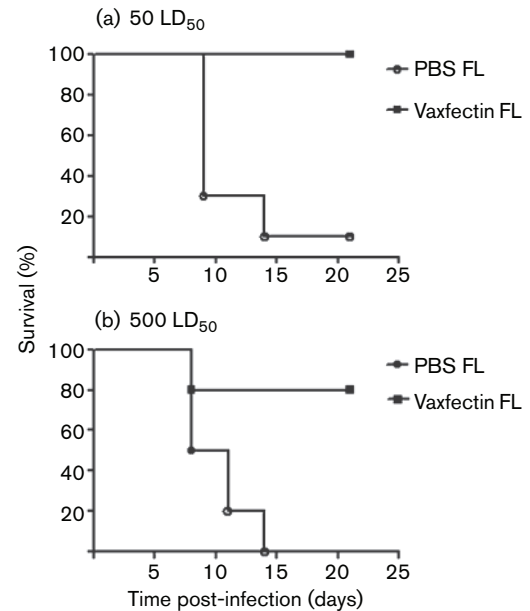
The use of a pDNA vaccine provides the advantage of allowing the evaluation of a full-length form, FL gD2$_{1-393}$, which includes CD4 and CD8 epitopes distributed throughout the protein (BenMohamed *et al.*, 2003; Chentoufi *et al.*, 2008; Kim *et al.*, 2008; Koelle *et al.*, 2003). There was no difference 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.
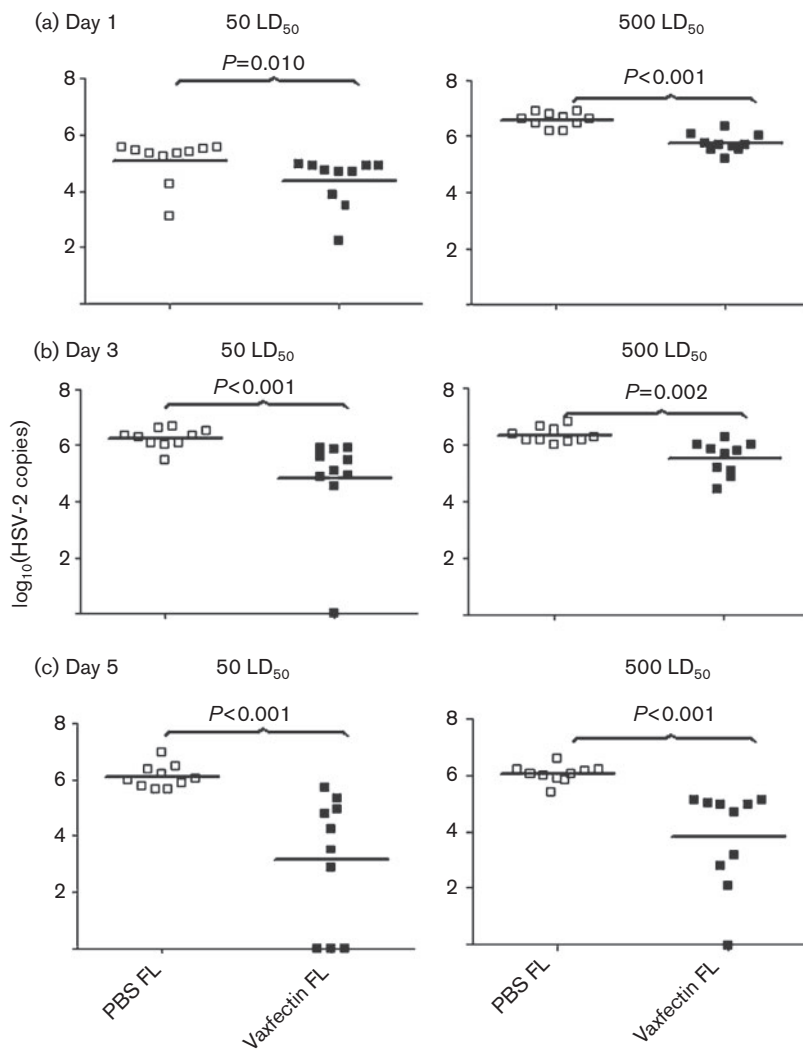
**Fig. 6.** Vaginal HSV-2 DNA copy number on day 1 (a), day 3 (b) and day 5 (c) following HSV-2 challenge. BALB/c mice (10 per group) were vaccinated three times intramuscularly with 100 µg FL gD2 pDNA (□, PBS FL; ■, Vaxfectin FL). At day 14 after the last vaccination, mice were inoculated vaginally with 50× $LD_{50}$ (left graphs) or 500× $LD_{50}$ (right graphs) of HSV-2. Vaginal swabs were collected and viral genomes were measured by qPCR for HSV-2 DNA. Each symbol represents an individual animal. The mean value for each group is shown by a black horizontal line. Values below the lower limit of detection are listed as zero.

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
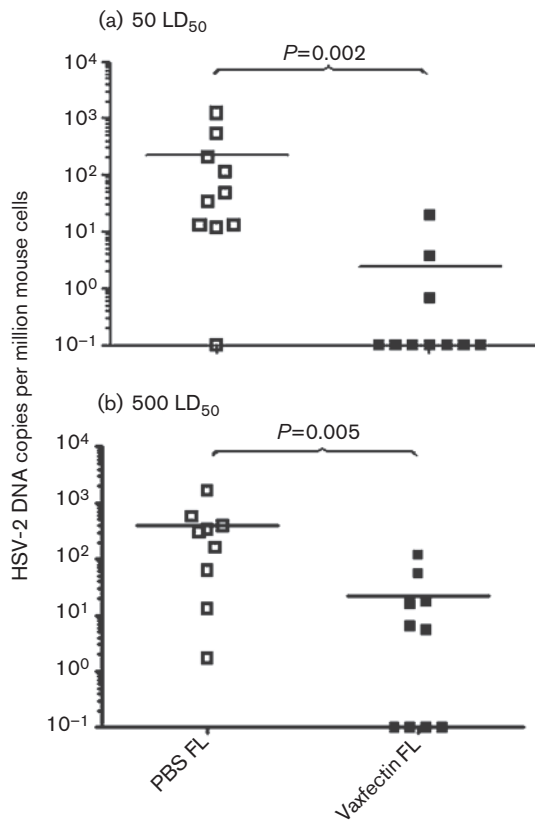
**Fig. 7.** DRG HSV-2 DNA copy number following HSV-2 challenge. BALB/c mice (10 per group) were vaccinated three times intramuscularly with 100 µg gD$_2$ pDNA. At day 14 after vaccination, mice were inoculated vaginally with 50 LD$_{50}$ (a) or 500 LD$_{50}$ (b) HSV-2. DRGs were collected 90 days after challenge and viral genome was detected using a qPCR assay. Each symbol represents an individual animal. The mean value for each group is shown by a black horizontal line. Viral genome numbers were normalized using mouse GAPDH DNA. Values below the lower limit of detection are indicated as 0.1.

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 LD$_{50}$). 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 LD$_{50}$ 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/cJ 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 synthesized 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 µg ml$^{-1}$ and washed. Bound IgG was detected with allophycocyanin-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 [( $\pm$ )-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(cis-9-tetradecenyloxy)-1-propanaminium bromide] and DPyPE (aminopropyl-dimethyl-myristoleyloxy-propanaminium bromide-diphytanoylphosphatidyl-ethanolamine) were resuspended in chloroform, mixed in 1 : 1 molar ratio, aliquotted 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 streaming 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 × 10$^6$ p.f.u. TK$^-$ HSV-2 strain 333 after Depo-provera (Pharmacia) pre-treatment (Coen et al., 1989).

**Anti-gD1$_{21-339}$ 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 gD2 are 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 gD1$_{21-339}$ ml$^{-1}$ 0.1 M Na$_2$CO$_3$ (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-

din–HRP (Pierce) and TMB peroxidase substrate (KPL). Plates were read at $A_{450}$ using a Victor3 1420 plate reader (Perkin Elmer). Assays were done in duplicate and mean $A_{450}$ values were used in subsequent calculations.

To calculate relative antibody titres, pooled serum from gD1$_{21-339}$-immunized mice was used as a positive control. In order to generate gD1$_{21-339}$ immune sera, four mice were immunized with 30 µg gD1$_{21-339}$ protein, mixed with Titermax adjuvant (CytRx Corporation), prepared according to the manufacturer's instructions. The animals were primed intramuscularly, followed by intraperitoneal boosts at 2 week intervals. Two weeks after the last immunization, the sera were collected and pooled for use as a standard in the ELISA. The dilution factor at which an $A_{450}$ value was two times above the background was assigned as the end-point relative titre. To calculate relative anti-gD1$_{21-339}$ IgG titres for each sample, we included a standard curve with the positive-control sera beginning at 1 : 2700 on each ELISA plate. Dilutions of the unknown sera with $A_{450}$ values falling within the linear portion of the positive-control $A_{450}$ vs dilution standard curve were used to calculate relative anti-gD1$_{21-339}$ IgG titres.

To confirm that animals surviving the HSV-2 challenge seroconverted to HSV-2, binding IgG antibodies to whole HSV-2 by ELISA were measured. Briefly, UV-treated HSV-2 stock (below) was diluted 1 : 1000 in 0.1 M Na$_2$CO$_3$ (pH 9.6) buffer and coated overnight at 4 °C. Mouse sera were applied to duplicate wells at 1 : 100 dilutions and binding IgG was detected as above. The raw $A_{450}$ was measured and animals with a mean $A_{450}$ greater than 2 × naïve sera controls were considered seropositive.

**Virus challenge.** Six days prior to the challenge, the animals were treated subcutaneously with 2 mg medroxyprogesterone (Depo-provera; Pharmacia) to confer susceptibility to infection (Parr et al., 1994). Two weeks after the third vaccination, the animals were challenged intravaginally with 1.55 × 10$^4$ or 1.55 × 10$^5$ p.f.u. of HSV-2 strain 186, corresponding to 50 LD$_{50}$ or 500 LD$_{50}$, respectively, determined in preliminary experiments in age-matched animals. The virus was thawed immediately before use and diluted in PBS and 0.1 % normal mouse serum prior to administration. Animals were anaesthetized with ketamine and xylazine. Vaginas were cleaned using a sterile calcium alginate fibre-tipped urethral swab (14-959-78; Thermo Fisher Scientific). Ten microlitres of diluted virus was gently injected into the vagina with a hand-held pipette and a 20 µl tip. Animals were placed in a supine position and awakened after several minutes. The animals were monitored twice daily, for 21 days, for signs of disease and survival. Animals with hind-limb paralysis, urine staining of the perineum, or other pre-morbid signs or symptoms were euthanized.

**HSV-2 replication.** On days 1, 3 and 5 post-infection, vaginal swabs were collected to assess the relative extent of virus replication. Using Copan polyester urethral swabs (185CS01; Copan), the vaginas of unanaesthetized animals were swabbed by gently turning the swab through 360°. Swabs were stored in digestion buffer (100 mM KCl, 10 mM Tris pH 8.0, 25 mM EDTA, 05 % Nonidet P-40) at −80 °C prior to use. DNA extraction and qPCR for HSV-2 DNA copy number were performed as described by Wald et al. (2003).

Animals that survived HSV-2 challenge were examined for virus replication in DRG 90 days after challenge. DRG from the bilateral lumbar and sacral regions were dissected as described by Malin et al. (2007) and collected into sterile tubes on dry ice. Pooled ganglia from each animal were washed carefully into 200 µl digestion buffer. DNA extraction and HSV-2 DNA copy number measurement were performed as above. We also estimated mouse cell number by measuring the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA copy number and dividing by two. Mouse DNA

genomes were measured with a qPCR primer-/probe cocktail (part 4308313) amplifying the GAPDH gene (ABI). Results are presented as HSV-2 DNA copies per million mouse cells.

**Statistical analysis.** Wilcoxon rank sum test or Student's *t*-test was used to compare $log_{10}$ IgG titres, 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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## REFERENCES

**Aumakhan, B., Hardick, A., Quinn, T. C., Laeyendecker, O., Gange, S. J., Beyrer, C., Cox, C., Anastos, K., Cohen, M. & other authors (2010).** Genital herpes evaluation by quantitative TaqMan PCR: correlating single detection and quantity of HSV-2 DNA in cervicovaginal lavage fluids with cross-sectional and longitudinal clinical data. *Virol J* **7**, 328.

**Balachandran, N., Bacchetti, S. & Rawls, W. E. (1982).** Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. *Infect Immun* **37**, 1132–1137.

**Belshe, R. B., Leone, P. A., Bernstein, D. I., Wald, A., Levin, M. J., Stapleton, J. T., Gorfinkel, I., Morrow, R. L., Ewell, M. G. & other authors (2012).** Efficacy results of a trial of a herpes simplex vaccine. *N Engl J Med* **366**, 34–43.

**BenMohamed, L., Bertrand, G., McNamara, C. D., Gras-Masse, H., Hammer, J., Wechsler, S. L. & Nesburn, A. B. (2003).** Identification of novel immunodominant CD4+ Th1-type T-cell peptide epitopes from herpes simplex virus glycoprotein D that confer protective immunity. *J Virol* **77**, 9463–9473.

**Bernstein, D. I., Aoki, F. Y., Tyring, S. K., Stanberry, L. R., St-Pierre, C., Shafran, S. D., Leroux-Roels, G., Van Herck, K., Bollaerts, A., Dubin, G. & Glaxo Smith Kline Herpes Vaccine Study Group (2005).** Safety and immunogenicity of glycoprotein D-adjuvant genital herpes vaccine. *Clin Infect Dis* **40**, 1271–1281.

**Bourne, N., Milligan, G. N., Schleiss, M. R., Bernstein, D. I. & Stanberry, L. R. (1996).** DNA immunization confers protective immunity on mice challenged intravaginally with herpes simplex virus type 2. *Vaccine* **14**, 1230–1234.

**Bourne, N., Pyles, R. B., Bernstein, D. I. & Stanberry, L. R. (2002).** Modification of primary and recurrent genital herpes in guinea pigs by passive immunization. *J Gen Virol* **83**, 2797–2801.

**Bourne, N., Milligan, G. N., Stanberry, L. R., Stegall, R. & Pyles, R. B. (2005).** Impact of immunization with glycoprotein D2/AS04 on herpes simplex virus type 2 shedding into the genital tract in guinea pigs that become infected. *J Infect Dis* **192**, 2117–2123.

**Cattamanchi, A., Posavad, C. M., Wald, A., Baine, Y., Moses, J., Higgins, T. J., Ginsberg, R., Ciccarelli, R., Corey, L. & Koelle, D. M. (2008).** Phase I study of a herpes simplex virus type 2 (HSV-2) DNA vaccine administered to healthy, HSV-2-seronegative adults by a needle-free injection system. *Clin Vaccine Immunol* **15**, 1638–1643.

**Chen, C. Y., Ballard, R. C., Beck-Sague, C. M., Dangor, Y., Radebe, F., Schmid, S., Weiss, J. B., Tshabalala, V., Fehler, G. & other authors (2000).** Human immunodeficiency virus infection and genital ulcer disease in South Africa: the herpetic connection. *Sex Transm Dis* **27**, 21–29.

**Chentoufi, A. A., Zhang, X., Lamberth, K., Dasgupta, G., Bettahi, I., Nguyen, A., Wu, M., Zhu, X., Mohebbi, A. & other authors (2008).** HLA-A*0201-restricted CD8+ cytotoxic T lymphocyte epitopes identified from herpes simplex virus glycoprotein D. *J Immunol* **180**, 426–437.

**Coen, D. M., Kosz-Vnenchak, M., Jacobson, J. G., Leib, D. A., Bogard, C. L., Schaffer, P. A., Tyler, K. L. & Knipe, D. M. (1989).** Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc Natl Acad Sci U S A* **86**, 4736–4740.

**Cohen, J. (2010).** Immunology. Painful failure of promising genital herpes vaccine. *Science* **330**, 304.

**Corey, L., Langenberg, A. G., Ashley, R., Sekulovich, R. E., Izu, A. E., Douglas, J. M., Jr, Handsfield, H. H., Warren, T., Marr, L. & other authors (1999).** Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. *JAMA* **282**, 331–340.

**Corey, L., Wald, A., Celum, C. L. & Quinn, T. C. (2004).** The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: a review of two overlapping epidemics. *J Acquir Immune Defic Syndr* **35**, 435–445.

**Dolan, A., Jamieson, F. E., Cunningham, C., Barnett, B. C. & McGeoch, D. J. (1998).** The genome sequence of herpes simplex virus type 2. *J Virol* **72**, 2010–2021.

**Donnelly, J. J., Ulmer, J. B. & Liu, M. A. (1997).** DNA vaccines. *Life Sci* **60**, 163–172.

**Fló, J. (2003).** Co-immunization with plasmids coding the full length and a soluble form of glycoprotein D of HSV-2 induces protective cellular and humoral immune response in mice. *Vaccine* **21**, 1239–1245.

**Hartikka, J., Sawdey, M., Cornefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M. & other authors (1996).** An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther* **7**, 1205–1217.

**Hartikka, J., Bozoukova, V., Ferrari, M., Sukhu, L., Enas, J., Sawdey, M., Wloch, M. K., Tonsky, K., Norman, J. & other authors (2001).** Vaxfectin enhances the humoral immune response to plasmid DNA-encoded antigens. *Vaccine* **19**, 1911–1923.

**Hermanson, G., Whitlow, V., Parker, S., Tonsky, K., Rusalov, D., Ferrari, M., Lalor, P., Komai, M., Mere, R. & other authors (2004).** A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. *Proc Natl Acad Sci U S A* **101**, 13601–13606.

**Higgins, T. J., Herold, K. M., Arnold, R. L., McElhiney, S. P., Shroff, K. E. & Pachuk, C. J. (2000).** Plasmid DNA-expressed secreted and nonsecreted forms of herpes simplex virus glycoprotein D2 induce different types of immune responses. *J Infect Dis* **182**, 1311–1320.

**Hosken, N., McGowan, P., Meier, A., Koelle, D. M., Sleath, P., Wagener, F., Elliott, M., Grabstein, K., Posavad, C. & Corey, L. (2006).** Diversity of the CD8+ T-cell response to herpes simplex virus type 2 proteins among persons with genital herpes. *J Virol* **80**, 5509–5515.

**Iijima, N., Linehan, M. M., Zamora, M., Butkus, D., Dunn, R., Kehry, M. R., Laufer, T. M. & Iwasaki, A. (2008).** Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med* **205**, 3041–3052.

**Jimenez, G. S., Planchon, R., Wei, Q., Rusalov, D., Geall, A., Enas, J., Lalor, P., Leamy, V., Vahle, R. & other authors (2007).** Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral

proteins protect mice against lethal viral challenge. *Hum Vaccin* **3**, 157–164.

Jing, L., McCaughey, S. M., Davies, D. H., Chong, T. M., Felgner, P. L., De Rosa, S. C., Wilson, C. B. & Koelle, D. M. (2009). ORFeome approach to the clonal, HLA allele-specific CD4 T-cell response to a complex pathogen in humans. *J Immunol Methods* **347**, 36–45.

Kask, A. S., Chen, X., Marshak, J. O., Dong, L., Saracino, M., Chen, D., Jarrahian, C., Kendall, M. A. & Koelle, D. M. (2010). DNA vaccine delivery by densely-packed and short microprojection arrays to skin protects against vaginal HSV-2 challenge. *Vaccine* **28**, 7483–7491.

Kemble, G. & Spaete, R. (2007). Herpes simplex vaccines. In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*, chapter 69. Edited by A. Arvin, G. Campadelli-Fiume, E. Mocarski, P. S. Moore, B. Roizman, R. Whitley & K. Yamanishi. Cambridge: Cambridge University Press.

Kim, M., Taylor, J., Sidney, J., Mikloska, Z., Bodsworth, N., Lagios, K., Dunckley, H., Byth-Wilson, K., Denis, M. & other authors (2008). Immunodominant epitopes in herpes simplex virus type 2 glycoprotein D are recognized by CD4 lymphocytes from both HSV-1 and HSV-2 seropositive subjects. *J Immunol* **181**, 6604–6615.

Klein, R. J. (1980). Effect of immune serum on the establishment of herpes simplex virus infection in trigeminal ganglia of hairless mice. *J Gen Virol* **49**, 401–405.

Koelle, D. M. & Corey, L. (2008). Herpes simplex: insights on pathogenesis and possible vaccines. *Annu Rev Med* **59**, 381–395.

Koelle, D. M., Benedetti, J., Langenberg, A. & Corey, L. (1992). Asymptomatic reactivation of herpes simplex virus in women after the first episode of genital herpes. *Ann Intern Med* **116**, 433–437.

Koelle, D. M., Liu, Z., McClurkan, C. L., Cevallos, R. C., Vieira, J., Hosken, N. A., Meseda, C. A., Snow, D. C., Wald, A. & Corey, L. (2003). Immunodominance among herpes simplex virus-specific CD8 T cells expressing a tissue-specific homing receptor. *Proc Natl Acad Sci U S A* **100**, 12899–12904.

Langenberg, A. G., Burke, R. L., Adair, S. F., Sekulovich, R., Tigges, M., Dekker, C. L. & Corey, L. (1995). A recombinant glycoprotein vaccine for herpes simplex virus type 2: safety and immunogenicity [corrected]. *Ann Intern Med* **122**, 889–898.

Long, D., Madara, T. J., Ponce de Leon, M., Cohen, G. H., Montgomery, P. C. & Eisenberg, R. J. (1984). Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. *Infect Immun* **43**, 761–764.

Malin, S. A., Davis, B. M. & Molliver, D. C. (2007). Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc* **2**, 152–160.

Manickan, E., Rouse, R. J., Yu, Z., Wire, W. S. & Rouse, B. T. (1995). Genetic immunization against herpes simplex virus. Protection is mediated by CD4$^+$ T lymphocytes. *J Immunol* **155**, 259–265.

Margalith, M. & Vilalta, A. (2006). Sustained protective rabies neutralizing antibody titers after administration of cationic lipid-formulated pDNA vaccine. *Genet Vaccines Ther* **4**, 2.

McClements, W. L., Armstrong, M. E., Keys, R. D. & Liu, M. A. (1996). Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease. *Proc Natl Acad Sci U S A* **93**, 11414–11420.

Muller, W. J., Dong, L., Vilalta, A., Byrd, B., Wilhelm, K. M., McClurkan, C. L., Margalith, M., Liu, C., Kaslow, D. & other authors (2009). Herpes simplex virus type 2 tegument proteins contain subdominant T-cell epitopes detectable in BALB/c mice after DNA immunization and infection. *J Gen Virol* **90**, 1153–1163.

Nass, P. H., Elkins, K. L. & Weir, J. P. (2001). Protective immunity against herpes simplex virus generated by DNA vaccination compared to natural infection. *Vaccine* **19**, 1538–1546.

Pan, C. H., Jimenez, G. S., Nair, N., Wei, Q., Adams, R. J., Polack, F. P., Rolland, A., Vilalta, A. & Griffin, D. E. (2008). Use of Vaxfectin adjuvant with DNA vaccine encoding the measles virus hemagglutinin and fusion proteins protects juvenile and infant rhesus macaques against measles virus. *Clin Vaccine Immunol* **15**, 1214–1221.

Parr, M. B., Kepple, L., McDermott, M. R., Drew, M. D., Bozzola, J. J. & Parr, E. L. (1994). A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. *Lab Invest* **70**, 369–380.

Perez, G., Skurnick, J. H., Denny, T. N., Stephens, R., Kennedy, C. A., Regivick, N., Nahmias, A., Lee, F. K., Lo, S. C. & other authors (1998). 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* **3**, 5–11.

Rajčáni, J., Mosko, T. & Rezuchová, I. (2005). Current developments in viral DNA vaccines: shall they solve the unsolved? *Rev Med Virol* **15**, 303–325.

Reyes, L., Hartikka, J., Bozoukova, V., Sukhu, L., Nishioka, W., Singh, G., Ferrari, M., Enas, J., Wheeler, C. J. & other authors (2001). Vaxfectin enhances antigen specific antibody titers and maintains Th1 type immune responses to plasmid DNA immunization. *Vaccine* **19**, 3778–3786.

Sawtell, N. M. (1998). The probability of in vivo reactivation of herpes simplex virus type 1 increases with the number of latently infected neurons in the ganglia. *J Virol* **72**, 6888–6892.

Sawtell, N. M., Poon, D. K., Tansky, C. S. & Thompson, R. L. (1998). The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. *J Virol* **72**, 5343–5350.

Sawtell, N. M., Thompson, R. L., Stanberry, L. R. & Bernstein, D. I. (2001). Early intervention with high-dose acyclovir treatment during primary herpes simplex virus infection reduces latency and subsequent reactivation in the nervous system in vivo. *J Infect Dis* **184**, 964–971.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L. & other authors (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331–335.

Simmons, A. & Nash, A. A. (1985). Role of antibody in primary and recurrent herpes simplex virus infection. *J Virol* **53**, 944–948.

Sin, J. I., Ayyavoo, V., Boyer, J., Kim, J., Ciccarelli, R. B. & Weiner, D. B. (1999). Protective immune correlates can segregate by vaccine type in a murine herpes model system. *Int Immunol* **11**, 1763–1773.

Sin, J., Kim, J. J., Pachuk, C., Satishchandran, C. & Weiner, D. B. (2000). DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4$^+$ T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. *J Virol* **74**, 11173–11180.

Sin, J. I., Kim, J. J., Zhang, D. & Weiner, D. B. (2001). Modulation of cellular responses by plasmid CD40L: CD40L plasmid vectors enhance antigen-specific helper T cell type 1 CD4$^+$ T cell-mediated protective immunity against herpes simplex virus type 2 in vivo. *Hum Gene Ther* **12**, 1091–1102.

Smith, L. R., Wloch, M. K., Ye, M., Reyes, L. R., Boutsaboualoy, S., Dunne, C. E., Chaplin, J. A., Rusalov, D., Rolland, A. P. & other authors (2010). Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. *Vaccine* **28**, 2565–2572.

**Stanberry, L. R., Spruance, S., Cunningham, A. L., Bernstein, D. I., Mindel, A., Sacks, S., Tyring, S., Aoki, F. Y., Slaoui, M. & other authors (2002).** Glycoprotein-D–adjuvant vaccine to prevent genital herpes. *N Engl J Med* **347**, 1652–1661.

**Sullivan, S. M., Doukas, J., Hartikka, J., Smith, L. & Rolland, A. (2010).** Vaxfectin: a versatile adjuvant for plasmid DNA- and protein-based vaccines. *Expert Opin Drug Deliv* **7**, 1433–1446.

**Ulmer, J. B., DeWitt, C. M., Chastain, M., Friedman, A., Donnelly, J. J., McClements, W. L., Caulfield, M. J., Bohannon, K. E., Volkin, D. B. & Evans, R. K. (1999).** Enhancement of DNA vaccine potency using conventional aluminum adjuvants. *Vaccine* **18**, 18–28.

**Vilalta, A., Shlapobersky, M., Wei, Q., Planchon, R., Rolland, A. & Sullivan, S. (2009).** Analysis of biomarkers after intramuscular injection of Vaxfectin-formulated hCMV gB plasmid DNA. *Vaccine* **27**, 7409–7417.

**Wald, A. & Corey, L. (2003).** How does herpes simplex virus type 2 influence human immunodeficiency virus infection and pathogenesis? *J Infect Dis* **187**, 1509–1512.

**Wald, A., Zeh, J., Selke, S., Warren, T., Ashley, R. & Corey, L. (2002).** Genital shedding of herpes simplex virus among men. *J Infect Dis* **186** (Suppl. 1), S34–S39.

**Wald, A., Huang, M. L., Carrell, D., Selke, S. & Corey, L. (2003).** Polymerase chain reaction for detection of herpes simplex virus (HSV) DNA on mucosal surfaces: comparison with HSV isolation in cell culture. *J Infect Dis* **188**, 1345–1351.

**Watson, R. J. (1983).** DNA sequence of the herpes simplex virus type 2 glycoprotein D gene. *Gene* **26**, 307–312.

**Xu, F., Sternberg, M. R., Kottiri, B. J., McQuillan, G. M., Lee, F. K., Nahmias, A. J., Berman, S. M. & Markowitz, L. E. (2006).** Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA* **296**, 964–973.