Vaccine 37 (2019) 6470–6477

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Intranasal nanoemulsion-adjuvanted HSV-2 subunit vaccine is effective as a prophylactic and therapeutic vaccine using the guinea pig model of genital herpes

David I. Bernstein [a,*], Rhonda D. Cardin [a], Fernando J. Bravo [a], Tarek Hamouda [b], Derek A. Pullum [a], Gary Cohen [c], Vira Bitko [b], Ali Fattom [b,*]

[a] Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[b] BlueWillow Biologics, Ann Arbor, MI, USA
[c] University of Pennsylvania, Philadelphia, PA, USA

ARTICLE INFO

Article history:
Received 24 June 2019
Received in revised form 16 August 2019
Accepted 29 August 2019
Available online 9 September 2019

Keywords:
Herpes simplex virus type 2
Genital herpes
Vaccine
NanoVax
Nanoemulsion

ABSTRACT

Genital herpes is a sexually transmitted disease representing a major global health concern. Currently, there is no approved vaccine and existing antiviral therapies exhibit limited efficacy. Herein, we describe an intranasal (IN) vaccine comprised of HSV-2 surface glycoproteins gD2 and gB2 formulated in a nanoemulsion adjuvant (NE01-gD2/gB2). Using the HSV-2 genital herpes guinea pig model, we demonstrate that IN NE01-gD2/gB2 induces higher levels of neutralizing antibody compared to a monovalent IN NE01-gD2 vaccine, but less than an intramuscular (IM) Alum/MPL-gD2 vaccine. Following intravaginal (IVag) challenge with HSV-2, the group immunized with IN NE01-gD2/gB2 exhibited significantly reduced acute and recurrent disease scores compared to placebo recipients. Significantly, latent virus was only detected in the dorsal root ganglia of 1 of 12 IN NE01-gD2/gB2-vaccinated animals compared to 11 of 12 placebo recipient. In the therapeutic model, IN NE01-gD2/gB2 immunized guinea pigs exhibited a significant reduction in the recurrent lesions scores (64%, $p < 0.01$), number of animal days with disease (64%, $p < 0.01$), number of animals with viral shedding (50%, $p < 0.04$) and reduction in virus positive vaginal swabs (56%, $p < 0.04$), These data suggests that the treatment may be effective in treating chronic disease and minimizing virus transmission. These results warrant advancing the development of IN NE01-gD2/gB2 as both a prophylactic and therapeutic vaccine against HSV-2.

1. Introduction

Herpes simplex virus type-2 (HSV-2) infection, commonly referred to as genital herpes, is a sexually-transmitted disease [1,2]. The disease may be asymptomatic or produce the formation of painful skin or mucosal lesions in the oral or genital areas [3,4]. In addition to these acute symptoms, the virus invades the trigeminal or lumbosacral dorsal root ganglia where it can reside over the course of a lifetime as a latent infection [5]. Reactivation of the latent virus is often manifested by the recurrence of lesions [2,3] but more commonly, by viral shedding in the absence of symptoms in the genital tract. This asymptomatic shedding is a major contributor to the transmission of the disease [6–8].

The transmission of HSV-2 via sexual activity is the primary contributor to the pervasive spread of the disease worldwide. As a consequence, adults between the ages 15 and 49 comprise the primary patient population, which was estimated at 417 million people in 2012 [9]. Adult infections are generally not life threatening but neonatal infections result in high morbidity and mortality [10]. HSV-2 infection can also markedly increase the risk of acquiring HIV [11–13]. In regions of high incidences of genital herpes, it's estimated that up to 50% of new HIV infections can be attributed to prior contraction of HSV-2 infections [14,15]. Further, in the United States, HSV-2 contributes an estimated $540 million total lifetime costs to the national healthcare system [16] even when the costs associated with neonatal infections and the role of HSV-2 in promoting the acquisition of HIV are not included.

Abbreviations: IM, intramuscular; IN, intranasal; gD2, glycoprotein D; gB2, glycoprotein B; NE, nanoemulsion; NE01, $W_{80}5EC$; DRG, dorsal root ganglia.

* Corresponding authors at: Cincinnati Children Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229, USA (D. Bernstein). 2311 Green Road, Suite A, Ann Arbor, MI 48105, USA (A. Fattom).

E-mail addresses: david.bernstein@cchmc.org (D.I. Bernstein), ali.fattom@bluewillow.com (A. Fattom).

https://doi.org/10.1016/j.vaccine.2019.08.077
0264-410X/

There are no current vaccines approved or available to prevent or treat HSV-2. Among the challenges limiting the development of a successful vaccine include the selection of appropriate antigen(s), establishing the ideal route of immunization and formulation optimization. The utility of HSV-2 antigenic viral surface glycoproteins (e.g. gB2, gD2, etc.) has been evaluated clinically [17], but this approach has thus far been ineffective. Recent research findings implicate the need for vaccination strategies that induce protective mucosal immunity, in addition to humoral immunity, to treat HSV-2 [18]. This is highlighted by the realization that genital herpes induces tissue T-cell immunological memory in genital mucosa [19,20]. Unfortunately, the route of intramuscular immunization of most HSV-2 vaccines does not generally induce a strong mucosal immune response.

We have developed and demonstrated the utility of using W_{80}5EC (NE01), a novel oil-in-water nanoemulsion (NE) adjuvant [21], for formulating viral antigens (e.g. RSV, HIV, Influenza, etc.) as vaccines for intranasal (IN) delivery [22–27]. In multiple applications, we have demonstrated the effectiveness of NE-formulated vaccines for promoting viral clearance. NE-based vaccines exhibit robust and complimentary humoral and mucosal responses, characterized by enhanced Th1 and Th17 immunity [28,29] without enhancement of inflammatory Th2-mediated immunopathology, resulting in a balanced Th1/Th2 immune response [29]. Mechanistically with respect to mucosal immunity, IN delivery in NE facilitates antigen uptake in epithelial cells within the nasal mucosa for efficient trafficking to systemic lymphoid tissues where potent activation of local T-cell immunity occurs [21]. This promotes local, tissue-specific acquisition of mucosal immune memory that facilitates long-term protection from future infections.

Robust mucosal immunity has been identified as a key in developing successful vaccines against sexually transmitted diseases [30–32]. Intranasal immunization induces mucosal immunity in the respiratory and genital tract mucosa [33]. The work presented here describes our investigation into the use of NE adjuvant to formulate antigenic HSV-2 glycoproteins as prophylactic and/or therapeutic vaccines for IN immunization of guinea pigs.

2. Methods

2.1. Animals

Female Hartley guinea pigs (250–350g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and housed under AAALAC approved conditions at Cincinnati Children's Hospital Medical Center. All procedures and protocols were approved by the Cincinnati Children's Hospital Research Foundation Animal Care and Use Committee.

2.2. Viruses and cells

HSV-2 strain MS (ATCC-VR540) was grown in low passage primary rabbit kidney cells maintained in 2% FBS BME (Gibco-Invitrogen) and titered on rabbit kidney cell monolayers as previously described [34].

2.3. Vaccine formulations

The recombinant proteins gB2 (727) and gD2 (306) were obtained from Gary H. Cohen, University of Pennsylvania. In brief, the proteins were prepared from Sf9 cells infected with a recombinant baculovirus expressing gD2 and gB2 protein as described earlier [35,36]. The vaccines utilized for both prophylactic and therapeutic studies were prepared at BlueWillow Biologics (formerly NanoBio Corporation) and shipped 2–3 days prior to vaccination. The univalent and bivalent formulations were prepared by mixing the respective antigen with 60% NE01 to a final vaccine formulation containing 20 μg of each antigen per dose. The NE01 concentration used was 20% for intranasal (IN) and intravaginal (IVag) vaccination and 5% for IM vaccination. The IN and IVag doses were 50 μl and the IM dose was 100 μl. For IVag immunizations, the vaginal mucosa was prepared using a wet swab followed by a dry swab. The intramuscular (IM) alum/MPL-gD2 vaccine was prepared by adding 20 μg of the gD2 antigen to 2% alhydrogel (InvivoGen) followed by MPL (Sigma) to yield a final concentration of 0.4% alum and 25 μg of MPL per dose (100 μl) per animal.

2.4. Vaccination studies

Experimental design:

For evaluation of prophylactic vaccination, forty-eight guinea pigs were randomized into four groups (N = 12): Group 1, no vaccine, Group 2, NE01-gD2, Group 3, NE01-gD2/gB2), Group 4, Alum/MPL-gD2. Animals were immunized either intranasally (IN) (groups 2 and 3) or intramuscularly (IM) (group 4) on days −63, −42, and −21 prior to viral challenge. IN vaccinated animals were anaesthetized using sodium pentobarbital intraperitoneally. Guinea pigs were challenged with 1×10^6 plaque forming units (pfu) of HSV-2 MS strain as previously described [37,38]. For IN vaccination, 50 μl (25 μl per nares) of the vaccine formulation was administered, and for IM vaccination 100 μl was injected into the hind leg. Guinea pigs were evaluated daily and primary genital skin disease was quantified using a lesion score-scale ranging from 0 representing no disease to 4 representing severe vesiculoulcerative skin disease of the perineum [26]. Vaginal washes were performed on days 2, 4, 6 and 8 following challenge. Samples were stored frozen (−80 °C) until assayed for virus on Vero cells grown in BME (Gibco-Invitrogen) and 10% FBS (Hyclone, Thermo Fisher Scientific).

Following recovery from primary infection, animals were examined daily from days 15–63 post each challenge for evidence of spontaneous recurrent herpetic lesions [26]. The number of lesion days (days on which a recurrent lesion was observed on the perineum) was recorded. Vaginal washings were performed on days 2, 4, 6 and 8 following challenge. Samples were stored frozen (−80 °C) until assayed for virus on Vero cells grown in BME (Gibco-Invitrogen) and 10% FBS (Hyclone, Thermo Fisher Scientific) [37–39].

At the end of the evaluation period for each challenge, the guinea pigs were sacrificed, and the dorsal root ganglia (DRG) were harvested aseptically. These tissues were stored frozen (−80 °C) until DNA was extracted from each animal for individual PCR evaluation of latent virus as previously described [38].

For evaluation of therapeutic vaccines, 60 animals were inoculated IVag with $1x10^6$ PFU HSV-2 strain MS. At 14 days post infection (dpi), based on primary disease scores, day 2 vaginal titers, and animal weights, the guinea pigs were randomized into four groups (N = 15): Group1, no vaccine, Group 2, IN NE01-gD2/gB2, Group 3 IVag NE01-gD2/gB2, Group 4, IM NE01-gD2/gB2. Animals were immunized on days 14, 21 and 35 after vaginal HSV-2 inoculation. Animals were observed daily for recurrent lesions (days 48–63) and vaginal swabs were collected from the no vaccine and IN NE01-gD2/gB2 groups for evaluation of viral shedding (days 46–57) as described above. Swabs were stored frozen (−80 °C) until they were processed for PCR analysis to determine the frequency of viral shedding into the genital tract.

2.5. gB2 and gD2 specific IgG responses

Vaccine induced antibodies against gB2 and gD2 were measured using ELISA. Recombinant HSV gB2 or gD2 proteins were

diluted in PBS to a concentration of 1 μg/mL, and coated onto 96 well MaxiSorp plates (Nunc) overnight at 4 °C. The unbound antigen was washed from the plates with PBS containing 0.33% Brij 35 (PBS-B35) and blocked with 2% nonfat dry milk in PBS at room temperature for 1.5 h. Serum samples from the vaccinated and naïve guinea pigs were diluted in 1% nonfat dry milk and 200 μl of the sample was transferred to the respective wells and diluted serially in 1% nonfat dry milk in PBS. The plates were incubated at room temperature for 1.5 h. The plates were washed three times with PBS-B35, followed by addition of anti-guinea pig IgG-HRP (Jackson ImmunoResearch) for 1.5 h and washed as described above. The bound antigen-antibody complex was detected by incubation with 3, 3′, 5, 5′- tetramethylbenzidine (TMB) substrate (Neogen) for 15 min at room temperature. The color development was stopped by addition of 1 N HCl solution in water. The plate was read at A450 nm using a spectrophotometer. The endpoint titer (EPT) is the inverse of the highest dilution that results in a cutoff (≥3X the background) OD.

2.6. Neutralization assays

Serum samples were diluted in EMEM (Eagle Minimum essential media, Life Sciences) containing 2% FBS. Pre-optimized volume of crude lysate virus (100 PFU/mL) was added to diluted serum samples. The serum/virus mixture was incubated at 37 °C for 1 h. As a negative control, virus was mixed with EMEM containing 2% FBS alone. The monolayer of Vero cells were washed twice with serum-free EMEM media, pre-warmed to 37 °C. The cells were then overlaid with 0.5 mL/well serum/virus mixture. Inoculated plates were incubated for 2 hrs at 37 °C, while rocking the plates every 40–45 min. After 2 hrs of incubation the serum/virus mixture was removed and replaced with 0.9% methylcellulose, pre-warmed at 34 °C. Plates were incubated for 2–3 days at 37 °C under 5% CO_2. Virus plaque forming units were detected by staining the infected cells with crystal violet. Plaques were counted using a light box and a magnifying glass mounted on a stand. To calculate PFU/mL, the plaque count from two replicate wells was used. The neutralization titer was defined as follows:

% Reduction = [(PFU in control sample − PFU in test sample)/PFU in control].

Sample dilutions were plotted against % reduction and the dilution with 50% reduction was reported as Neutralization Unit 50 (NU 50).

2.7. HSV-2 DNA isolation and quantitative PCR

Vaginal swabs and DRG were stored at −80 °C until DNA was isolated from 200 μl of vaginal swab media or DRG homogenates using QIAamp DNA Mini Kit (Qiagen #51306) according to manufacturer's protocol, HSV-2 gG2 gene detection was performed by quantitative PCR. The gG2 primer and probe sequences were as followed: Forward: 5′- CGG/AGA/CAT/TCG/AGT/ACC/AGA/TC -3′; reverse: 5′- GCC/CAC/CTC/TAC/CCA/CAA/CA -3′; and probe: 5′-FAM- ACC/CAC/GTG/CAG/CTC/GCC/G -tamRA-3′ [38,40].

Each PCR reaction contained 100 ng of DNA, 50 μM of each primer, 0.10 μM of FAM/TAMRA fluorescent probe, and 10 μl of Taqman Gene Expression Master Mix (ABI) in a total volume of 20 μl reaction. PCR amplification of HSV-2 DNA was performed on a 7500 Fast Real-Time PCR system (ABI) using the following conditions: pre-incubation at 50 °C for 2 min and 95 °C for 10 min followed by 50 cycles consisting of a denaturation step at 95 °C for 15 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 10 s. A standard curve was generated with ten-fold serial dilutions of purified HSV-2 DNA (ATCC) containing 10^5–10^0 HSV-2 copies in 50 ng of uninfected guinea pig brain DNA. The limit of detection for HSV-2 was determined to be between 10^0 and 10^1 copies, with excellent linearity ($R \geq 0.98$) over 5 logs of HSV genomic DNA content.

2.8. Statistical analysis

Antibody titers and gene copy statistics were compared using Mann-Whitney nonparametric test for unpaired data. Data are presented as mean and 95% CI. The severity of disease and viral titers were analyzed using two-tailed Student's t tests for comparisons with equal variance. A P value < 0.05 was considered significant. Days of disease observed in each group were compared to placebo group using the chi-square test.

3. Results

3.1. Prophylactic immunization studies

3.1.1. Vaccine immunogenicity

All vaccines tested elicited detectable serum antibodies against gD2 (Fig. 1A). The bivalent formulation IN NE01-gD2/gB2 induced significantly higher anti-gD2 antibodies compared to the monovalent formulation IN NE01-gD2 vaccine. The intramuscular alum-formulated gD2 vaccine elicited approximately 10-fold more anti-gD2 antibodies than IN NE01-gD2/gB2. As expected, the addition of the glycoprotein B2 (gB2) to the NE01-gD2/gB2 formulation lead to induction of gB2 antibodies after intranasal immunization.

A similar trend was observed in the induction of neutralizing antibodies (Fig. 1B). Significantly higher neutralizing antibody titers were measured after immunization with the bivalent vaccine IN NE01-gD2/gB2 compared to IN NE01-gD2, but titers were still significantly lower than intramuscular immunization with IM alum/MPL-gD2.

3.1.2. Primary genital disease

As shown in Fig. 2A, 11 of 12 animals in the control group developed primary disease with a cumulative acute mean lesion score of 9.38. All vaccinated groups exhibited a significant reduction in mean lesion scores compared to the control group ($p < 0.003$). Three out of 12 animals developed lesions in the IN NE01-gD2/gB2 group (acute cumulative mean lesion score of 0.21), two out of 12 animals developed lesions in the IM Alum/MPL-gD2 group (acute cumulative mean lesion score of 0.42), and 7 out of 12 animals developed lesions in the IN NE01-gD2 group (cumulative mean lesion score of 2.4). The acute cumulative mean lesion score of the bivalent vaccine IN NE01-gD2/gB2 group was also significantly lower ($p = 0.003$) compared to the single antigen formulation IN NE01-gD2 group. A slight improvement (not statistically significant) in the acute cumulative mean lesion score was observed with IN NE01-gD2/gB2 versus intramuscular (IM alum/MPL-gD2) vaccination indicating similar efficacy of the two vaccines for prophylaxis against HSV infections.

3.1.3. Primary vaginal replication following HSV-2 challenge

Analysis of vaginal viral shedding post-challenge showed that animals vaccinated with either IN NE01-gD2/gB2 or IM Alum/MPL-gD2 had significantly less virus shed on days 2 and 4 post challenge. Virus detection in these 2 groups was near the limit of detection on Day 8 post challenge (Fig. 3).

3.1.4. Recurrent genital disease

Extended monitoring of the guinea pigs was performed to assess recurrent disease, day15-63. Data in Fig. 2B show that the bivalent intranasal IN NE01-gD2/gB2 vaccine exhibited significant improvement in reducing number of animals with recurrent lesions and the cumulative recurrent lesion scores compared to

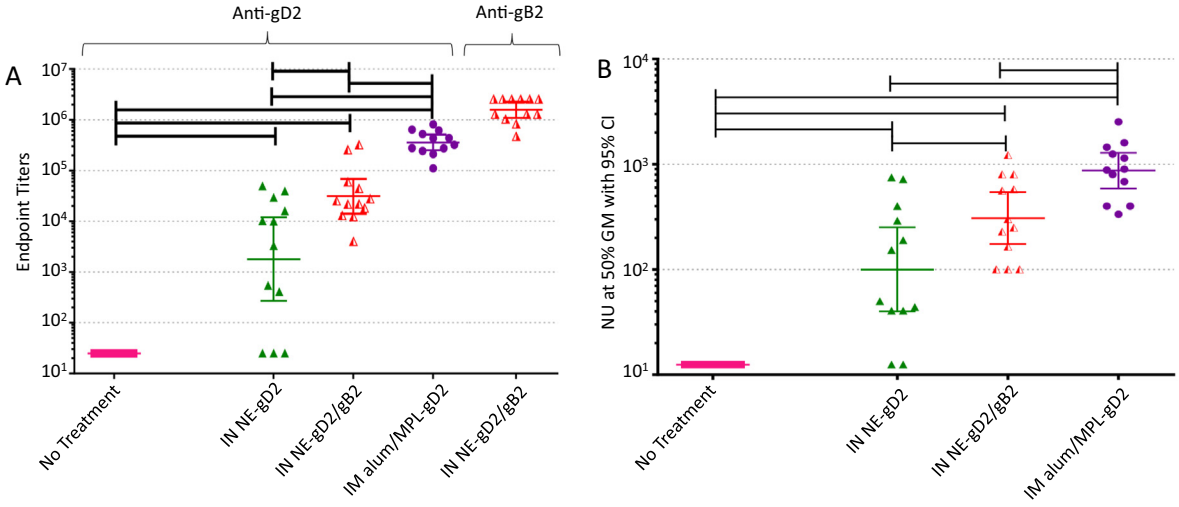

Fig. 1. Humoral immune response following three vaccinations. Humoral immune responses elicited after immunization with various vaccine formulations but prior to HSV-2 challenge as determined from measurements of: (A) Serum IgG antibodies against gD2 and gB2 glycoproteins and, (B) serum neutralizing antibodies. Connecting bars indicate statistical differences ($p < 0.05$) in measured levels. Error bars are 95% CI.

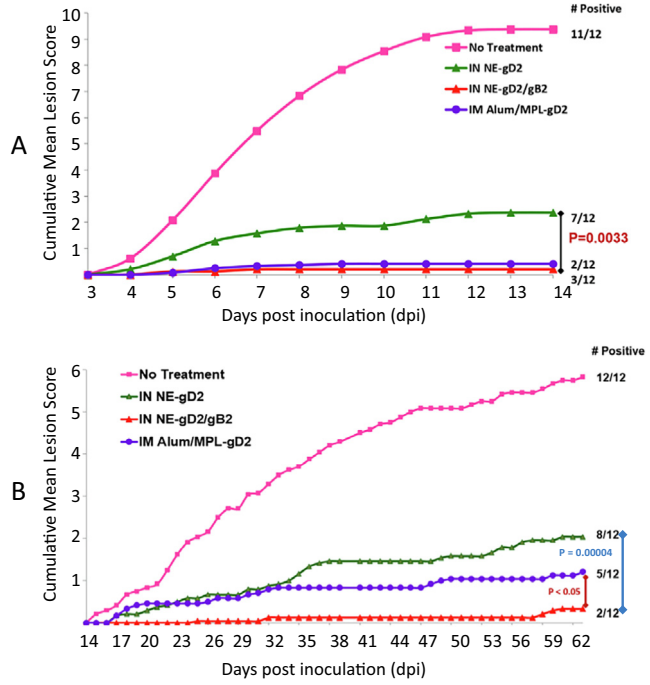

Fig. 2. Acute and recurrent herpetic lesion score. Cumulative mean lesion scores in guinea pigs immunized with various vaccine formulations prior to HSV-2 challenge. (A) Acute mean lesion scores (days 1–14). Numbers to the right indicate number of animals developing any lesion. Bars to the right show significant differences in the cumulative mean lesion score between vaccinated groups. (B) Recurrent mean lesion scores. Numbers to the right are the number of animals developing any recurrent lesions. Bars to the right show significant differences in the cumulative mean lesion score between vaccinated groups. All vaccinated groups developed reduced scores compared to placebo for both acute and recurrent disease.

the monovalent vaccine IN NE01-gD2 ($p = 0.00004$). Most notably, a remarkable and significant improvement in the reduction of the cumulative recurrent lesion scores was observed for the IN NE01-gD2/gB2 vaccinated animals compared to animals treated with the IM alum/MPL-gD2 vaccine ($p < 0.05$). We examined animals for herpetic lesion daily from day 15 to day 63 (last day of follow up). Data in Table 1 show that the number of days that animals developed recurrent lesions was significantly less in the IN and IM vaccinated groups compared to the placebo group ($p < 0.01$). The intranasal immunization demonstrated significantly higher protection compared to the IM route, ($p < 0.05$). Similarly, the number of animals with recurrent disease was significantly reduced in the IN group compared to the IM group $p < 0.01$.

3.1.5. Latent viral load in dorsal root ganglion

At the conclusion of the prophylactic immunization study, animals were sacrificed, and dorsal root ganglia (DRG) were collected to measure the latent DNA viral load using qPCR. As shown in Fig. 4, HSV-2 DNA was detected in 92% (11/12) of guinea pigs in the control group, but in only one guinea pig in the IN NE01-gD2/gB2 group (8.3%, 1/12). This reduction was also significant compared to the IN NE01-gD2 group (50%, 6/12), ($p = 0.041$). Viral DNA was detected in 2 of 12 animals in the IM alum/MPL-gD2 group (NS compared to either of the IN groups). DNA viral load was significantly reduced in all vaccinated groups, particularly in animals immunized with IN NE01-gD2/gB2 and IM alum/MPL-gD2vaccines, respectively.

3.2. Therapeutic immunization studies

3.2.1. Antibody responses in infected animals vaccinated IM, IN or IVag with NE01-gD2/gB2 vaccine

The high level of prophylactic protection afforded by the IN NE01-gD2/gB2 vaccine prompted us to evaluate the potential of this intranasal vaccine as a therapeutic option against recurrent disease. To evaluate whether post infection immunization can influence the recurrence rate, HSV-2 infected animals received either IN gB2/gD2/NE01, IVag gB2/gD2/NE01, or IM gD2/gB2 NE01 (5%) or were not vaccinated. Data presented in Fig. 5 show that the viral neutralization activity was not significantly increased after immunization regardless of the route. There was a trend toward more robustness in the immunized animals compared to the nonimmunized group; nonetheless, it was not statistically significant.

3.2.2. Recurrent genital disease

As shown in Fig. 6, intranasal delivery induced the greatest reduction (64%) in the recurrent lesion scores, $p = 0.006$ after last immunization, compared to the unimmunized control group. The

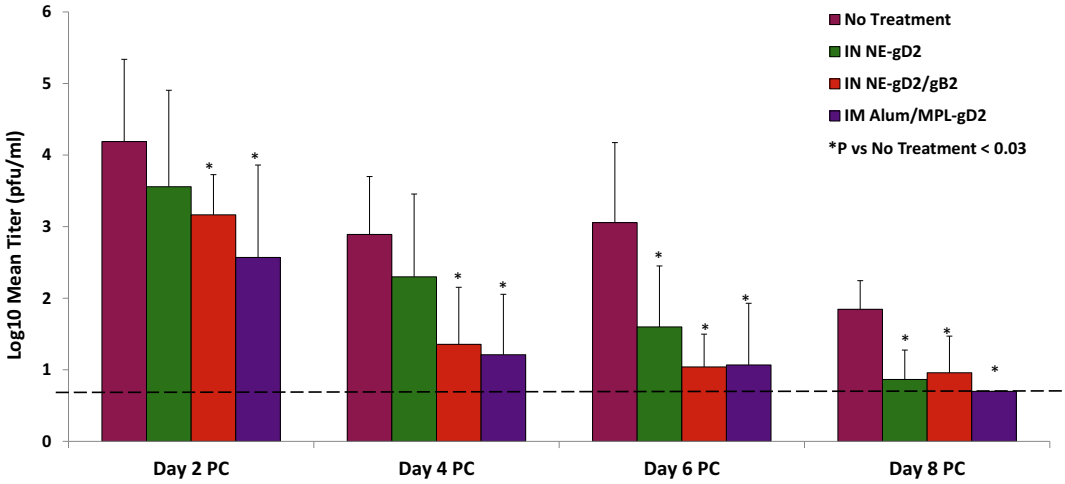

Fig. 3. Viral titer post challenge. Vaginal viral shedding from swabs collected from guinea pigs post challenge. Vaginal swabs were obtained on days 2, 4, 6 and 8 following challenge with HSV-2 virus.

Table 1
Prophylactic vaccination and HSV2 challenge in guinea pig model. Number of animal days positive for herpetic lesions during the chronic infection follow up, days 15–63.

	No Treatment	IN NE-gD2/gB2	IM NE-gD2/gB2
Number positive animals/Total	12/12	2/12	5/12
Days with positive lesions	84	5	17
Days with no lesions	492	571	559
Total observations	576	576	576
Efficacy (% lesion reduction)		94%	80%
Chi-square vs. No Treatment		P < 0.01	P < 0.01

Chi-square: IN vs IM P < 0.01.

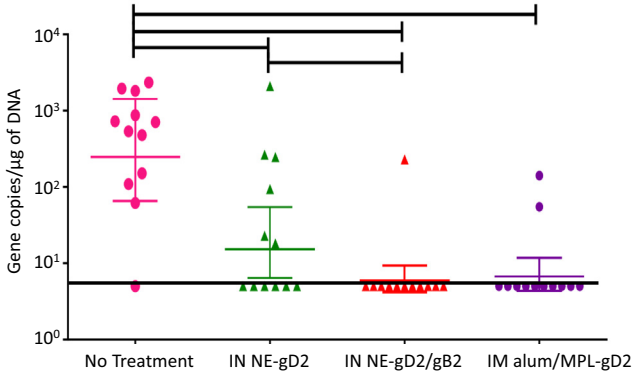

Fig. 4. Dorsal root ganglion viral gene copies (Latent Virus). HSV-2 viral load measured in dorsal root ganglia (DRG) of guinea pigs vaccinated prior to HSV-2 intravaginal challenge. DRG were collected at the end of the observation period and assess by qPCR. Bars at the top of figure indicate significance (P < 0.05 differences).

other routes of vaccination, IM or IVag, resulted in a nonsignificant reduction in lesion scores (27% and 33%, respectively). We also evaluated the number of positive disease days following the last immunization, days 48–63. Similar efficacy was observed using the number of days with recurrent lesion (Table 2). Compared to the placebo group, vaccination resulted in reduction of number of disease days of 58% in the IN group (p < 0.0001), 33% in the IM group (p < 0.03), and 38% in the IVag group (p < 0.02).

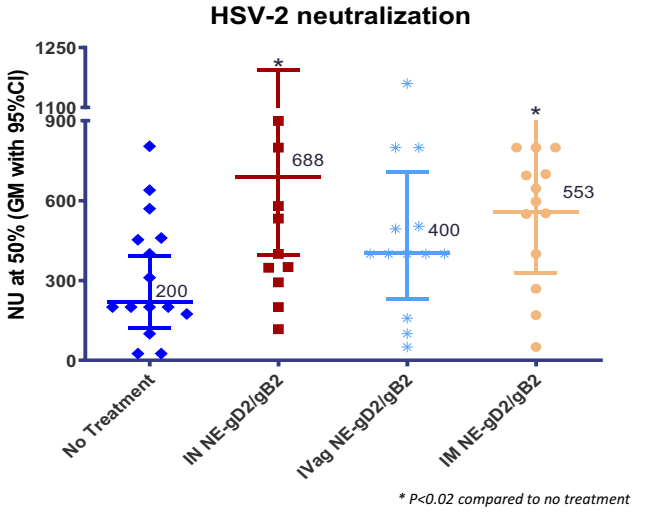

Fig. 5. Therapeutic study: Virus neutralization titers prior to 3rd immunization (Day35). Virus neutralization titers prior to 3rd therapeutic vaccine immunization. Animals were infected intravaginally with herpes virus on day 0, followed by 3 doses vaccine on days 14, 21 and 35. Sera were collected on day 35 and tested for neutralizing antibodies. Error bars are 95% CI.

3.2.3. Recurrent viral shedding following therapeutic vaccination

Recurrent viral shedding was also measured to gain insight into the potential of the intranasal vaccine for reducing transmission. Although vaginal swabs were collected for all the groups, only the group with the greatest reduction in recurrent disease, IN NE01-gD2/gB2, and the no vaccine group were analyzed. Compared to non-vaccinated guinea pigs, the IN NE01-gD2/gB2 group showed 50% reduction in numbers of animals shedding virus (p = 0.035, Fig. 7), coinciding with a 56% reduction in the number of positive swabs (p = 0.034).

4. Discussion

The most widely explored antigens for vaccine development against HSV-1 and HSV-2 have been the glycoproteins expressed on the viral surfaces [17]. In particular, gD2 has received the most attention due to its ability to generate the highest neutralizing antibody activity. However, vaccine development strategies focused on gD2 formulations have not been successful in the clinic

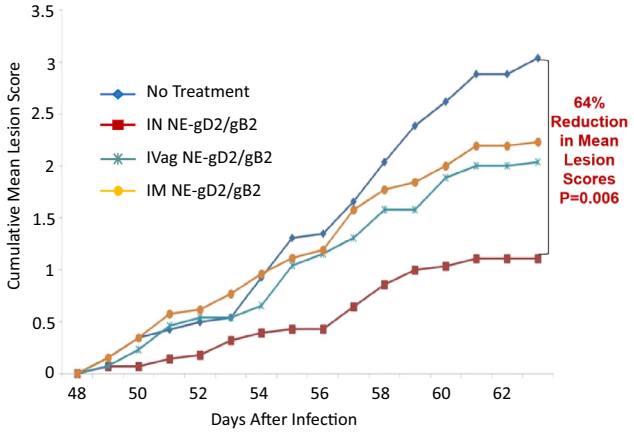

Fig. 6. Therapeutic study: Recurrent Lesion Score. Cumulative mean recurrent lesion scores in guinea pigs receiving therapeutic vaccination after HSV-2 infection. Each animal received three vaccinations; the final being administered on Day 35 post- infection. Recurrences were evaluated daily from day 48 (2 weeks after final vaccination) until day 63. The bars to the right show the % reduction and the significance between IN NE01-gD2/gB2 and No treatment group.

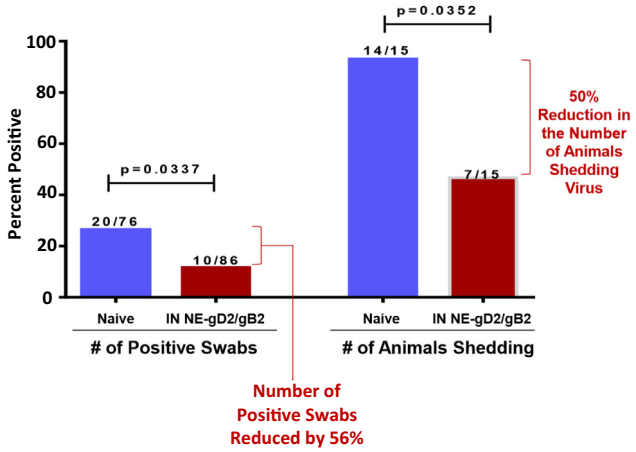

Fig. 7. Therapeutic study: recurrent viral shedding. The percent of recurrent vaginal swabs and the number of animals with any recurrent shedding was evaluated. Swabs were collected 3x/week from day 46–57.

[17]. Other strategies have combined other antigenic targets formulated with gD2, including gB [41] in hopes that multiple antigens will facilitate sufficient protection from the infection and/or eliminate or reduce the spread of the disease. While induction of a strong and robust humoral immune response to the multiple viral antigens is important, current thinking has centered on the need to elicit other immune responses that are important for protection against sexually transmitted diseases, specifically mucosal [18].

The rationale for evaluating intranasal immunization with NE01-formulated vaccines is based on our prior experience with NE01 adjuvanted recombinant viral proteins in successfully formulating vaccines against multiple pathogens [22–27]. In addition to observing potent antiviral activity in multiple applications, we have documented the ability of the NE vaccines to induce robust humoral and mucosal immunity, the latter facilitates long-term tissue immune memory and protection. Homing of immune cells including T-cells and IgG and IgA producing B-cells following mucosal intranasal immunization with NE01-formualted vaccines to mucosal tissues, respiratory and genital, has been shown in several animal models [22,29,42]. In a recent study, macaques immunized intranasally with NE01-gp120 showed a higher number of gp120-specific IgG B-cells, and INFγ-, and IL17 producing T cells in the vaginal mucosa compared to IM immunized animals (unpublished data). Plans for demonstrating these attributes in the guinea pig model are planned.

In the studies presented here, we compared HSV-2 vaccines formulated with either gD2 alone or a combination of gD2/gB2 and NE01 and evaluated prophylactic intranasal immunization. These vaccines were compared to intramuscular vaccination with alum/MPL-gD2 (control). While all vaccinated animals demonstrated vaccine-induced humoral immunogenicity, the addition of gB2 to the NE-formulated gD2 vaccine (i.e., IN NE01-gD2/gB2) increased the induction of neutralizing antibody compared to the monovalent IN NE01-gD2 vaccine. However, even the bivalent combination did not elicit levels of anti-gD2 antibodies as high as IM alum/MPL-gD2.

Despite lower serum antibody levels, the bivalent IN NE01-gD2/gB2 exhibited equivalent prophylactic protection against vaginal virus replication and acute HSV disease compared to the IM alum/MPL-gD2 vaccine. Perhaps, most importantly, there was a dramatic reduction in subsequent recurrent disease in IN NE01-gD2/gB2 vaccinated guinea pigs, correlating to the almost complete (11/12 animals) absence of detectable virus in the dorsal root ganglia. Reductions in recurrent disease and virus detection in the DRG was as good as or better than the gD2 alum/MPL vaccine. This combination of equivalent protection with decreased systemic antibody levels compared to IM immunization indicates the probable induction of mucosal immunity and its importance in protection from genital HSV infections. Studies to evaluate mucosal immunity after intranasal immunization in the guinea pig and other models are being developed.

For evaluation of the IN NE01-gD2/gB2 as a therapeutic vaccine, we compared efficacy against NE01-gD2/gB2 intravaginally and intramuscularly (i.e., IVag NE01-gD2/gB2 and IM NE01-gD2/gB2). Intranasal delivery of the bivalent vaccine exhibited significantly reduced recurrent lesion scores and the number of days with recurrent herpetic lesions compared to the no vaccine group as well as the other routes of administration. Similarly, the number of animals developing recurrent disease was significantly reduced to the other route of vaccination in spite of the fact that the intranasal vaccine was administered using a suboptimal schedule due to model limitation. Thus, in order to allow enough time for evaluation of recurrent disease post immunization, animals are

Table 2
Therapeutic vaccination in HSV2 challenged guinea pig animals. Number of days positive for herpetic lesion following the third vaccination, days 42–63.

	No Treatment	IN NE-gD2/gB2	IM NE-gD2/gB2	IVag NE-gD2/gB2
Number positive animals/Total	12/13	8/14	8/13	10/13
Days with positive lesions	50	21	33	32
Days with no lesions	132	175	149	150
Total observations	182	196	182	182
Efficacy (% lesion reduction)		58%	34%	36%
Chi-square vs. No Treatment		$p < 0.0001$	$P < 0.03$	$P < 0.02$

Chi-square: IN vs. IM P = 0.04; IN vs IVag, P = 0.054.

vaccinated in a compressed weekly, non-ideal schedule, of 14/21/35 days. The other routes of immunization, intramuscular and intravaginal vaccination, produced similar levels of serum neutralizing antibodies but had lower but significant therapeutic effect. These data suggest that in addition to serum neutralizing antibodies, intranasal immunization with NE01-gD2/gB2 probably induced an effective mucosal immune response that helped control recurrent virus shedding and recurrent HSV disease. This is an important attribute for any therapeutic HSV vaccine as vaccination should also ultimately reduce the transmission of the virus in addition to reduction of recurrent disease.

In summary, prophylactic NE01-gD2/gB2 intranasal vaccine induced almost complete protection against genital herpetic lesions, dorsal root ganglia (DRG) infection, and prevention of chronic recurrent infection. As a therapeutic vaccine, it reduced recurrent lesions and recurrent virus shedding in already chronically infected animals. These data clearly support further evaluation and development of this intranasal vaccine for clinical evaluation in humans. Ongoing plans to expand further the characterization of the mucosal immunity responses and to optimize this vaccine should enhance the advancement in the development of this HSV-2 vaccine to address this major public health problem and the huge unmet medical need for reducing the burden of HSV-2 disease.

Author contributions

DB, RC, TH, VB and AF, contributed to concept, protocol designs, data analysis and interpretation, FB and DP executed the animal work and data collection. GC provided the antigens and participated in interpretation of data. DB, RC, TH and AF contributed to the writing of the manuscript and approved the final version of the manuscript.

Source of funding

This work was supported by the National Institute of Allergy and Infectious Diseases/National Institutes of Health Contract No. HHSN272201700017I and HHSN27200003 to Cincinnati Children Hospital Medical Center (CCHMC).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TH, VB & AF, are full time employees at BlueWillow Biologics.

References

[1] Centers for Disease C, Prevention. Seroprevalence of herpes simplex virus type 2 among persons aged 14-49 years – United States, 2005–2008. MMWR Morb Mortal Wkly Rep. 2010;59(15):456–9. Epub 2010/04/24. PubMed PMID: 20414188.

[2] Koelle DM, Corey L. Herpes simplex: insights on pathogenesis and possible vaccines. Annu Rev Med 2008;59:381–95. Epub 2008/01/12. doi: 10.1146/annurev.med.59.061606.095540. PubMed PMID: 18186706.

[3] Gupta R, Warren T, Wald A. Genital herpes. Lancet 2007;370(9605):2127–37. Epub 2007/12/25. doi: 10.1016/S0140-6736(07)61908-4. PubMed PMID: 18156035.

[4] Whitley RJ, Roizman B. Herpes simplex virus infections. Lancet 2001;357(9267):1513–8. Epub 2001/05/30. doi: 10.1016/S0140-6736(00)04638-9. PubMed PMID: 11377626.

[5] Brugha R, Keersmaekers K, Renton A, Meheus A. Genital herpes infection: a review. Int J Epidemiol 1997;26(4):698–709. Epub 1997/08/01. doi: 10.1093/ije/26.4.698. PubMed PMID: 9279600.

[6] Tronstein E, Johnston C, Huang ML, Selke S, Magaret A, Warren T, et al. Genital shedding of herpes simplex virus among symptomatic and asymptomatic persons with HSV-2 infection. JAMA 2011;305(14):1441–9. Epub 2011/04/14. doi: 10.1001/jama.2011.420. PubMed PMID: 21486977; PubMed Central PMCID: PMCPMC3144252.

[7] Schiffer JT, Abu-Raddad L, Mark KE, Zhu J, Selke S, Magaret A, et al. Frequent release of low amounts of herpes simplex virus from neurons: results of a mathematical model. Sci Transl Med. 2009;1(7):7ra16. Epub 2010/02/18. doi: 10.1126/scitranslmed.3000193. PubMed PMID: 20161655; PubMed Central PMCID: PMCPMC2818652.

[8] Hofstetter AM, Rosenthal SL, Stanberry LR. Current thinking on genital herpes. Curr Opin Infect Dis 2014;27(1):75–83. Epub 2013/12/18. doi: 10.1097/QCO.0000000000000029. PubMed PMID: 24335720.

[9] Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. PLoS ONE 2015;10(1). e114989 Epub 2015/01/22. doi: 10.1371/journal.pone.0114989. PubMed PMID: 25608026; PubMed Central PMCID: PMCPMC4301914.

[10] Thompson C, Whitley R. Neonatal herpes simplex virus infections: where are we now? Adv Exp Med Biol 2011;697:221–30. Epub 2010/12/02. doi: 10.1007/978-1-4419-7185-2_15. PubMed PMID: 21120729; PubMed Central PMCID: PMCPMC3433171.

[11] Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. AIDS 2006;20(1):73–83. Epub 2005/12/06. doi: 10.1097/01.aids.0000198081.09337.a7. PubMed PMID: 16327322.

[12] Johnson KE, Sherman ME, Ssempiija V, Tobian AA, Zenilman JM, Duggan MA, et al. Foreskin inflammation is associated with HIV and herpes simplex virus type-2 infections in Rakai, Uganda. AIDS 2009;23(14):1807–15. Epub 2009/07/09. doi: 10.1097/QAD.0b013e32832efdf1. PubMed PMID: 19584700; PubMed Central PMCID: PMCPMC2752438.

[13] Rebbapragada A, Wachihi C, Pettengell C, Sunderji S, Huibner S, Jaoko W, et al. Negative mucosal synergy between Herpes simplex type 2 and HIV in the female genital tract. AIDS 2007;21(5):589–98. Epub 2007/02/23. doi: 10.1097/QAD.0b013e328012b896. PubMed PMID: 17314521.

[14] Freeman EE, Orroth KK, White RG, Glynn JR, Bakker R, Boily MC, et al. Proportion of new HIV infections attributable to herpes simplex 2 increases over time: simulations of the changing role of sexually transmitted infections in sub-Saharan African HIV epidemics. Sex Transm Infect 2007;83(Suppl 1):i17–24. Epub 2007/04/05. doi: 10.1136/sti.2006.023549. PubMed PMID: 17405782.

[15] Masese L, Baeten JM, Richardson BA, Bukusi E, John-Stewart G, Graham SM, et al. Changes in the contribution of genital tract infections to HIV acquisition among Kenyan high-risk women from 1993 to 2012. AIDS 2015;29(9):1077–85. Epub 2015/07/01. doi: 10.1097/QAD.0000000000000646. PubMed PMID: 26125141; PubMed Central PMCID: PMCPMC4576156.

[16] Owusu-Edusei Jr K, Chesson HW, Gift TL, Tao G, Mahajan R, Ocfemia MC, et al. The estimated direct medical cost of selected sexually transmitted infections in the United States, 2008. Sex Transm Dis 2013;40(3):197–201. Epub 2013/02/14. doi: 10.1097/OLQ.0b013e318285c6d2. PubMed PMID: 23403600.

[17] Belshe RB, Leone PA, Bernstein DI, Wald A, Levin MJ, Stapleton JT, et al. Efficacy results of a trial of a herpes simplex vaccine. N Engl J Med 2012;366(1):34–43. Epub 2012/01/06. doi: 10.1056/NEJMoa1103151. PubMed PMID: 22216840; PubMed Central PMCID: PMCPMC3287348.

[18] Johnston C, Gottlieb SL, Wald A. Status of vaccine research and development of vaccines for herpes simplex virus. Vaccine 2016;34(26):2948–52. Epub 2016/03/15. doi: 10.1016/j.vaccine.2015.12.076. PubMed PMID: 26973067.

[19] Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. Nature 2012;491(7424):463–7. Epub 2012/10/19. doi: 10.1038/nature11522. PubMed PMID: 23075848; PubMed Central PMCID: PMCPMC3499630.

[20] Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8αα+ skin-resident T cells in human herpes virus infection. Nature 2013;497(7450):494–7. Epub 2013/05/10. doi: 10.1038/nature12110. PubMed PMID: 23657257; PubMed Central PMCID: PMCPMC3663925.

[21] Makidon PE, Belyakov IM, Blanco LP, Janczak KW, Landers J, Bielinska AU, et al. Nanoemulsion mucosal adjuvant uniquely activates cytokine production by nasal ciliated epithelium and induces dendritic cell trafficking. Eur J Immunol 2012;42(8):2073–86. Epub 2012/06/02. doi: 10.1002/eji.201142346. PubMed PMID: 22653620; PubMed Central PMCID: PMCPMC3929939.

[22] Passmore C, Makidon PE, O'Konek JJ, Zahn JA, Pannu J, Hamouda T, et al. Intranasal immunization with W 80 5EC adjuvanted recombinant RSV rF-ptn enhances clearance of respiratory syncytial virus in a mouse model. Hum Vaccin Immunother 2014;10(3):615–22. Epub 2013/12/12. doi: 10.4161/hv.27383. PubMed PMID: 24326268; PubMed Central PMCID: PMCPMC4130273.

[23] Makidon PE, Bielinska AU, Nigavekar SS, Janczak KW, Knowlton J, Scott AJ, et al. Pre-clinical evaluation of a novel nanoemulsion-based hepatitis B mucosal vaccine. PLoS ONE 2008;3(8):e2954. Epub 2008/08/14. doi: 10.1371/journal.pone.0002954. PubMed PMID: 18698426; PubMed Central PMCID: PMCPMC2496893.

[24] Bielinska AU, Janczak KW, Landers JJ, Makidon P, Sower LE, Peterson JW, et al. Mucosal immunization with a novel nanoemulsion-based recombinant anthrax protective antigen vaccine protects against Bacillus anthracis spore challenge. Infect Immun 2007;75(8):4020–9. Epub 2007/05/16. doi: 10.1128/IAI.00070-07. PubMed PMID: 17502384; PubMed Central PMCID: PMCPMC1952013.

[25] Bielinska AU, Chepurnov AA, Landers JJ, Janczak KW, Chepurnova TS, Luker GD, et al. A novel, killed-virus nasal vaccinia virus vaccine. Clin Vaccine Immunol 2008;15(2):348–58. Epub 2007/12/07. doi: 10.1128/CVI.00440-07. PubMed PMID: 18057181; PubMed Central PMCID: PMCPMC2238057.
[26] Bielinska AU, Janczak KW, Landers JJ, Markovitz DM, Montefiori DC, Baker Jr JR. Nasal immunization with a recombinant HIV gp120 and nanoemulsion adjuvant produces Th1 polarized responses and neutralizing antibodies to primary HIV type 1 isolates. AIDS Res Hum Retroviruses 2008;24(2):271–81. Epub 2008/02/12. doi: 10.1089/aid.2007.0148. PubMed PMID: 18260780.
[27] Myc A, Kukowska-Latallo JF, Bielinska AU, Cao P, Myc PP, Janczak K, et al. Development of immune response that protects mice from viral pneumonitis after a single intranasal immunization with influenza A virus and nanoemulsion. Vaccine. 2003;21(25–26):3801–14. Epub 2003/08/19. doi: 10.1016/s0264-410x(03)00381-5. PubMed PMID: 12922114.
[28] Bielinska AU, Makidon PE, Janczak KW, Blanco LP, Swanson B, Smith DM, et al. Distinct pathways of humoral and cellular immunity induced with the mucosal administration of a nanoemulsion adjuvant. J Immunol 2014;192(6):2722–33. Epub 2014/02/18. doi: 10.4049/jimmunol.1301424. PubMed PMID: 24532579; PubMed Central PMCID: PMCPMC3948110.
[29] Lindell DM, Morris SB, White MP, Kallal LE, Lundy PK, Hamouda T, et al. A novel inactivated intranasal respiratory syncytial virus vaccine promotes viral clearance without Th2 associated vaccine-enhanced disease. PLoS ONE 2011;6(7):e21823. Epub 2011/07/27. doi: 10.1371/journal.pone.0021823. PubMed PMID: 21789184; PubMed Central PMCID: PMCPMC3137595.
[30] Roth K, Ferreira VH, Kaushic C. HSV-2 vaccine: current state and insights into development of a vaccine that targets genital mucosal protection. Microb Pathog 2013;58:45–54. Epub 2012/11/20. doi: 10.1016/j.micpath.2012.11.001. PubMed PMID: 23159485.
[31] Anjuere F, Bekri S, Bihl F, Braud VM, Cuburu N, Czerkinsky C, et al. B cell and T cell immunity in the female genital tract: potential of distinct mucosal routes of vaccination and role of tissue-associated dendritic cells and natural killer cells. Clin Microbiol Infect 2012;18(Suppl 5):117–22. Epub 2012/08/14. doi: 10.1111/j.1469-0691.2012.03995.x. PubMed PMID: 22882377.
[32] Stary G, Olive A, Radovic-Moreno AF, Gondek D, Alvarez D, Basto PA, et al. A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells. Science 2015;348(6241):aaa8205. Epub 2015/06/20. doi: 10.1126/science.aaa8205. PubMed PMID: 26089520; PubMed Central PMCID: PMCPMC4605428.
[33] Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11(4 Suppl):S45–53. Epub 2005/04/07. doi: 10.1038/nm1213. PubMed PMID: 15812489.
[34] Bourne N, Stanberry LR, Kern ER, Holan G, Matthews B, Bernstein DI. Dendrimers, a new class of candidate topical microbicides with activity against herpes simplex virus infection. Antimicrob Agents Chemother 2000;44(9):2471–4. Epub 2000/08/22. doi: 10.1128/aac.44.9.2471-2474.2000. PubMed PMID: 10952597; PubMed Central PMCID: PMCPMC90087.
[35] Hensel MT, Marshall JD, Dorwart MR, Heeke DS, Rao E, Tummala P, et al. Prophylactic Herpes Simplex Virus 2 (HSV-2) Vaccines Adjuvanted with Stable Emulsion and Toll-Like Receptor 9 Agonist Induce a Robust HSV-2-Specific Cell-Mediated Immune Response, Protect against Symptomatic Disease, and Reduce the Latent Viral Reservoir. J Virol 2017;91(9). Epub 2017/02/24. doi: 10.1128/JVI.02257-16. PubMed PMID: 28228587; PubMed Central PMCID: PMCPMC5391472.
[36] Bernstein DI, Earwood JD, Bravo FJ, Cohen GH, Eisenberg RJ, Clark JR, et al. Effects of herpes simplex virus type 2 glycoprotein vaccines and CLDC adjuvant on genital herpes infection in the guinea pig. Vaccine 2011;29(11):2071–8. Epub 2011/01/18. doi: 10.1016/j.vaccine.2011.01.005. PubMed PMID: 21238569; PubMed Central PMCID: PMCPMC3082315.
[37] Stanberry LR, Bernstein DI, Burke RL, Pachl C, Myers MG. Vaccination with recombinant herpes simplex virus glycoproteins: protection against initial and recurrent genital herpes. J Infect Dis 1987;155(5):914–20. Epub 1987/05/01. doi: 10.1093/infdis/155.5.914. PubMed PMID: 3031173.
[38] Bernstein DI, Cardin RD, Bravo FJ, Earwood J, Clark JR, Li Y, et al. Topical SMIP-7.7, a toll-like receptor 7 agonist, protects against genital herpes simplex virus type-2 disease in the guinea pig model of genital herpes. Antivir Chem Chemother 2014;23(5):189–96. Epub 2012/12/13. doi: 10.3851/IMP2499. PubMed PMID: 23232327.
[39] Bernstein DI, Farley N, Bravo FJ, Earwood J, McNeal M, Fairman J, et al. The adjuvant CLDC increases protection of a herpes simplex type 2 glycoprotein D vaccine in guinea pigs. Vaccine 2010;28(21):3748–53. Epub 2009/10/28. doi: 10.1016/j.vaccine.2009.10.025. PubMed PMID: 19857450; PubMed Central PMCID: PMCPMC2862079.
[40] Bernstein DI, Pullum DA, Cardin RD, Bravo FJ, Dixon DA, Kousoulas KG. The HSV-1 live attenuated VC2 vaccine provides protection against HSV-2 genital infection in the guinea pig model of genital herpes. Vaccine 2019;37(1):61–8. Epub 2018/11/26. doi: 10.1016/j.vaccine.2018.11.042. PubMed PMID: 30471955.
[41] Corey L, Langenberg AG, Ashley R, Sekulovich RE, Izu AE, Douglas Jr JM, et al. Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group JAMA 1999;282(4):331–40. Epub 1999/08/04. doi: 10.1001/jama.282.4.331. PubMed PMID: 10432030.
[42] Wang SH, Smith D, Cao Z, Chen J, Acosta H, Chichester JA, et al. Recombinant H5 hemagglutinin adjuvanted with nanoemulsion protects ferrets against pathogenic avian influenza virus challenge. Vaccine 2019;37(12):1591–600. Epub 2019/02/24. doi: 10.1016/j.vaccine.2019.02.002. PubMed PMID: 30795941.