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Title: Trivalent nucleoside-modified mRNA vaccine yields durable memory B cell protection against genital herpes in preclinical models

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Abstract

Nucleoside-modified mRNA vaccines have gained global attention because of COVID-19. We evaluated a similar vaccine approach for preventing a chronic latent genital infection rather than an acute respiratory infection. We used animal models to compare an HSV-2 trivalent nucleoside-modified mRNA vaccine with the same antigens prepared as proteins with an emphasis on antigen-specific memory B cell responses and immune correlates of protection. In guinea pigs, serum neutralizing antibody titers were higher at one month and declined far less by eight months in mRNA- than protein-immunized animals. Both vaccines protected against death and genital lesions when infected one month after immunization; however, protection was more durable in the mRNA than protein group when infected after eight months, an interval representing >15% of the animal’s lifespan. Serum and vaginal neutralizing antibody titers correlated with protection against infection as measured by genital lesions and vaginal virus titers two days post infection. In mice, the mRNA vaccine generated more antigen-specific memory B cells than the protein vaccine at early times post immunization that persisted for up to one year. High neutralizing titers and robust B cell immune memory likely explain the more durable protection by the HSV-2 mRNA vaccine.
Introduction

Nucleoside-modified mRNA-lipid nanoparticle (LNP) vaccines have gained global recognition as a delivery platform because of their impressive success preventing serious COVID-19 infections (1-3). Questions remain about the durability of protection and whether the mRNA-LNP technology will be successful for other pathogens, particularly those that establish latent or persistent infection. We previously compared immunity and efficacy of a nucleoside-modified mRNA-LNP vaccine to an adjuvanted baculovirus protein vaccine for preventing genital herpes in mice and guinea pigs (4). Both vaccines protected well against genital lesions; however, the mRNA vaccine was superior in preventing latent infection. Those studies involved intravaginal infection one month after the third (final) immunization and did not address durability. Here, we compare the two vaccines in guinea pigs one or eight months after the final immunization, a timeframe that represents >15% of the lifespan of a Hartley strain guinea pig (5).

HSV-2 encodes more than 70 proteins (6). Selecting the appropriate proteins as vaccine antigens is crucial. Past prophylactic HSV-2 vaccine efforts focused on virus entry proteins and achieved partial success (7-9). The first large human phase 3 trial included HSV-2 entry molecules glycoproteins B and D (gB2, gD2) (7). Protection against genital herpes was not durable in that it persisted for only five months. The second trial contained gD2 protein alone (8). The vaccine was effective in HSV-1/HSV-2 double seronegative women but not in HSV-1 seropositive women or men whether HSV-1 seropositive or seronegative. The third trial administered gD2 protein only to double seronegative women (9). The vaccine failed to protect double seronegative women against HSV-2 infection in this repeat trial but was effective against HSV-1 genital infection. These results support the rationale for including one or more entry proteins as immunogens but also suggest additional antigens are needed.
Attenuated live virus, subunit protein and nucleic acid vaccines for genital herpes are currently in preclinical development or human trials (10-14). Our candidate mRNA-LNP vaccine encodes three glycoproteins, gC2, gD2, and gE2 that are expressed on the virus envelope and at the surface of infected cells during virus replication (4). Glycoprotein gD2 is an entry molecule while gC2 and gE2 are immune evasion molecules (15, 16). Glycoprotein gC2 binds complement component C3b to inhibit complement activation (17-19). Glycoprotein gE2 binds the IgG Fc domain including the Fc domain of antibodies targeting the virus via their variable domains (20-23). The IgG Fc domain mediates antibody effector functions including complement activation and antibody-dependent cellular cytotoxicity. Antibodies produced by the trivalent mRNA vaccine block virus entry by gD2 and immune evasion from antibody and complement by gC2 and gE2 (4, 24). Antibodies to gC2 also neutralize virus even in the absence of complement, while antibodies to gD2 and gE2 block cell-to-cell spread (25, 26).

The primary goals of the current study were to assess the durability of immunity and protection provided by the mRNA vaccine compared to the same antigens produced as baculovirus proteins, to define immune correlates of protection, and to evaluate memory B cell responses to the vaccines. Secondary goals included evaluating different routes and frequency of immunization and determining whether prior intranasal (IN) HSV-1 infection impairs protection by the mRNA vaccine (8).
Results

Experimental design of guinea pig studies

Ninety Hartley strain female guinea pigs were divided into nine groups of 10 animals each (Table 1) to address several issues. i) To evaluate the durability of the mRNA and protein vaccines by comparing immune responses and infection outcomes when animals were challenge at one month (short-term) or eight months (long-term) after the final immunization (Table 1, groups 1-6). ii) Within these groups, we compared two with three immunizations (Table 1, groups 1 & 2), and intradermal (ID) with intramuscular (IM) routes (Table 1, groups 4 & 5). iii) To assess whether prior IN HSV-1 infection interferes with immunity and protection induced by the HSV-2 mRNA vaccine (Table 1, groups 7 & 8). The HSV-1 experiments were performed because a genital herpes human vaccine trial reported poor efficacy of an HSV-2 gD2 vaccine in individuals previously infected with HSV-1 (8). We included age-matched naïve animals that served as unimmunized controls (Table 1, group 9).

The immunizing dose selected for the mRNA vaccine was 20 µg of each mRNA and 10 µg of each protein, which was based on excellent protection by the mRNA and protein vaccines against HSV-2 intravaginal infection in guinea pigs infected one month after the final immunization in our prior report (4). In pilot studies, we evaluated higher doses of 50 µg of each mRNA or 20 µg of each protein. We noted that the mRNA administered ID at 50 µg produced erythema and some ulcers at the inoculation site in 2/10 guinea pigs, while protection in guinea pigs immunized with 20 µg of each protein was markedly inferior to 10 µg. At 20 µg, 8/10 (80%) animals developed genital lesions on 21/500 (4.2%) observation days compared to results using 10 µg in our previous study that reported 0/10 animals had genital lesions on 0/520
observation days (4). Therefore, we selected a dose of 20 µg for each mRNA and 10 µg for each protein immunogen.

Two mRNA immunizations as effective as three, ID as effective as IM

To compare two with three mRNA immunizations and IM with ID routes, guinea pigs were immunized with the mRNA vaccine two or three times and challenged one month after the final immunization (Table 1, groups 1 & 2), or immunized ID or IM and challenged eight months after the final immunization (Table 1, groups 4 & 5). Serum and vaginal neutralizing antibodies and protection against HSV-2 infection were not significantly different comparing guinea pigs immunized two or three times (Suppl. Table 1) or comparing ID and IM routes (Suppl. Table 2). Therefore, for subsequent analyses we combined the two and three immunization groups when analyzing results of animals challenged at one month, and the ID and IM routes at eight months.

Durability of antibody responses in mRNA- or protein-immunized animals

a) Serum ELISA IgG titers: Comparing groups challenged at one or eight months, IgG ELISA titers to gC2, gD2, and gE2 declined 2- to 3-fold for both the mRNA and protein vaccines (Fig 1A). Comparing mRNA with protein vaccines, the only significant difference was that gE2 titers were higher in the protein group at one and eight months (Fig 1A).

b) Serum neutralizing antibody titers: Comparing challenge at one and eight months, serum neutralizing titers declined 2.2-fold in the mRNA groups from 1:5,888 at one month to 1:2,624 at eight months (Fig 1B). In the protein groups, serum neutralizing antibody titers declined 6.2-fold from a lower initial titer of 1:1,696 at one month to 1:272 at eight months (Fig 1B). Comparing mRNA with protein vaccines, mRNA titers were 3.5-fold higher at one month and 9.6-fold higher at eight months.
c) Vaginal neutralizing antibody titers: The mRNA vaccine titers declined 3.6-fold from 1:59 at one month to 1:16.5 at eight months, while the protein group had titers of 1:9 at one month that declined to undetectable levels (<1:10) at eight months (Fig 1C). The mRNA vaginal titers were 6.5-fold higher than the protein titers at one month and remained higher by an indeterminant amount at eight months.

We conclude that serum IgG ELISA, serum neutralizing, and vaginal neutralizing titers declined somewhat in the mRNA and protein groups, but the most notable results were: i) much higher serum and vaginal neutralizing titers at one and eight months in the mRNA than protein group; and ii) a much steeper decline in serum neutralizing antibodies at eight months in the protein group.

Efficacy of the mRNA vaccine outperforms the protein vaccine

Guinea pigs were infected intravaginally with HSV-2 at 5x10^5 PFU (25 LD_{50}) one or eight months after the final mRNA or protein immunization, while the naïve (control) animals were infected at the same time and at approximately the same age. Only 1/10 naïve animals survived (Fig 2A). All mRNA-immunized animals challenged at one or eight months survived as did all animals in the protein group challenged at one month; however, 3/10 animals in the protein group required humane euthanasia when challenged at eight months.

We next evaluated genital lesions, urinary retention, and weight loss as indicators of HSV-2 clinical disease (27). In animals vaccinated with mRNA, some decline in protection against genital lesions was noted comparing one and eight months, although protection remained potent at eight months based on detecting genital lesions in 3/20 (15%) animals on 4/880 (0.5%) observation days at eight months compared with 0/20 animals on 0/880 observation days at one month (Fig 2B). Urinary retention and weight followed a similar pattern of modest decline in the
mRNA-immunized animals at eight months (Figs 2C, D). In the protein group, the decline in protection was more precipitous. Genital lesions appeared in 8/10 (80%) animals on 62/353 (17.6%) observation days at eight months compared to 0/10 animals on 0/440 observation days at one month (Fig 2B). Protection against urinary retention and weight loss also waned at eight months (Figs 2C, D). In general, protection against disease waned considerably less in the mRNA than the protein group at eight months. Although protection declined in the protein group, it remained better than in naïve controls.

More durable protection by mRNA than protein vaccine against subclinical infection

In humans, transmission of genital herpes to intimate partners often occurs during episodes of subclinical (asymptomatic) infection (28). As markers of HSV-2 subclinical infection, we measured vaginal virus titers in guinea pigs on days two and four post infection; we assessed HSV-2 reactivation from latency by measuring HSV-2 DNA copy number in vaginal swabs obtained daily on days 28 to 48 post infection (recurrent phase of infection); and we determined whether vaginal swabs that contained HSV-2 DNA also contained replication competent virus.

Day two and day four vaginal virus titers post infection (measured by plaque assay): For the mRNA vaccine, some waning of durability was apparent based on an increase in mean day two and day four vaginal swab virus titers (Figs 2E, F). A similar pattern was noted for the protein vaccine (Figs 2E, F). These titer changes in the mRNA and protein groups from one to eight months did not reach statistical significance; however, titers were lower in the mRNA than protein group at each time point (Figs 2E, F).

Vaginal shedding of HSV-2 DNA on days 28 to 48 post infection as an indicator of reactivation from latency: For the mRNA vaccine, a significant decline in durability was apparent based on the number of days with HSV-2 DNA shedding at eight months (Fig 2G, Suppl Fig 1). For the
protein vaccine, days with shedding HSV-2 DNA did not increase at eight months, perhaps because the three sickest animals succumbed to infection prior to monitoring for HSV-2 DNA shedding on day 28 (Figs 2A, G, Suppl Fig 1). Comparing the mRNA and protein groups at one month, animals in the mRNA group had significantly fewer days of HSV-2 DNA shedding than the protein group (Fig 2G, Suppl Fig 1). The mean DNA copy number was similar in all groups on days animals shed HSV-2 DNA, despite the mRNA group having fewer days of HSV-2 DNA shedding at one month (Fig 2G). As an additional indicator of latent infection, we measured HSV-2 DNA copy number in DRG and spinal cord in guinea pigs that survived until the end of the experiment (day 48 post infection). HSV-2 DNA shedding in vaginal secretions was the most sensitive assay for detecting animals with latent infection; however, some animals were positive for HSV-2 DNA only in DRG or spinal cord samples (Suppl Table 3). Overall, 7/20 (35%) animals in the mRNA group developed latent infection at one month and 12/20 (60%) at eight months compared to 8/10 (80%) in the protein group at one month and 6/7 (86%) surviving animals at eight months (Suppl Table 3).

Replication competent virus in vaginal secretions days 28 to 48 post infection: We previously reported in guinea pigs that replication competent virus was isolated from 0/636 (0%) swabs that were negative for HSV-2 DNA, while replication competent virus was isolated from 8/75 (10.7%) swabs that contained HSV-2 DNA (24). Our guinea pig results align well with studies in humans with recurrent genital herpes that reported isolating virus from 49/32,056 (0.2%) HSV DNA negative swabs, while 1038/4464 (23.3%) were positive when HSV DNA was detected (29). Based on our prior guinea pig results, we performed virus cultures only on vaginal swabs that were positive for HSV-2 DNA in the current study (24). The mRNA groups did not shed replication competent virus at one month or eight months despite shedding HSV-2 DNA on five
and 30 days, respectively (Fig 2G). In contrast, the protein groups shed replication competent virus on 3/23 (13%) DNA shedding days at one month and 2/11 (18%) days at eight months (Fig 2G, green symbols, Suppl Fig 1 stippled symbols). In total, virus was recovered on 0/35 (0%) DNA shedding days in the mRNA groups and significantly more often on 5/34 (14.7%) days in the protein groups (Fig 2G), suggesting that mRNA-immunized animals were at very low risk for shedding replication competent virus during the recurrent phase of infection.

Prior HSV-1 IN infection does not interfere with mRNA vaccine immunity or protection

A prophylactic genital herpes vaccine is intended for subjects not previously infected with HSV-2. Many of those individuals will have prior oral HSV-1 infection that may possibly impair efficacy of a genital herpes vaccine (8). We infected 20 guinea pigs IN with HSV-1 at 1x10^6 PFU to address whether prior HSV-1 infection interferes with the mRNA vaccine. One month after IN HSV-1 infection, serum was obtained and tested for HSV-1 glycoprotein G (gG1) IgG to confirm HSV-1 infection (30). All 20 animals were gG1 seropositive (titers ≥1:50) (Fig 3A), although one animal had a titer of 1:50 (Fig 3A, green symbol) that we considered borderline positive (30). That animal was clearly HSV-1-infected based on cross-reacting antibodies to HSV-2 gC2, gD2, and gE2 detected in serum evaluated eight months after HSV-1 infection (Fig 3B, green symbols). We conclude that all 20 animals were infected with HSV-1.

Antibody responses: The 20 HSV-1 infected animals were randomly assigned to either remain unimmunized or receive three immunizations with the mRNA vaccine. Antibody studies in both groups were performed on samples obtained one month after the final mRNA immunization. The mRNA vaccine significantly boosted serum ELISA and neutralizing antibody titers in animals with prior HSV-1 infection (the naïve animals are the same animals as in Fig 2) (Figs 3B, C). Vaginal neutralizing antibody titers also significantly boosted in HSV-1 infected animals (Fig
3D). We conclude that prior HSV-1 infection did not interfere with immune responses to the mRNA vaccine. In fact, animals with prior HSV-1 infection attained serum neutralizing antibody titers that were 1.6-fold higher and vaginal neutralizing antibody titers that were 6.8-fold higher than in animals not previously infected with HSV-1 (compare Figs 3C, D with 1B, C).

Protection against HSV-2 vaginal infection: The 20 HSV-1 seropositive guinea pigs were infected intravaginally with HSV-2 at 5x10^5 PFU (25 LD_{50}) and compared to the naïve group that was infected at the same time (same naïve animals as in Fig 2). Comparing HSV-1 seropositive with naïve animals, 3/10 (30%) HSV-1 seropositive (unimmunized) animals required humane euthanasia compared to 9/10 (90%) in the naïve group (Fig 3E), indicating partial protection against the most serious outcome in HSV-1 seropositive animals. Partial protection was also noted comparing genital lesions, urinary retention, weight loss and day two vaginal titers post infection (Figs 3F-I). In HSV-1 infected animals immunized with the mRNA vaccine, protection against HSV-2 challenge was greatly enhanced compared to unimmunized, HSV-1 infected animals (Figs 3E-K) and protection was as potent as in naïve animals immunized with the mRNA vaccine (compare HSV-1+ & mRNA in Figs 3E-K to mRNA 1 mo. in Figs 2A-G). We conclude that prior IN HSV-1 infection offers partial protection against genital HSV-2 and does not impair mRNA vaccine efficacy (27, 31).

Serum and vaginal neutralizing antibody titers are immune correlates of protection

We previously reported that serum neutralizing antibody titers correlated with protection against genital disease in guinea pigs immunized with a gD2 protein vaccine (32). We now expand the scope of our analysis based on 90 guinea pigs in the current study compared to 25 evaluated previously. We first evaluated serum neutralizing antibody titers in 75 animals that survived genital HSV-2 infection and 15 that required humane euthanasia. All animals with a neutralizing
titer above 1:160 survived while 15/25 (60%) animals with titers 1:160 or lower succumbed to infection, establishing serum neutralizing titers above 1:160 as a threshold for survival (Fig 4A). We next evaluated the correlation between serum neutralizing titers and genital disease (Fig 4B). A neutralizing titer above 2,560 was the threshold above which no guinea pig developed genital disease, a titer that was much higher than required to prevent death (Fig 4B). Although a high titer was needed to totally prevent genital disease, a strong correlation existed between high serum neutralizing antibody titers and fewer days with genital disease (Fig 4E).

Serum neutralizing antibody titers were significantly higher in animals that had negative day two vaginal virus titers than positive titers (Fig. 4C). No threshold value for total protection was noted, although a strong correlation was apparent between high serum neutralizing titers and low vaginal virus titers on day two (Fig 4F). Serum neutralizing titers were also significantly higher in animals with no shedding of HSV-2 DNA on days 28 to 48 post infection than animals with shedding (Fig 4D). A correlation was noted between serum neutralizing antibody titers and vaginal shedding of HSV-2 DNA (Fig 4G), although the correlation was weak, perhaps because many of the animals with low neutralizing titers succumbed to infection prior to obtaining the shedding samples.

We next evaluated the correlation between serum and vaginal neutralizing antibody titers. A very strong correlation was noted (Fig 4H). This observation led us to assess the correlation between vaginal neutralizing antibody titers and genital disease ($r=0.6291$), day two vaginal virus titers ($r=0.6791$), and vaginal shedding of HSV-2 DNA on days 28 to 48 ($r=0.3107$) (P values ranged from $P<0.0001$ to $P=0.0067$). Serum neutralizing titers had a stronger correlation than vaginal neutralizing titers with genital disease and day two vaginal virus titers, while vaginal neutralizing antibody titers had a stronger correlation with vaginal shedding of HSV-2 DNA.
Vaginal virus titers day two post infection correlate with disease and HSV-2 DNA shedding

Vaginal virus titers generally peak one to two days post infection (33). We evaluated whether day two vaginal virus titers correlated with survival, genital disease, or vaginal shedding of HSV-2 DNA (Fig 5). A day two titer < 3.41 log_{10} was the threshold value for survival. Below that titer, 0/56 (0%) animals died while 15/34 (44%) animals with higher titers required humane euthanasia (Fig 5A). For genital lesions, a day two titer <1.75 log_{10} was the threshold value for total protection. Below that titer, 0/31 (0%) animals developed genital lesions, while 28/59 (47%) with higher titers had genital lesions (Fig 5B). We detected a strong correlation between high day two virus titers and genital disease (Fig 5D). A day two virus titer <1.62 log_{10} was the threshold value for almost total prevention of vaginal HSV-2 DNA shedding (Fig 5C). Below that virus titer, only 1/31 (3%) animals had vaginal shedding, while 30/44 (68%) with higher virus titers had shedding (Fig 5C, E). We conclude that day two vaginal virus titers correlated with whether animals survived, developed genital disease, or shed HSV-2 DNA between days 28 to 48. Low day two vaginal virus titers were an important indicator of vaccine efficacy.

mRNA vaccine stimulates potent antigen-specific memory B cell responses in mice

Our results indicate that serum and vaginal neutralizing antibody titers were significantly higher at one and eight months in mRNA than protein groups (Figs 1B, C) and that neutralizing titers correlated with protection (Figs 4E-G). We previously reported in BALB/c mice that the mRNA vaccine stimulates more robust CD4^{+} T follicular helper (T_{FH}) cell and germinal center (GC) B cell responses than the protein vaccine when evaluated 10 days after one or two immunizations (4). We now assessed whether the T_{FH} and GC B cells produce more potent antigen-specific memory B cell responses. BALB/c mice (n=5/group) were immunized with mRNA, protein, or Poly(C) RNA as a control. Splenocytes were harvested at day 17 (short-term) or day 40
(intermediate) after a single immunization, while in a second experiment, splenocytes were harvested one year (long-term) after two immunizations with mRNA or three immunizations with the protein vaccine (our preferred immunization schedule for these vaccines) (4, 34, 35). Consistent with our previous report, the number of CD4+ T_{FH} cells was greatly increased (2.7-fold) in the mRNA compared to the protein group 17 days post immunization (short-term) (Fig 6A, and Suppl Fig 2A for gating strategy and 2B for a representative flow cytometry display) (4). We evaluated gD2-specific class-switched B cells and detected large differences between the mRNA and protein groups with 118-fold higher gD2-specific class-switched B cells in the mRNA group (Fig 6B) (Suppl Fig 2C, day 17). These class-switched B cells were 90% GC cells in the mRNA group compared to 25% in the protein group (Fig 6C). At the intermediate time of 40 days, the T_{FH} response remained significantly higher (2-fold) in the mRNA than protein group (Fig 6D) The difference in gD2-specific B cell response was 7-fold (Fig 6E) (an example is shown in Suppl Fig 2C, day 40). Thirty percent of the gD2-specific B cells in the mRNA group were GC B cells compared to 5% in the protein group (Fig 6F). Approximately 90% of the remaining gD2-specific B cells in the mRNA and protein groups had a memory B cell phenotype (GL7-CD38+; PDL2+ and/or CD80+), resulting in 5.1-fold more memory B cells in the mRNA than protein group (Fig 6G) (36-38). At one year (long-term), the number of gD2-specific memory B cells was 3.4-fold higher for the mRNA than protein group based on the total number of gD2-specific B cells (Fig 6H and Suppl Fig 2C) and the percent of gD2-specific B cells that were memory cells (Fig 6I). We evaluated bone marrow cells for gD2-specific antibody secreting cells (ASC) by ELISpot (Fig 6J). The number of ASC producing gD2 IgG1, IgG2a and IgG2b was approximately 2.2-fold higher in the mRNA than protein group. We conclude that in mice the mRNA vaccine produced a more robust
antigen-specific B cell memory response than the protein vaccine (summarized in Suppl Table 4), which likely explains marked differences between the two vaccines in durability of immunity and protection in guinea pigs.
**Discussion**

We demonstrated outstanding protection with a nucleoside-modified mRNA-LNP vaccine when guinea pigs were infected one or eight months after the final immunization. Key outcomes comparing the mRNA and protein vaccines were: i) serum and vaginal neutralizing antibody titers were significantly higher in the mRNA than protein groups at one and eight months; ii) both the mRNA and protein groups had no clinical disease at one month; however, at eight months major differences emerged, including some deaths in the protein group and more animals in that group developed genital lesions; iii) fewer animals in the mRNA than protein group at one month had latent infection; and iv) during the recurrent phase of infection (days 28 to 48), fewer guinea pigs in the mRNA groups had genital shedding of replication competent virus. The improved durability of the mRNA compared to protein vaccine is important because poor durability has hampered two prior HSV-2 human vaccine trials that used protein-based vaccines, one that had initial low serum neutralizing titers that rapidly waned and another where protection persisted for only 5 months (7, 9). Despite durable protection by the mRNA vaccine at eight months, we detected waning serum and vaginal neutralizing antibody titers, some breakthrough genital lesions, and more days with HSV-2 DNA shedding. These results suggest that a booster dose of the mRNA vaccine may be required to maintain potent protection for individuals at risk of acquiring genital herpes over many years, similar to emerging considerations for COVID-19 (39, 40).

Protection by the mRNA vaccine was comparable in animals immunized two or three times, and ID or IM. The IM route is more widely used than ID for human vaccines; therefore, we plan to pursue the IM route in future studies. The lack of interference by IN HSV-1 infection confirms reports in guinea pigs immunized with a gC2 and gD2 protein vaccine or live, attenuated
replication-defective HSV-2, yet conflicts with a gD2 vaccine study in humans where vaccine protection occurred in double seronegative women but not in HSV-1 seropositive women (8, 27, 31). A possible explanation for the results of the human study is that the sample size was calculated based on infection rates in double seronegative rather than HSV-1 seropositive subjects and very few infections occurred in the seropositive individuals (8). Our results indicate that prior HSV-1 infection partially protected guinea pigs against genital HSV-2 disease, which is consistent with observations in humans (41). The likelihood that prior HSV-1 infection will provide partial protection for subjects in the placebo arm of a trial will need to be considered in future genital herpes efficacy trials.

We detected a strong correlation between serum neutralizing antibody titers and vaginal neutralizing antibody titers, genital disease, and infection as measured by day two vaginal virus titers supporting the importance of neutralizing antibody titers as an immune correlate of protection for the trivalent mRNA vaccine. We detected a weaker correlation between serum or vaginal neutralizing antibody titers with vaginal shedding of HSV-2 DNA, suggesting the possibility that some immune responses not measured here, such as antibody-dependent cellular cytotoxicity, CD4 or CD8 T cells may be important for preventing HSV-2 DNA shedding (4, 42, 43). Day two vaginal virus titers post infection were key predictors of survival, genital disease, and vaginal shedding of HSV-2 DNA. Our results establish high serum and vaginal neutralizing antibody titers in guinea pigs as correlates of low day two virus titers, and low day two virus titers as a predictor of an efficacious vaccine.

We evaluated B cell immune responses in mice to explain the superior durability of the mRNA than protein vaccine. We postulated that the mRNA vaccine would stimulate a more robust and persistent antigen-specific memory B cell response than the protein vaccine (44, 45). We
assessed B cell memory responses in mice rather than guinea pigs because better reagents are available for mice and our prior results demonstrated that the mRNA vaccine was highly protective in mice (4, 16). Major differences emerged between the mRNA and protein vaccines that favored the mRNA vaccine in stimulating $T_{FH}$, antigen-specific GC B cells and antigen-specific memory B cells over the course of one year. The potent and persistent memory B cell response contributes to the outstanding durability of the mRNA vaccine. Our results are similar to those reported for antigen-specific memory B cell responses in mice and humans that received SARS-CoV-2 nucleoside-modified mRNA-LNP vaccines (44, 46). We measured memory B cells in splenocytes, and ASCs derived from bone marrow. Whether these cells can be identified in genital tract tissues as demonstrated in human biopsy samples remains to be determined (47). B cells circulate to genital tract tissues in response to a second exposure to genital HSV-2 (48). Our results suggest that prior IM or ID immunization with the mRNA vaccine may produce a similar genital tract response after the first exposure to HSV-2.

A potential limitation of our study is that we used a high concentration of 20 $\mu$g of each mRNA for immunization (60 $\mu$g total). Our study was initiated prior to the COVID-19 pandemic. Subsequent experience with mRNA-LNP vaccines for COVID-19 indicated that more severe side-effects occurred at higher mRNA-LNP concentrations (49, 50). Further studies will be required to determine the lowest concentration of gC2, gD2, and gE2 mRNA that will achieve outstanding protection, and to confirm that the mRNA vaccine outperforms the protein vaccine at these lower concentrations. Another possible shortfall is that the COVID-19 pandemic interrupted our immunization schedule because all laboratory work was halted for approximately 3 months. The impact was that some immunizations were separated by a longer time frame than initially intended. Nevertheless, our conclusions accurately represent the performance of the
mRNA and protein vaccines when administered on a similar schedule. A third potential concern is that we evaluated protein antigens prepared in baculovirus. Perhaps protection by protein vaccines may be improved using antigens prepared in mammalian instead of insect cells or using different adjuvants. Despite these possible limitations, we consider the trivalent nucleoside-modified mRNA-LNP vaccine to be an outstanding candidate for human trials because of the potency of the serum and vaginal neutralizing antibody responses and the durability of immunity and protection.
Materials and Methods

Immunization protocols

Guinea pigs: Experiments were performed in female Hartley strain guinea pigs (Charles River) to address durability of protection, two versus three immunizations, ID versus IM routes, and whether prior HSV-1 infection interfered with the HSV-2 mRNA vaccine. HSV-1 infection was performed by IN inoculation of $5 \times 10^5$ PFU HSV-1 strain NS into each nostril (total $1 \times 10^6$ PFU) (27). Female Hartley strain guinea pigs were immunized IM (hind limb hip muscle) or ID (denuded back) two or three times with gC2, gD2, gE2 nucleoside-modified mRNA-LNP containing 20 µg of each mRNA (total 60 µg), or IM three times containing 10 µg each (30 µg total) of the same three glycoprotein antigens administered as baculovirus proteins with 100 µg CpG and 150 µg alum, or IM three times with 20 µg Poly(C) RNA-LNP (control). The mRNA and baculovirus constructs have been previously described (4, 24). The LNP was prepared by Acuitas (4, 25, 27). Animals were bled prior to intravaginal infection, and all animals were infected on the same day using $5 \times 10^5$ PFU (25 LD$_{50}$) HSV-2 strain MS (4). Animals were monitored for survival, genital lesions, urinary retention, weight loss, vaginal virus titers on days two and four post infection, vaginal shedding of HSV-2 DNA and replication competent virus on days 28 to 48 post infection, and HSV-2 DNA in DRG and spinal cord at the end of the experiment (4). Weight loss was measured on days 1-14 post infection. Animals that succumbed before day 14 were assigned their last recorded weight for subsequent days. Scoring for days with lesions was performed by two investigators blinded to group, and the score assigned was by consensus. A score of one was assigned each day with one or more genital lesions.

Mice: T$_{FH}$, GC and memory B cell studies were performed in female BALB/c mice (Charles River) that were immunized with 10 µg each gC2, gD2, gE2 mRNA-LNP (total 30 µg), or 5 µg
each gC2, gD2, gE2 protein (total 15 µg) with 50 µg CpG and 75 µg alum or 10 or 30 µg Poly(C) RNA-LNP as a control and evaluated for T<sub>FH</sub>, GC and memory B cell responses. These doses were considered optimal for both mRNA and protein based on our prior studies (4).

Antibody assays:

Serum IgG ELISA for gC2, gD2, and gE2 was performed using baculovirus proteins (24). Serum gG1 IgG ELISA was performed using gG1 (Abcam, ab43048) at 100 ng per well, and serial 2-fold dilutions of guinea pig serum starting at 1:50. Serum and vaginal neutralizing assays used 100 PFU of HSV-2 strain MS and 5% human serum as source of complement obtained from an HSV-1/HSV-2 seronegative volunteer. Vaginal secretions for neutralizing titers were obtained using an eye spear swab (BVI) that was placed in 100 µl PBS and centrifuged to elute the antibodies prior to removing the swab. The number of virus plaques was determined on Vero cells (ATCC CCL-81). The endpoint titer was considered the dilution that reduced the virus plaque number by 50%. (25).

Vaginal swabs and virus titers

Vaginal swabs were obtained on days two and four and days 28 to 48 post infection and placed in 1 ml of complete Dulbecco’s modified Eagle’s medium (DMEM, containing HEPES, L-glutamine, and antibiotics) and 5% fetal bovine serum (FBS). Serial 10-fold dilutions were evaluated by plaque assays on Vero cells using 300 µl in the first well resulting in a limit of detection of 3.3 PFU/mL. Vaginal virus titers <1:10 were assigned a titer of 1:5 when calculating mean titers for day two and day four samples. Virus cultures for replication competent virus on days 28 to 48 were only performed on samples that were positive for HSV-2 DNA.

qPCR for HSV-2 DNA in vaginal secretions, DRG and spinal cord
Vaginal swabs were collected daily from days 28 to 48 post infection as above. Two hundred microliters were used for DNA purification (QiaCube HT) and five µl of purified DNA were processed for DNA amplification (Roche LightCycler 96) using primers and probe for HSV-2 Us9 DNA (34). Samples with less than one copy of HSV-2 DNA by 40 cycles were considered negative while positive samples were confirmed in duplicate. The limit of detection of the assay is 200 copies of HSV-2 DNA/mL. DRG were stored in 1 mL DMEM containing 5% FBS. The media was removed and lysis buffer (Qiagen) added overnight at 56°C then 200 µl processed for DNA purification (QiaCube HT) (51). Spinal cord samples were handled similarly, except samples were homogenized prior to lysis. Five microliters of purified DNA from DRG or spinal cord were amplified in duplicate (Roche LightCycler 96) using primers and probes for Us9 DNA and copy number calculated as log10 DNA copies per 10^6 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (27).

Fluorescent labeling of gD2

Baculovirus gD2 protein was conjugated to either PE or Alexa-fluor 647 using Lightning Link (LL) chemistry (Novus Biologicals, 703-0010 and 336-0005). The gD2 was diluted to 0.2 mg/mL in PBS and 20 µg reacted in the presence of 1:10 (v/v) of LL-modifier for three hours at RT. The labeling reaction was stopped in the presence of 1:10 (v/v) LL-quencher for 30 min. and stored at 4°C.

Flow cytometry for T<sub>FH</sub>, GC and memory B cells in BALB/c mice

Splenocytes were isolated at 17 days, 40 days, or one-year post immunization, stained with gD2 antigen probes and antibodies, and analyzed on a BD LSR II flow cytometer (BD Biosciences) (52). The gating schemes for T<sub>FH</sub> cells, gD2<sup>+</sup> B cells, gD2<sup>+</sup> GC B cells and gD2<sup>+</sup> memory B cells
are shown in Suppl Fig 2 (36-38). The following antibodies were used: CD80 BV650 clone 16-10A1, IgM PECF594 clone R6-60.2, and CD8 PECy5 clone 53-6.7 (BD Biosciences), CD38 AF700 clone 90, CD19 PECy5.5 clone eBio103, and F4/80 PECy5 clone BM8 (Thermo Fisher), PD-L2 BV421 clone TY25, CD23 FITC clone B3B4, IgD BV711 clone 11-26c.2a, CD21 APC-Cy7 clone 7E9, GL7 PECy7 clone GL7, CD11c BV605 clone N418, B220 BV785 clone RA3-6B2, CD4 PECy5 clone H129.19, Gr-1 PECy5 clone RB6-8C5, and Zombie Aqua viability dye (BioLegend).

**ASC ELISpot**

ELISpot plates (Millipore, MSIPN4W50) were coated with gD2 protein antigen at 10 µg/mL at pH 9.6 and for one hour at 37°C. Wells were blocked with RPMI and 10% FBS for 30 min. Bone marrow was harvested from femurs and tibia, placed into FACS buffer consisting of PBS (Roche) and 0.1% BSA (Sigma) and filtered through a 63 µm Nitex nylon mesh (Genesee Scientific). Red blood cells were lysed in ACK buffer (Scripps), and the remaining cells were serially diluted starting with one million cells and incubated overnight in RPMI and 10% FBS. Biotinylated detection antibody (Southern Biotech, 1050-08, 1060-08, 1070-08, 1080-08, 1090-08, 1100-08; Biolegend, RMA-1: 400703, RMM-1: 406504) was added at RT for one hour followed by streptavidin-alkaline phosphatase at RT for 30 min (Sigma, E2636). BCIP/NBT single solution (Sigma, B1911) was added until spots were visible that were then quenched in 1M sodium phosphate monobasic solution. Plates were dried overnight, scanned, and counted using CTL Immunospot hardware and software (44, 53).

**Statistics**

P values are displayed in the figures, figure legends, and tables if P ≤ 0.05 or in some cases P values >0.05 are shown to indicate that an analysis was performed. We used the term significant
to indicate a P value ≤0.05. The methods used to calculate P values are noted in the figure legends. We used the two-tailed Mann-Whitney test for nonparametric distribution, unpaired t test for parametric distribution, and the two-tailed Fisher’s exact test to compare the event ratio of two groups. We used the Kruskal-Wallis test with Dunn’s correction for multiple comparisons or ordinary one-way ANOVA with multiple comparisons. We used the log-rank test for survival, and Spearman correlation to calculate r and P values in correlation graphs, and Mann-Whitney-Wilcoxon Test with Holm adjustment for multiple comparisons to calculate area under the curve for weight loss P values. Analyses were performed using GraphPad Prism version 9.1.0 (GraphPad Software Inc.), and R software version 4.0.2 (R Core Team, 2020) for weight loss.

**Study approval**

The guinea pig and mouse studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee under protocol 805187.
Author contributions: S.A. designed and performed the guinea pig immunizations, infections, scoring, DRG and spinal cord tissue harvests, ELISA and neutralizing antibody assays, prepared some figures. J.J.K. performed the T\textsubscript{FH}, GC and memory B cell studies, prepared the flow cytometry figures and graphs, and wrote the methods section for the flow cytometry studies. A.D. prepared the Graphic Abstract, immunized and sustained the mice for the one-year B cell studies and assisted with the B cell ELISpot assay. M-G.A. labeled the gD2 protein with fluorophores and assisted J.L.K. in the B cell flow cytometry studies. J.M.L., L.M.H. and K.P.E. assisted S.A. in the guinea pig studies. L.M.H. prepared the final version of the figures. A.N. performed the qPCR studies. B.T. G. performed the ASC ELISpot studies, prepared the ELISpot graphs and wrote the methods section for the ELISpot studies. Y.K.T. was responsible for the LNP encapsulation of the mRNA constructs. N.P. prepared mRNA constructs and helped with ID guinea pig immunizations. D.A. supervised B.T.G. and assisted in the interpretation of data. E.T.L.P and M.P.C supervised J.J.K. and advised on interpretation of the B cell flow cytometry studies. D.W. supervised the preparation of nucleoside-modified mRNA and advised on experimental design. G.H.C. supervised the preparation of protein immunogens, participated in experimental design and editing the manuscript. H.M.F. oversaw the entire project, participated in planning the experiment, assisted with the guinea pig studies, performed the statistical analyses in collaboration with P.S. and G.C., prepared many of the figures and wrote the first draft and final version of the manuscript.

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Competing interests: In accordance with the University of Pennsylvania policies and procedures and our ethical obligations as researchers, we report that H.M.F., S.A., and G.H.C. are named on patents that describe the use of multiple subunit glycoprotein antigens for HSV vaccines. H.M.F., S.A., G.H.C. and D.W. are named on a patent that uses nucleoside-modified mRNA as a vaccine for HSV. D.W. is named on patents that describe the use of nucleoside-modified mRNA as a platform to deliver therapeutic proteins. D.W., N.P. and Y.K.T. are named on a patent describing the use of nucleoside-modified mRNA in LNP as a vaccine platform. We have disclosed those interests fully to the University of Pennsylvania, and we have in place an approved plan for managing any potential conflicts arising from licensing of our patents. Y.K.T. is an employee of Acuitas Therapeutics, a company developing LNP for delivery of nucleic acid-based drugs.
References


Figure 1. Serum and vaginal antibody titers in guinea pigs. A. gC2, gD2 and gE2 serum IgG ELISA titers of naïve (unimmunized) animal samples obtained five months after entering the animal colony or mRNA- and protein-immunized animals one or eight months after the final immunization. B-C. Serum and vaginal fluid neutralizing antibody titers one or eight months after the final immunization. n=10/group for naïve, protein 1 mo. and protein 8 mo.; n=20 for mRNA 1 mo. and mRNA 8 mo. P values were calculated by the two-tailed Mann-Whitney test for gC2 and gD2 ELISA, and by the Kruskal-Wallis test with Dunn’s correction for multiple comparisons for gE2 ELISA, serum, and vaginal neutralizing titers.
Figure 2. Enhanced efficacy of mRNA compared to protein vaccine in guinea pigs. A. Survival. P values compare groups with 100% survival with protein 8 mo. or naïve. B. Percent days with genital disease. Actual number of days with genital disease shown above graph. C. Days with urinary retention measured days 1-20 post infection. D. Weight loss: P=0.1064 comparing naïve and protein at eight months; P=0.0038 comparing mRNA and protein at eight months; P= 0.0029 comparing protein at one and eight months; P= 0.0205 comparing mRNA at one and eight months. E-F. Day two and day four vaginal virus titers post infection. G. Vaginal shedding HSV-2 DNA days 28 to 48 post infection. Numbers above the data points represent the number of days HSV-2 shedding was detected (numerator) and the total number of days sampled (denominator). Numbers below the data points represent the number of days replication competent virus was isolated (numerator) and the total number of days of HSV-2 DNA shedding (denominator). The green symbol represents an HSV-2 DNA sample with replication competent virus. n=10/group for naïve, protein 1 mo. and 8 mo., n=20/group for mRNA 1 mo. and 8 mo. P values were calculated by the Log-rank test in A; by the Kruskal-Wallis test with Dunn’s correction for multiple comparisons in B, C, E and F; by the Mann-Whitney-Wilcoxon Test with Holm adjustment for multiple comparisons in D; and by two-tailed Fisher’s exact test for numbers above or below graphs in B and G.
Figure 3. mRNA vaccine is immunogenic and efficacious in guinea pigs previously infected IN with HSV-1. A. gG1 ELISA titers from four animals prior to HSV-1 infection and 20 animals one month after HSV-1. B. Serum IgG ELISA titers. C-D. Serum and vaginal neutralizing antibody titers. E-K. Survival, genital disease, urinary retention, weight loss, day two and day four vaginal virus titers, and vaginal shedding of HSV-2 DNA and replication competent virus days 28 to 48 post infection. Weight loss in H: P=0.0524 comparing naïve and HSV-1+; P=0.0294 comparing HSV-1+ to HSV-1+ & mRNA; P<0.0001 comparing naïve to HSV-1+ & mRNA. Numbers above the data points in K represent days with HSV-2 shedding and total days sampled. Green symbol indicates the sample contained replication competent virus. n=10 animals/group for B-K, except naïve group in K, n=1 survivor. P values calculated by the two-tailed Mann-Whitney test A-D; the Log-rank test in E; the Kruskal-Wallis test with Dunn’s adjustment for multiple comparisons in F, G, I, J; Mann-Whitney-Wilcoxon Test with Holm adjustment for multiple comparisons in H; and two-tailed Fisher’s exact test in K.
Figure 4. Serum neutralizing antibody titers in guinea pigs are immune correlates of protection. A-D. Serum neutralizing titers in animals that survived infection, developed no genital disease, had negative day two vaginal cultures post infection, or did not shed HSV-2 DNA in vaginal secretions days 28 to 48 post infection. Dotted lines in A and B represent threshold values for protection. E-H. Correlation of serum neutralizing antibody titers with genital lesions, day two vaginal virus titers, days with vaginal shedding of HSV-2 DNA, and vaginal neutralizing antibody titers. The size and color of symbols reflect the number of overlapping values at that point. P values were calculated by the two-tailed Mann-Whitney test in A-D, and by Spearman correlation in E-H.
Figure 5. Day two vaginal virus titers in guinea pigs correlate with survival, genital disease, and vaginal shedding of HSV-2 DNA. A-C. Threshold values of vaginal virus titers on day two post infection for survival, genital disease, and vaginal shedding of HSV-2 DNA on days 28 to 48. Dotted lines represent the threshold value below which no animal or only one animal developed the outcome. D. Correlation of day two vaginal virus titers with genital disease E. Correlation of day two vaginal virus titers with HSV-2 DNA vaginal shedding. P values were calculated by the two-tailed Mann-Whitney test in A-C, and by Spearman correlation in D & E.
Figure 6. **TFH cell, gD2-specific GC B cell and memory B cell responses are more potent for the mRNA than protein vaccine in mice.**

A. TFH response on day 17 after one immunization with mRNA, protein, or Poly(C) as a control. B. gD2+ B cells on day 17. C. Percent gD2+ B cells with GC phenotype on day 17. D-F. TFH cells, gD2+ B cells and percent gD2+ B cells with GC phenotype 40 days after one immunization. G. Frequency of CD80 and PD-L2 expression in gD2+ memory (GL7-CD38+) B cells on day 40. H. gD2+ B cells one year after two mRNA or three protein immunizations. I. Frequency of CD80 and PD-L2 expression in gD2+ memory B cells at one year. J. IgG1, IgG2a, IgG2b gD2-antibody secreting cells (ASC) at one year detected by ELISpot. P values were calculated by Ordinary one-way ANOVA in A, B, D, E, H, J, and by unpaired t test in C, F.
Table 1. Schema of guinea pig studies

<table>
<thead>
<tr>
<th>Groups (n=10/group)</th>
<th>Purpose</th>
<th>Immunization schedule</th>
<th>HSV-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. mRNA, 2</td>
<td>Short-term protection &amp; compare 2 mRNA with 3 mRNA immunizations (group 2)</td>
<td>0 mo. ID, 3 mo. IM (mixed ID &amp; IM)</td>
<td>1 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. mRNA, 3</td>
<td>Short-term protection &amp; compare 2 mRNA (group 1) with 3 mRNA immunizations</td>
<td>0, 1 mo. ID, 4 mo. IM (mixed ID &amp; IM)</td>
<td>1 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Protein, 3</td>
<td>Short-term protection to compare with mRNA groups 1 &amp; 2</td>
<td>0, 1, 4 mo. IM (only IM)</td>
<td>1 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. mRNA, 3 ID</td>
<td>Long-term protection &amp; compare mRNA ID with mRNA IM immunization (group 5)</td>
<td>0, 1, 2 mo. ID (only ID)</td>
<td>8 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. mRNA, 3 IM</td>
<td>Long-term protection &amp; compare mRNA ID (group 4) with mRNA IM immunization</td>
<td>0, 1, 2 mo. IM (only IM)</td>
<td>8 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Protein, 3</td>
<td>Long-term protection to compare with mRNA groups 4 &amp; 5</td>
<td>0, 1, 2 mo. IM (only IM)</td>
<td>8 mo.</td>
</tr>
<tr>
<td>immunizations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. HSV-1 infection,</td>
<td>Determine if HSV-1 infection alters mRNA vaccine (compare to group 8)</td>
<td>None</td>
<td>1 mo.</td>
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<tr>
<td>no vaccine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>8. HSV-1 infection,</td>
<td>Infect with HSV-1, then immunize with mRNA (compare to group 7)</td>
<td>3, 4 mo. ID, 7 mo. IM (mixed ID &amp; IM)</td>
<td>1 mo.</td>
</tr>
<tr>
<td>then mRNA x 3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>9. Unimmunized</td>
<td>Naïve controls</td>
<td>None</td>
<td>All groups challenged same time</td>
</tr>
<tr>
<td>controls</td>
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</table>

*Intravaginal HSV-2 challenge was performed one or eight months (mo.) after the final immunization.