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## Nucleoside-modified mRNA Encoding HSV-2 Glycoproteins C, D, E Prevents Clinical and Subclinical Genital Herpes

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### Abstract

The goals of a genital herpes vaccine are to prevent painful genital lesions and reduce or eliminate subclinical infection that risks transmission to partners and newborns. We evaluated a trivalent glycoprotein vaccine containing herpes simplex virus type 2 (HSV-2) entry molecule glycoprotein D (gD2) and two immune evasion molecules, glycoprotein C (gC2) that binds complement C3b, and glycoprotein E (gE2) that blocks IgG Fc activities. The trivalent vaccine was administered either as baculovirus proteins with CpG and alum, or the identical amino acids were expressed using nucleoside-modified mRNA in lipid nanoparticles. Both formulations completely prevented genital lesions in mice and guinea pigs. Differences emerged when evaluating subclinical infection. The trivalent protein vaccine prevented dorsal root ganglia infection and day 2 and 4 vaginal cultures were negative in 23/30 (73%) mice compared with 63/64 (98%) in the mRNA group ( $P=0.0012$ ). In guinea pigs, 5/10 (50%) animals in the trivalent subunit protein group had vaginal shedding of HSV-2 DNA on 19/210 (9%) days compared with 2/10 (20%) animals in the

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Author Contributions: S.A. designed and performed the murine studies, many of the immunology studies, prepared many of the figures, and performed some of the statistical analysis in collaboration with P.S. in the Department of Biostatistics; L.M.H. assisted with the murine studies, performed epitope mapping and prepared the final version of figures; N.P. prepared the nucleoside-modified mRNA, performed cell transfections and assisted with murine studies; F.W. performed qPCR for HSV-2 DNA; A.M. and M.P.C. performed the Tfh and GC B cell studies; G.H.C. supervised preparation of subunit proteins and epitope mapping; D.W. supervised preparation and planning of nucleoside-modified mRNA-LNP studies; and H.M.F. oversaw the entire project, performed some of the statistical analysis in collaboration with P.S. and wrote the manuscript.

Competing Interests: In accordance with the University of Pennsylvania policies and procedures and our ethical obligations as researchers, we report that Harvey Friedman, Sita Awasthi, and Gary Cohen are named on patents that describe the use of multiple subunit glycoprotein antigens for HSV vaccines. Harvey Friedman, Sita Awasthi and Drew Weissman have submitted a patent claim that uses nucleoside-modified mRNA as a vaccine for HSV. Drew Weissman is named on patents that describe the use of nucleoside-modified mRNA as a platform to deliver therapeutic proteins. Drew Weissman and Norbert Pardi are also named on a patent describing the use of nucleoside-modified mRNA in lipid nanoparticles 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.

Data and materials availability

All data are available within the article and will be made available upon request.

Access to subunit antigens must be obtained through a MTA.

mRNA group that shed HSV-2 DNA on 5/210 (2%) days ( $P=0.0052$ ). Immunology assays were performed in mice. The trivalent mRNA vaccine was superior to trivalent proteins in stimulating serum and vaginal ELISA IgG antibodies, serum neutralizing antibodies, antibodies that bind to crucial gD2 epitopes involved in entry and cell-to-cell spread, CD4<sup>+</sup> T cell responses, and T follicular helper and germinal center B cell responses. The trivalent nucleoside-modified mRNA-LNP vaccine is a promising candidate for human trials.

### One Sentence Summary:

Improved efficacy using mRNA for a herpes vaccine

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### Introduction

Herpes simplex virus type 2 (HSV-2) is a common sexually transmitted infection with 14% prevalence among 15 to 49 year old persons in the USA and 11% globally (1). Individuals remain infected for life, with periodic clinical recurrences that may be painful or subclinical recurrences that risk transmission of infection to intimate partners and newborns (2). The incidence of neonatal herpes is approximately 14,000 cases annually worldwide (3) and results in substantial morbidity and mortality because of encephalitis, pneumonia and hepatitis even with antiviral therapy (4, 5). Genital herpes increases the risk of acquisition and transmission of HIV by 3–4-fold, further highlighting the need for an effective prophylactic vaccine (6).

Previous HSV-2 prophylactic human vaccine efforts included immunizing with two essential virus entry molecules, HSV-2 glycoproteins B (gB2) and D (gD2) (7), or gD2 alone (8, 9). In the gB2/gD2 trial, vaccine recipients had delayed onset of infection, but overall were not protected (7). In the first of two reports using gD2 alone, a subgroup analysis indicated that HSV-1/HSV-2 doubly seronegative women, but not men were protected against HSV-2 genital lesions (8). A repeat study performed in doubly seronegative women failed to confirm this result but noted 58% protection against genital lesions caused by HSV-1. Sixty percent of the cases of genital herpes in the control group were caused by HSV-1 (9). ELISA antibodies to gD2 correlated with protection against genital HSV-1 infection (10). Maternal and neonatal antibodies that neutralize HSV or that mediate antibody-dependent cellular cytotoxicity also correlate with protection against severe neonatal herpes (11–13). These studies suggest that antibodies are important for prevention of herpes infection.

Our vaccine strategy is designed to produce potent antibody responses and includes an entry molecule, gD and two additional HSV glycoproteins, gC and gE. HSV-1 and HSV-2 gC and gE are immune evasion molecules that block the effectiveness of antibody responses (14–16). HSV gC binds complement component C3b to inhibit complement activation, while gE binds the IgG Fc domain of antibodies targeting HSV antigens to block IgG Fc activities, including complement activation and antibody-dependent cellular cytotoxicity (ADCC) (17–20). Antibodies produced to the three glycoproteins perform multiple antiviral activities, including neutralizing virus (gC2 and gD2), blocking cell to cell spread (gD2 and gE2), and preventing immune evasion from antibody and complement (gC2 and gE2) (16, 21–24).

No genital herpes vaccine has prevented both clinical and subclinical infection. Our prior vaccine studies in mice and guinea pigs used baculovirus-produced gC2, gD2, and gE2 subunit proteins with CpG and alum as adjuvants. The vaccine provided strong protection against clinical lesions in mice (100%) and guinea pigs (98%) but it did not prevent vaginal virus replication on days 2 and 4 post-infection in mice and 17/36 (47%) guinea pigs developed subclinical infection detected by vaginal shedding of HSV-2 DNA between days 28 and 49 post-infection (16, 22). Here we evaluated whether an immunization strategy that uses nucleoside-modified mRNA to encode the same three HSV-2 glycoproteins improves vaccine efficacy.

Nucleoside-modified mRNA vaccines have recently emerged as highly promising for infectious disease vaccine development (25). Modifications in mRNA increase translation and reduce inflammatory side effects. Modifications include altering the 5' cap, the 5' and 3'-UTRs, poly A tails, replacing uridine with 1-methylpseudouridine, and removing double stranded RNA contaminants. Non-inflammatory mRNA is beneficial because innate immune sensors recognize mRNA, release type I interferons and activate interferon-inducible genes that inhibit translation (26). Various formulations protect nucleoside-modified mRNA from RNases and facilitate efficient *in vivo* delivery, including lipid nanoparticles (LNPs) (27, 28). Here we demonstrate that the HSV-2 gC2, gD2 and gE2 trivalent nucleoside-modified mRNA-LNP vaccine outperformed the trivalent protein vaccine given with CpG and alum and achieved outstanding vaccine efficacy in mice and guinea pigs.

## Results

The primary goal was to determine whether the trivalent nucleoside-modified mRNA-LNP vaccine provides better protection than the trivalent protein-CpG/alum vaccine. A secondary goal was to determine whether the trivalent mRNA-LNP vaccine outperformed gD2 nucleoside-modified mRNA-LNP alone as support for adding gC2 and gE2 mRNA to the vaccine.

### Characterization of the truncated gC2, gD2 and gE2 proteins produced in cells transfected with nucleoside-modified mRNA-LNP

Nucleoside-modified mRNA was prepared to encode gC2 amino acids 27–426, gD2 amino acids 26–331, and gE2 amino acids 24–405, each truncated prior to their transmembrane domains. Expression of the glycoproteins was assessed in HEK 293 cells that were transfected with the mRNAs or with PolyC RNA as a control. The proteins in the mRNA-transfected cells reacted with gC2, gD2 or gE2 antibodies by Western blot and were of the expected size when accounting for different glycosylation patterns in mammalian cells (Figs. S1a–c). Supernatant fluids from the transfected cells contained the glycoprotein antigens when assayed by ELISA (Figs. S1d–f). We conclude that cells transfected with each nucleoside-modified mRNA synthesized and secreted the truncated glycoprotein.

### Vaccine efficacy in mice

We infected mice intravaginally with either  $5 \times 10^3$  or  $5 \times 10^4$  PFU of HSV-2 to assess protection over a 10-fold titer range.

### Protection against $5 \times 10^3$ PFU (275 LD<sub>50</sub>) intravaginal HSV-2 after intradermal immunization with nucleoside-modified mRNA-LNP

BALB/c mice were immunized intradermally (ID) twice at a 28-day interval with nucleoside-modified mRNA-LNP consisting of 10 $\mu$ g of gD2-LNP mRNA, 10 $\mu$ g each of gC2, gD2 and gE2 mRNA combined in an LNP, or 10 $\mu$ g of PolyC RNA-LNP as a control. For comparison, mice were immunized intramuscularly (IM) three times at two-week intervals with 5 $\mu$ g each of gC2, gD2 and gE2 baculovirus-expressed proteins combined with CpG/alum. These concentrations and dosing interval were chosen based on our prior studies in mice using baculovirus subunit protein antigens and mRNA-LNP studies performed with other pathogen vaccines (21, 22, 29–31). Twenty-eight days after the last immunization, mice were treated with medroxyprogesterone to synchronize and thin the vaginal epithelium and five days later infected intravaginally with  $5 \times 10^3$  PFU of HSV-2 strain MS (275 LD<sub>50</sub>). All mice in the PolyC control group were euthanized by day 10, while no other animals died (Fig. 1a).

No animal in the trivalent mRNA, trivalent protein, or gD2 mRNA group developed ruffled fur, hunched posture, abnormal gait or lethargy as disease indicators. Weights of animals in the trivalent mRNA, trivalent protein or gD2 mRNA group did not differ significantly from one another, yet each group differed significantly from the PolyC RNA controls (Fig. 1b). No genital disease developed in the trivalent mRNA or trivalent protein group. Mild genital disease occurred in one animal in the gD2 mRNA group. Overall, these three groups did not differ significantly from one another, while extensive genital disease developed in the PolyC RNA mice (Fig. 1c).

Day 2 and 4 vaginal cultures measure acute viral infection and represent a stringent test of vaccine efficacy. Strikingly, 39/39 animals in the mRNA trivalent group had negative vaginal cultures on day 2, which was significantly different from 7/10 in the trivalent protein group and 16/20 in the gD2 mRNA group (Fig. 1d). Significant differences between the trivalent mRNA and the trivalent protein or the gD2 mRNA group were also detected on day 4 post-infection (Fig. 1d). We assessed day 2 and day 4 vaginal HSV-2 DNA copy number by qPCR to further evaluate indicators of virus infection. Approximately 5 log<sub>10</sub> HSV-2 DNA was detected in the trivalent mRNA group on day 2 and approximately 4 log<sub>10</sub> on day 4 despite recovering no replication competent virus on either day, which suggests that the virus was coated with neutralizing antibodies and/or degraded (Fig. 1e). By day 4, the HSV-2 DNA copy number was significantly lower in the trivalent mRNA mice than the trivalent protein or gD2 mRNA alone mice (Fig. 1e).

Peak HSV-2 DNA copy number in dorsal root ganglia (DRG) develops on day 4 or 5 post-challenge in naïve mice (22). All 10 mice in the PolyC RNA group had HSV-2 DNA detected in DRG on day 4, compared with 3/10 in the gD2 mRNA group, 1/19 in the trivalent mRNA group and 5/10 in the trivalent subunit protein group. Differences between trivalent mRNA and trivalent subunit protein were significant, while trivalent mRNA and gD2 mRNA differences were not significant (Fig. 1f).

### **Protection against $5 \times 10^4$ PFU (2,750 LD<sub>50</sub>) intravaginal HSV-2 in mice**

As a more stringent test of vaccine efficacy, five mice were immunized ID twice with 10 $\mu$ g each of trivalent mRNA-LNP and infected with a 10-fold higher HSV-2 dose,  $5 \times 10^4$  PFU strain MS (2,750 LD<sub>50</sub>). We did not include the trivalent protein group because breakthrough infections occurred at the lower challenge dose. All 5 mice survived, had no genital disease, had negative vaginal cultures on days 2 and 4 and had no HSV-2 DNA detected in DRG at the end of the experiment on day 28 (Fig. 1g). Therefore, no breakthrough infections occurred even at a 10-fold higher challenge dose.

### **Vaccine efficacy in mice: Protection against intravaginal HSV-2 after IM immunization with nucleoside-modified mRNA-LNP**

Our trivalent subunit protein vaccine studies in mice use IM immunization (22). We evaluated the trivalent mRNA vaccine given IM to compare the two vaccine formulations using the same immunization route. Mice were immunized as in Figure 1 except one additional group received trivalent mRNA-LNP using 2 $\mu$ g of each immunogen to assess protection at lower mRNA concentrations. Animals were challenged with  $5 \times 10^3$  HSV-2 strain MS (275 LD<sub>50</sub>). All PolyC control animals were euthanized by day 11, while all other animals survived (Fig. 2a). No animal in the 2 $\mu$ g or 10 $\mu$ g trivalent mRNA or trivalent subunit protein group developed ruffled fur, hunched posture, abnormal gait or lethargy and no significant differences in weight loss were detected comparing these three groups, while the PolyC controls had extensive weight loss (Fig. 2b). The PolyC group developed severe genital disease, while the other groups were totally protected (Fig. 2c). Day 2 vaginal cultures were positive in all 15 mice in the PolyC RNA group, 1/10 in the trivalent subunit protein group, 4/5 in the 2 $\mu$ g trivalent mRNA group and 0/20 in the 10 $\mu$ g trivalent mRNA group (Fig. 2d). All 20 mice in the 10 $\mu$ g trivalent mRNA group also had negative vaginal cultures on day 4, as noted in ID immunized animals (Figs. 2d). DRG were harvested at the time of euthanasia in nine mice from the PolyC group or at the end of the experiment on day 28 in the other groups. All nine animals in the PolyC group had HSV-2 DNA detected in DRG, while 0/10 were positive in the trivalent subunit protein group, 1/5 in the 2 $\mu$ g trivalent mRNA group, and 0/20 in the 10 $\mu$ g trivalent mRNA group (Fig. 2e). We conclude that dose-response protection was apparent in the trivalent mRNA mice immunized IM based on significantly better protection at 10 $\mu$ g than 2 $\mu$ g against vaginal infection on day 2 and slightly better protection of DRG. Although 10 $\mu$ g trivalent mRNA outperformed trivalent subunit protein when evaluating days 2 and 4 vaginal cultures, differences were not statistically significant.

### **Combining the ID and IM results in mice**

We combined the results of IM and ID for the 10 $\mu$ g trivalent mRNA-immunized mice because both routes produced virtually identical results. The trivalent subunit protein and trivalent mRNA mice were both totally protected against clinical disease as measured by death, genital lesions and signs of generalized illness (Table 1). Differences between the two vaccine formulations emerged when evaluating subclinical infection measured by days 2 and 4 vaginal cultures and HSV-2 DNA in DRG (Table 1). Based on clinical and subclinical infection, immunization ID or IM with 10 $\mu$ g trivalent mRNA achieved sterilizing immunity

in 63/64 (98%) mice compared with 23/30 (77%) in the trivalent subunit protein and 15/20 (75%) in the gD2 mRNA group, while 0/25 (0%) in the PolyC RNA group had sterilizing immunity (Table 1). The breakthrough infections in the trivalent subunit protein group included 5/10 animals that had HSV-2 DNA in DRG, which represents more DRG infection than we reported in a prior study (22). In the current study we used a more stringent criterion for a negative result based on normalizing the HSV-2 DNA copy number to  $10^5$  adipsin genes rather than  $10^4$  previously.

### Transmission studies in mice

Transmission studies were performed using day 2 vaginal secretions collected from 10 mice immunized ID with 10 $\mu$ g trivalent mRNA-LNP or PolyC RNA-LNP as a control and infected with  $5 \times 10^3$  PFU (275 LD<sub>50</sub>). The vaginal secretions (20 $\mu$ l) from each mouse were inoculated intravaginally into a naïve mouse. An additional five naïve mice were inoculated with  $5 \times 10^3$  HSV-2 as a positive control and as expected, all five mice became ill and were euthanized. Three of 10 mice inoculated with vaginal secretions from the PolyC RNA group developed disease and were euthanized compared with 0/10 from the trivalent-mRNA group (Fig. S2a). No animal in the trivalent mRNA group lost weight, while 6/10 in the PolyC and 5/5 in the HSV-2 control group lost weight (Fig. S2b). All naïve animals in the HSV-2-inoculated group and 6/10 in the PolyC RNA group developed genital disease compared with 0/10 in the trivalent mRNA group. Differences were also noted comparing days 2 and 4 vaginal titers of mice inoculated with vaginal secretions from trivalent mRNA or PolyC RNA mice with 6/10 mice in the PolyC RNA group positive on both days compared to 0/10 in the trivalent mRNA group (Fig. S2c–e). We did not evaluate transmission using vaginal fluids from subunit vaccine-immunized animals; however, 4/20 mice in this group had positive day 2 titers compared with 0/64 in the modified mRNA group. Therefore, we postulate that more mice in the baculovirus protein group would transmit virus. HSV-2 DNA was detected in the day 2 vaginal fluids of most mice in the trivalent mRNA group yet no replication competent virus was detected and no transmission of infection occurred to naïve mice. This result is consistent with the concept that on day 2 the virus was either coated with neutralizing antibody or degraded. Overall, no naïve mouse inoculated with 20 $\mu$ l of vaginal secretions from the 10 $\mu$ g trivalent mRNA group died, developed genital disease, or had positive cultures on days 2 or 4.

### Vaccine efficacy in guinea pigs

The guinea pig genital infection model is generally viewed as a more stringent test of vaccine efficacy and more biologically relevant in that guinea pigs develop recurrent genital lesions and recurrent vaginal shedding of HSV-2 DNA, while mice do not. Genital HSV-2 shedding indicates reactivation from latency and is a marker of subclinical infection when it occurs in the absence of genital lesions. Nucleoside-modified mRNA-LNP vaccines have not previously been evaluated in guinea pigs; therefore, we decided to use three immunizations, rather than two as in mice. We chose a dose of 20 $\mu$ g per nucleoside-modified mRNA rather than the 10 $\mu$ g dose used in mice based on the larger body size of guinea pigs. Female Hartley strain guinea pigs (10 animals per group) were immunized ID three times at one month intervals with 20 $\mu$ g each of gC2, gD2 and gE2 nucleoside-modified mRNA-LNP; IM with 10 $\mu$ g each of gC2, gD2 and gE2 baculovirus subunit proteins with CpG/alum; IM with

10 $\mu$ g PolyC RNA-LNP as a control; or left unimmunized. One month after the final immunization, animals were infected intravaginally with 5 $\times$ 10<sup>5</sup> PFU (50 LD<sub>50</sub>) HSV-2 MS, which is the dose we used in prior studies with the trivalent baculovirus protein vaccine (16). The group that was not immunized were not infected. Animals were observed for survival, weight loss, and genital lesions. Eight of 10 animals in the PolyC RNA-LNP group became ill and were euthanized by day 20 post-infection, while no animal in the trivalent mRNA, trivalent protein, or uninfected group died (Fig. 3a), developed weight loss (Fig. 3b), hind limb weakness or urinary retention. Animals in the PolyC RNA-LNP group had extensive genital lesions, while no genital lesions appeared in the other groups over 60 days (Fig. 3c).

We performed vaginal cultures on days 2 and 4 post-infection. Nine of 10 guinea pigs in the PolyC RNA-LNP group had positive titers on day 2. The negative day 2 titer in one animal indicates that animal did not get infected, which is consistent with the observation that one animal in the PolyC RNA group did not lose weight or develop genital lesions. In the trivalent mRNA and trivalent subunit protein groups, 5/10 animals had positive vaginal titers on day 2. The positive vaginal cultures represent the mean of two determinations in a total of 300 $\mu$ l of vaginal fluid. Two animals in the trivalent mRNA group and one animal in the trivalent subunit protein group had 1 virus plaque detected (Fig. 3d). Vaginal titers remained positive on day 4 in 9/10 animals in the PolyC group and 2/10 in the trivalent subunit protein group. In contrast, all 10 animals in the trivalent mRNA group had negative vaginal cultures on day 4 (Fig. 3d). Therefore, no animal in the trivalent mRNA group had a positive vaginal virus titer beyond day 2. We evaluated day 2 and day 4 HSV-2 DNA copy number by qPCR. No significant differences were detected comparing trivalent mRNA with trivalent subunit protein animals although DNA copy numbers were generally lower in the trivalent mRNA group (Fig. 3e). Some guinea pigs had high copy numbers of HSV-2 DNA despite negative virus cultures, suggesting that the virus was neutralized and/or degraded, as in the murine studies.

The inoculation titer used for the guinea pig experiments was 5 $\times$ 10<sup>5</sup> PFU, which is 10-fold to 100-fold higher than the inoculum used in mice. The low positive day 2 titers in the trivalent mRNA and trivalent protein groups may represent residual input virus and/or local replication of virus in genital tissues. An important goal of the vaccine is to prevent the virus from reaching sites where virus establishes latency in ganglia that serve as a source for reactivation infection. We performed daily vaginal swabs for 21 days starting on day 28 post-infection to detect vaginal shedding of HSV-2 DNA as a marker of reactivation infection. Two animals in the PolyC RNA group survived until day 21, but only one was infected based on day 2 titers. We did not detect HSV-2 DNA in vaginal swabs from either the infected or uninfected animal. No animal in the uninfected group shed HSV-2 DNA. In the trivalent subunit protein group, 5/10 animals shed HSV-2 DNA on 19/210 (9.0%) days. In contrast, 2/10 guinea pigs shed HSV-2 DNA in the trivalent nucleoside-modified mRNA-LNP group on a total of 5/210 (2.4%) days (P=0.0052) (Fig. 3f). Virus cultures were performed to detect replication competent virus on days that animals had vaginal shedding of HSV-2 DNA. We performed virus cultures only on samples that were positive for HSV-2 DNA because we consistently fail to recover replication competent virus on days animals are not shedding HSV-2 DNA (32). In the trivalent mRNA group, 0/5 HSV-2 DNA positive samples yielded replication competent virus, compared with 1/19 in the trivalent protein

group. Spinal cord and DRG were evaluated for HSV-2 DNA at the end of the experiment. One of two animals in the PolyC RNA group was positive for HSV-2 DNA in DRG and spinal cord (the animal with disease), while 1/10 in the trivalent mRNA group was positive for HSV-2 DNA in spinal cord, but not DRG. The positive animal was the guinea pig that had vaginal shedding of HSV-2 DNA on 4 days from days 47 to 50. In the trivalent subunit protein group, 0/10 animals was positive for HSV-2 DNA in spinal cord or DRG, suggesting that detecting HSV-2 DNA in vaginal secretions is a more sensitive assay for subclinical infection than assays performed at the end of the experiment to detect HSV-2 DNA in DRG or spinal cord (Fig. 3g). Key guinea pig results are summarized in Fig. 3h.

### Immunology Assays in Mice

In both mice and guinea pigs, vaccine efficacy was better with the trivalent nucleoside-modified mRNA-LNP formulation than with the trivalent subunit protein vaccine. We performed immunologic analyses in mice to evaluate the mechanisms of the enhanced protection.

#### Serum IgG antibody responses in mice

ELISA endpoint IgG titers were evaluated prior to infection on sera collected 28 days after the first and second (final) mRNA ID immunization or 28 days after the third (final) trivalent subunit protein IM immunization. ELISA titers increased comparing the first and second immunization for each nucleoside-modified mRNA (Fig. 4a–c). The nucleoside-modified mRNA produced significantly higher ELISA endpoint titers to each immunogen after the second immunization compared to the baculovirus-expressed subunit proteins after the third immunization (Fig. 4a–c). After the final immunization, the ratio of IgG2a to IgG1 in mice immunized with trivalent mRNA was 0.89, while the ratio in animals immunized with trivalent subunit proteins was 1.04, suggesting a balanced  $T_H1/T_H2$  response for both (Fig. S3) (33).

#### Vaginal wash IgG antibody responses in mice

Vaginal mucosa fluids were obtained prior to infection at four weeks after the final immunization. IgG vaginal mucosa antibody responses to gC2 and gE2 were significantly higher in mice immunized with trivalent mRNA-LNP than trivalent subunit proteins (Figs. 4d, f). Titers were also higher to gD2 in the trivalent mRNA mice, although differences were not significant (Fig. 4e).

#### Serum neutralizing antibodies in mice

HSV-2 neutralizing antibody titers in the presence of complement were evaluated after the second (final) mRNA immunization or third (final) subunit protein immunization. The neutralizing antibody titers in the trivalent mRNA group were higher than in the trivalent subunit protein group (Fig. 4g). Neutralizing titers were also higher in the trivalent mRNA group than in the gD2 mRNA group, indicating that gC2 and/or gE2 antibodies enhance neutralization (Fig. 4g) (16, 21, 22). HSV-1 is a frequent cause of genital herpes (9); therefore, we evaluated the same sera for neutralizing antibody titers against HSV-1. Each of the vaccine formulations produced high titers of neutralizing antibodies against HSV-1 (Fig.



4h). The trivalent mRNA vaccine produced higher HSV-1 neutralizing antibody titers than trivalent subunit proteins or gD2 mRNA, although the differences did not reach statistical significance (Fig. 4h).

### **Antibody-dependent cellular cytotoxicity responses in mice**

The control PolyC RNA sera failed to stimulate ADCC activity mediated by murine Fc $\gamma$ RIII, while sera from the trivalent mRNA, the trivalent subunit protein and the gD2 mRNA mice produced significantly greater ADCC responses (Fig. 4i). ADCC responses did not differ comparing trivalent mRNA and trivalent subunit protein, suggesting that ADCC does not explain the superior performance of the trivalent mRNA vaccine (Fig. 4i).

### **Antibodies that block immune evasion domains on gC2 and gE2 in mice**

We reported that immunization with trivalent subunit proteins produced higher titers of antibodies that blocked gC2 binding to C3b than produced by HSV-2 infection (34). Here we assessed whether trivalent mRNA immunization produces higher titers of blocking antibodies than trivalent subunit proteins. We compared multiple concentrations of IgG purified from sera obtained four weeks after the final immunization with trivalent mRNA-LNP or trivalent subunit proteins. The IgG was incubated with 40ng of gC2 to determine whether the antibodies block gC2 binding to C3b on an ELISA plate (16, 21). IgG from gD2 mRNA-immunized mice was included as a negative control. At 10 $\mu$ g/ml or 20 $\mu$ g/ml IgG, the trivalent mRNA-LNP significantly outperformed trivalent subunit proteins in blocking gC2 binding to C3b (Fig. 5a). At 50 $\mu$ g/ml IgG, both trivalent mRNA and trivalent subunit protein totally blocked gC2 binding to C3b (Fig. 5a). The results indicate that both trivalent mRNA and trivalent subunit proteins produce antibodies that block gC2 binding to C3b, but that the trivalent mRNA produces higher titers of blocking antibodies.

HSV-2 gE2 functions as an IgG Fc receptor (22, 35). We evaluated whether gE2 antibody produced by immunization with trivalent mRNA is more effective than trivalent subunit proteins at blocking gE2 protein binding to non-immune IgG Fc (16, 22). As a control, we assessed whether gE2 antibodies are more effective than gD2 antibodies at blocking gE2 binding to non-immune IgG. This control takes into consideration that gE2 antibodies can bind to gE2 protein by their F(ab')<sub>2</sub> domain and/or Fc domain, while antibodies to gD2 protein can only bind by their Fc domain. We used 4 or 2 $\mu$ g/ml of gE2 protein that was incubated with 62.5 $\mu$ g/ml of IgG from immunized mice. Comparing trivalent mRNA with trivalent subunit proteins at a gE2 protein concentration of 4 $\mu$ g/ml, IgG from the trivalent mRNA mice blocked gE2 binding better than trivalent subunit proteins, suggesting that the trivalent mRNA produced higher titers of gE2 IgG Fc-blocking antibodies (Fig. 5b). These differences were not apparent at a lower gE2 concentration of 2 $\mu$ g/ml, although both vaccine formulations blocked gE2 protein binding to IgG Fc significantly better than gD2 antibody (Fig. 5b). These results indicate that immunization with both trivalent mRNA and trivalent subunit proteins produce antibodies that block gE2 binding to IgG Fc, but higher titers of blocking antibodies are produced by trivalent mRNA immunization.

### Antibodies to gD2 epitopes involved in virus entry and cell-to-cell spread in mice

We recently evaluated epitope-specific antibody responses to 3 linear and 4 conformational gD2 epitopes that are crucial for gD2 function (23). The gD2 epitopes are involved in virus binding to herpesvirus entry mediator (HVEM) and/or nectin-1 receptors, gD2 interaction with HSV-2 downstream entry molecules glycoproteins H and L (gH2/gL2), or cell-to-cell spread (36–38). We reported that guinea pigs immunized with gD2-CpG/alum produced a range of responses to crucial epitopes. Some animals produced antibodies to few epitopes, while others produced antibodies to most or all epitopes. The more crucial epitopes blocked, the better the protection was against intravaginal HSV-2 challenge (23).

Here we evaluated antibody responses in mice to six gD2 epitopes that are involved in receptor binding, interaction with gH2/gL2 or cell-to-cell spread. The assay places monoclonal antibodies (MAbs) that recognize each of the gD2 crucial epitopes on a biosensor chip. Individual sera obtained after the second immunization with trivalent mRNA or third immunization with trivalent subunit proteins were incubated with gD2 protein and floated over the MAb-coated chip. Sera from mice immunized with PolyC RNA served as a control. Blocking gD2 protein binding to MAb on the biosensor chip indicates that antibody is present in the mouse serum to the epitope recognized by the MAb. The percent blocking of gD2 binding to prototype MAbs that recognize the six crucial epitopes is shown in Figs. 5c–h. The trivalent mRNA immunized mice produced antibodies that blocked gD2 binding significantly better than trivalent subunit proteins to 3/6 crucial epitopes. Although significant, the differences were fairly small for two of these three epitopes, 77S and MC5, but more impressive for the MC14 epitope (blocks cell-to-cell spread) (Figs. 5d, f, h). These results indicate that most mice immunized with either vaccine formulation produced antibodies to the gD2 epitopes, although trivalent mRNA outperformed trivalent subunit proteins by producing higher titers of blocking antibodies to 3/6 epitopes.

### CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in mice

Splenocytes were harvested two weeks after the second trivalent mRNA or third trivalent subunit protein immunization. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were stimulated *in vitro* with a single gD2 overlapping peptide pool, and gC2 or gE2 overlapping peptides each divided into two pools. The peptide pools span the entire amino acid sequences of the subunit proteins. As a control, T cells were stimulated with DMSO, the vehicle used to dissolve the overlapping peptides. CD4<sup>+</sup> T cells in mice immunized with trivalent mRNA significantly increased production of IFN $\gamma$  alone or both IFN $\gamma$  and TNF $\alpha$  when stimulated with gD2, gC2 or gE2 peptide pools, compared with DMSO alone (Figs. 6a, b). Trivalent subunit protein immunization was less effective at stimulating CD4<sup>+</sup> T cell cytokine production (Figs. 6d, e). The trivalent mRNA and subunit protein groups did not produce robust CD8<sup>+</sup> T cell responses. In the mRNA mice, only gE2 stimulated a significant increase in CD8<sup>+</sup> IFN $\gamma$  production, while in the protein subunit group, gD2 pool 1 stimulated an increase in CD8<sup>+</sup> IFN $\gamma$  production that approached statistical significance (Fig. 6c, f). We conclude that mice immunized with trivalent mRNA stimulated a more robust CD4<sup>+</sup> T cell response than mice immunized with trivalent subunit proteins, and a marginally better CD8<sup>+</sup> T cell response.

## T follicular helper (Tfh) and germinal center (GC) B cell responses in mice

Tfh cells are important for forming and maintaining GCs, stimulating class switching and B cell affinity maturation, and developing memory B cells and long-lived plasma cells (39, 40). Nucleoside-modified mRNA-LNP vaccines are potent inducers of CD4<sup>+</sup> Tfh and GC B cell responses (30). We evaluated the number of splenic CD4<sup>+</sup> Tfh cells and GC B cells 10 days after a single immunization with trivalent mRNA-LNP or trivalent subunit proteins-CpG/alum. The flow cytometer gating strategy for Tfh and GC B cells is shown in Figs. S4a,b. Tfh cell responses were significantly higher in animals immunized with trivalent mRNA than trivalent subunit proteins (Fig. 6g). The Tfh response in the trivalent subunit protein group was not significantly higher than in the CpG/alum controls. Germinal center B cell responses were also significantly higher in the trivalent mRNA than subunit protein mice, while differences between CpG/alum and trivalent subunit proteins were not significant (Fig. 6h). We performed an additional experiment that included PolyC RNA-LNP as a control to assess the effects of LNP. Mice were immunized with PolyC RNA-LNP and responses compared with naïve mice or mice immunized with trivalent mRNA-LNP. Splenocytes were harvested 10 days after the second immunization. Trivalent mRNA-LNP produced significantly higher Tfh and GC B cell responses than PolyC RNA-LNP mice (Fig. 6i, j). These results indicate that trivalent mRNA-LNP induces a more potent Tfh and GC B cell response than trivalent subunit proteins with CpG/alum. Overall, where differences in immune responses were detected comparing trivalent mRNA with trivalent subunit protein, responses favored the trivalent mRNA vaccine.

## Discussion

The virus titer that results in genital herpes infection in humans is unknown making it difficult to choose the optimal challenge dose in animals. We selected a high challenge dose by using 275 or 2,750 LD<sub>50</sub> of HSV-2 in mice and 50 LD<sub>50</sub> in guinea pigs. Despite these high doses, no mouse immunized ID or IM with 10µg trivalent nucleoside-modified mRNA-LNP and no guinea pig immunized with 20µg trivalent nucleoside-modified mRNA-LNP developed genital disease, although it is possible that at even higher challenge doses some breakthrough infection may occur. Mice and guinea pigs were also well-protected against subclinical infection. Commonly used indicators of subclinical infection in mice include vaginal swab cultures on days 2 and 4 and HSV-2 DNA in DRG, while a useful marker for subclinical infection in guinea pigs is vaginal shedding of HSV-2 DNA after resolution of the acute infection (41–43). By these criteria, only 1/64 mice and 2/10 guinea pigs developed subclinical infection. Overall, guinea pigs shed HSV-2 DNA on 2.4% of days and no replication competent virus was recovered in the vaginal secretions, suggesting that the risk of transmission is very low in these animals. Our decision to immunize with three antigens was based on our prior studies that reported adding gC2 to gD2 subunit protein improved protection in mice and guinea pigs, and although performed in different studies, we noted that adding gE2 to gC2/gD2 subunit proteins further improved protection in mice (16, 21, 22). These results are consistent with our current study demonstrating that trivalent mRNA-LNP provides better protection than gD2 mRNA-LNP alone.

Trivalent nucleoside-modified mRNA-LNP immunization outperformed the same immunogens administered as subunit proteins with CpG/alum in mice and guinea pigs. Our choice of 10µg of each nucleoside-modified mRNA in mice was partially guided by dose-response studies using nucleoside-modified mRNA to express firefly luciferase and vaccine studies of Zika, influenza and HIV antigens in mice (29, 30, 44). The choice of 10µg seems appropriate based on the nearly complete protection provided by trivalent mRNA at this concentration and lower efficacy at 2µg. Immunization with 20µg of each trivalent nucleoside-modified mRNA in guinea pigs was based on the larger body mass of guinea pigs than mice. Our choice of 5µg for subunit proteins in mice and 10µg in guinea pigs was based on our prior results demonstrating excellent protection in mice and guinea pigs against genital lesions at these concentrations (16, 22). A limitation of our study is that we did not evaluate whether higher concentrations of trivalent modified mRNA-LNP or trivalent subunit proteins will further improve vaccine efficacy. We also did not assess the durability of immunity and protection. The potent Tfh cell and GC B cell responses produced by the trivalent mRNA immunogens suggest that durable protection is likely. Impressive durability was demonstrated for HIV, influenza and Zika when animals were immunized by the same nucleoside-modified mRNA-LNP platform as used here (29, 30). The high antibody titers and potent Tfh and GC B cell responses noted with other nucleoside modified mRNA-LNP immunogens, including influenza, Zika, and HIV was a key reason we chose to evaluate nucleoside-modified mRNA as a delivery mechanism for our candidate HSV-2 vaccine (25, 30).

We performed multiple immunology assays to assess mechanisms by which the trivalent nucleoside-modified mRNA outperformed the trivalent protein vaccine. Where differences emerged, mRNA was superior including: i) serum and vaginal IgG ELISA titers; ii) HSV-2 neutralizing antibody titers; iii) antibodies that block gC2 and gE2 immune evasion domains; iv) antibody responses to gD2 epitopes involved in binding to receptors, interaction with gH2/gL2 and cell-to-cell spread; v) CD4<sup>+</sup> T cell responses; and vi) Tfh cell and GC B cell responses. It seems likely that a combination of these immune responses accounts for the enhanced performance of the trivalent mRNA vaccine. The prolonged expression of antigens using nucleoside-modified mRNA-LNP delivery and the Tfh adjuvant activity of LNPs may explain the more potent immune responses (44, 45). Although trivalent nucleoside-modified mRNA outperformed trivalent subunit protein CpG/alum, it is possible that new adjuvants may improve the efficacy of subunit protein vaccines.

Few preclinical HSV-2 vaccine studies have been published that evaluated the same antigens and adjuvants that were subsequently used in human trials. One exception is the gD2 subunit protein vaccine administered with MPL/alum that was evaluated in a guinea pig model of genital herpes. The gD2-MPL/alum vaccine prevented genital lesions in 9/10 guinea pigs in one study and 10/12 in another, but all animals developed subclinical infection as measured by vaginal shedding of HSV-2 DNA (46, 47). In our current study, the trivalent nucleoside modified mRNA-LNP vaccine prevented genital lesions in 100% of guinea pigs and subclinical genital shedding of HSV-2 DNA in 80%, which surpasses the results obtained using an immunogen that entered phase 3 human trials. We conclude that the trivalent nucleoside-modified mRNA-LNP vaccine is a promising candidate for future human trials.

## Materials and Methods

### Study Design

The primary goal was to assess whether the trivalent nucleoside-modified mRNA-LNP vaccine outperformed the same antigens expressed as baculovirus subunit proteins administered with CpG/alum. A secondary goal was to evaluate whether adding gC2 and gE2 nucleoside-modified mRNA-LNP to gD2 nucleoside-modified mRNA-LNP improved the protection provided by gD2. Mice and guinea pigs were immunized with nucleoside-modified mRNA-LNP immunogens or baculovirus subunit protein-CpG/alum antigens. Serum and vaginal wash fluids were obtained approximately one month after the final immunization and animals were then infected Ivag with HSV-2 strain MS. Mice: Vaginal swabs were obtained for cultures on days 2 and 4 post-infection; animals were weighed daily for 2 weeks and scored for genital disease for 4 weeks; DRG were obtained either on day 4 post-infection or at the end of the experiment. Guinea pigs: Vaginal swabs were obtained for cultures on days 2 and 4 post-infection; animals were scored for genital disease for 60 days; swabs were obtained for vaginal shedding of HSV-2 DNA for 21 days beginning 28 days post-infection; DRG and spinal cord were harvested for HSV-2 DNA at the end of the experiment. Scoring for genital disease in mice was performed without blinding, while scoring in guinea pigs was done blinded.

### Ethics statement

All animal studies were conducted under protocol 805187 approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

### Production of bac-gC2, bac-gD2 and bac-gE2 subunit proteins, production of mRNA, formulation in LNPs, and Western blots

The baculovirus proteins were produced as previously described (22, 48, 49). The methods used to synthesize trivalent nucleoside-modified mRNA-LNP and to perform Western blots are described in the legend to Fig. S1.

### ELISA, neutralizing antibody, and ADCC titers

Serum IgG ELISA titers and IgG1/IgG2a subtype titers were evaluated as previously described (16, 50). ELISA vaginal wash fluid titers were assessed by adding 30 $\mu$ l of DMEM with 5% fetal bovine serum to the vaginal cavity and retrieving the fluid several seconds later. This procedure was repeated once. Mucosal IgG antibody in vaginal fluid was measured at a 1:50 dilution. Neutralizing antibody titers were reported as the serum dilution that reduced the number of virus plaques by 50% (21). ADCC was measured using the murine Fc $\gamma$ RIII ADCC Reporter Bioassay (Promega Corporation) that binds murine IgG1 and IgG2a (23, 51).

### Blocking C3b binding to gC2 and IgG Fc binding to gE2

Serum (10 $\mu$ l) from each mouse in a group was pooled and the IgG purified on a NAb Protein G Spin Column (Pierce Biotechnology). Blocking gC2 binding to C3b was performed by coating ELISA plates with 200ng of purified C3b protein (Complement Technologies),

incubating 40ng of purified gC2 protein with varying concentrations of purified IgG, and detecting bound gC2 (34). Blocking gE2 binding to IgG Fc was evaluated using HSV-1/HSV-2 seronegative human IgG (1mg/well) to coat ELISA wells, and incubating 62.5µg/ml of purified IgG from immunized mice with 2 or 4µg/ml bac-gE2(24–405t) for 15 min at room temperature, and detecting bound gE2 (16).

### **Biosensor-based antibody competition assay**

The Cytiva Microfluidics continuous flow microspotter surface plasmon resonance imaging (CFM/SPRi) system was used to detect epitope-specific antibody responses to crucial gD2 epitopes involved in virus entry and cell-to-cell spread (23, 24).

### **CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses and Tfh and GC B cell responses**

Spleens were pooled from 5 animals per group and 10<sup>6</sup> splenocytes were stimulated at 37°C with gC2, gD2 or gE2 peptide pools consisting of 15 amino acids with 11 overlapping amino acids (JPT Innovative Peptide Solutions). After 1 h, brefeldin A (10µg/ml) (BD Pharmingen) was added for 16 h at 37°C. Splenocytes were stained with aqua blue (Invitrogen) to distinguish live-dead cells, Pacific blue-conjugated anti-CD8 mouse MAb (Biolegend) and R-phycoerythrin–cyanine 5.5 (PE-Cy5.5) anti-CD4 mouse MAb (BD Pharmingen). Cells were permeabilized with Cytotfix/Cytoperm (BD Pharmingen), stained with Alexa Fluor 700-conjugated anti-IFNγ mouse MAb, PE-Cy7 anti-TNFα mouse MAb, and allophycocyanin-Cy7 anti-CD3 mouse MAb. Splenocytes were fixed with 1% paraformaldehyde and analyzed by FACS using an 18-color LSR II flow cytometer and FlowJo flow cytometry analytic software (21, 52, 53). Details of the staining and gating strategy for Tfh and GC B cells are presented in the legend to Fig. S4.

### **Immunizations, vaginal infections and scoring**

BALB/c mice (Charles River): The gC2, gD2, and gE2 baculovirus subunit proteins were individually incubated at RT for two h with 16.7µg of CpG oligonucleotide 5'-TCCATGACGTTCCCTGACGTT-3' (Coley Pharmaceutical) and 25µg of alum/µg protein (Alhydrogel; Accurate Chemical and Scientific Corp.) and combined prior to immunization in a volume of 50µl (22). Mock IM immunizations were performed with CpG and alum without proteins. The trivalent (gC2, gD2, and gE2) mRNAs were combined prior to LNP encapsulation. ID immunizations with trivalent mRNA-LNP, polyC RNA-LNP or gD2 mRNA-LNP were administered in four sites on the flank using a 29-gauge insulin syringe containing 30µl for each site, while IM immunizations with LNP-containing immunogens were given in 50µl into the gastrocnemius muscle. Mice were infected Ivag one month after the final immunization and scored for genital disease (22).

Hartley strain guinea pigs (Charles River): 10µg of gC2, gD2, gE2 subunit proteins were mixed individually and then combined to form a final concentration of 100µg CpG (5'-TCGTCGTTGTCGTTTGTGTCGTT-3') (Trilink Inc.) per guinea pig and 150µg of alum (16). ID immunizations and Ivag infections one month after the final immunization were performed as in mice, except 40µl was inoculated ID at each site (16).

### **Virus cultures from vaginal swabs**

Vaginal swabs were placed in one ml Dulbecco's modified Eagle's medium containing 5% fetal bovine serum supplemented with 25µg/ml vancomycin. For viral titers, 150µl was evaluated in mice and 300µl in guinea pigs. Serial 10-fold dilutions were added to Vero cells to assess virus titers by plaque assay (21).

### **HSV-2 DNA isolation, real-time qPCR for HSV-2 DNA copy number in DRG, spinal cord and in vaginal swab samples**

Mouse DRG samples were analyzed in duplicate for HSV-2 DNA as previously described, except separate reactions were used to amplify HSV-2 DNA and the mouse adipsin gene and DRG HSV-2 DNA copy number was expressed as log<sub>10</sub> DNA copies per 10<sup>5</sup> adipsin genes instead of 10<sup>4</sup> (22). HSV-2 DNA copy number in guinea pig DRG and spinal cord samples were calculated as log<sub>10</sub> DNA copies per 10<sup>6</sup> GAPDH genes (54). HSV-2 DNA in day 2 and 4 vaginal swab samples in mice and guinea pigs and in vaginal samples obtained on 21 days between days 28–50 in guinea pigs were run as single samples and expressed as HSV-2 DNA copies per ml (16). Samples with <1 copy by 40 cycles were considered negative.

### **Statistical analysis**

P values are displayed in the figures for primary endpoints (trivalent mRNA-LNP compared with trivalent subunit protein-CpG/alum) and secondary endpoints (trivalent mRNA-LNP compared with gD2 mRNA-LNP). We used the Mann-Whitney test for these two-group comparisons. Some P values are also noted in the figure legends. For some comparisons, the mice in one treatment group all had the same response (e.g. zero as the lower limit of quantification) and the two-tailed Fisher's exact test was used to compare the values. For comparisons looking across several time points, we used two-way ANOVA followed by Tukey adjustment for significance. We used the log-rank test to compare survival distributions between groups. We used Wilcoxon signed-rank test to compare samples from the same animal at different time points. All significance tests were two-sided and performed at P<0.05. Analyses were done using GraphPad Prism version 7.0 (GraphPad Software, Inc.).

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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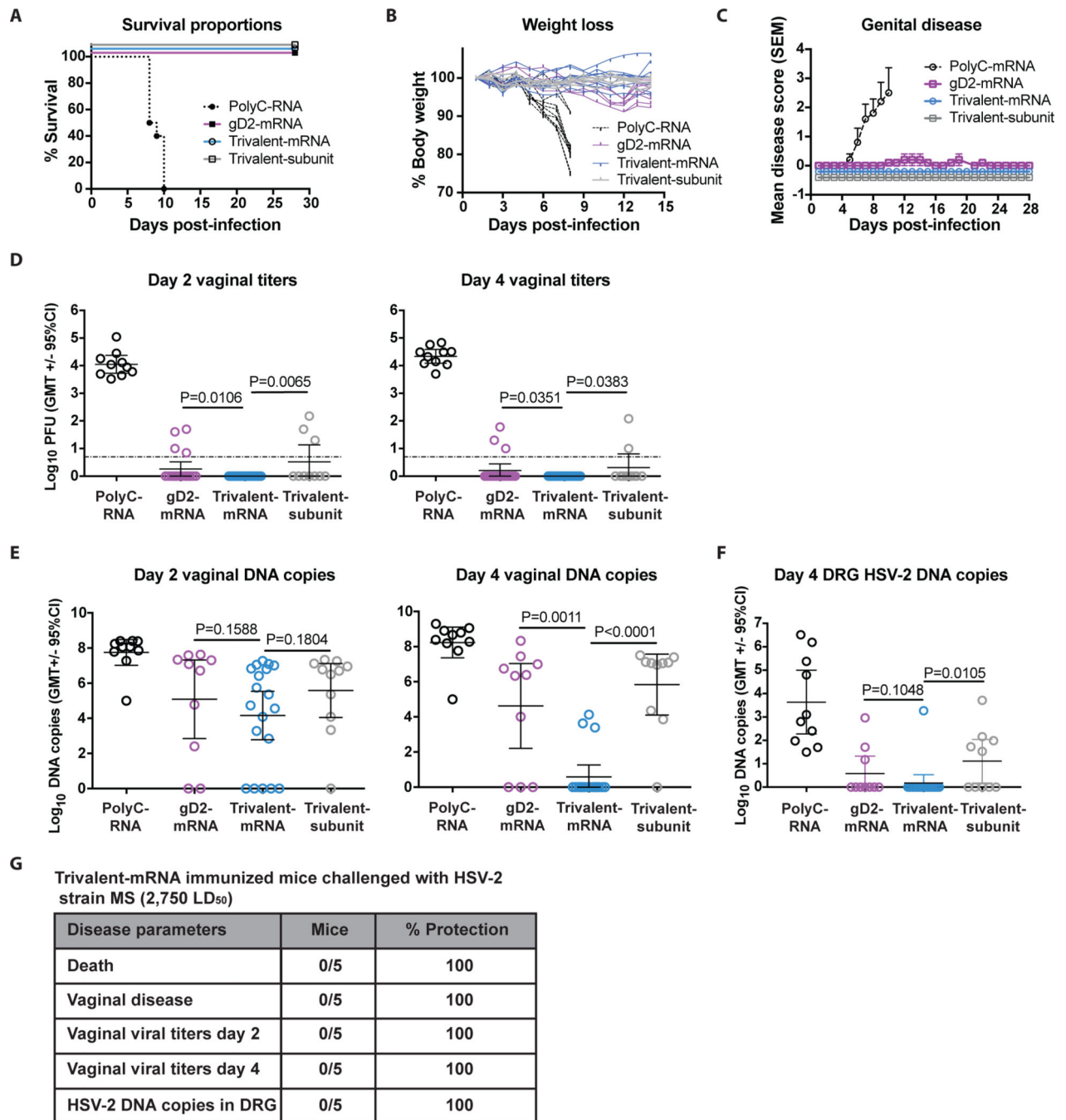
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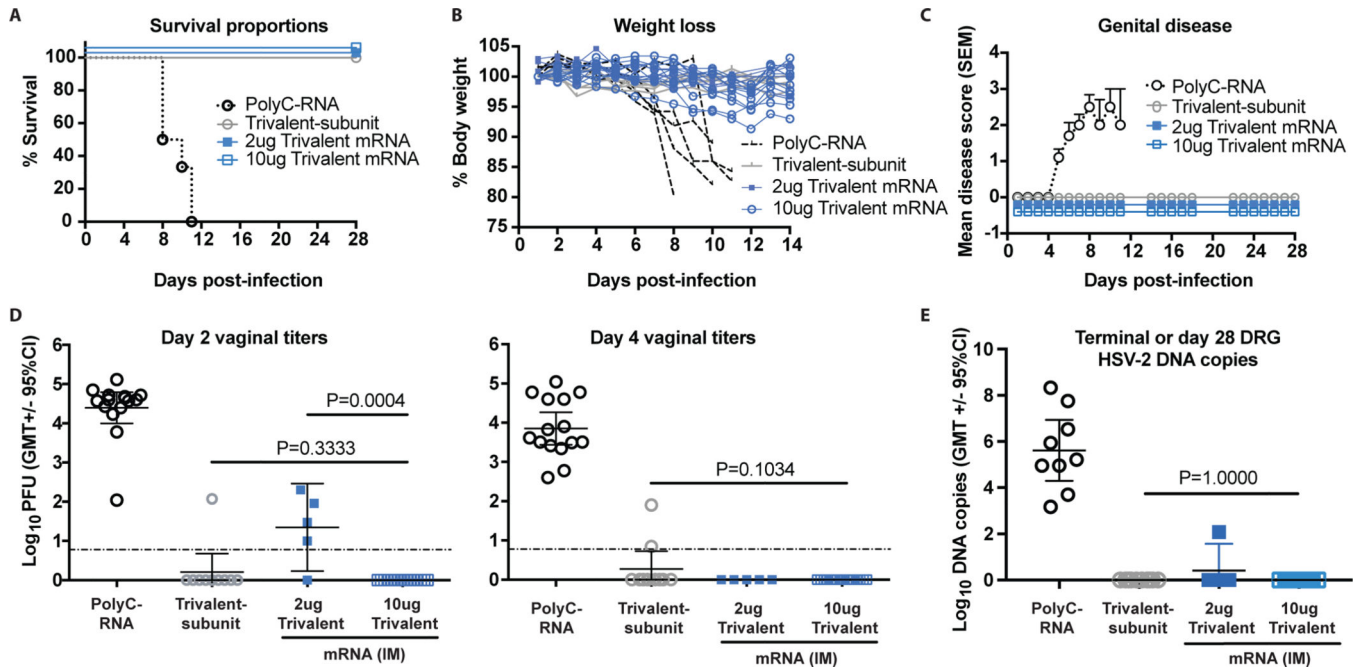
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**Figure 1. ID immunization with nucleoside-modified trivalent mRNA-LNP protects against genital HSV-2 in mice.**

(a-f) BALB/c mice were immunized twice with PolyC RNA-LNP, gD2 nucleoside-modified mRNA-LNP, trivalent nucleoside-modified mRNA-LNP, or three times with trivalent subunit protein-CpG/alum and challenged one month after the final immunization intravaginally with  $5 \times 10^3$  HSV-2. (a) Survival curves:  $P < 0.001$  comparing PolyC RNA with each other group. P values were calculated by the log-rank test. (b) The mean weight loss each day post-infection of animals in the trivalent mRNA, trivalent subunit protein, or gD2 mRNA group was compared with PolyC RNA controls;  $P < 0.0001$ . (c) Mean genital disease scores

post-infection comparing trivalent mRNA, trivalent subunit protein or gD2 mRNA with PolyC RNA,  $P < 0.0001$ . P value in (b) and (c) was calculated by two-way ANOVA followed by Tukey for significance. Error bars in (c) represent SEM. (d) Day 2 and day 4 vaginal titers. Dotted line indicates the limit of assay detection at 6.7 PFU/ml. (e) Day 2 and day 4 vaginal DNA copy number by qPCR. (f) Day 4 DRG HSV-2 DNA copy number by qPCR. P values in (d-f) are shown on the figures for the primary endpoint comparison between trivalent mRNA and trivalent protein or secondary endpoint comparison between trivalent mRNA and gD2 mRNA and were calculated by the two-tailed Fisher's exact test. Sample size: (a-c and f),  $n=10$  per group, except trivalent mRNA group,  $n=20$  for (a-c) and  $n=19$  for (f); (d),  $n=10$  in PolyC RNA,  $n=20$  in gD2 mRNA,  $n=39$  in trivalent mRNA and  $n=10$  in trivalent subunit protein; (e),  $n=10$  per group except trivalent mRNA  $n=20$ . (g) Challenge of trivalent mRNA-immunized mice with  $5 \times 10^4$  PFU (2,750 LD<sub>50</sub>).



**Figure 2. IM immunization with nucleoside-modified trivalent mRNA-LNP protects against genital HSV-2 in mice.**

BALB/c mice were immunized as in Figure 1 except one group received 2 $\mu$ g trivalent mRNA. Mice were challenged one month after the final immunization intravaginally with  $5 \times 10^3$  HSV-2 (275 LD<sub>50</sub>). (a) Survival curves:  $P < 0.0001$  comparing PolyC RNA with each other group. P values were calculated by the log-rank test. (b) Weight loss of individual animals:  $P < 0.0001$  comparing PolyC RNA with the other three groups. (c) Genital disease:  $P < 0.0001$  comparing PolyC RNA with the other three groups. P values in (b-c) were calculated by two-way ANOVA followed by Tukey for significance. Sample size: (a-c),  $n = 10$  in PolyC RNA group for (a) and (c),  $n = 5$  for (b),  $n = 10$  in trivalent subunit protein,  $n = 5$  in 2 $\mu$ g trivalent mRNA, and  $n = 10$  in 10 $\mu$ g trivalent mRNA. Error bars in (c) represent SEM. P values in (d-e) are shown on the figures for the primary endpoint comparison between trivalent mRNA and trivalent protein or the comparison between 2 $\mu$ g and 10 $\mu$ g trivalent mRNA. Other P values are noted below. (d) Day 2 vaginal titers: comparing 10 $\mu$ g trivalent mRNA or trivalent subunit protein with PolyC RNA,  $P < 0.0001$ ; comparing 2 $\mu$ g trivalent mRNA with PolyC RNA,  $P = 0.2500$ . Day 4 vaginal titers: comparing the number of animals with positive day 4 cultures in the 10 $\mu$ g trivalent mRNA, the 2 $\mu$ g trivalent mRNA or the trivalent subunit protein group with PolyC RNA,  $P < 0.0001$ . Sample size:  $n = 15$  in PolyC RNA group,  $n = 10$  in trivalent subunit protein group,  $n = 5$  in the 2 $\mu$ g trivalent mRNA group and  $n = 20$  in the 10 $\mu$ g trivalent mRNA group. Dotted line indicates the limit of assay detection at 6.7 PFU/ml. (e) DRG HSV-2 DNA copy number at time of euthanasia of PolyC RNA group or on day 28 in the other groups;  $P < 0.0001$  comparing the number of animals with positive HSV-2 DNA copy number in the PolyC RNA with the 10 $\mu$ g trivalent mRNA or the trivalent subunit protein group;  $P = 0.0050$  comparing the number of animals with positive HSV-2 DNA copy in the PolyC RNA with the 2 $\mu$ g trivalent mRNA group. Other comparisons were not significant. Sample size:  $n = 9$  in the PolyC group,  $n = 10$  in the trivalent

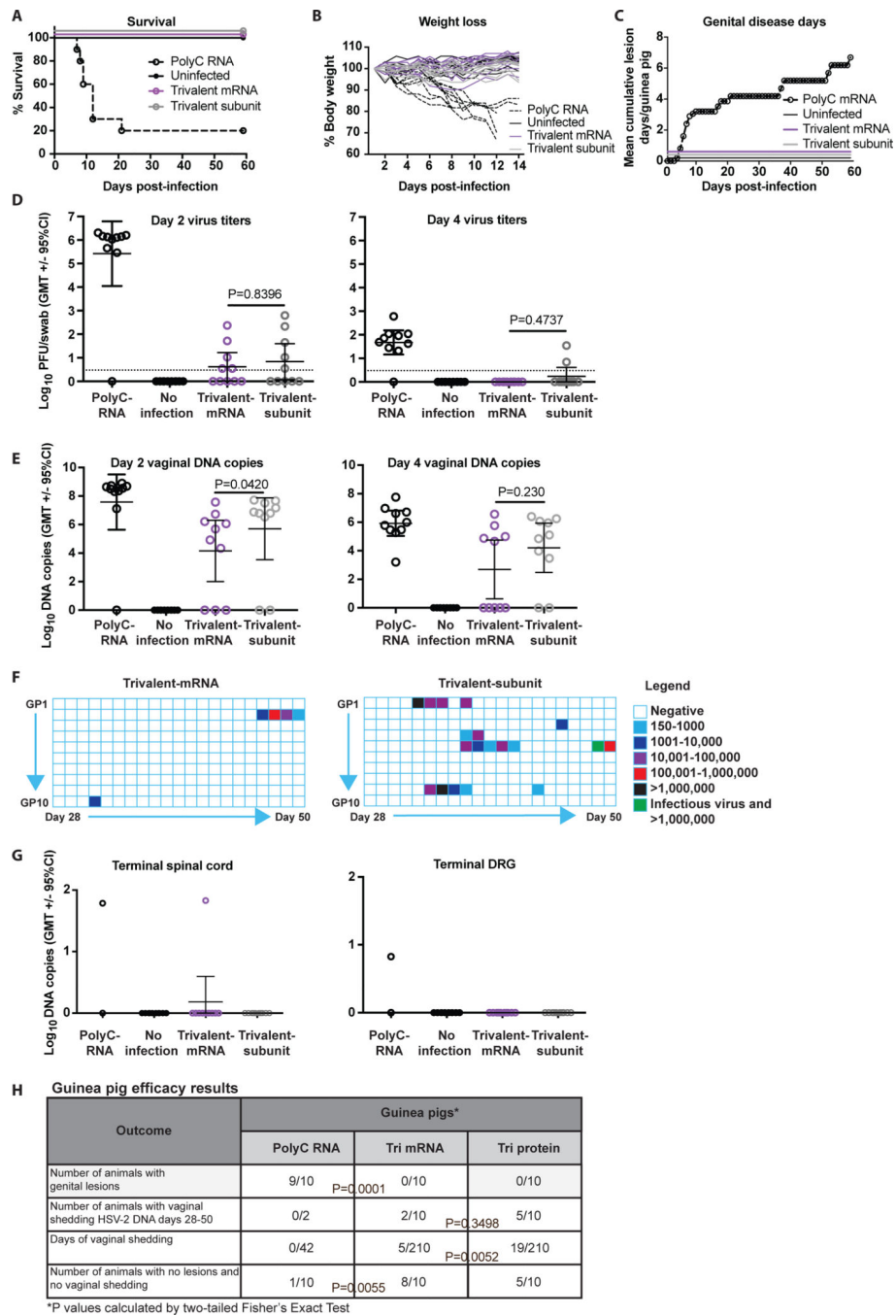
subunit protein group, n=5 in the 2 $\mu$ g trivalent mRNA group and n=10 in the 10 $\mu$ g trivalent mRNA group. P values for (d-e) were calculated by the two-tailed Fisher's exact test.

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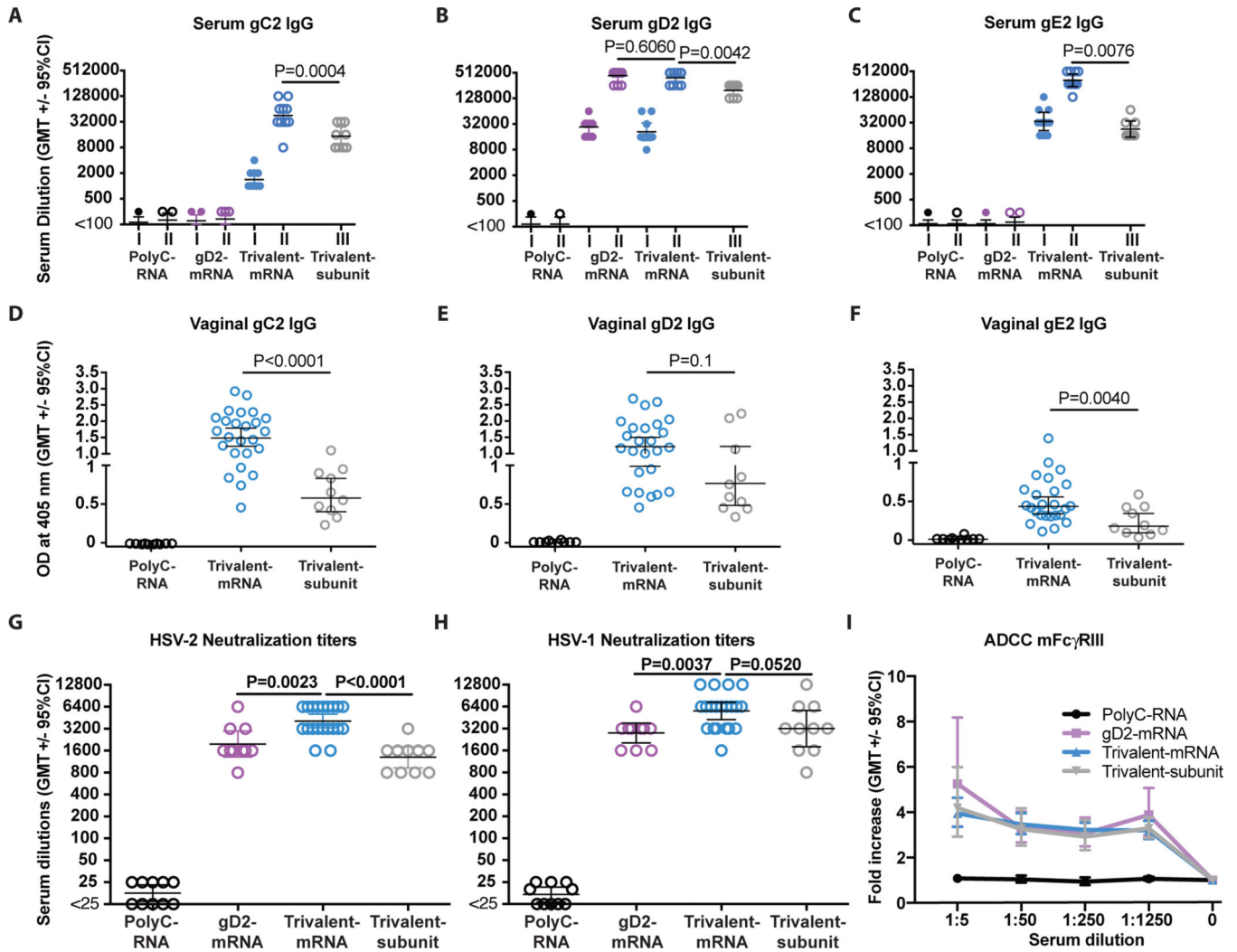


**Figure 3. ID immunization with nucleoside-modified trivalent mRNA-LNP protects against genital HSV-2 in guinea pigs.**

(a-f) Guinea pigs were immunized three times monthly IM with 10 $\mu$ g PolyC RNA-LNP; ID with 20 $\mu$ g each trivalent nucleoside-modified mRNA-LNP; IM with 10 $\mu$ g each trivalent subunit protein-CpG/alum; or left unimmunized (n=10/group). One month after the final immunization, all immunized animals were infected intravaginally with 5 $\times$ 10<sup>5</sup> HSV-2 (50 LD<sub>50</sub>), while unimmunized animals remained uninfected. (a) Survival curves: P<0.0001 comparing PolyC RNA with each other group. P values calculated by the log-rank test. (b) The mean weight loss each day post-infection of animals in the trivalent mRNA, trivalent



subunit protein, or uninfected group was compared with PolyC RNA controls;  $P < 0.0001$ . (c) Cumulative mean genital disease score per guinea pig is shown for each day post-infection. No animal developed genital lesions in the trivalent mRNA, trivalent subunit protein or uninfected group. Comparing trivalent mRNA, trivalent subunit protein or uninfected with PolyC RNA,  $P < 0.0001$ . The P value in (b) and (c) was calculated by two-way ANOVA followed by Tukey for significance. (d-e) P values shown on the figures are for the primary endpoint comparison between trivalent mRNA and trivalent protein. Other P values are noted below. (d) Day 2 and day 4 vaginal titers: Day 2,  $P = 0.0013$  and day 4  $P = 0.0010$  comparing PolyC RNA with trivalent mRNA. Dotted line indicates the limit of assay detection at 3.3 PFU/ml. (e) Day 2 and day 4 vaginal DNA copy number by qPCR. P value in (d) and (e) was calculated by the two-tailed Mann-Whitney, except the day 4 comparison between trivalent mRNA and trivalent subunit proteins was performed by the two-tailed Fisher's exact test. (f) Vaginal shedding of HSV-2 DNA over 21 days from day 28–50 post-infection. One animal in the trivalent subunit protein group had replication competent virus recovered on a day HSV-2 DNA shedding was detected (marked in green). Comparing days of shedding in the trivalent mRNA and trivalent subunit protein group,  $P = 0.0052$ , calculated by the two-tailed Fisher's exact test. (g) HSV-2 DNA copy number/ $10^6$  copies of GAPDH in spinal cord and DRG at the end of the experiment;  $n = 2$  in PolyC RNA and  $n = 10$  in the 3 other groups. (h) Table summarizing key results of guinea pig studies.



**Figure 4. ELISA, neutralizing and ADCC antibody responses.** Mice were immunized ID with PolyC RNA-LNP, gD2 mRNA-LNP, 10µg trivalent mRNA-LNP, or IM with trivalent subunit protein-CpG/alum. Sera were obtained 4 weeks after the first and second immunizations in the mRNA-LNP groups and 4 weeks after the third immunization in the subunit protein group. P values are shown on the figures for the primary endpoint comparison between trivalent mRNA and trivalent protein or secondary endpoint comparison between trivalent mRNA and gD2 mRNA. Other P values are noted below. (a) Serum gC2 IgG: P=0.0058 comparing first and second trivalent mRNA immunization. (b) Serum gD2 IgG: P=0.6060 comparing final immunizations of trivalent mRNA with gD2 mRNA; P=0.0085 comparing first and second gD2 mRNA immunization; P=0.0056 comparing first and second trivalent mRNA immunization. (c) Serum gE2 IgG: P=0.0058 comparing first and second trivalent mRNA immunization. n=9 animals per group. (a-c) P values comparing first with second immunization were calculated by the two-tailed Wilcoxon signed-rank test. P values comparing final immunizations were performed by the two-tailed Mann-Whitney test. (d-f) Vaginal gC2 IgG, gD2 IgG or gE2 IgG ELISA titers: n=10 for PolyC and gD2 mRNA, n=25 for trivalent mRNA, n=10 for trivalent subunit

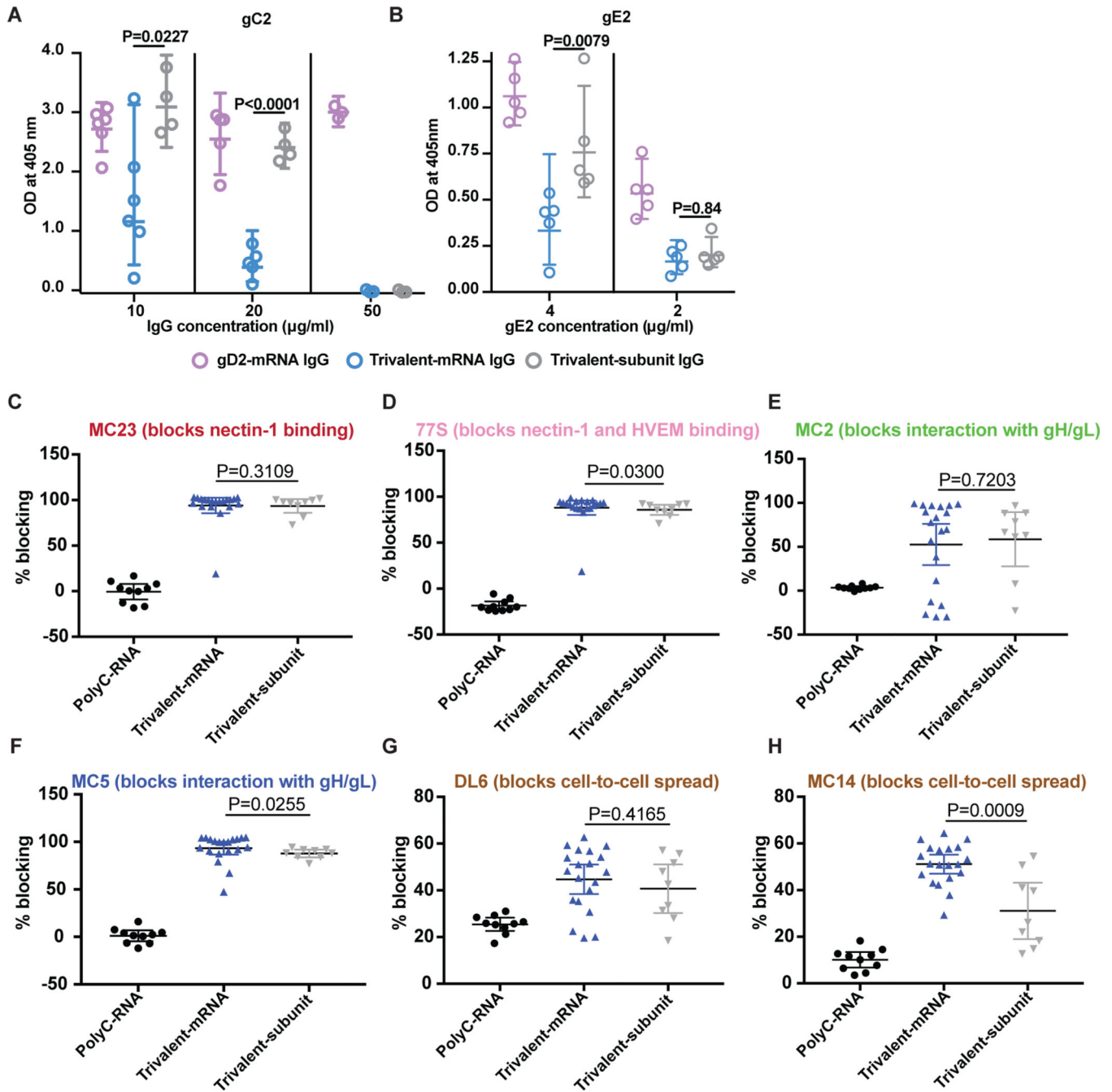
protein. (g) HSV-2 strain MS neutralizing titers in the presence of 10% HSV-1/HSV-2 seronegative human serum as a source of complement. (h) HSV-1 strain NS neutralizing antibody titers in the presence of 10% seronegative human serum as a source of complement. n=10 in PolyC, n=10 in gD2, n=20 in trivalent mRNA and n=10 in trivalent subunit groups in (g-h). (d-h) P values comparing trivalent mRNA with trivalent subunit protein or with gD2 mRNA were calculated by the two-tailed Mann-Whitney test. (i) ADCC using sera from PolyC RNA, gD2 mRNA, trivalent mRNA and trivalent subunit mice. Results shown represent 5–6 separate sera tested per group.  $P < 0.001$  comparing each curve with PolyC RNA as calculated by two-way ANOVA with Tukey for significance.

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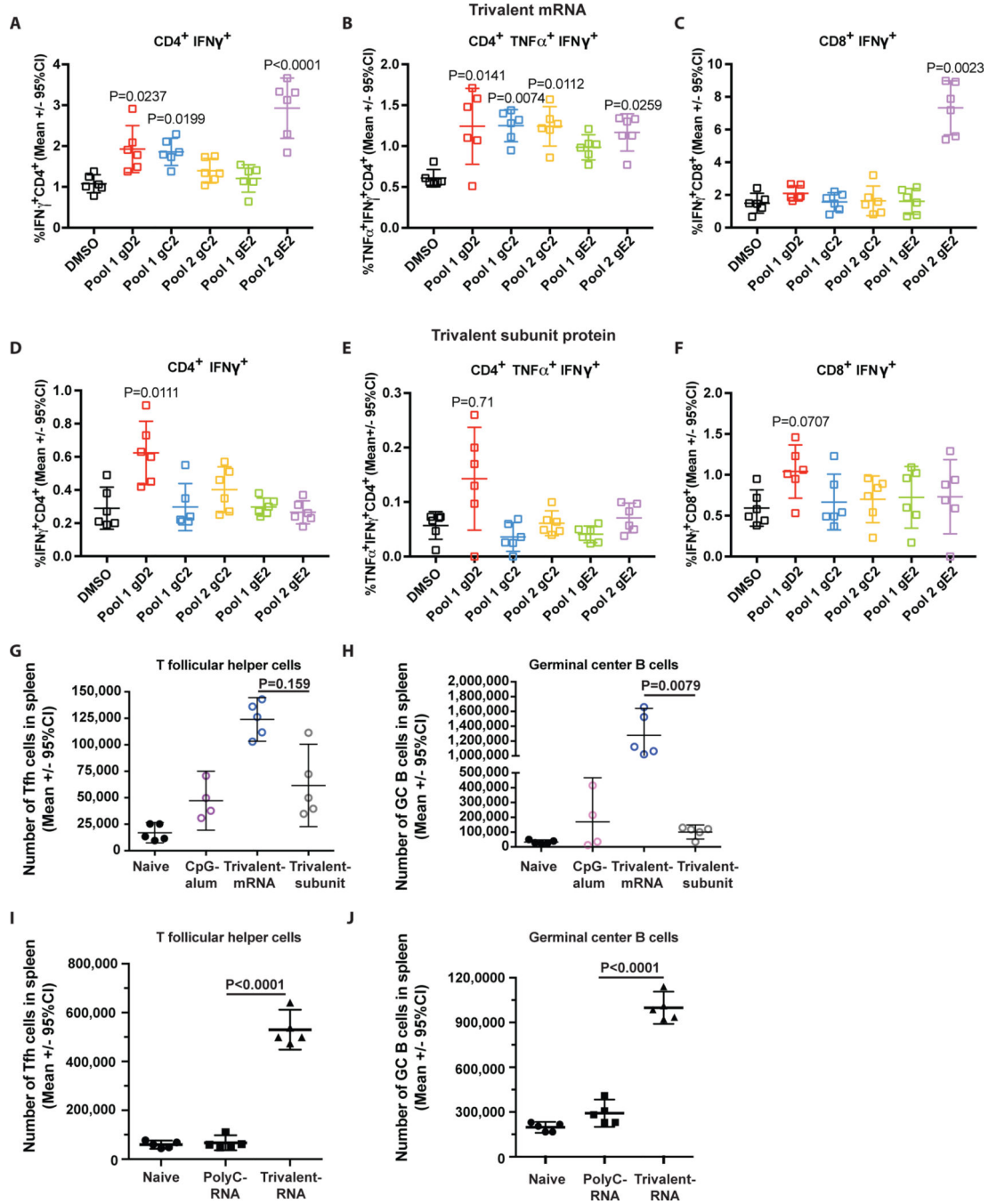
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**Figure 5. Antibodies that block gC2 and gE2 immune evasion domains and that bind gD2 epitopes that mediate crucial functions.**

P values are shown on the figures for the primary endpoint comparison between trivalent mRNA and trivalent protein. Other P values are noted below. (a) Antibodies produced by trivalent mRNA and trivalent subunit protein block C3b binding to gC2. Error bars represent geometric mean and 95% confidence intervals. Comparing trivalent mRNA with gD2 mRNA, P=0.0220 at 10µg/ml; P<0.0001 at 20 and 50µg/ml. Comparing trivalent subunit protein with gD2 mRNA, P<0.0001 at 50µg/ml; P not significant at other IgG concentrations. (b) Blocking gE2 binding to IgG Fc. Comparing trivalent mRNA with gD2

mRNA at 4 $\mu$ g/ml, P=0.0079. Comparing trivalent subunit protein with gD2 mRNA at 4 $\mu$ g/ml, P=0.1508; at 2 $\mu$ g/ml, P=0.0079. Results shown in (a-b) are geometric means with 95% CI. IgG was purified from pooled sera of 10 animals/group in (a-b). Each entry in (a-b) represents results from a single assay. P values in (a-b) were determined by the two-tailed Mann-Whitney test. (c-h) Sera were evaluated for blocking gD2 binding to prototype MAbs that recognize crucial gD2 epitopes. n= 10 for PolyC RNA, n=20 for trivalent mRNA, n=9 for trivalent subunit protein. Results in (c-g) represent means with 95% CI. P values comparing trivalent mRNA with trivalent subunit protein were calculated using the two-tailed Mann-Whitney test.



**Figure 6. CD4<sup>+</sup>, CD8<sup>+</sup>, Tfh<sup>+</sup> T cell responses and germinal center B cell responses.** Splenocytes for CD4<sup>+</sup> and CD8<sup>+</sup> T cell assays were pooled from 5 animals and the responses of 6 replicate wells are shown. (a) CD4<sup>+</sup> T cell IFN $\gamma$  responses in mice immunized with trivalent mRNA or (b) CD4<sup>+</sup> polyfunctional IFN $\gamma$  and TNF $\alpha$  responses in mice immunized with trivalent mRNA. (c) CD8<sup>+</sup> T cell IFN $\gamma$  responses in mice immunized with trivalent mRNA. (d) CD4<sup>+</sup> T cell IFN $\gamma$  responses in mice immunized with trivalent subunit protein or (e) CD4<sup>+</sup> polyfunctional IFN $\gamma$  and TNF $\alpha$  responses in mice immunized with trivalent subunit protein. (f) CD8<sup>+</sup> T cell IFN $\gamma$  responses in mice immunized with

trivalent subunit protein. (a-f) Each peptide-stimulated CD4<sup>+</sup> or CD8<sup>+</sup> T cell response was compared with DMSO vehicle-treated controls. The CD4<sup>+</sup> and CD8<sup>+</sup> T cell assays for trivalent mRNA and trivalent subunit protein were run on separate days, which accounts for the variability in the DMSO control results. The percent of cytokine positive cells is based on the total number of CD4<sup>+</sup> T cells counted in the trivalent mRNA and subunit protein assays. The mean number of CD4<sup>+</sup> T cells counted in the trivalent mRNA experiments was  $4680 \pm 714$ , and in the trivalent subunit protein was  $7850 \pm 960$ . The mean number of CD8<sup>+</sup> T cells counted in the trivalent mRNA experiments was  $981 \pm 232$ , and in the trivalent subunit protein  $1118 \pm 206$ . All values with  $P < 0.05$  are shown in the figures. P values were calculated by the Kruskal-Wallis test with Dunn's for multiple comparisons. (g-h) Tfh and GC B cell responses that include CpG/alum as a control. Splenocytes were harvested for Tfh and GC B cell assays from individual mice 10 days after a single immunization with CpG/alum alone, trivalent mRNA-LNP, trivalent subunit protein-CpG/alum, or no immunization. (g-h) P values are shown on the figures for the primary endpoint comparison between trivalent mRNA and trivalent protein. Other P values are noted below. (g) Tfh cells:  $P=0.532$  comparing CpG/alum with trivalent subunit protein-CpG/alum. (h) Germinal center B cells:  $P=0.90$  comparing CpG/alum with trivalent subunit protein. (g-h):  $n=5$  animals per group, except  $n=4$  for CpG/alum. P values were calculated using the two-tailed Mann-Whitney test. Error bars represent geometric means and 95% confidence intervals. (i-j) Tfh and GC B cell responses that include PolyC RNA-LNP as a control. Splenocytes were harvested for Tfh and GC B cell assays from individual mice 10 days after the second immunization with PolyC RNA-LNP, trivalent mRNA-LNP, or no immunization. P values are shown on the figures and were calculated by the two-tailed Mann-Whitney test. (i-j):  $n=5$  animals per group.

**Table 1.**

## Combined ID and IM efficacy studies

Outcome	Number of animals with outcome*			
	PolyC RNA	gD2 mRNA	Tri mRNA	Tri protein
<b>Clinical disease:</b>	25/25	1/10	0/44	0/20
Death, genital lesions or generalized illness		P=0.1852	P=1.0000	
<b>Subclinical infection:</b>	25/25	4/20	0/64	4/20
• Day 2 vag. titers		P=0.0025	P=0.0025	
• Day 4 vag. titers	25/25	3/20	0/64	4/20
		P=0.0120	P=0.0025	
• HSV-2 DNA in DRG	19/19	3/10	1/39	5/20
		P=0.0231	P=0.0143	
<b>Sterile immunity:</b>	0/25	15/20	63/64	23/30
No clinical or subclinical infection		P=0.0025	P=0.0012	

\*The primary endpoint is the comparison between trivalent mRNA-LNP and trivalent subunit protein. The secondary endpoint is the comparison between trivalent mRNA-LNP and gD2 mRNA-LNP. P values were calculated by two-tailed Fisher's exact test.