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Boosting of vaginal HSV-2-specific B and T cell responses by intravaginal therapeutic immunization results in diminished recurrent HSV-2 disease

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ABSTRACT Boosting herpes simplex virus (HSV)-specific immunity in the genital tissues of HSV-positive individuals to increase control of HSV-2 recurrent disease and virus shedding is an important goal of therapeutic immunization and would impact HSV-2 transmission. Experimental therapeutic HSV-2 vaccines delivered by a parenteral route have resulted in decreased recurrent disease in experimental animals. We used a guinea pig model of HSV-2 infection to test if HSV-specific antibody and cell-mediated responses in the vaginal mucosa would be more effectively increased by intravaginal (Ivag) therapeutic immunization compared to parenteral immunization. Therapeutic immunization with HSV glycoproteins and CpG adjuvant increased glycoprotein-specific IgG titers in vaginal secretions and serum to comparable levels in Ivag- and intramuscular (IM)-immunized animals. However, the mean numbers of HSV glycoprotein-specific antibody secreting cells (ASCs) and IFN-γ SCs were greater in Ivag-immunized animals demonstrating superior boosting of immunity in the vaginal mucosa compared to parenteral immunization. Therapeutic Ivag immunization also resulted in a significant decrease in the cumulative mean lesion days compared to IM immunization. There was no difference in the incidence or magnitude of HSV-2 shedding in either therapeutic immunization group compared to control-treated animals. Collectively, these data demonstrated that Ivag therapeutic immunization was superior compared to parenteral immunization to boost HSV-2 antigen-specific ASC and IFN-γ SC responses in the vagina and control recurrent HSV-2 disease. These results suggest that novel antigen delivery methods providing controlled release of optimized antigen/adjuvant combinations in the vaginal mucosa would be an effective approach for therapeutic HSV vaccines.

IMPORTANCE HSV-2 replicates in skin cells before it infects sensory nerve cells where it establishes a lifelong but mostly silent infection. HSV-2 occasionally reactivates, producing new virus which is released back at the skin surface and may be transmitted to new individuals. Some HSV-specific immune cells reside at the skin site of the HSV-2 infection that can quickly activate and clear new virus. Immunizing people already infected with HSV-2 to boost their skin-resident immune cells and rapidly control the new HSV-2 infection is logical, but we do not know the best way to administer the vaccine to achieve this goal. In this study, a therapeutic vaccine given intravaginally resulted in significantly better protection against HSV-2 disease than immunization with the same vaccine by a conventional route. Immunization by the intravaginal route resulted in greater stimulation of vaginal-resident, virus-specific cells that produced antibody and produced immune molecules to rapidly clear virus.

KEYWORDS HSV-2, recurrent HSV-2 disease, therapeutic vaccine, guinea pig, vaginal mucosa

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Worldwide, there are greater than 19 million new herpes simplex virus (HSV) infections a year (1). Severe disease can result from HSV-2 infection of immunocompromised individuals or of neonates (2–4), and genital HSV-2 infections are the leading cause of genital ulcer disease worldwide (5–7). The disruption of the epithelial barrier resulting from HSV-2 infection and recurrent disease may increase the incidence of infection with other pathogens, including HIV (8).

HSV-2 replicates initially in epithelial cells before gaining access to sensory nerve endings where it is transported to and replicates in nerve cell bodies in the sensory ganglia. The virus establishes a lifelong latent infection in sensory neurons. Following incompletely defined triggering events, HSV-2 reactivates from latency resulting in anterograde transport of new virions to the original epithelial site of infection, where it infects epithelial cells at the neuro-epithelial junction and is shed from the epithelial surface. Recurrent HSV-2 shedding happens very frequently (9), and most HSV-2 transmission occurs during periods of asymptomatic shedding (10, 11). Most shedding events are of short duration which suggests rapid immune control of viral replication following emergence from sensory neurons (12).

Populations of HSV-specific resident memory T cells at sites of previous HSV infection have been found in humans, mice, and guinea pigs (13–18) that resolve HSV lesions and control recurrent HSV-2 disease and virus shedding following reactivation from latency (17). The goal of therapeutic immunization is to boost these cell populations that control recurrent disease and virus shedding and therefore diminish the risk of HSV-2 transmission. Mathematical modeling of an imperfect HSV-2 therapeutic vaccine estimated that new infections could be substantially reduced through use of a therapeutic vaccine (19), and a recent clinical phase I/II trial of a candidate therapeutic vaccine showed reduced recurrent disease and virus shedding (20, 21). Although these results are encouraging, improvements are needed in the design of HSV therapeutic vaccines to increase control of HSV-2 recurrences.

Augmentation of genital-resident immunity by immunization is complex and incompletely understood. Experimental therapeutic HSV-2 vaccines have predominantly been delivered by a parenteral route, although boosting of vaginal mucosa-resident, HSV-specific immune cells by this route is likely not the most efficient approach. The vagina is lined by stratified squamous epithelium and permeability of this barrier to non-infectious antigens appears dependent on hormonal influence (22, 23). Additionally, circulating memory immune cells elicited by parenteral immunization do not easily enter and reside in the vaginal mucosa (24). Approaches to boost HSV-specific vaginal T cell responses and control HSV disease by therapeutic immunization at genital or non-genital mucosal sites have been used to induce infiltration of virus-specific memory T cells into the vaginal mucosa (25, 26). Genital immunization with HSV-2 ribonucleotide reductase protein reduced both HSV disease and virus shedding compared to parenteral delivery (26). However, in a separate study, intranasal therapeutic immunization with a nanoemulsion-adjuvanted HSV-2 vaccine containing glycoprotein D (gD) and glycoprotein B (gB) significantly reduced recurrent lesion scores compared to either parenteral or Ivag immunization (25). Recently, a "prime and pull" approach in which specific chemokines are applied to the vaginal epithelium of parenterally immunized animals has been shown to attract vaccine-specific T cells into the vagina, resulting in establishment of HSV-specific resident memory $CD8^+$ T cells in the vaginal epithelium (27). Incorporation of this approach into a therapeutic vaccination against HSV-2 utilizing a trivalent HSV vaccine as the "prime" and Ivag imiquimod treatment as the "pull" resulted in a significant reduction in HSV-2 disease, but not shedding, compared to the same vaccine given with subcutaneous imiquimod treatment (28). The results of these studies demonstrate some success for mucosal therapeutic immunization, but differences in immunization protocols and vaccine antigens/adjuvants make direct comparison complicated. It is also unclear if these approaches will augment both vagina-resident, HSV-specific B and T cell responses. In this study, we directly tested the boosting of vaginal mucosa-resident HSV-reactive B and T cells by parenteral or vaginal delivery of therapeutic HSV glycoprotein vaccines

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and tested the ability to diminish HSV-2 recurrent disease and virus shedding resulting from virus reactivation. Our results demonstrate that in the setting of pre-existing HSV-specific vaginal immune cells, Ivag immunization increased the numbers of both vaccine antigen-specific antibody secreting cells (ASCs) and IFNγ secreting cells (SCs) in the vaginal mucosa and significantly diminished recurrent HSV-2 disease compared to IM immunization.

RESULTS

Ivag therapeutic immunization results in increased vaginal and serum anti-HSV-2 glycoprotein titers

We previously demonstrated the presence of HSV-specific IFN-γ secreting cells and HSV-specific antibody secreting cells in the vaginal mucosa of guinea pigs several months after Ivag HSV-2 infection (16). We hypothesized that vaginal immunity could be boosted by direct therapeutic immunization of the vaginal mucosa of guinea pigs previously Ivag infected with HSV-2. Guinea pigs were control treated by Ivag instillation of CpG adjuvant only or were immunized with recombinant HSV-2 glycoprotein B plus CpG adjuvant (gB2/CpG) as described in Materials and Methods and Fig. 1A. As shown in Fig. 1B, the mean endpoint titer of anti-gB2 IgG antibodies in vaginal secretions in gB2/CpG-immunized animals increased nearly sixfold compared to CpG controls and was significantly greater compared to unimmunized controls or to CpG controls ($P < 0.01$, $P < 0.04$, respectively; Welch's ANOVA with Dunnet's T3 multiple comparisons test). The serum anti-gB2 antibody endpoint titer was not significantly different between the CpG and gB2/CpG immunization groups (Fig. 1C, Welch's *t*-test). We extended these findings in a second experiment by utilizing therapeutic immunization with recombinant HSV-2 glycoprotein D plus CpG (gD2/CpG). As shown in Fig. 1D, the anti-gD2 titer in vaginal secretions of gD2/CpG-immunized animals increased greater than 21-fold after the first immunization series and were not further increased by the second immunization series. Final titers were greater than 13-fold higher in these animals compared to CpG controls after the second immunization series ($P < 0.0001$, Welch's *t*-test). Endpoint titers in serum were measured only after the second immunization and were significantly higher in gD2/CpG-immunized animals compared to CpG only animals (Fig. 1E, $P < 0.01$, Welch's *t*-test). In a subsequent experiment, therapeutic immunization with either 1.0 µg or 10.0 µg gD2/CpG resulted in significantly higher endpoint anti-gD2 IgG titers compared to CpG-only controls in both vaginal secretions and serum (Fig. S1).

Increased gB2-specific ASC in the vaginal mucosa following Ivag therapeutic immunization

HSV-2 therapeutic vaccines tested in animal models and in human clinical trials have been commonly delivered by the intramuscular route. We tested if local antibody responses to gB2/CpG following Ivag immunization were of greater magnitude than antibody responses resulting from parenteral (IM) therapeutic immunization. Previously Ivag-infected guinea pigs were control treated or therapeutically immunized by the Ivag or IM route, and vaginal lavages were collected 2 weeks following each immunization (Fig. 2A). As shown in Fig. 2B, anti-gB2 IgG titers in vaginal lavages were greatest after the second immunization. Immunization by either route resulted in significantly increased anti-gB2 IgG titers compared to CpG controls ($P < 0.02$ and $P < 0.0004$ for IM and Ivag groups, respectively; Welch's ANOVA with Dunnet's T3 multiple comparisons test). Serum antibody titers in CpG-treated and Ivag gB2/CpG-immunized animals were not different; however, serum endpoint anti-gB2 titers in IM-immunized animals were significantly higher compared to either group (Fig. 2C, $P < 0.002$ versus Ivag, $P < 0.003$ versus CpG; Welch's ANOVA with Dunnet's T3 multiple comparisons test).

We previously showed that HSV-specific ASCs are present in the vaginal mucosa for long periods of time following Ivag HSV-2 infection (16, 29). We hypothesized that Ivag

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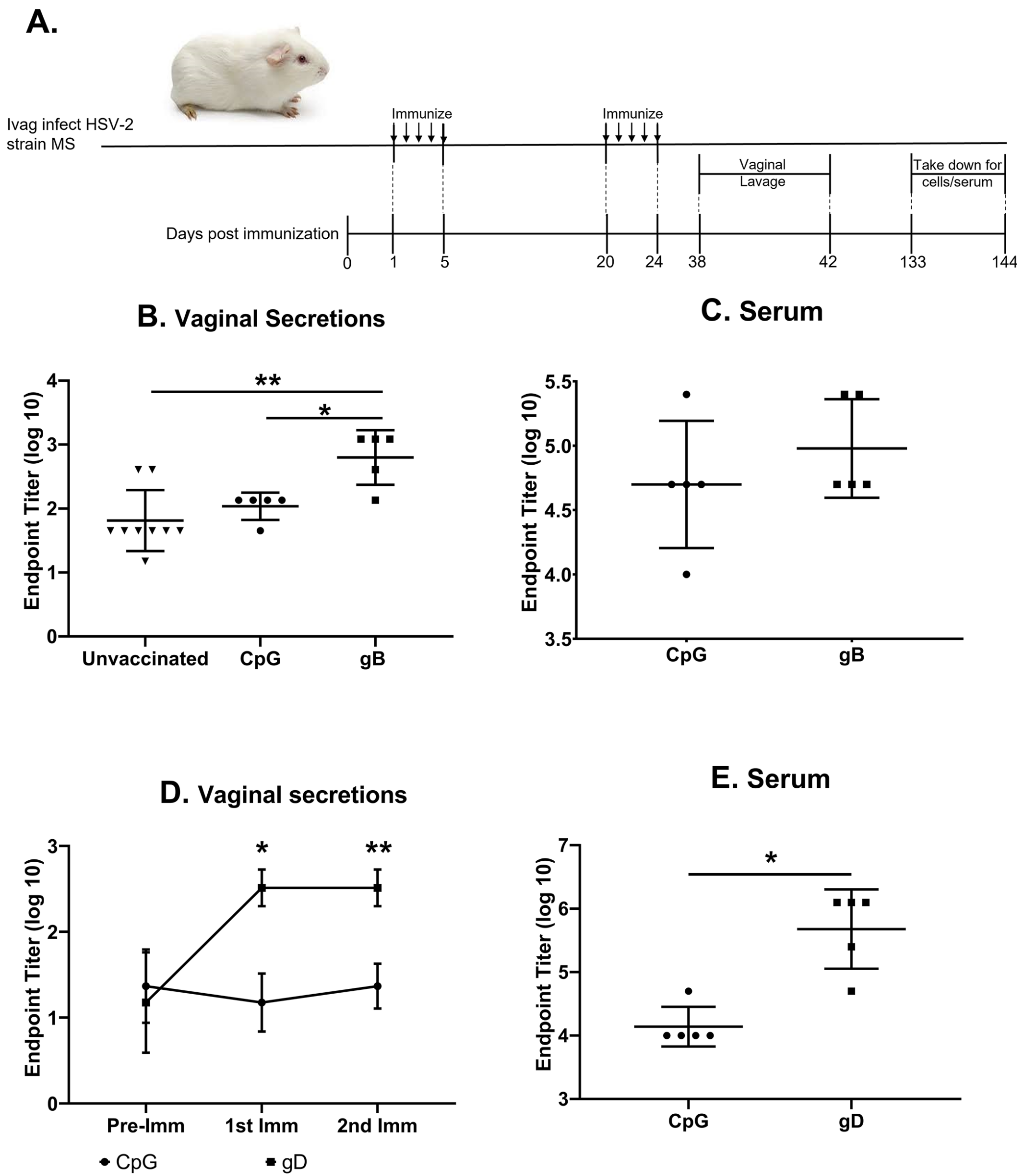

FIG 1 Ivag therapeutic immunization with gB2/CpG or gD2/CpG resulted in increased HSV2 glycoprotein-specific IgG titers in vaginal secretions and serum. (A) Experimental design. (B) Vaginal lavages from non-immunized, HSV-infected guinea pigs (N = 9), CpG-treated, HSV-2-infected guinea pigs (N = 5), and gB2/CpG-immunized, HSV-infected guinea pigs (N = 5) were collected and IgG titers determined by enzyme-linked immunosorbent assay (ELISA). Results are expressed as the mean endpoint titer ± SD, (**P < 0.01, *P < 0.04, Welch's ANOVA test with Dunnet's T3 multiple comparisons test). (C) Mean serum gB2-specific endpoint titers ± SD from CpG-treated and gB2/CpG-immunized groups (NS, P > 0.05, Welch's t-test). (D) Vaginal secretions from CpG-treated, HSV-infected guinea pigs (N = 5), and gD2/CpG-immunized, HSV-infected guinea pigs (N = 5) were collected prior to therapeutic immunization, following the first immunization period or following the second immunization and IgG titers determined by ELISA. Results are expressed as the mean endpoint titer ± SD (*P < 0.001, **P < 0.0001, compared to CpG-treated group values from the same immunization, Welch's t-test). (E) Mean gD2-specific endpoint titers ± SD from serum of CpG-treated and gD2/CpG-immunized groups (*P < 0.01, Welch's t-test).

therapeutic immunization would increase the number of these cells compared to IM immunization. Lymphocytes from the vaginal mucosa and spleen were obtained at the termination of the experiment (Fig. 2A), and gB2-specific ASCs were quantified. As shown in Fig. 2D, the mean number of vaginal gB2-specific ASCs was approximately 10-fold greater in Ivag-immunized animals compared to either CpG animals (P < 0.001; Welch's

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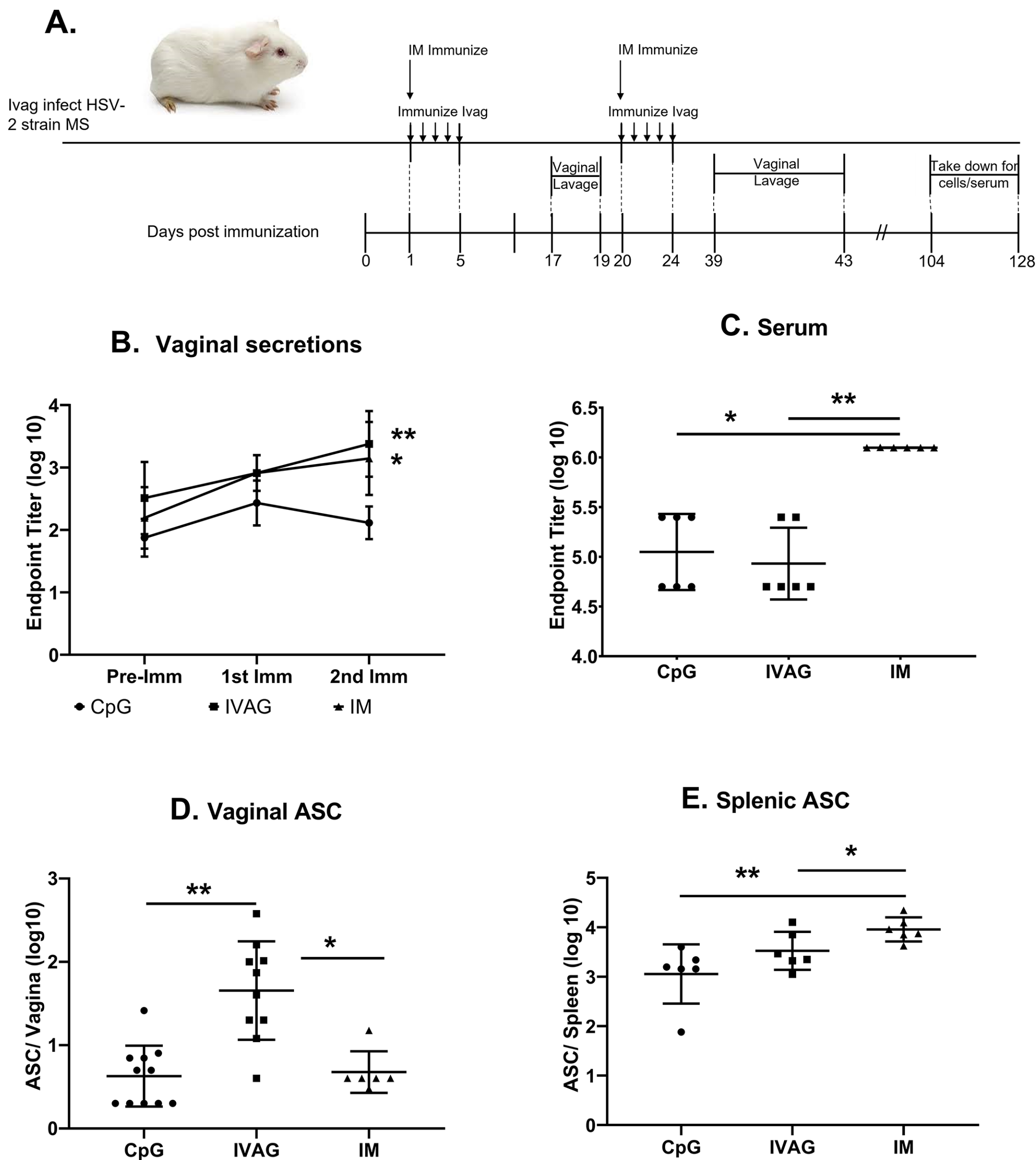

FIG 2 IM therapeutic immunization resulted in comparable HSV glycoprotein-specific IgG titers in vaginal mucosa and serum but significantly lower vaginal HSV gB2-specific ASC responses compared to Ivag immunization. (A) Experimental design. (B) HSV gB2-specific IgG endpoint titers of vaginal lavages from CpG treated ($N = 6$), Ivag immunized ($N = 6$), or IM immunized ($N = 6$) taken before, or on the indicated days after the first and second therapeutic immunizations. Results are expressed as the mean endpoint titer ± SD (*$P < 0.02$, **$P < 0.004$ compared to same date CpG group values, Welch's ANOVA with Dunnet's T3 multiple comparisons test). (C) Anti-gB2 IgG endpoint titers from serum 2 weeks after second immunization. Mean endpoint titer ± SD (*$P < 0.003$, **$P < 0.002$ Welch's ANOVA with Dunnet's T3 multiple comparisons test). (D) HSV gB2-specific ASC in vaginal mucosa from CpG treated ($N = 11$), Ivag immunized ($N = 10$), or IM immunized ($N = 6$). Results from CpG- and Ivag-immunized animals are from two pooled experiments and from IM animals are from a single experiment and are expressed as the mean gB2-specific ASC per vaginal tract ± SD (*$P < 0.002$; **$P < 0.001$, Welch's ANOVA with Dunnet's T3 multiple comparisons test). (E) HSV gB2-specific ASC in spleens of six animals per group from a single experiment. Results are expressed as the mean gB2-specific ASC per spleen ± SD (*$P = 0.048$; **$P = 0.0011$, Welch's ANOVA with Dunnet's T3 multiple comparisons test).

ANOVA with Dunnet's T3 multiple comparisons test) or IM-immunized animals ($P < 0.002$; Welch's ANOVA with Dunnet's T3 multiple comparisons test). In agreement with the significantly higher anti-gB2 titers in the serum of IM-immunized mice (Fig. 2C), the number of gB2-specific ASCs in the spleen of IM-immunized animals was significantly

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higher compared to CpG (Fig. 2E, $P = 0.011$; Welch's ANOVA with Dunnet's T3 multiple comparisons test) or Ivag-immunized animals (Fig. 2E, $P = 0.048$). Together, these data indicate that although Ivag and IM immunization resulted in comparable anti-gB2 IgG titers in vaginal secretions, Ivag immunization resulted in a significantly greater number of vaginal gB2-specific ASCs.

HSV-2 recurrent disease and virus shedding following therapeutic immunization by the Ivag or IM routes

The increased number of antigen-specific ASCs in the vagina following Ivag immunization suggested that local adaptive immune responses were being boosted by the therapeutic immunization. Therefore, we tested if Ivag therapeutic immunization would provide superior protection against HSV-2 recurrent disease and virus shedding compared to IM immunization. Guinea pigs previously infected with HSV-2 were immunized with gD2 + gB2/CpG by the Ivag or IM route or control treated with CpG and then were observed for genital HSV-2 disease and vaginal swabs collected for determination of HSV-2 shedding as shown in Fig. 3A. The cumulative mean lesion day data for each group for days 18–63 post infection (PI) are shown in Fig. 3B. Cumulative mean lesion day curves from each group are similar from the initiation of observation on day 18 PI until the end of the first immunization period at day 28 PI. Thereafter, the curves differ among groups through day 63 PI. Animals in the Ivag immunization group experienced fewer lesions compared to the CpG or IM groups. The incidence of animals experiencing recurrent disease was also impacted by Ivag therapeutic immunization (Fig. 3C). From the last day of the first Ivag immunization (d28 PI) through day 63, all 24 CpG controls experienced at least 1 day of HSV-2 disease, whereas 8 of 24 Ivag-immunized animals did not experience recurrent disease ($P < 0.004$ compared to CpG group, Fisher's exact test), suggesting Ivag immunization prevented recurrent disease in 33% of the animals over the course of the study. By contrast, the recurrent disease incidence was not different between IM-immunized and control-treated groups. Vaginal swabs were collected from animals in each group on days 31–35 and 45–54 post infection, and the frequency and magnitude of HSV-2 shedding were determined by quantitative PCR (qPCR) (30). Therapeutic immunization by any route apparently did not affect virus shedding. There was no significant difference in the incidence of shedding among the immunization groups (Fig. 3D; $P > 0.05$, Fisher's exact test). The percentage of days in which HSV-2 genomes were detected in vaginal swabs was 5.6%, 6.9%, and 6.1% for CpG controls, Ivag-immunized, and IM-immunized animals, respectively. Similarly, there was no difference among groups in the mean number of HSV-2 genomes detected in individual animals on days virus was shed (Fig. 3E, $P > 0.05$, Welch's ANOVA with Dunnet's T3 multiple comparisons test).

Vaginal and systemic anti-HSV-2 glycoprotein immune responses following therapeutic immunization by the Ivag or IM routes

We tested if the increased therapeutic vaccine efficacy of Ivag-immunized animals reflected increased vaccine antigen-specific antibody and cell-mediated responses in the vaginal mucosa following therapeutic immunization. For antibody present in vaginal secretions, the gB2-specific IgG titers of both Ivag and IM immunization groups were significantly greater than in CpG-treated animals (Fig. 4B, $P < 0.004$ and $P < 0.02$, respectively; Welch's ANOVA with Dunnet's T3 multiple comparisons test). Similar results were obtained for anti-gD2 IgG titers in vaginal secretions (Fig. 4C). The endpoint titers in vaginal secretions for both anti-gB2 and anti-gD2 IgG were not different between the Ivag and IM immunization groups (Fig. 4B and C). Serum anti-gB2 (Fig. 4D) and anti-gD2 titers (Fig. 4E) increased compared to CpG-treated animals following immunization, although the difference did not reach significance for gD2-specific titers (Fig. 4E). There was no difference between serum titers of Ivag and IM immunization groups for either anti-gB2 (Fig. 4D) or anti-gD2 (Fig. 4E).

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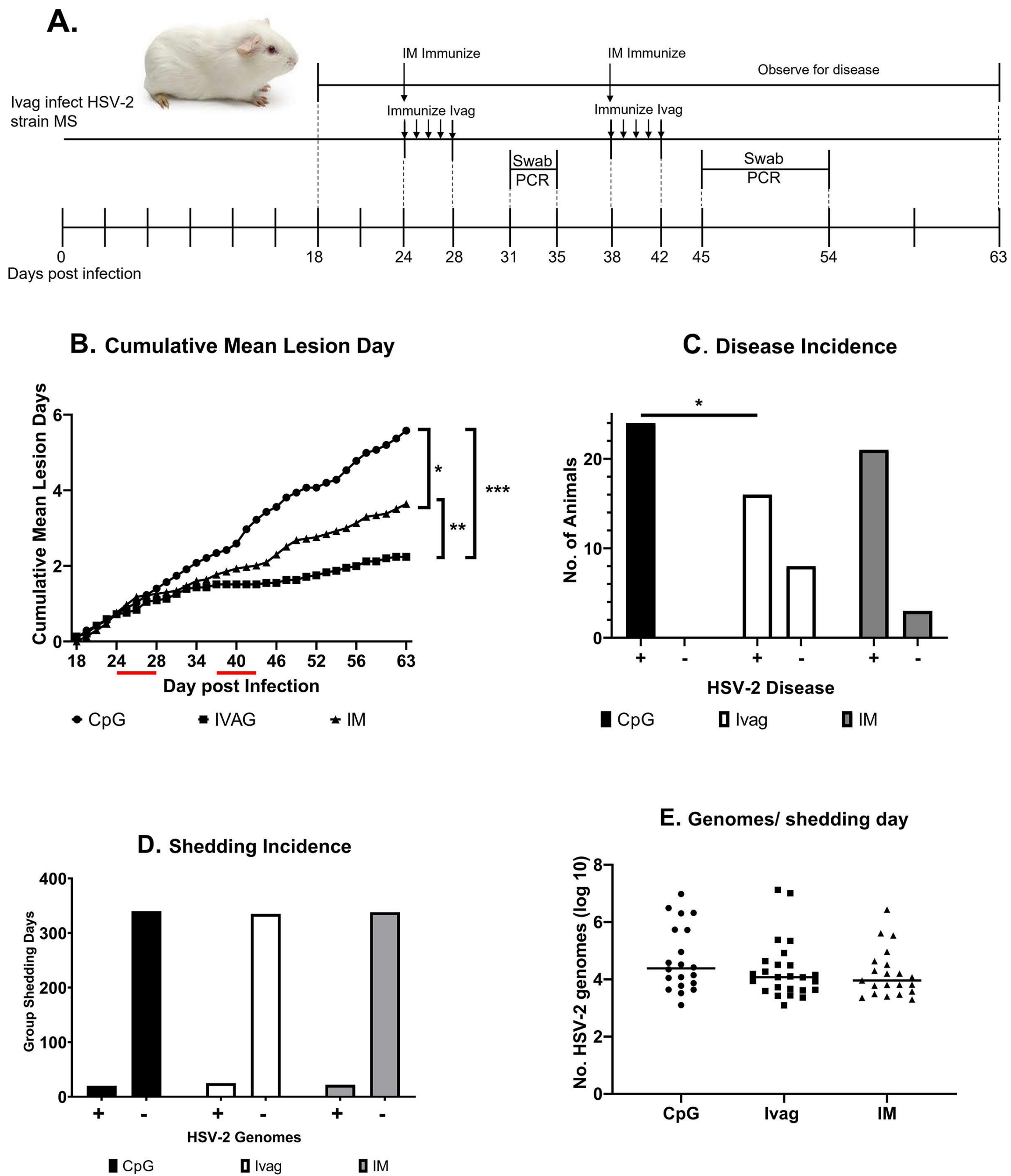

FIG 3 Decreased recurrent disease, but not HSV-2 shedding, in Ivag therapeutically immunized guinea pigs. (A) Experimental design. Guinea pigs were infected intravaginally with HSV-2 strain MS, then control treated intravaginally with CpG, or were Ivag immunized with gB2 + gD2/CpG by two sets of five-daily immunizations on the indicated days relative to infection. For IM immunization, two single injections of gB2 + gD2/CpG were given in the quadriceps muscle on the indicated days. $N = 24$ for all groups. Animals were observed daily for clinical disease between days 18 and 63 PI, and vaginal swabs were taken on the indicated days for detection of HSV-2 genomes in the vaginal mucosa by qPCR. (B) Cumulative mean lesion days for each treatment group ($N = 24$/group). Days of therapeutic immunization are shown in red on the *x*-axis. Statistical differences in mean lesion days are indicated (*$P < 0.02$; **$P < 0.0001$; ***$P < 0.009$; Welch's ANOVA with Dunnet's T3 multiple comparisons test). (C) Incidence of recurrent disease. Data show the number of animals experiencing recurrent lesions versus animals in which recurrent lesions were not detected for each immunization group on days 28–63 PI (*$P < 0.004$, Fisher's exact test). (D) Incidence of shedding detected by genome-positive vaginal swabs on days 31–35 and days 45–54 PI. The incidence of shedding is not significant among groups ($P > 0.05$, Fisher's exact test). (E) The data show the mean HSV-2 genomes in HSV-2 genome-positive vaginal swabs for each group. The mean number of genomes per shedding day is not different among groups ($P > 0.05$, Fisher's exact test).

Therapeutic immunization by either the Ivag or IM routes resulted in a significant increase in the number of gB2- and gD2-specific ASCs in vaginal tissue compared to CpG-treated animals (Fig. 5B, $P < 0.002$, Welch's ANOVA with Dunnet's T3 multiple

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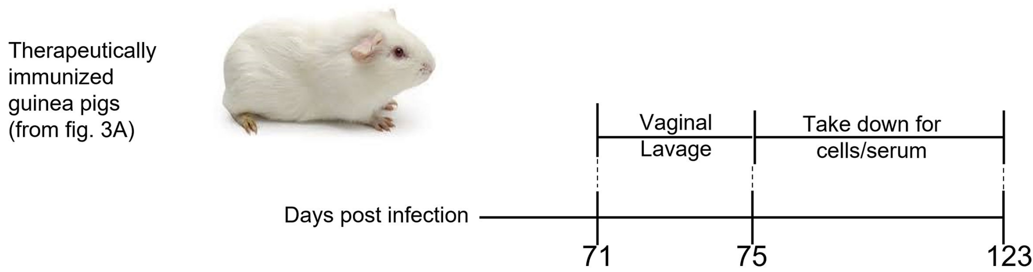

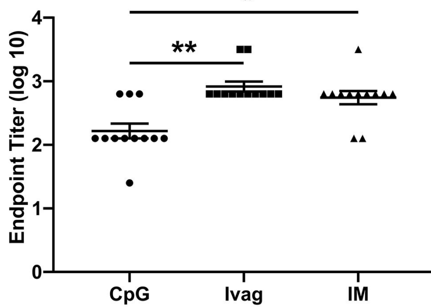

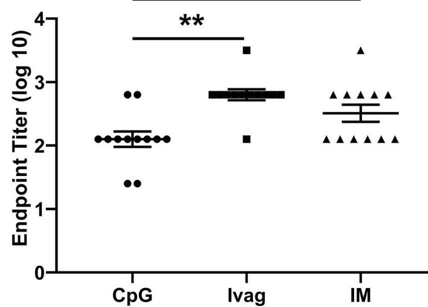

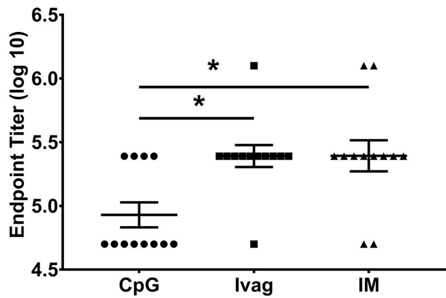

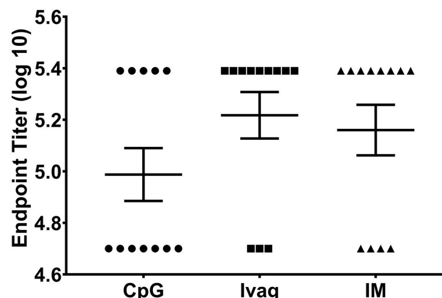

FIG 4 Increased HSV-glycoprotein-specific IgG titers following Ivag or IM therapeutic immunization. (A) Experimental design. Panels (B) and (C) show titers in vaginal secretions for gB2- (B) or gD2-specific (C) IgG. Panels (D) and (E) show serum titers for gB2- (D) or gD2-specific (E) IgG. Significant differences are shown as follows: panel (B), $*P < 0.02$, $**P < 0.004$; panel (C), $*P < 0.04$; $**P < 0.001$; panel (D), $*P < 0.02$; panel (E) no differences $P > 0.05$, Welch's ANOVA with Dunnet's T3 multiple comparisons test. $N = 12$ for each group.

comparisons test). Approximately 16-fold greater glycoprotein-specific ASCs were detected in Ivag-immunized compared to CpG-treated animals. Additionally, the number of glycoprotein-specific ASCs was significantly greater (>fourfold) in Ivag- compared to IM-immunized animals ($P < 0.003$, Welch's ANOVA with Dunnet's T3 multiple comparisons test). There was no difference in the number of HSV glycoprotein-specific ASCs detected in the spleens of any immunization group (Fig. 5C). For cell-mediated responses in the vaginal mucosa, therapeutic immunization by either route significantly increased the number of HSV glycoprotein-specific IFN-γ secreting cells compared to CpG-treated animals (Fig. 5D, for Ivag, $P = 0.0002$; for IM, $P = 0.006$, Welch's ANOVA with Dunnet's T3

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multiple comparisons test). The mean number of vaginal, antigen-specific IFN-γ secreting cells in Ivag-immunized animals was approximately 43-fold higher than in CpG controls and trended greater (approximately threefold) compared to IM-immunized animals, although the number did not reach significance. There was no difference in the number of glycoprotein-specific IFN-γ secreting cells detected in the inguinal lymph nodes of any immunization group (Fig. 5E).

DISCUSSION

Relatively weak antigen-specific antibody responses were reported in early efforts to immunize mice with non-infectious antigens by the Ivag route (22, 23). However, the use of more effective adjuvants and thinning of the vaginal epithelia by progesterone treatment in subsequent studies resulted in more vigorous increases in antigen-specific vaginal antibody titers and T cell numbers following Ivag immunization (31–33). In contrast, HSV-specific B and T cell responses including virus-specific memory $CD4^+$ and $CD8^+$ T cells and long-lived plasma cells are readily detected in the vaginal mucosa of humans and experimental animals for months after Ivag infection with HSV-2 (16, 17, 29, 34–36). In the current studies, we tested the ability of Ivag therapeutic immunization with an HSV-2 glycoprotein/CpG adjuvant vaccine to boost pre-existing HSV-2-specific immune responses in the vaginal mucosa. We found that Ivag therapeutic immunization boosted both HSV-2 glycoprotein-specific B and T cell responses in the vaginal mucosa in the absence of exogenous hormone conditioning of the vaginal epithelia.

Antigen-specific IgG responses in serum and vaginal secretions were significantly increased following Ivag immunization with either gD2- or gB2/CpG compared to CpG-treated controls (Fig. 1). Comparable increases in HSV-2 glycoprotein-specific IgG responses in vaginal secretions were also detected following IM immunization (Fig. 2B). However, the mean number of HSV-2 glycoprotein-specific ASCs in the vaginal mucosa of Ivag-immunized animals was significantly higher than the IM group (Fig. 2D and 5B) suggesting that at least some of the increased glycoprotein-specific IgG in vaginal secretions was produced in the vaginal mucosa and entered the vaginal lumen by FcRN-mediated transport (37). The identity of the specific B cell population(s) responding to the Ivag therapeutic boost is unclear but may involve HSV glycoprotein-specific memory B cells present in the vaginal mucosa as a result of the initial HSV-2 infection. Alternatively, or in addition, activation of circulating memory B cells that infiltrated the vagina in response to immune signals from the vaccinated vaginal mucosa (38) may also explain our findings. In contrast, the glycoprotein-specific ASC response in the vaginal mucosa of IM-immunized guinea pigs was inconsistent, ranging from comparable to CpG-treated animals (Fig. 2D) to increased responses in four of eight animals (Fig. 5B). These results suggest that a larger proportion of the increased glycoprotein-specific IgG in vaginal secretions of the IM group originated from serum transudation.

A role for antibody in limiting recurrent disease and virus shedding is uncertain, particularly, given the ability of HSV to evade antibody-mediated immunity (39). However, HSV-specific ASCs in the vaginal mucosa may play a role in limiting spread of newly reactivated virus from the nerve ending to surrounding epithelial cells (40) or in presenting antigen to HSV-specific resident memory T cells (41, 42). A recent study of HSV-2 lesion biopsies demonstrated the presence of ASC that co-localized with $CD4^+$ T cells in perivascular tissues (34). Furthermore, HSV-specific antibodies were detected in both active and healing lesions. The concentration of HSV-specific IgG at the junction of sensory nerve endings and surrounding epithelial cells was not determined in the present study, and it is unclear how the concentration of HSV-specific IgG in vaginal secretions might correlate with antibody levels at this site. However, it seems possible that increased HSV-specific ASCs in the vaginal mucosa following therapeutic immunization might provide a means to directly increase the amount of neutralizing HSV-specific antibody including antibodies capable of blocking virus entry and cell-to-cell spread (43) at the neuro-epithelial junction.

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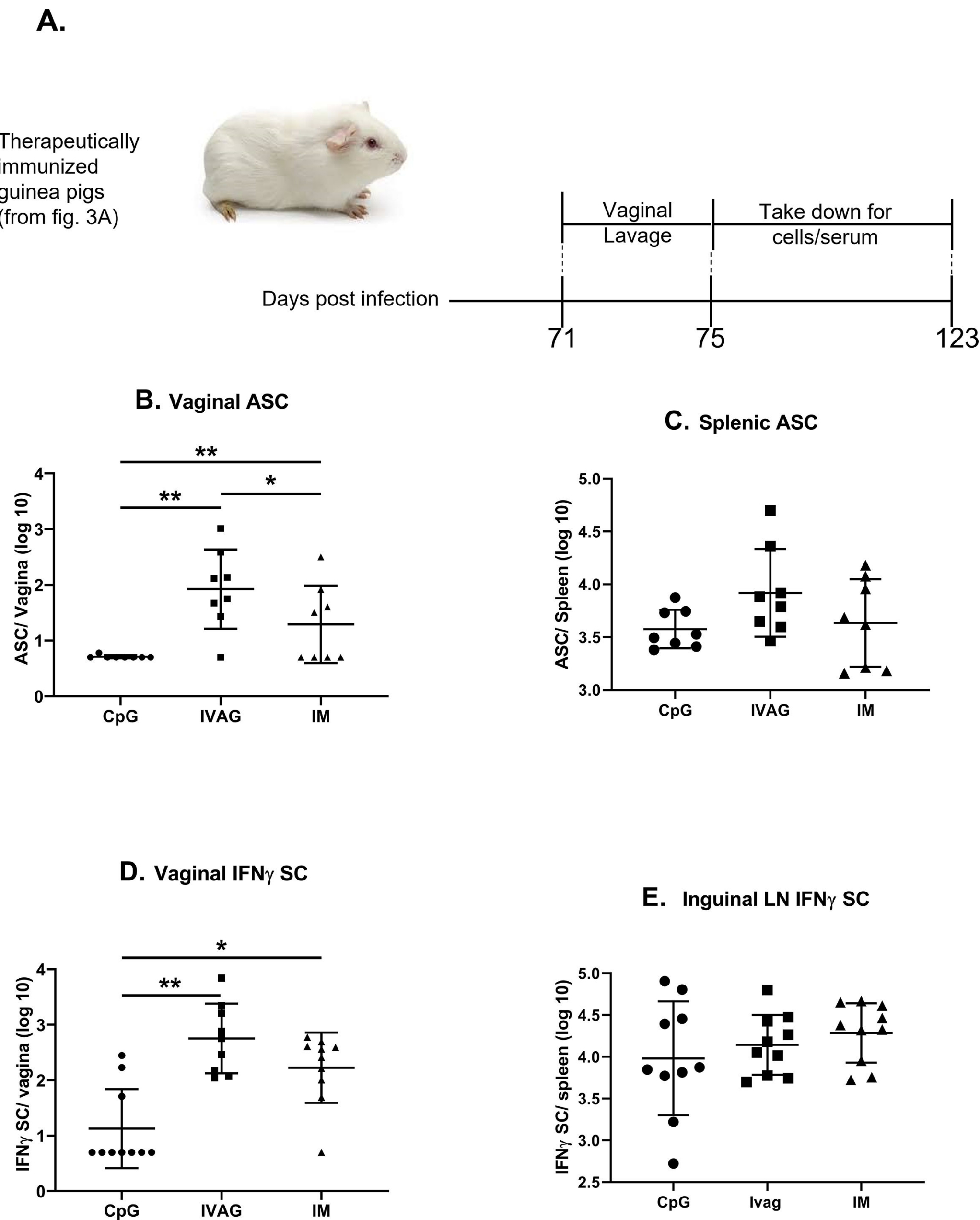

FIG 5 Greater numbers of HSV glycoprotein-specific ASCs and IFNγ SCs in the vaginal mucosa following Ivag therapeutic immunization. (A) Experimental design. Cells were taken to quantify HSV-glycoprotein-specific ASCs between days 73 and 103 post immunization. Cells were taken to quantify IFNγ SC between days 113 and 123 post immunization. (B) HSV glycoprotein-specific ASCs in vaginal mucosa (*$P < 0.003$, **$P < 0.002$, Welch's ANOVA with Dunnet's T3 multiple comparisons test; $N = 8$ for all groups). (C) HSV glycoprotein-specific ASCs in spleens (NS, $P > 0.05$; $N = 8$ per group). (D) HSV glycoprotein-specific IFNγ SC in the vaginal mucosa (*$P = 0.006$, **$P = 0.0001$, Welch's ANOVA with Dunnet's T3 multiple comparisons test; $N = 10$ per group). (E) HSV glycoprotein-specific IFNγ SC in the inguinal lymph nodes (NS, $P > 0.05$; $N = 10$ per group).

There is evidence that cell-mediated responses are highly involved in limiting HSV disease and virus shedding following HSV reactivation (18, 35, 36, 44). In guinea pigs, both $CD8^+$ and $CD4^+$ IFNγ secreting cells can be detected in the vaginal mucosa several months after Ivag HSV-2 infection (16). Depletion of the $CD4^+$ T cell subset from guinea pigs experiencing HSV-2 reactivations has been shown to play a role in limiting recurrent HSV shedding (45). In the present studies, therapeutic immunization of previously infected guinea pigs by either Ivag or IM routes resulted in significantly increased

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numbers of vaccine antigen-specific IFNγ secreting cells in the vaginal mucosa compared to control-treated animals (Fig. 5D). The T cell subpopulations enhanced by immunization were not directly identified, but given the recombinant antigens and adjuvant mixture used for immunization, the T cell response was likely predominated by $CD4^+$ T cells. Greater numbers of vaccine glycoprotein-specific IFNγ secreting cells were detected in the vaginal mucosa of Ivag compared to IM-immunized animals; however, the difference did not reach significance likely due to the variability in the response of individual animals.

In the current study, increased local immune responses following Ivag therapeutic immunization were detected in the setting of increased protection against recurrent HSV-2 disease following reactivation from latency (Fig. 5B through E). Ivag immunization resulted in significantly decreased recurrent HSV-2 disease, measured as a decrease in cumulative mean lesion days (Fig. 3B), compared to either IM immunization or control CpG treatment. Our results are consistent with those of others in which therapeutic immunization with experimental therapeutic HSV vaccines resulted in differentially diminished recurrent disease with little or no effect on HSV shedding (28, 46, 47). The reason for the lack of impact on virus shedding is unclear but may reflect the relatively low shedding rate in this experiment (ranging in all groups between 5.6% and 6.9%) or the relatively short time interval in which shedding was measured compared to recurrent disease (Fig. 3A). Alternatively, our recombinant protein vaccine likely primarily boosted the $CD4^+$ T cell response and not the HSV-specific $CD8^+$ T cell response required for optimal control of shedding. Utilization of vaccines capable of boosting both T cell subpopulations is likely required to control HSV-2 shedding.

In order to increase adaptive immune responses in the vagina, investigators have used therapeutic immunization of mucosal sites to induce vaginal mucosa-homing immune cells and have shown increased levels of antibody and, in some cases, increased numbers of vaginal antigen-specific T cells. Direct vaginal immunization with a therapeutic HSV-2 vaccine resulted in increased numbers of antigen-specific $CD4^+$ and $CD8^+$ T cells in the genital tract (26) and significantly diminished recurrent disease and HSV-2 shedding. Somewhat disparate results were reported by Bernstein et al. (25). Although these investigators demonstrated increased efficacy of a therapeutic HSV-2 vaccine delivered by the intranasal route compared to the Ivag route, comparison of these results to previous studies (26) and to the present study is complicated; in that, quantification of local vaginal immune responses resulting from the different immunization routes was not reported (25). In related studies, parenteral vaccination with an HSV-2 therapeutic vaccine followed by genital application of the TLR7 stimulant imiquimod resulted in greater numbers of vaginal T cells and a significant reduction in recurrent disease compared to imiquimod treatment or IM immunization alone (28). Although the antigen specificity of the T cell infiltrate responding to local imiquimod treatment was not defined, it is likely that both antigen-specific and non-specific T cells were recruited to the vaginal epithelium by this method, and both may have contributed to the increased efficacy of the vaccine.

In the present study, direct vaginal immunization, in the absence of hormonal conditioning, resulted in boosting both antibody and cell-mediated immunity in the vaginal mucosa. Furthermore, increased numbers of vaginal-resident HSV-specific ASCs were detected in one experiment greater than 100 days after therapeutic immunization (Fig. 1), and HSV glycoprotein-specific IFNγ SCs were detected between 70 and 80 days after the last therapeutic immunization (Fig. 3). Whether these cells are maintained indefinitely in the vagina or represent transient populations that ultimately return to pre-immunization levels requires further investigation. Vaginal immunization will require careful optimization of release of vaccine components as well as minimizing vaginal inflammatory responses. The use of novel technologies such as pod-based intravaginal rings (48, 49) may prove useful for therapeutic immunization in the future to provide controlled release of vaccine antigens/

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adjuvants necessary for optimal activation of protective immune cell populations. Given concerns that intravaginal immunization or "prime and pull" approaches may result in infiltration of inflammatory immune cells that serve as targets for HIV, these rings may also be able to release anti-retroviral compounds for additional protection during immunization.

MATERIALS AND METHODS

Virus

Working stocks of HSV-2 strain MS were prepared using Vero cell monolayers and stored at −80°C as previously described (50).

Guinea pig model of genital herpes

Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in an AAALAC-approved vivarium and allowed to acclimate prior to experimentation. All studies were humanely conducted with the approval of the UTMB Institutional Animal Care and Use Committee with oversight of staff veterinarians.

Animals were inoculated Ivag with 6.0 log_{10} plaque forming units (pfu) HSV-2 strain MS as described previously (47). Following HSV-2 inoculation, animals were examined daily, and the severity of primary disease and the frequency and severity of spontaneous recurrent disease were scored as previously described (51).

Vaginal swab samples were collected from HSV-2-infected guinea pigs into collection media on days 31–35 and 45–54 PI, and HSV-2 genomes were quantified by qPCR as described previously (30).

Immunization

HSV-2-infected guinea pigs included in experiments described in Fig. 1 and 2; Fig. S1 were immunized by Ivag instillation of 10.0 µg recombinant gD2 or gB2 plus 10.0 µg of the class B CpG oligonucleotide, OPN 2007 (Invivogen, San Diego, CA, USA) (gD2/CpG or gB2/CpG) per day for five sequential days. Following a 2-week rest, animals were again immunized with the same dose of gD2/CpG or gB2/CpG for five sequential days. For IM immunization, two single injections of gD2/CpG or gB2/CpG were administered in the quadriceps muscle 3 weeks apart. Control treatment was accomplished by Ivag instillation of 10.0 µg CpG on the same days as Ivag immunization. For therapeutic immunization described in Fig. 3, animals received two Ivag immunizations with 5.0 µg gD2 + 5.0 µg gB2/10.0 µg CpG or 10.0 µg CpG only per day for five sequential days with a 1-week rest between immunizations. For IM immunization, a single immunization of 5.0 µg gD2 + 5.0 µg gB2/10.0 µg CpG was given in the quadriceps muscle on two occasions, 2 weeks apart.

HSV glycoprotein-specific ELISpot

Quantification of HSV glycoprotein-specific ASCs from the vaginal mucosa and spleen was performed by ELISpot as previously described (16) on recombinant gD2- and/or gB2-coated plates. Background spots detected on OVA-coated wells were routinely subtracted from total spots detected on HSV-2 glycoprotein-coated plates.

ELISA

For ELISA measurement of HSV-2 glycoprotein-specific IgG antibodies in serum and vaginal lavages, serial dilutions of samples were plated in duplicate on gD2- or gB2-coated plates and incubated at 4°C overnight. IgG titers were detected by incubation with HRP-goat anti-guinea pig IgG (Abcam, Cambridge, MA, USA) and developed as described previously (29). The optical density at 405 nm (OD_{405}) was determined using SoftmaxPro 7.0.3 software. The endpoint titer was defined as the sample dilution

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resulting in an OD_{405} reading greater than 0.1 and greater than three standard deviations above media blank values.

IFNγ ELISpot

HSV gD2- and gB2-specific IFNγ secreting cells were quantified by ELISpot from populations of lymphocytes enriched from the vaginal mucosa or inguinal lymph nodes of immunized and control-treated animals as previously described (16, 45). Mesenteric lymph node populations from individual animals served as antigen presenting cells and were pulsed with either recombinant gD2 + gB2 proteins or control treated with medium only and cultured with spleen or vaginal mucosa lymphocytes on anti-guinea pig IFNγ-coated ELISpot plates. Spots representing IFNγ SCs were developed as described previously (16).

Statistics

For all groups, data were analyzed using Prism software (v8.2; GraphPad, La Jolla, CA, USA). Comparisons between multiple groups were made using Welch's ANOVA with Dunnet's T3 multiple comparisons test. Comparisons between the means of two groups were made using Welch's *t*-test. Comparison of the slopes of cumulative virus shedding curves was made between groups using linear regression analysis and area under the curve. Comparison of shedding incidence was made using Fisher's exact test. For all comparisons, a *P* value of 0.05 was used to designate significance.

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Nigel Bourne, Conceptualization, Data curation, Investigation, Methodology, Writing – review and editing | Celeste A. Keith, Investigation, Methodology | Aaron L. Miller, Data curation, Formal analysis, Investigation | Richard B. Pyles, Data curation, Methodology, Resources | Gary Cohen, Resources | Gregg N. Milligan, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review and editing

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Figure S1 Legend (JVI00669-23-s0001.docx). Full legend of Fig. S1.
Fig. S1 (JVI00669-23-s0002.tif). Increased vaginal and serum anti-gD2 IgG titers following therapeutic vaccination with 1.0 or 10.0 µg gD2/CpG.

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