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EEHV1A glycoprotein B subunit vaccine elicits humoral and cell-mediated immune responses in mice

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ABSTRACT

Asian elephants are an endangered species facing many threats, including severe hemorrhagic disease (HD) caused by the elephant endotheliotropic herpesvirus (EEHV). EEHV-HD is the leading cause of death in captive juvenile Asian elephants in North America and Europe, and also affects elephants in their natural range countries. Significant challenges exist for successful treatment of EEHV-HD, which include timely recognition of disease onset and limited availability of highly effective treatment options. To address this problem, our goal is to prevent lethal disease in young elephants by developing a vaccine that elicits robust and durable humoral and cell-mediated immunity against EEHV. EEHV glycoprotein B (gB) is a major target for cellular and humoral immunity in elephants previously exposed to EEHV. Therefore, we generated a vaccine containing recombinant EEHV1A gB together with a liposome formulated TLR-4 and saponin combination adjuvant (SLA-LSQ). CD-1 mice that received one or two vaccinations with the vaccine elicited significant anti-gB antibody and polyfunctional $CD4^+$ and $CD8^+$ T cell responses, while no adverse effects of vaccination were observed. Overall, our findings demonstrate that an adjuvanted gB protein subunit vaccine stimulates robust humoral and cell-mediated immune responses and supports its potential use in elephants.

1. Introduction

Elephant endotheliotropic herpesvirus (EEHV) can cause lethal hemorrhagic disease (HD) in both Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants [1]. EEHV-HD is the leading cause of death in juvenile captive Asian elephants in North America and Europe with calves between 1 and 8 years old being most susceptible to disease [1]. Moreover, EEHV-HD is increasingly being recognized in both captive and wild Asian elephants in their natural range countries [1]. While documented cases of EEHV-HD in African elephants were rare prior to 2019, recent morbidity and mortality in captive African elephants in North American zoological institutions has been observed and is a growing concern [2].

Several EEHV species within the the *Proboscivirus* genus (proposed *Deltaherpesvirinae* subfamily) have been identified and appear to be endemic within Asian and African elephants. EEHV species 1A, 1B, 4, and 5 affect Asian elephant populations, while EEHVs 2, 3A, 3B, 6, and 7 circulate in African elephants [1,3]. In Asian elephants, EEHV1A and EEHV1B chimeric variants are responsible for most EEHV-related deaths [1], while EEHV3 has caused most African elephant EEHV-HD cases [2]. To better monitor elephants for EEHV viremia, our laboratory developed real-time PCR assays to detect all species of EEHV in captive elephant herds [4–6]. While detection has been an effective tool, viremia can occur in infected elephants with no visible signs of disease, and recognition of clinical signs often occurs when infection has progressed to stages where treatment may be less effective. Treatment options consist largely of supportive care and include fluids, plasma from elephants experienced with prior infection, and anti-herpesvirus drugs [7]. To date, the effectiveness of anti-herpesvirus drugs against EEHV remains unknown.

Our goal is to develop an immunogenic EEHV vaccine to protect juvenile elephants against lethal EEHV-HD, with initial efforts

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focused on EEHV1A in endangered Asian elephants. However, current obstacles for achieving this goal include the inability to grow the virus in culture, a lack of neutralizing antibody assays, and the absence of an appropriate model for challenge studies. Despite these limitations, the urgency of the situation underscores the need to generate an effective EEHV vaccine.

Previous data have shown that both humoral and cellular-mediated immunity (CMI) are important for protection and clearance of herpesvirus infections [8–13]. We have identified EEHV glycoprotein B (gB), E40, and the major capsid protein (MCP) as immunoprevalent T cell targets in adult Asian elephants chronically infected with EEHV [14]. Our laboratory has also shown that EEHV specific maternal antibodies are prenatally transferred to juvenile elephants [15,16] and that waning of these antibodies correlates with vulnerability to primary EEHV infection and disease susceptibility [15,17]. Due to many EEHV-HD fatalities being seronegative with the EEHV type causing infection, and the importance of CMI in controlling human herpesvirus infections, we postulate that a protective EEHV vaccine will need to stimulate robust humoral and T cell immunity, particularly against previously identified EEHV antigens that are targeted by these responses.

Because of the inability to grow EEHV in culture, inactivated or attenuated virus vaccine strategies are not possible. Therefore, we have chosen to focus on an adjuvanted subunit vaccine platform targeting EEHV1A glycoprotein gB. Specifically, we chose to incorporate gB into our EEHV1A subunit vaccine because: 1) it is highly conserved across all EEHV species, 2) it has high antibody cross-reactivity between all Asian elephant EEHVs, 3) it is an immunodominant target for CMI in chronically infected adult elephants, and 4) low levels of gB specific antibodies can correlate with increased risk of EEHV-HD [14,15,17]. Furthermore, glycoproteins have also been shown to elicit robust and long-lasting humoral and CMI responses in the context of herpesviruses [18]. Subunit-based vaccines have good safety records in animal and human systems, and numerous glycoprotein subunit vaccines are already licensed or currently being evaluated [19–21]. In humans, the Shingrix vaccine composed of viral glycoprotein E and Lipid A-based adjuvants has been effective against shingles by inducing strong VZV specific CMI [22]. Many veterinary herpesvirus vaccines incorporate glycoproteins, including glycoprotein B for Guinea pig CMV [23], glycoproteins B and G for pseudorabies virus in piglets [24], and glycoprotein D in bovine herpesvirus 5 [25].

In this study, we demonstrate *in vivo* safety and immunogenicity of an adjuvanted EEHV1A gB subunit vaccine in a mouse model. We are unaware of any vaccine developed specifically for elephants or of any extensive testing of vaccines used for other species being tested in mice as a precursor to evaluate their use in elephants. Therefore, we are in uncharted waters. The rational for using mice was to assess immunogenicity of our vaccine in an animal or whether any general concerning side-effects might be induced. At the current time, it is unclear whether any other animal model would yield helpful information (i.e., about the efficacy of our vaccine) beyond what we learn in a mouse model system, especially since there is not available challenge model. Our data shows that the adjuvanted gB vaccine elicits robust gB-specific antibody production and CMI responses, indicating that it holds promise as a vaccine for preventing fatal EEHV-HD in juvenile endangered elephants.

2. Materials and methods

2.1. Production and purification of gB protein.

The gB protein was expressed and purified as previously described [17]. Briefly, EEHV1A gB ectodomain sequences (residues 43–685) were codon-optimized and cloned into the pFRT expression plasmid with a C-terminal Strep tag. Seven amino acid substitutions were made in the fusion loops and furin cleavage site to enhance protein expression. Plasmid expressing gB was transfected into HEK293F cells and cell culture supernatants were harvested after 5 days. Supernatants were centrifuged at low speed to remove cellular debris and gB protein was purified with Strep-Tactin Sepharose beads. Purified gB protein was run on protein gels and protein levels were measured by quantitative densitometry and compared to a BSA standard curve.

2.2. Mouse immunizations

Six to 8-week-old female CD-1 mice were used in this study based on the hypothesis that outbred animals may respond to vaccination within a range that is representative of responses that might be seen in an elephant population of heterogeneous individuals. All mice were purchased from the Center for Comparative Medicine (CCM) at Baylor College of Medicine. A detailed overview of the vaccination schedule and study design is described in Fig. 1 and Table 1. Mice were separated into two treatment groups ($n = 6$) that received injections of gB and adjuvant or saline and adjuvant. The adjuvant used in these studies consisted of a liposome encapsulated combination of synthetic monophosphoryl lipid A (SLA) toll-like receptor 4 (TLR4) ligand and saponin (QS-21) (IDRI-LS530, Infectious Disease Research Institute). The gB with adjuvant group received 100 μL of 1 μg gB and 5 mg SLA/2mg QS-21 liposome formulated adjuvant in sterile saline. The saline with adjuvant group received 100 μL of 5 mg SLA/2mg QS-21 liposome formulated adjuvant in sterile saline. Mice received up to two intraperitoneal injections at 4 week intervals, yielding prime vaccinated and prime-boost vaccinated groups. Six mice per treatment group were euthanized 4 weeks after each injection, serum was harvested for measuring antibody production, and spleens were processed for cellular staining and flow cytometric analysis as described below. All procedures used in this study were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC) protocol AN-7938. Vaccinated mice were euthanized in accordance with IACUC and CCM Euthanasia in Rodents Policy consisting of carbon dioxide gas (CO_2) asphyxiation and a bilateral thoracotomy (secondary method).

2.3. *Immunologic assays for evaluation of gB-specific immune responses*

(i)gB-specific Luciferase Immunoprecipitations System (LIPS) antibody assay.

Preparation of GLuc-antigen fusion constructs were performed as previously described [15,26]. To show specificity for gB antibody responses, antibodies against an additional EEHV1A glycoprotein, gH, were also measured. Briefly, codon-optimized sequences for EEHV1A strain Kimba gB and gH ectodomains were synthesized (GenScript) and subcloned into the pGaus3 expression plasmid. These sequences included amino acid residues 1–731 for gB and 1–705 for gH. Expression plasmids were transfected into HEK 293 T cells, and supernatants and cell extracts were harvested and stored at −80 °C. LIPS analysis was performed as previously described [15]. Briefly, serum samples were diluted 1:10 in Buffer A (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM Mg Cl2, 1 % Triton X-100) for storage up to a month at 4 °C. For use in the assay, samples were diluted 1:5 in the same buffer. Relative luminescence units (RLU) of stored GLuc extracts were determined on the day of the assay and a mastermix of each GLuc extract containing $1x10^7$ LU per 50 μL was made. Fifty μL of GLuc extract mastermix was added to 50 μL of diluted serum (in duplicate) and incubated on a shaker at room temperature (RT) for 1 h. After 1 h, GLuc extract-diluted

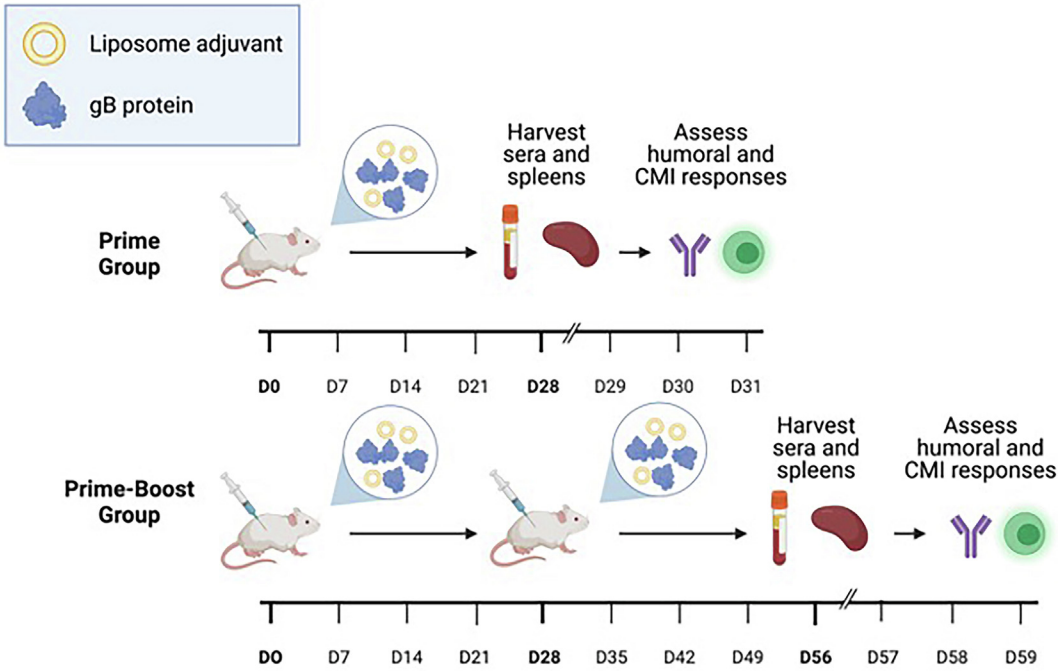

Fig. 1. Mouse gB subunit vaccine timeline. Mice were separated into prime or prime-boost cohorts and received one or two injections with gB protein subunit and liposomal adjuvant. Mouse sera and spleens were harvested 4 or 8 weeks post-injection(s). Sera was used to assess gB-specific antibody production. Splenocytes were processed and stimulated with gB peptides to induce T cell activation and assessed by flow cytometry.
This figure was created using BioRender.com.

Table 1
Overview of vaccination groups and immunization schedules.

Group	Mice (*n*)	Treatment 1 (Day 0)	Treatment 2 (4 weeks)	Harvesting
Prime	6	gB + Adjuvant[a]	N/A	4 weeks
	6	Saline + Adjuvant[b]		
Prime-boost	6	gB + Adjuvant[a]	gB + Adjuvant[a]	8 weeks
	6	Saline + Adjuvant[b]	Saline + Adjuvant[b]	

[a] 1μg gB, 5 mg SLA, 2 mg QS-21 in 100 μL.
[b] 5mg SLA, 2 mg QS-21 in 100 μL.

serum was added to 5 μL of a 30 % Protein A-G bead suspension (diluted in PBS) in a 96-well filter plate (Millipore). Samples were incubated with the beads for 1 h at RT with constant shaking. Beads were then washed five times with Buffer A and two times with PBS using a 96-well vacuum manifold (Millipore). An OmniStar automatic plate luminometer (BMG Labtech) was used to inject 50 μL of Renilla luciferase assay substrate into well, shake for 2 s, and record RLU values for 5 s. Values reported represent the average RLUs over 5 s post-injection.

(ii)gB-specific T cell responses.

Mouse spleens were processed by trimming excess fat, cutting spleens into multiple pieces, and mashing spleen remnants though a 40 μm filter. Red blood cells were lysed and splenocytes were centrifuged at 350 × g for 5 min before resuspending in complete RPMI-1640 media supplemented with 10 % FBS, penicillin/streptomycin, and 2-mercaptoethanol. Cells were pelleted at 350 × g for 5 min and resuspended in 10 mL RPMI-1640 media. After isolation, $2x10^6$ splenocytes were plated on 96-well round bottom plates and stimulated at 37 °C overnight with an EEHV1A gB peptide pool at 1ug/mL in complete RPMI-1640 medium. The EEHV1A glycoprotein B peptides were synthesized and purified by Mimotopes at 1 mg per vial. Peptide libraries are consecutive 15-mers overlapping by 11 amino acids spanning the length of gB (210 peptides). After peptide stimulation, Brefeldin A (BD Bioscience) and Monensin (2 μg/mL) were added, and the cells were incubated an additional 6 h at 37 °C. At the end of the stimulation period, cells were washed and incubated with Fc Block (BD Biosciences) for 15 min at 4 °C.

After fixation, splenocytes were permeabilized with 1 × perm/wash solution (BD Biosciences) before intracellular cytokine staining. Cells were stained with intracellular cytokine specific antibodies diluted in a 1:1 mixture of Brilliant Stain Buffer (BD Biosciences) and 2 × perm/wash buffer at 4 °C for 30 min in the dark. Stimulated spleen cells were washed in perm/wash buffer and resuspended in analysis buffer (PBS without Mg^{2+} and Ca^{2+}, 2 % FBS, 2 mM EDTA, 25 mM HEPES) prior to flow cytometric analysis. Dead cells were excluded using GhostRed780 kit (Tonbo). The fluorochrome-conjugated, anti-mouse antibodies used are described in Table 2. Cell staining was analyzed using an LRSII (BD Biosciences) and data analysis was performed using the FlowJo software version 10.7 (BD Biosciences). Background responses in negative controls were subtracted from stimulated samples.

Table 2
Specific antibodies used to assess CMI in vaccinated mice.

Antibody Target	Fluorophore	Dilution	Manufacturer	Catalog #	Clone
CD3e	BV711	1:40	BioLegend	100,241	17A2
CD4	BUV395	1:400	BD Biosciences	563,790	GK1.5
CD8a	FITC	1:800	Tonbo	35–0081-U500	53–6.7
IFNγ	BV421	1:50	BD Biosciences	563,376	XMG1.2
IL-2	APC	1:200	Tonbo	20–7021-U100	JES6-5H4
TNFα	PE	1:40	BioLegend	506,306	MP6-XT22
IL-4	BV605	1:25	BioLegend	504,126	11B11
Viability	GhoseDye780	1:50	Tonbo	13–0865-T500	N/A

2.4. Statistical analysis

Statistical analysis was done with GraphPad Prism software version 9 (San Diego). LIPS assays were performed in duplicate, with at least 2 independent experiments for each sample. For LIPS, results for different groups are presented as geometric means ± the standard deviation (SD) on $\log_{10}$ values. Normality of the data sets were determined using the Shapiro-Wilk test. Significance of normally distributed data were determined by unpaired t-tests with a p value ≤ 0.05 denoting statistical significance. The cut-off level for determining sensitivity and specificity for each viral antigen was derived from the mean antibody titer of the no serum control samples plus 5 SD. Additional details describing establishment of the EEHV-specific LIPS assay have been published previously [15]. For flow cytometry assays, results are presented as mean ± the SD of percent of positive cells. Data is presented as the sum of percent positive cells from all gB peptide pools per mouse. Normality of the data sets were determined using the Shapiro-Wilk test. Significance of normally distributed data were determined by two-way ANOVA with Tukey's multiple comparisons test with a p value ≤ 0.05 denoting statistical significance. Outliers were identified by ROUT analysis and removed.

3. Results

3.1. Prime-boost gB subunit vaccine elicits robust humoral immunity

CD-1 mice were divided into prime and prime-boost cohorts, receiving either one or two vaccinations 4 weeks apart, and were monitored biweekly for signs of distress including hunched or abnormal posture, decreased movement or activity, changes in grooming or appearance, and excessive licking or scratching. Signs of distress were chosen based on the Guide for the Care and Use of Laboratory Animals and Recognition and Alleviation of Distress in Laboratory Animals. Importantly, no signs of illness, discomfort, or side effects were observed in vaccinated mice throughout the study. Four weeks post-vaccination, mouse sera were harvested to measure humoral responses using the LIPS assay as previously described [15,26]. Mice that received a single adjuvanted gB vaccine produced gB-specific antibodies while mice that received saline with adjuvant had no detectable anti-gB antibodies (Fig. 2**A**). No antibody responses were detected against gH in either group of vaccinated mice (Fig. 2**B**). These data indicate that a single dose of the adjuvanted gB subunit vaccine elicits strong humoral immunity specific for the gB antigen. Mice boosted at 4 weeks with the adjuvanted gB subunit vaccine continued to produce high levels of gB-specific antibodies that were slightly increased over levels observed with the single prime vaccine (14297 RLUs versus 9809 RLUs) (Fig. 2**C**). Mice that received a second vaccination with saline plus adjuvant had no detectable anti-gB antibodies. Both groups of prime-boost vaccinated mice had no detectable antibodies against the unrelated gH antigen (Fig. 2**D**). Taken together, these data indicate that even a single dose of adjuvanted gB vaccine induces maximal or near maximal anti-gB antibody responses.

3.2. Adjuvanted ***gB subunit vaccines induce strong general T cell responses in mice***

Splenocytes harvested from gB vaccinated or control mice were used to measure CMI responses post-vaccination for both prime and prime-boost vaccinated groups. Spleen cells were isolated and stimulated with gB peptide pools representative of the entire gB protein [14], and cytokine producing T cells were measured by flow cytometry. Live cells were distinguished using a live/dead cellular stain, and gated based on $CD3^+$, $CD4^+$, and $CD8^+$ cellular markers. Resulting $CD4^+$ and $CD8^+$ populations were analyzed for intracellular cytokine production. Intracellular cytokines indicative of T_H1 responses included interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), or interleukin-2 (IL-2). Total $CD4^+$ and $CD8^+$ T cells expressing at least one T_H1 marker were significantly higher in mice that received a prime vaccine compared to mice receiving the adjuvant only (Fig. 3**A**). Similar results were seen for mice that received a booster vaccine. When analyzed separately, mice vaccinated with gB and adjuvant had higher $CD4^+$ T cells expressing at least one T_H1 cytokine (up to 40 %) than those inoculated with saline with adjuvant after a single priming vaccine (Fig. 3**B**). $CD8^+$ cytokine producing T cells were increased in splenocytes of mice vaccinated with adjuvanted gB (up to 35 %) compared to that of mice vaccinated with saline and adjuvant (Fig. 3**C**). While $CD4^+$ and $CD8^+$ total populations appeared to increase following a booster inoculation, the background levels in adjuvant only vaccinated mice tended to be slightly higher as well. Regardless, these data indicate that our adjuvanted gB subunit prime-boost vaccine platform elicits robust, general T_H1-type responses.

3.3. gB subunit vaccines induce polyfunctional $CD4^+$ T cell responses in mice

In addition to measuring total T cell cytokine production, we also quantified the percentages of IFNγ, TNFα, and IL-2 positive T cells. Splenocytes from the prime vaccinated mice generated greater $IFN\gamma^+$ $CD4^+$ T cells compared to the adjuvant only vaccinated group (20 % versus 10 %) (Fig. 4**A**). However, this increase was not observed in the prime-boost vaccinated group. Mice primed with the gB vaccine had significantly higher $CD4^+$ cells expressing TNFα or IL-2 than the adjuvant only control (5 % versus 1 % and 0.75 % versus 0.25 %) inoculated mice (Fig. 4**B and C**). In contrast to IFNγ expression, mice receiving a booster vaccine continued to express higher levels of TNFα or IL-2 than adjuvant only vaccinated mice. Small populations of $CD4^+$ T cells were also polyfunctional and expressed multiple T_H1 cytokines following initial vaccination. Splenocytes from gB vaccinated mice showed a trend of increasing $IFN\gamma^+/TNF\alpha^+$ and $TNF\alpha^+/IL\text{-}2^+$ $CD4^+$ T cells compared to splenocytes from adjuvant only vaccinated mice, although no differences were seen for $IFN\gamma^+/IL\text{-}2^+$ (Fig. 4**D-F**). However, after a booster vaccination, significantly higher levels of $IFN\gamma^+/TNF\alpha^+$, $IFN\gamma^+/IL\text{-}2^+$, and $TNF\alpha^+/IL\text{-}2^+$ expressing $CD4^+$ T cells were observed compared to mice boosted with adjuvant only. We also measured triple positive ($IFN\gamma^+/TNF\alpha^+/IL\text{-}2^+$) $CD4^+$ and $CD8^+$ T cells and saw

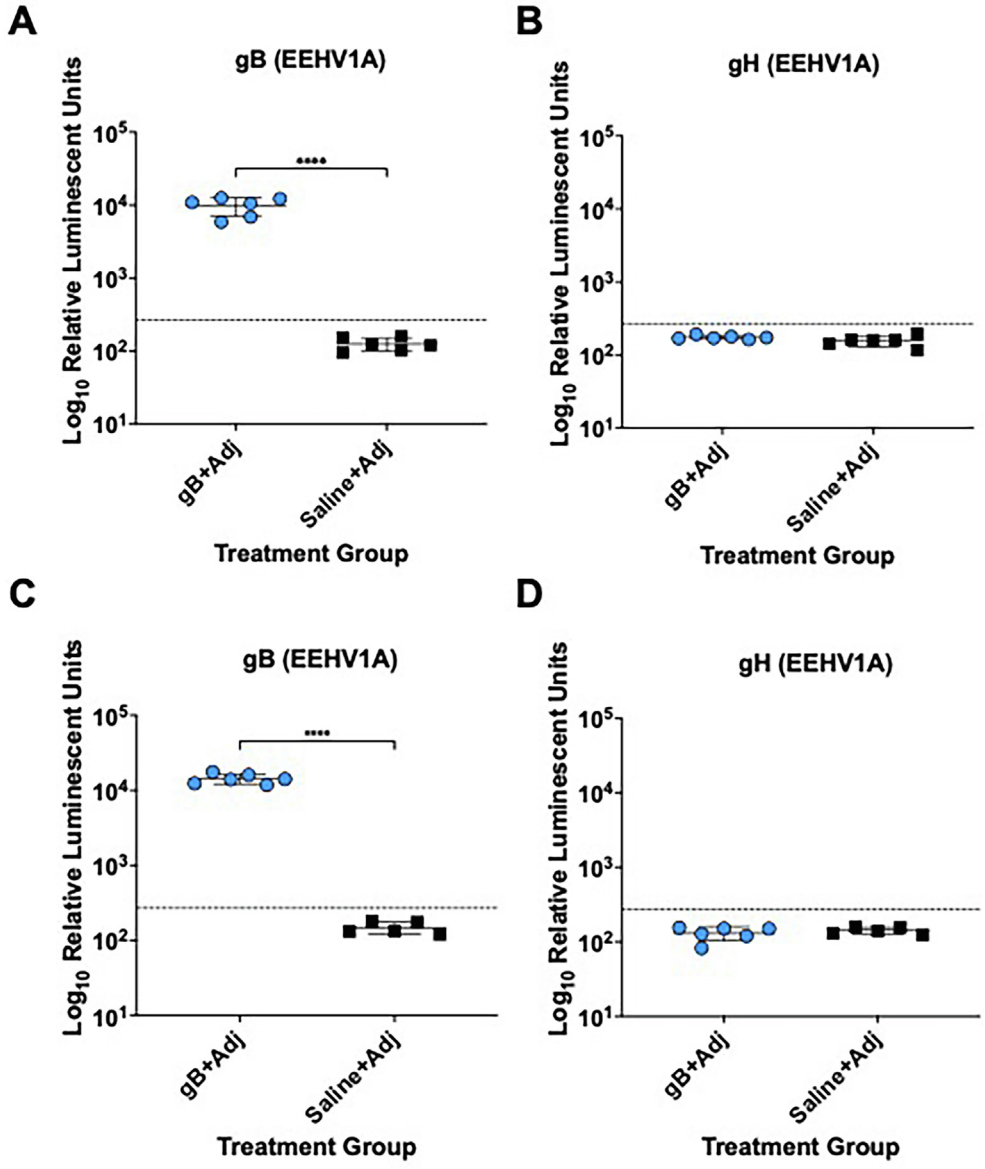

Fig. 2. Prime and prime-boost injections with gB induces anti-gB antibody responses in mice. Specific antibody titers for gB or gH from prime (**A, B**) and prime-boost (**C, D**) vaccinated mice. Sera from mice inoculated with gB with adjuvant and saline with adjuvant are represented by blue circles and black squares, respectively. Antibody levels are expressed in RLUs and plotted on a $\log_{10}$ scale. Mean ± SD values for each are shown, with each symbol representing the mean result for one animal with two replicates. Asterisks (****) indicate the statistically significant difference ($p \leq 0.0001$) between immunized groups, as determined by unpaired t-tests. The dashed line indicates the cut-off level for determining the sensitivity and specificity for each viral antigen, and is derived from the mean antibody titer of seronegative serum samples or no serum controls plus 5 SD.

an increasing trend after the booster vaccination, although the differences were not statistically significant (data not shown). Importantly, we found no detectable IL-4$^+$ CD4$^+$ T cells after the prime and prime-boost vaccines, suggesting that the vaccine does not induce significant T_H2-type responses (Fig. 4**G**). Taken together, these data show that the prime-boost adjuvanted gB vaccine regimen induces a robust polyfunctional and T_H1-specific CD4$^+$ T cell responses in mice.

3.4. gB subunit vaccines induce polyfunctional CD8$^+$ T cell responses in mice

Activation of CD8$^+$ T cells was measured additionally from mouse splenocytes stimulated with gB peptide pools. Splenocytes from gB vaccinated mice yielded significantly higher percentages of IFNγ^+ CD8$^+$ T cells (~10 %) compared with splenocytes from adjuvant only vaccinated mice (~5%) (Fig. 5**A**). This difference was increased after the prime-boost vaccines (~13 % versus ~ 10 %) but was not statistically significant. Additionally, more TNFα^+ CD8$^+$ T cells were observed from gB vaccinated mice compared to adjuvant only vaccinated mice regardless of whether they received a single (prime) or booster vaccine (prime-boost) (Fig. 5**B**). In contrast, no significant differences were observed for any group regarding IL-2 expression (Fig. 5**C**). Double positive, polyfunctional CD8$^+$ T cell populations were also assessed. While IFNγ^+/TNFα^+ CD8$^+$ T cells were generally increased in the gB vaccinated group compared to adjuvant only vaccinated groups, the differences were not significant until the mice received the booster vaccination (Fig. 5**D**). Similar trends were observed for CD8$^+$ T cell populations expressing IFNγ^+/IL-2$^+$ and TNFα^+/IL-2$^+$ (Fig. 5**E-F**). Importantly, like the results seen for CD4$^+$ cells, there was no discernable IL-4$^+$ CD8$^+$ T cell populations across prime and prime-boost vaccination groups indicating no substantial T_H2 response profile (Fig. 5**G**). Overall, these data indicate that our adjuvanted

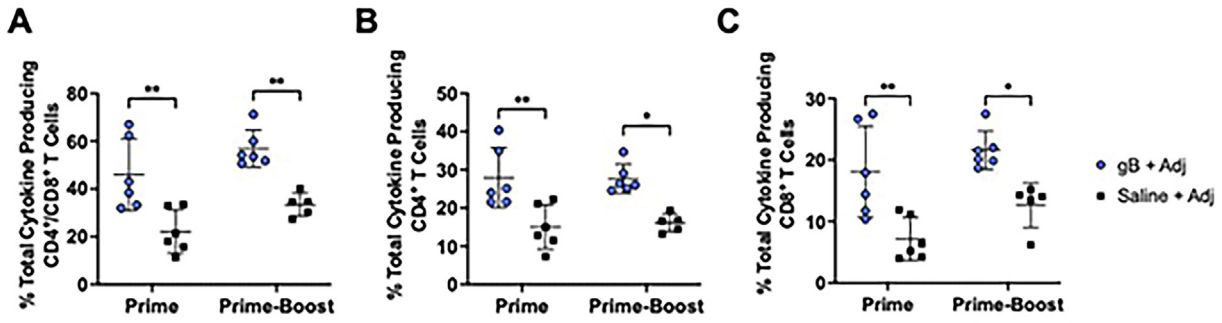

Fig. 3. Induction of gB-specific T cell responses after prime and prime-boost immunizations. Splenic T cells from immunized mice were assessed for cytokine production after stimulation with gB peptide pools. Splenic T cells from animals immunized with gB and adjuvant or saline and adjuvant are represented by blue circles and black squares, respectively. (**A**) Percentage of $CD4^+$ and $CD8^+$ cells producing at least one of three cytokines IFNγ, TNFα, or IL-2. (**B**) Percentage of $CD4^+$ cells producing at least one of three cytokines IFNγ, TNFα, or IL-2. (**C**) Percentage of $CD8^+$ cells producing at least one of three cytokines IFNγ, TNFα, or IL-2. Mean values ± SD values for each group are shown as horizontal lines and error bars. Asterisks (*/**) indicate statistically significant differences ($p \leq 0.05/p \leq 0.01$) between immunization groups and number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test. A p value ≤ 0.05 denotes significance.

gB subunit prime-boost vaccine induces an activated, polyfunctional, and T_H1-specific $CD8^+$ T cell response in mice.

4. Discussion

In this study, we generated a TLR4 ligand/saponin adjuvanted subunit vaccine approach containing the gB surface glycoprotein from the most lethal EEHV subtype, EEHV1A. The gB protein was chosen as it is a known immunoprevalent target for CMI and elicits robust antibody responses in elephants [14,15,17]. Vaccinated mice produced significant humoral and polyfunctional $CD4^+$ and $CD8^+$ T cell responses against EEHV1A gB (Figs. 2-5). Importantly, no signs of illness or adverse effects were observed in vaccinated mice. Taken together, these findings support the potential use of our adjuvanted gB subunit vaccine in elephants.

The induction of robust antibody responses in this study is similar to those observed with other adjuvanted gB vaccines for human and guinea pig CMV, which like EEHV, are members of the betaherpesvirus subfamily [27–29]. In addition, anti-gB antibody levels elicited in mice averaged almost 2 logs above the cut-off, which is similar to levels observed in EEHV1A experienced healthy adult elephants [15]. While induction of neutralizing antibodies was observed in these other studies [27–29], it was also noted that non-neutralizing antibodies may have significant roles for contributing to vaccine efficiency. However, because no system exists for cultivating EEHV at present, we are unable to evaluate potential induction of neutralizing antibodies or investigate potential mechanisms involving non-neutralizing antibodies such as antibody dependent cell-mediated phagocytosis. Nonetheless, these prior studies predict that our adjuvanted EEHV1A gB subunit vaccine has the potential for eliciting similar functional antibody responses.

Our adjuvanted gB subunit vaccine elicited significant levels of $CD4^+$ and $CD8^+$ T cell responses in mice (Figs. 3-5). Previous studies have also shown that both $CD4^+$ and $CD8^+$ T cell responses against gB are generated following infection by other herpesviruses [30–34]. An interesting and potentially beneficial feature of responses observed in this current study was the polyfunctionality of antigen-specific T cells following vaccination. These cytokines play vital roles in protection and generation of memory immune responses [35]. TNFα plays an important role for protection against infectious pathogens and for maintenance of the microarchitecture in lymphoid organs while IL-2 is critical for generation of antiviral CD8 responses. $CD4^+$ T cells have recently been described as having cytotoxic functions as well and may also contribute to cellular killing [31,36–38]. Additionally, we showed that the adjuvanted gB subunit vaccine stimulates a strong and singularly T_H1-specific response, which allays concerns about potential detrimental immune responses generated by the vaccine.

How durable will the immune response from this vaccine be in elephants? We are unaware of any studies in which persistence of immunity following vaccination in mice can be used to predict persistence of immunity in an elephant. Another complication is that different vaccines will elicit immunity that lasts to varying degrees for a variety of reasons (vaccine platform, immunogenicity of the vaccine antigens, prior infection with related pathogens etc etc). We intend to monitor immune responses from our vaccine in elephants over time to better gauge this question in the future.

An important component of this study was comparing the immunogenicity of a prime only and prime-boost subunit vaccine regimen. While the antibody responses were similar following the prime or boost inoculations, the CMI responses were markedly increased after receiving a booster vaccination. Generally, apart from IFNγ, there was an increased population of polyfunctional $CD4^+$ T cells after the booster vaccine. We noted similar changes in $CD8^+$ T cell polyfunctionality across all cytokines measured except IL-2. We also found that there were higher overall percentages of activated $CD4^+$ T cells than $CD8^+$ T cells. It is possible that the differences in activated $CD4^+$ or $CD8^+$ T cells populations could be attributed to the saponin and TLR4 ligand components of adjuvant used in this study. Saponin can modulate CMI and antibody responses [39], while TLRs can indirectly stimulate T cell responses through enhanced antigen presentation, costimulatory molecule expression, and pro-inflammatory cytokine production [40]. However, TLR4 may specifically elicit a T cell receptor independent mechanism of bystander $CD4^+$ T cell activation [41]. Therefore, it is possible that the enhanced CD4-specific CMI responses are influenced by using the saponin and TLR4 ligand-based adjuvant.

While gB subunit vaccines against CMV have shown 50 % effectiveness in humans and some protection in animal systems [42], others have shown that additional glycoproteins involved in attachment and fusion activities, such as gH and gL, may also elicit significant protective antibody responses [43–45]. In light of these results, we postulate that multiple EEHV antigens, in particular gH and gL, may be necessary in addition to gB to induce protection from EEHV-HD in vulnerable elephants. Additionally, previous studies have indicated that a combination of multiple vaccine platforms, or a heterologous prime-boost approach, often induces more robust immune responses than a homologous prime-boost regimen [46–48]. In this regard, we have generated a recombinant Modified Vaccinia Ankara virus expressing EEHV1A gB, which might provide a suitable partner vaccine for this type of approach [49]. When this current study was initiated,

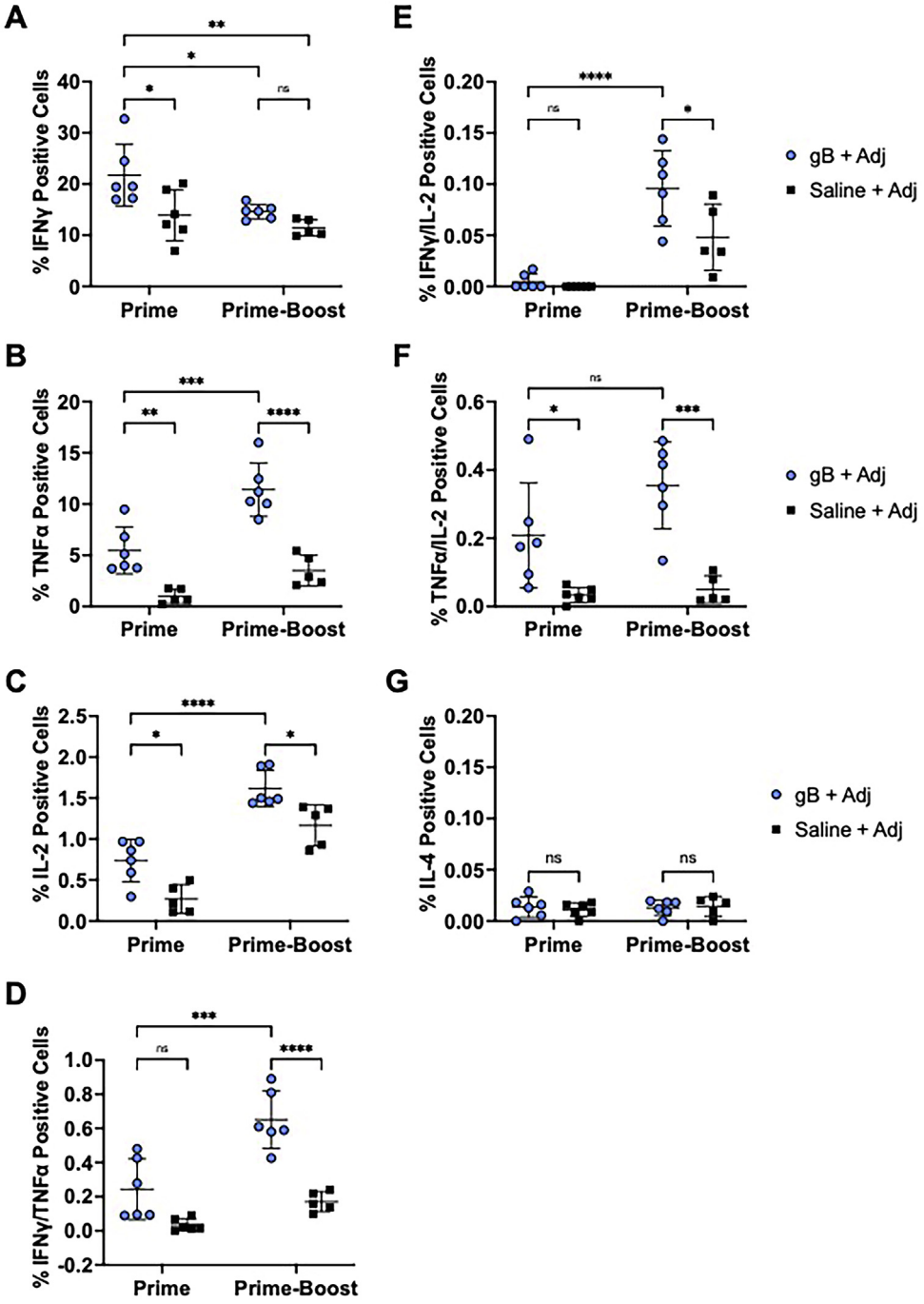

Fig. 4. Prime and prime-boost gB vaccination induces polyfunctional CD4⁺ T cell responses in mice. CD4⁺ T cells isolated from splenocytes of immunized mice were assessed for cytokine production after gB peptide pool stimulation. Percentage of CD4⁺ T cells positive for single cytokine activation markers IFNγ (**A**), TNFα (**B**), or IL-2 (**C**), or double positive cytokine activation markers IFNγ/TNFα (**D**), IFNγ/IL-2 (**E**), or TNFα/IL-2 (**F**). IL-4 was also included to measure induction of a T_H2 response (**G**). Splenic T cells from animals immunized with gB and adjuvant or saline and adjuvant are represented by blue circles and black squares, respectively. Mean values ± SD values for each group are shown as horizontal lines and error bars. Asterisks (*/**/***/****) indicate statistically significant differences ($p \leq 0.05/p \leq 0.01/p \leq 0.001/p \leq 0.0001$) between immunization groups and number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test. A p value ≤ 0.05 denotes significance.

the efficacy of mRNA vaccines was somewhat theoretical. However, the widespread success of mRNA vaccines against SARS CoV2 provides sufficient evidence that generation of EEHV mRNA vaccines should be considered. Future studies will be directed towards combining the subunit and viral vectored or mRNA vaccine platforms with multiple EEHV target antigens to assess immunogenicity using a heterologous prime-boost vaccine approach.

In summary, we sought to develop an immunogenic subunit vaccine that elicited strong and specific humoral and CMI against EEHV1A. Based on the data presented herein, our adjuvanted gB subunit prototype vaccine stimulates robust antibody and cellular

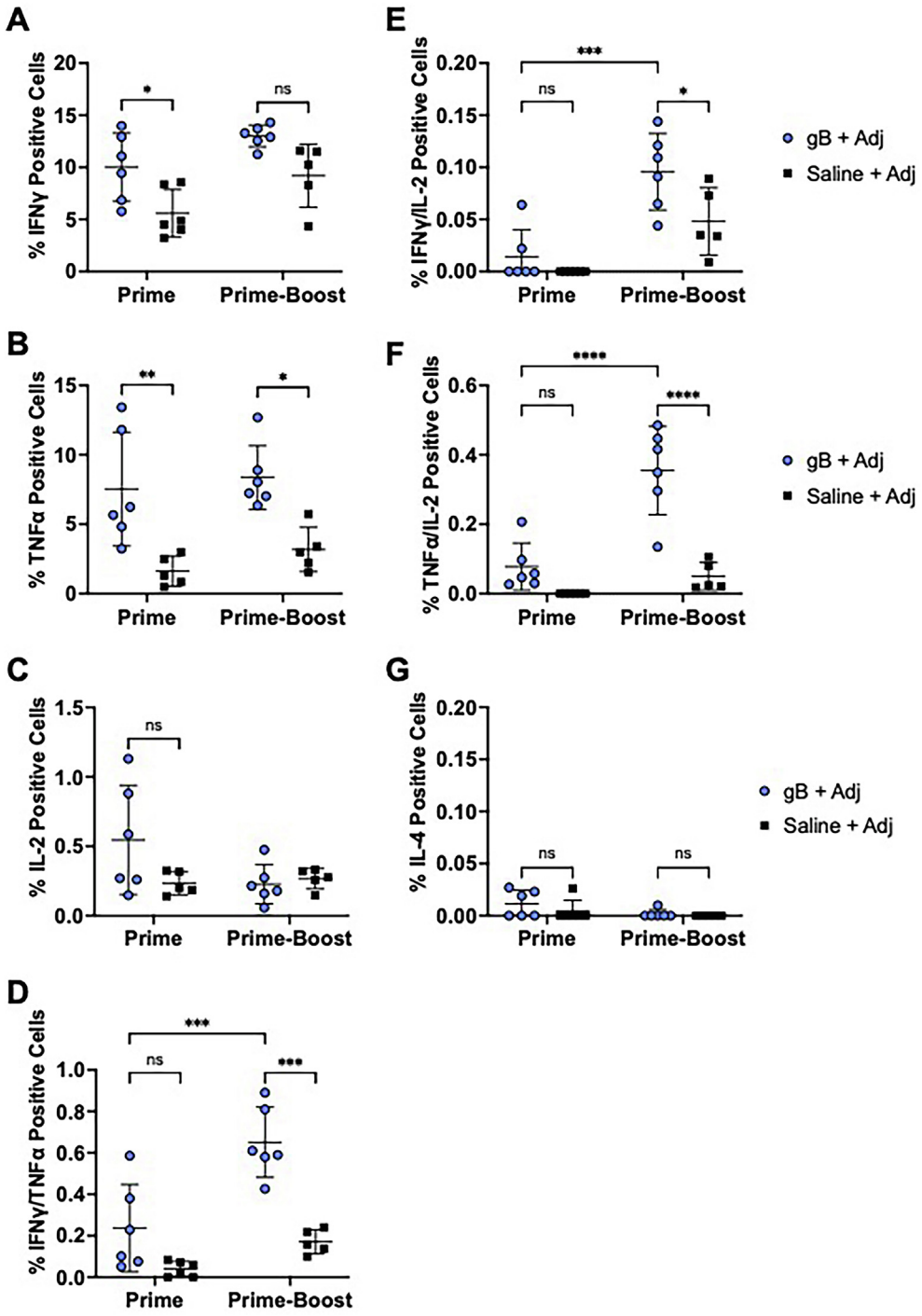

Fig. 5. Prime and prime-boost gB vaccination induces polyfunctional CD8⁺ T cell responses in mice. $CD8^+$ T cells isolated from splenocytes of immunized mice were assessed for cytokine production after gB peptide pool stimulation. Percentage of $CD4^+$ T cells positive for single cytokine activation markers IFNγ (**A**), TNFα (**B**), or IL-2 (**C**), or double positive cytokine activation markers IFNγ/TNFα (**D**), IFNγ/IL-2 (**E**), or TNFα/IL-2 (**F**). IL-4 was also included to measure induction of a T_H2 response (**G**). Splenic T cells from animals immunized with gB and adjuvant or saline and adjuvant are represented by blue circles and black squares, respectively. Mean values ± SD values for each group are shown as horizontal lines and error bars. Asterisks (*/**/***/****) indicate statistically significant differences ($p \leq 0.05/p \leq 0.01/p \leq 0.001/p \leq 0.0001$) between immunization groups and number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test. A p value ≤ 0.05 denotes significance.

immune responses in a mouse model. Taken together, these findings indicate that EEHV-specific immunoreactivity is elicited by our candidate gB subunit vaccine and supports its potential use in elephants.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data statement

Raw data files for the data presented in this work are available upon request.

Authorship statement

All authors attest they meet the ICMJE criteria for authorship.

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