The EEHV1A gH/gL complex elicits humoral and cell-mediated immune responses in mice

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Abstract

Elephant endotheliotropic herpesvirus (EEHV) causes lethal hemorrhagic disease (HD) in Asian and African elephants. Although rapid detection of viremia and supportive treatments may improve survival rates, an effective vaccine would mitigate the devastating effects of this virus. In elephants, chronic infection with EEHV leads to adaptive immunity against glycoproteins gB and gH/gL, the core entry machinery for most herpesviruses. We previously evaluated two EEHV gB vaccines in mice but not a gH/gL vaccine. Here, we found that inoculation of mice with an adjuvanted EEHV gH/gL subunit vaccine induced a significant antibody response that was similar to the response observed in elephants chronically infected with EEHV. Moreover, the gH/gL heterodimer elicited polyfunctional T cells with a Th1 phenotype but no detectable Th2 response. These results suggest that gH/gL, possibly in combination with gB, may be suitable immunogens for a vaccine comprising herpesvirus glycoproteins that are known to mediate cell entry and infection.

Keywords: Elephant endotheliotropic herpesvirus; Herpesvirus; Vaccine; Elephants

1. Introduction

Hemorrhagic disease (HD) caused by elephant endotheliotropic herpesvirus (EEHV) presents a significant threat to endangered Asian (*Elephas maximus*) and African Savannah (*Loxodonta africana*) elephants [1]. Although most adult Asian elephants are chronic carriers, EEHV can cause lethal HD, particularly in calves 1–8 years old [1,2]. EEHV-HD is the leading cause of juvenile Asian elephant deaths in North America and Europe, and over 100 cases have been documented in captive and wild Asian elephants in their natural range countries [1]. Although EEHV-HD occurs primarily in Asian elephants, there have been several recent EEHV-HD cases in African elephants residing in Western zoos, raising concerns about EEHV in this species [3-5].

Several related EEHV species in the Betaherpesviridae subfamily and the novel *Proboscivirus* genus are endemic in populations of each species of elephant. EEHV 1 A, 1B, 4, 5 A, and 5B are endemic to Asian elephants, and EEHV types 2, 3 A, 3B, 6, and 7 circulate in African

savannah elephants [1,6-9]. EEHV 1 A causes the most deaths in Asian elephants [1] while EEHV 2 (Erin Latimer, personal communication) and EEHV 3 [4] have been linked to the majority of deaths in African elephants. EEHV viremias progress rapidly, often with little or no clinical signs of disease [2]. Without regular monitoring for EEHV viremia, the disease is recognized only after the infection has progressed to a stage where treatments may be ineffective. Our lab has developed diagnostics to monitor EEHV viremias [10-12]; however, there are no vaccines for EEHV-HD in at-risk elephant populations. Therefore, our goal is to develop an immunogenic EEHV vaccine to protect elephants against lethal EEHV-HD.

No in vitro culture system is available for EEHV; thus, whole inactivated or attenuated vaccines are not feasible at the current time. Therefore, approaches such as a subunit vaccine or molecular biological approaches using viral vectors, DNA, or mRNA vaccines are the most feasible platforms. Because both humoral and cell-mediated immunity are required to prevent and control herpesvirus infections [13-17], we sought a vaccine platform using EEHV proteins that would induce robust and sustained levels of humoral and cell-mediated responses to prevent lethal disease. Several human and animal studies of the Betaherpesviridae subfamily, particularly human cytomegalovirus (hCMV), have shown that gB alone induces partial protective immunity, whereas the pentamer (gH/gL/pUL128–131) either alone or in combination with gB induces more potent immune responses than gB alone [14,18,19]. The viral fusion protein gB and the heterodimer gH/gL represent a core set of viral glycoproteins required for entry of all herpesviruses [20]. Several human and animal alphaherpesvirus or gammaherpesvirus gB and gH/gL vaccines also induce neutralizing antibodies, cell-mediated immunity, and protection in some challenge models [21-32].

For EEHV, prior infection with EEHV species other than EEHV1A (i.e., EEHV1B, EEHV4, or EEHV5) may not protect against clinical illness from EEHV1A [33-35]. However, it has not been observed that EEHV1A causes illness in elephants with previous evidence of EEHV1A infection. Thus, it appears that infection with EEHV1A protects an elephant from subsequent disease by EEHV1A, even if the first infection did not produce a clinical illness. This suggests that EEHV1A possesses unique properties to induce protective immunity against EEHV1A that are not shared with the other EEHV types. Although gB is well conserved among EEHV species, gH and gL are more diverged [1,7,36,37], serving as candidate biomarkers for distinguishing antibodies generated from prior infection with different EEHV species [35].

We showed previously that there is significant transplacental transfer of antibodies, including anti-EEHV antibodies, in Asian and African elephants [5,33,38], and the decline of these antibodies correlates with susceptibility to clinical or lethal illness [5,33,35]. Thus, the passive transfer of anti-EEHV antibodies likely provides important protection against serious EEHV disease. In addition to humoral immunity, cell-mediated immunity is important for protection against and control of herpesvirus infections. We showed previously that gB, the major capsid protein (MCP), and a putative regulatory protein E40 were immunoprevalent antigens for cell-mediated immunity in EEHV-infected elephants [39]. Thus, it seems likely that an effective EEHV1A vaccine that includes gB and gH/gL would induce strong humoral and cellular immunity to protect immunologically naïve elephants from serious EEHV1A disease. Our findings here that gH/gL heterodimer elicited robust immunity without illness, discomfort, or side effects in mice is an important step in moving gB and gH/gL protein subunit vaccines into trials in elephants.

2. Materials and methods

2.1. Production and purification of gH/gL proteins

The gH and gL proteins were expressed and purified as described previously [34]. Briefly, codon-optimized cDNAs encoding EEHV1A gH ectodomain sequences (residues 30–706) and gL (residues 57–304) were cloned into the pFRT expression plasmid in frame with a C-terminal StrepTag (gH) or HisTag (gL). Plasmids encoding EEHV1A gH-3xStrepTag and gL-6xHisTag were co-transfected into HEK293F cells, and cell culture supernatants were harvested after 5 days. Supernatants were centrifuged at low speed to remove cellular debris, and the gH/gL heterodimer was purified using Strep-Tactin Sepharose beads. Purified gH/gL was run on protein gels, and protein levels were measured by quantitative densitometry using a bovine serum albumin standard curve.

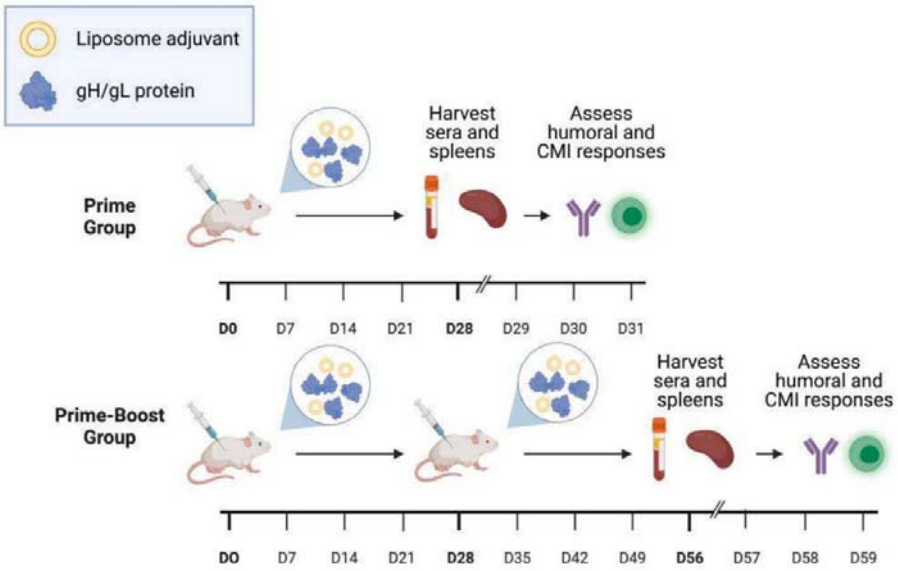

Fig. 1. Mouse gH/gL subunit vaccine timeline. Mice received either one or two injections of the gH/gL subunit vaccine and adjuvant or saline and adjuvant only. Mouse sera and spleens were harvested 4- or 8-weeks post-injection(s) to assess antibodies and T cell activation, respectively. This figure was created using BioRender.com.

2.2. Mouse immunizations

Outbred 6- to 8-week-old female CD-1 mice were used in this study to mimic the genetic diversity of elephants, our ultimate target host. A single sex of outbred animals responds to vaccination within a range that is hypothesized to be representative of responses that might be seen in a heterogeneous elephant population. All mice were purchased from the Center for Comparative Medicine (CCM) at Baylor College of Medicine. Mice were separated into two treatment groups (n = 6) that received injections of gH/gL and saline or an adjuvant comprised 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 gH/gL with adjuvant group received 100 µL doses containing 1 µg gH/gL and 5 mg SLA with 2 mg QS-21 liposome adjuvant in sterile saline. The saline with adjuvant group received 100 µL of 5 mg SLA with 2 mg QS-21 liposome adjuvant in sterile saline. Mice

received up to two intraperitoneal injections at 4-week intervals, yielding prime vaccinated and prime-boost vaccinated groups. Mice were monitored biweekly for physical signs of discomfort for the duration of the study. Six mice per treatment group were euthanized 4 weeks after each injection, and serum was harvested to measure antibody production. Spleens were processed for cellular staining and flow cytometric analysis, as described below. An overview of the vaccination schedule and study design is described in Fig. 1. 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 to evaluate gH/gL-specific immune responses

(i) **gH/gL ELISA.**

ELISA plates were coated with 40 ng recombinant EEHV1A gH/gL per well, washed, and blocked as described previously [34]. Pre-coated plates were frozen and stored at −20 °C until use. Freezing of plates were shown not to affect assay performance (data not shown). Briefly, mouse sera were serially diluted 1: 100, 1: 400, and 1: 1600 in blocking buffer (PBS, 3% BSA, 0.1% Tween-20), after which 100 µL sample was added to the wells and incubated for 1 h. Plates were washed and subsequently incubated with 100 µL HRP-conjugated recombinant Protein A/G (0.5 µg/mL diluted in blocking buffer; Pierce). After washing, 100 µL/well TMB Substrate (Surmodics, Eden Prairie, MN, USA) was added, plates were incubated for 10 min in the dark, and the reaction was stopped by adding 100 µL 12.5% H_2SO_4. Optical density (OD) was measured at 450 nm in an EL-808 ELISA reader (BioTEK, Winooski, VT, USA). For each sample, the antigen-specific signal (signal in wells containing antigen) and serum-specific background signal (signal in wells lacking antigen) was assessed simultaneously, and the ΔOD value (OD value antigen coated well minus OD value uncoated well) was reported.

(ii) **gH and gL-specific Luciferase Immunoprecipitation System (LIPS) assay.**

Gaussia luciferase (GLuc)-antigen fusions were constructed as described previously [33]. To show specificity for gH and gL antibody responses, antibodies against another EEHV1A glycoprotein, gB, were also measured as a negative control (data not shown). Briefly, codon optimized EEHV1A Kimba sequences (GenBank accession KC618527.1) for the gH ectodomain (1–706), gL (57–305), and gB [33] were synthesized (GenScript) and subcloned into the pGaus3 expression plasmid. A signal sequence from GLuc was added to the amino terminus of the gL protein. Expression plasmids were transfected into HEK293T cells, and supernatants and cell extracts were harvested and stored at −80 °C. Supernatants were used for gB and gL, as most of the luciferase activity was secreted, whereas most of the gH activity was associated with the cell lysates. LIPS assays were performed as described previously [33]. Briefly, serum samples from vaccinated mice and elephant populations were diluted 1: 10 in Buffer A (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) and stored for up to a month at 4 °C. Elephant sera was obtained from healthy adult elephants at the Houston Zoo who had been previously exposed to EEHV and determined to be sero-positive towards EEHV by LIPS (33). For 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 to create a master mix of each GLuc extract containing 1×10^7 LU per 50 µL. Fifty µL of GLuc extract master mix 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 serum was added to 5 µL of

a 30% Protein A-G bead suspension diluted in phosphate-buffered saline (PBS) in a 96-well filter plate (Millipore). Samples were incubated with the beads for 1 h at RT with constant shaking. Beads were washed five times with Buffer A and twice 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 each well, shake the plate for 2 s, and record RLU values for 5 s. Values were the average RLUs over 5 s post-injection.

(iii) **gH and gL-specific T cell responses.**

gH- and gL-specific T cell responses in vaccinated mice were measured as described previously for gB-vaccinated mice [40]. Mouse spleens were trimmed of excess fat, cut into multiple pieces, and mashed through a 40 μm filter. Red blood cells were lysed, and splenocytes were centrifuged at 350 ×*g* for 5 min before resuspending them in complete RPMI-1640 media supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 2-mercaptoethanol. Cells were pelleted at 350 ×g for 5 min and resuspended in 10 mL RPMI-1640 medium. After isolation**,** 2×10^6 splenocytes were plated on 96-well round bottom plates and stimulated at 37 °C overnight with EEHV1A gH and gL peptide pools at 1 μg/mL in complete RPMI-1640 medium. The EEHV1A gH and gL peptides were synthesized and purified by Mimotopes at 1 mg per vial. Peptide libraries were consecutive 15-mers overlapping by 11 amino acids spanning the length of gH (182 peptides) or gL (77 peptides). Four peptide pools (denoted gH1, gH2, gH3, or gL) were made containing 60–61 (gH1–3) or 77 (gL) individual peptides per pool and reconstituted in dimethyl sulfoxide at 10 mg/mL [39]. After peptide stimulation, Brefeldin A (BD Bioscience) and Monensin (2 μg/mL) were added, and the cells were incubated for 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 blocking, splenocytes were stained with fluorochrome-conjugated, extracellular-specific antibodies and a viability dye for 30 min at 4 °C in Brilliant Stain Buffer (BD Biosciences) in the dark. Cell surface marker antibodies and viability dye included CD3e-BV711 (BioLegend, #100241, 1: 40), CD4-BUV395 (BD Biosciences, #563790, 1: 400), CD8a-FITC (Tonbo, #35–0081-U500, 1: 800), and GhostRed780 (Tonbo, #13–0865-T500, 1: 50). Cells were fixed in cytofix/cytoperm solution (BD Biosciences) for 20 min at 4 °C and permeabilized with 1× perm/wash solution (BD Biosciences) before intracellular cytokine staining. Cells were stained with four 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. Cytokine-specific antibodies included interferon-γ (IFNγ)-BV421 (BD Biosciences, #563376, 1: 50), interleukin-2 (IL-2)-APC (Tonbo, #20–7021-U100, 1: 200), tumor necrosis factor-α (TNFα)-PE (BioLegend, #506306, 1: 40), and interleukin-4 (IL-4)-BV605 (BioLegend, #504126, 1: 25). Stimulated and stained 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. Staining cells were analyzed using an LRSII flow cytometer (BD Biosciences), and data analysis was performed using the FlowJo software version 10.7 (BD Biosciences). Dead cells were excluded from the analysis. Background responses in negative controls were subtracted from stimulated samples.

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 two independent experiments for each sample. For LIPS, results for different groups were presented as geometric means ± the standard deviation (SD) based on $\log_{10}$ values. The normality of the data sets was determined using the Shapiro-

Wilk test. The significance of normally distributed data was 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. For flow cytometry assays, results are presented as mean ± the SD of the percentage of positive cells. Data are presented as the sum of the percentage of positive cells from all gH or gL peptide pools per mouse. The normality of the data sets was determined using the Shapiro-Wilk test. The significance of normally distributed data was 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 gH/gL subunit vaccine elicits robust humoral immunity

The adjuvanted EEHV1A gH/gL subunit vaccine was administered to CD-1 mice with either a single prime vaccination or a prime and booster vaccination (denoted P—B in the figures) four weeks apart. No signs of pain or discomfort in vaccinated mice were observed for the study duration. Antibody responses in vaccinated mice were first measured by ELISA assay using the gH/gL heterodimer that was also used as vaccine antigen. Antigen-specific antibodies against gH/gL were readily detected after the prime vaccination and further increased after the boost, demonstrating the effectiveness of the booster vaccination (Fig. 2A and B). Because the vaccine proteins had Strep-and His-tags, it's unclear whether antibodies were generated to the tags and how much they contributed to the observed responses. Thus, LIPS assays for EEHV gH and gL were used in addition to measure antibody responses in vaccinated mice. As we were unable to generate complexes of gH-GLuc proteins with gL, the LIPS assay evaluated responses against the individual glycoproteins fused to GLuc. No responses to gH were detected at 4 weeks after the initial vaccination, however, a > 10-fold increase in antibodies against gH was observed in mice boosted 4 weeks later (Fig. 2C and D). Half of the mice generated responses against gL after the prime, that increased substantially in all of the mice following the boost (Fig. 2E and F). As gH and gL were expressed independently for LIPS assays, differential antibody responses may be due to improper gH folding and presentation of critical gH epitopes in the absence of gL. To determine whether the magnitude of the response was similar to elephants previously infected with EEHV, we compared antibody levels to gH and gL from 10 adult elephants at several institutions vs. three EEHV-seronegative elephants [33]. We found that antibody responses in elephants previously infected with EEHV were slightly higher against gH but somewhat lower against gL compared to the vaccinated mice (Fig. 2G and H).

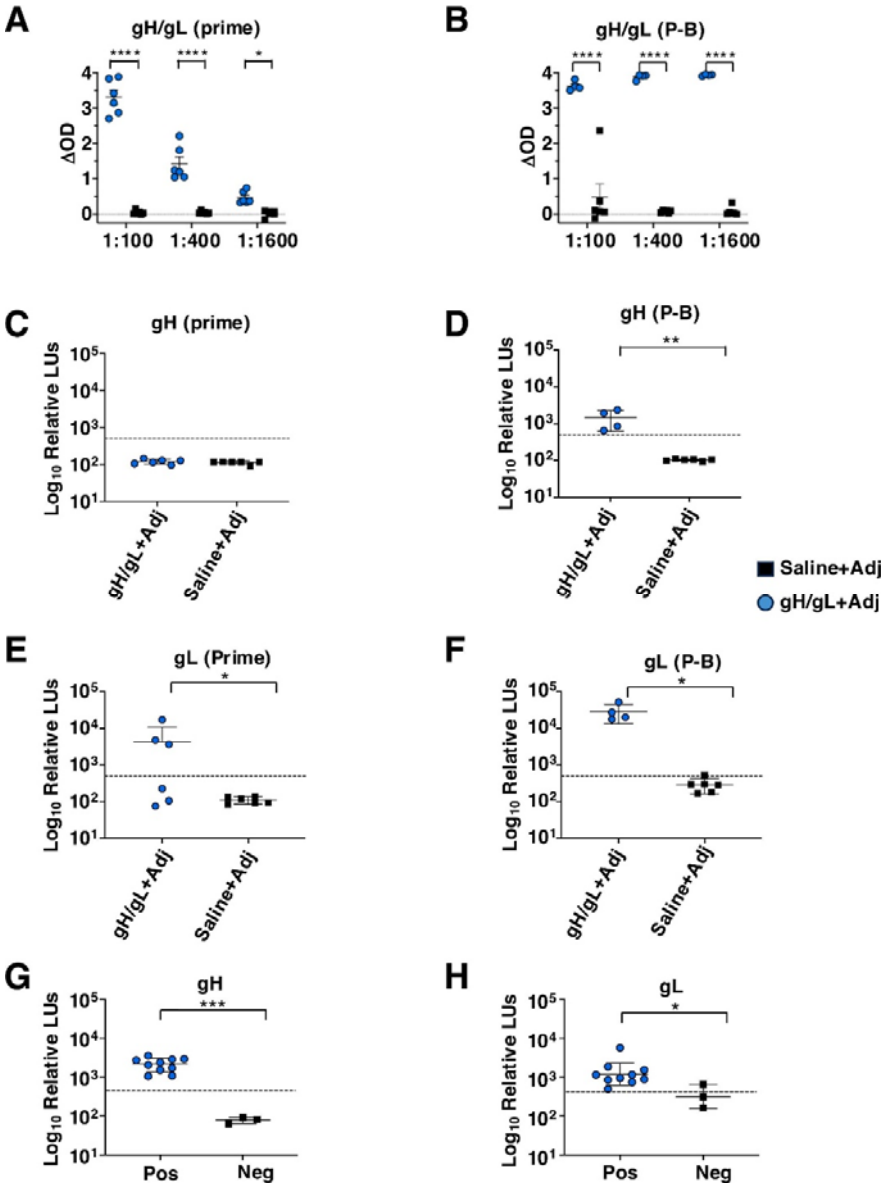

Fig. 2. **Prime and prime-boost injections with gH/gL induce anti-gH and gL antibody responses in mice. (A, B)** Specific gH/gL-specific antibody levels for prime and prime-boost vaccinated mice, measured at a 1: 100, 1: 400, and 1: 600 serum dilution by ELISA. ΔOD values were obtained by subtraction of serum-specific background signals from antigen-specific signals. The dashed line indicates a ΔOD value of zero. Specific antibody titers for gH or gL from prime (**C, D**) and prime-boost (**E, F**) vaccinated mice measured by LIPS assay. Sera from mice inoculated with gH/gL with an adjuvant or saline with an adjuvant are represented by blue circles or 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. Antibody titers against gH and gL from EEHV-experienced elephants (blue circles) or EEHV-seronegative elephants (black squares) were assessed (**G, H**). 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. Asterisks (*/**/***) indicate the statistically significant difference ($P \leq 0.05$/$P \leq 0.01$/$P \leq 0.0001$) between immunized groups, as determined by unpaired *t*-tests (LIPS) or Kruskall-Wallis test, including pairwise comparisons (ELISA).

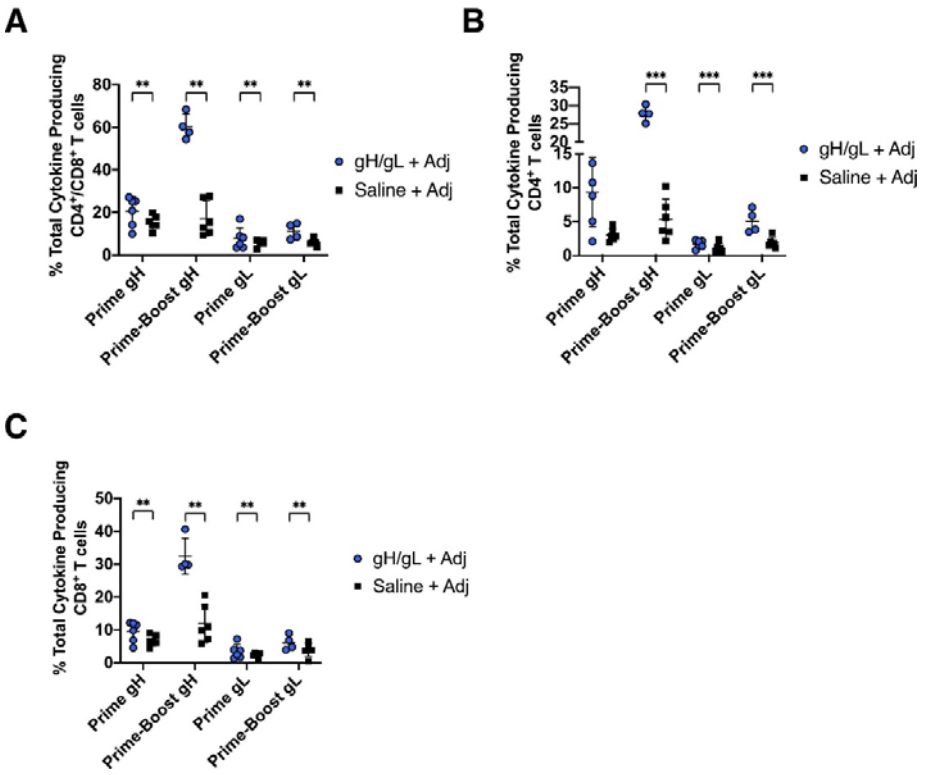

Fig. 3. **Induction of gH/gL-specific T cell responses after prime and prime-boost immunizations.** Splenic T cells from immunized mice were assessed for cytokine production after stimulation with gH or gL peptide pools. Splenic T cells from animals immunized with gH/gL with an adjuvant or saline with an adjuvant are represented by blue circles or 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 ($P \leq 0.05/P \leq 0.01/P \leq 0.0001$) between immunization groups and the number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test.

3.2. Adjuvanted gH/gL subunit vaccines induce T cell responses in mice

To determine whether the EEHV gH/gL vaccine induced a T cell response, we measured the production of three representative Th1 cytokines (IFNγ, TNFα, and IL-2) following ex vivo stimulation with peptide pools representing gH or gL in EEHV-specific CD4 and CD8 T cells from vaccinated or unvaccinated mouse splenocytes. Populations of CD4 or CD8 T cells expressing at least one Th1 cytokine were measured. Following the first vaccination, we found a small but significant increase in cytokine-producing gH-reactive T cells, and the numbers of gH-specific T cells increased substantially following the booster vaccine (Fig. 3A). We also found small but statistically significant increases in gL-reactive T cells, although the booster vaccine had little effect compared to the initial inoculation (Fig. 3A). Both CD4 and CD8 cells, analyzed separately, were reactive against gH and gL; however, cells from the prime-boost group gave the greatest response to gH with only a modest response to gL in either the prime-only or prime-boost groups (Fig. 3B and C). Some background induction of T cell responses was observed in the control groups, as the SLA-LSQ adjuvant was included with saline for control mice vaccinations. SLA-LSQ consists of liposome-encoated TLR4 ligand and saponin, and are intended to boost immunogenicity during antigen vaccination. Therefore, it is not surprising that some high background levels of T cell induction were observed in the saline

with adjuvant vaccinated groups. These data indicate that our adjuvanted gH/gL subunit prime-boost vaccine platform elicited a Th1-type response.

3.3. gH/gL subunit vaccines induce bifunctional T cell responses in mice

Activation of individual T cell subsets was measured by the expression of individual Th1 cytokines. There was an increase in both gH- and gL-specific CD4 cells that produced IFNγ and IL-2; however, only the gH-specific CD4 cells producing TNFα and the prime-boost gL cells producing IL-2 reached statistical significance (Fig. 4A, B, and C). T cell bifunctionality was also assessed by measuring CD4 and CD8 populations expressing multiple cytokine combinations. Double positive populations of each combination of IFNγ, TNFα, and IL-2 Th1 cytokines were assessed. We found gH- and gL-specific CD4 cells producing both TNFα and IL-2 (Fig. 4F), whereas no other combination reached statistical significance (Fig. 4D, E, and G). To assess Th2 responses, we measured IL-4 expression in spleen cells. We found no significantly positive CD4 IL4-producing cells, indicating that T cell responses in the vaccinated mice were predominantly Th1. For CD8 cells (Fig. 5A, B, C, D, E, F, and G), we found, as for CD4, significant levels of TNFα producing gH-specific CD8 cells (Fig. 5B). In contrast to CD4 cells, no CD8 cells were positive for any dual combination of the three cytokines (Fig. 5D, E, and F). No IL-4-producing CD8 cells above the background were detected (Fig. 5G). Thus, our adjuvanted gH/gL subunit vaccine induced a limited bifunctional Th1-specific T cell response in mice, consisting predominantly of bifunctional CD4 T cells.

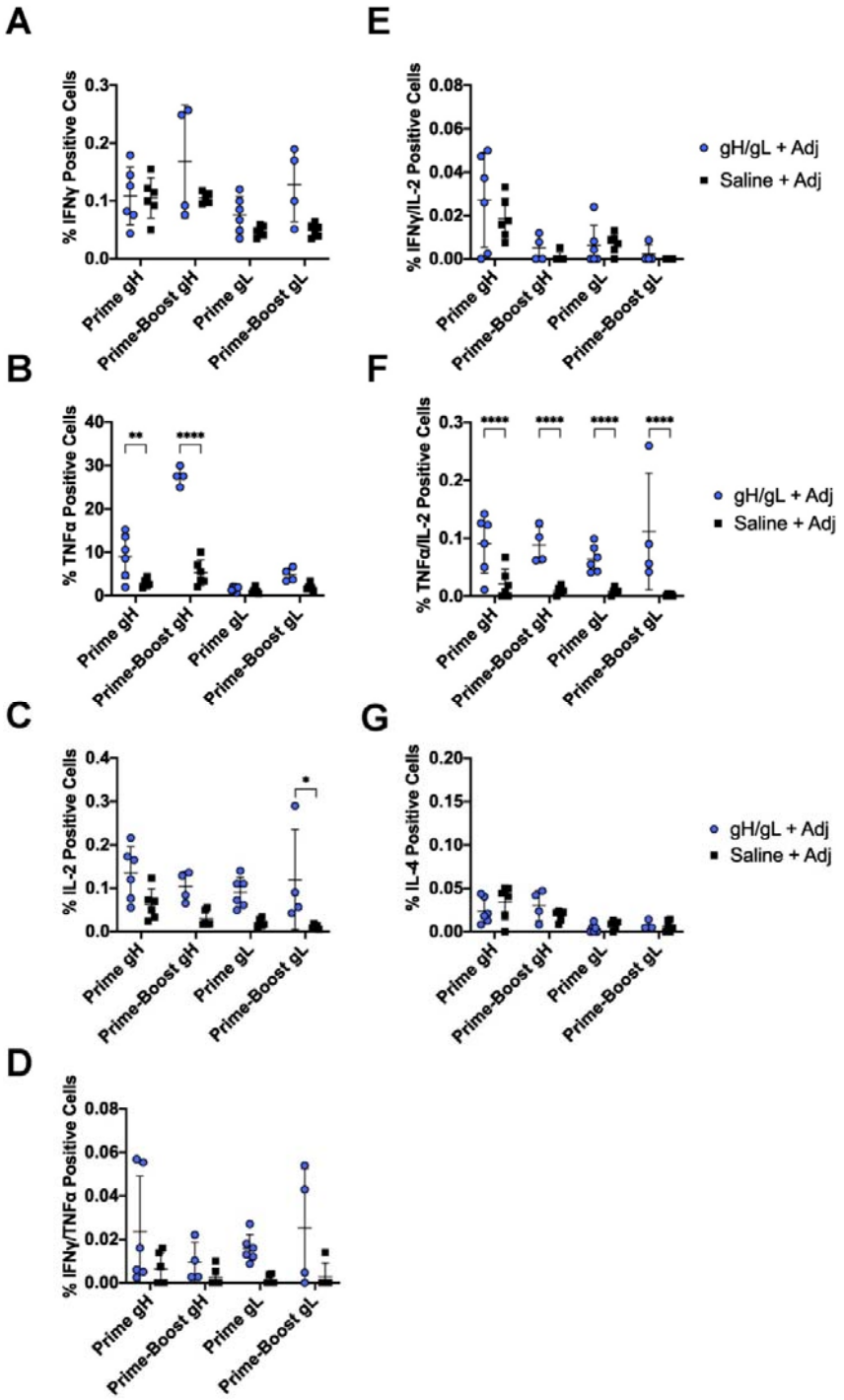

Fig. 4. **Prime and prime-boost gH/gL vaccination induces bifunctional CD4$^+$ T cell responses in mice.** CD4$^+$ T cells isolated from splenocytes of immunized mice were assessed for cytokine production after gH or gL peptide pool stimulation. The percentage of CD4$^+$ T cells positive for single cytokine 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**) are shown. IL-4 was included to measure the induction of a Th2 response (**G**). Splenic T cells from animals immunized with an adjuvant or saline with an adjuvant are represented by blue circles or black squares, respectively. Mean values ± SD values for each group are shown as horizontal lines and error bars. Asterisks (*/**/****) indicate statistically significant differences ($P \leq 0.05/P \leq 0.01/P \leq 0.0001$) between immunization groups and the number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test.

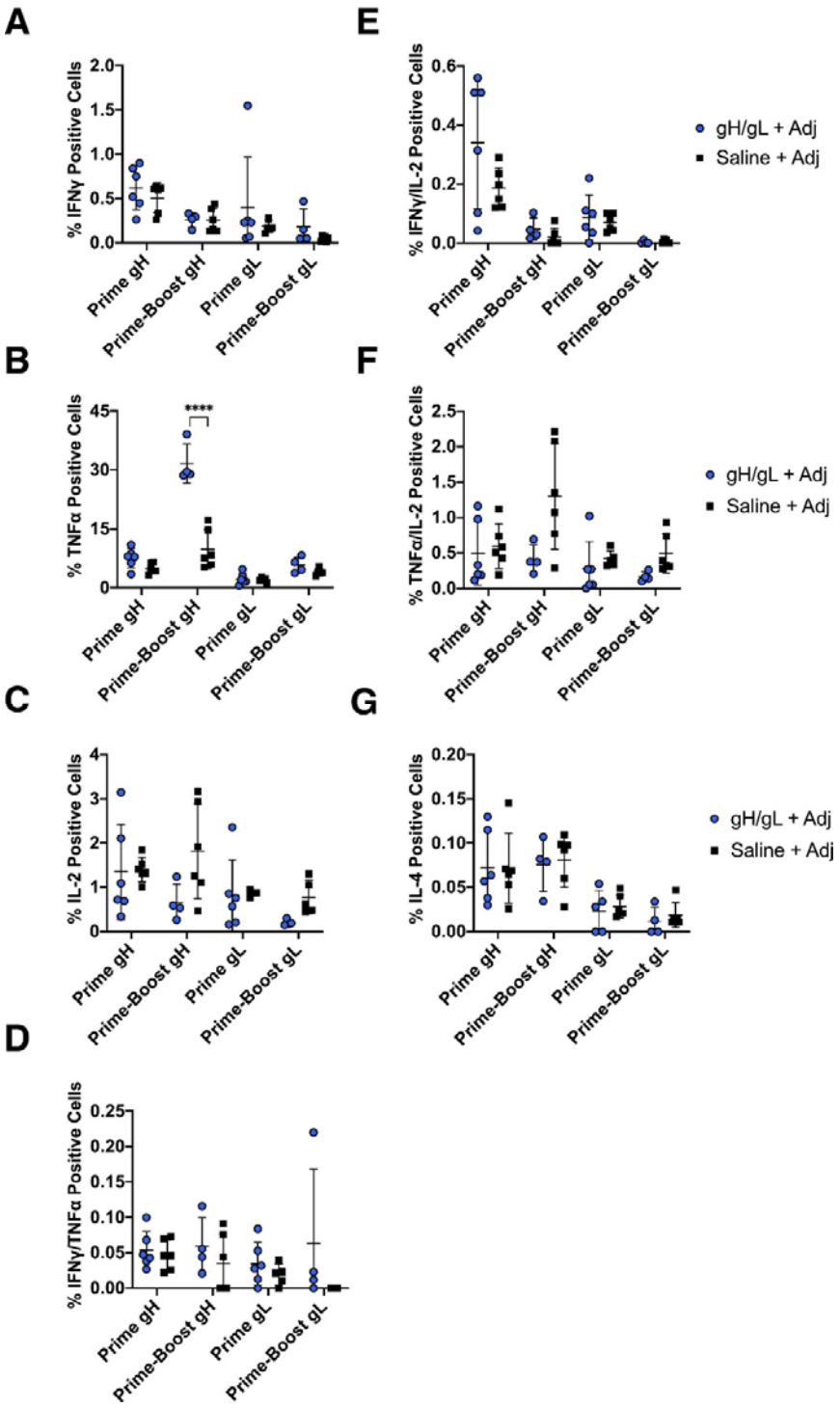

Fig. 5. Prime and prime-boost gH/gL vaccination induces a limited CD8$^+$ T cell response in mice. CD8$^+$ T cells isolated from splenocytes of immunized mice were assessed for cytokine production after gH or gL peptide pool stimulation. Percentage of CD4$^+$ T cells positive for single cytokine activation 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 included to measure the induction of a Th2 response (**G**). Splenic T cells from animals immunized with an adjuvant or saline with an adjuvant are represented by blue circles or black squares, respectively. Mean values ± SD values for each group are shown as horizontal lines and error bars. Asterisks (****) indicate statistically significant differences ($P \leq 0.0001$) between immunization groups and the number of vaccinations, as determined by two-way ANOVA with Tukey's multiple comparisons test. A P-value ≤0.05 denotes significance.

4. Discussion

Our experiments show that inoculation of mice with an adjuvanted EEHV1A gH/gL glycoprotein heterodimer subunit vaccine induced antibody production and CD4 bifunctional T cell responses to both proteins and the magnitude of the antibody responses approached those found in EEHV-infected adult elephants. Therefore, components of the core conserved herpesvirus attachment and fusion machinery may induce humoral and cellular immunity levels sufficient to protect young elephants from severe illness after infection with EEHV1A, the most lethal subspecies.

Vaccination with the gH/gL heterodimer resulted in high antibody levels against the vaccine antigens as determined by ELISA. One advantage of the ELISA assay is that it detects antibodies from vaccinated mice directed against both the individual proteins and possible conformation epitopes formed by association of these two proteins together. On the other hand, it remains unknown how much conformational epitopes are affected by the lack of a transmembrane domain and association with cellular membranes. Additionally, the contribution of antibodies generated against the epitope tags to this response is difficult to evaluate. Therefore, LIPS assays were used as a complementary measure of specific antibodies towards these proteins. In the LIPS assays, the antibody response to gL was robust, whereas the response to gH was modest. This contrasts with the higher response to gH in infected elephants and a much lower response to gL (Fig. 2G and H). It is not clear to what extent the difference in the gH-specific antibody levels are related to gH being expressed in the absence of gL in the LIPS assay. Prior data from other herpesvirus systems indicate that gL is expected to assist gH in its folding [41,42]. Indeed, release of gH from cells was only observed when the ectodomain of gH was expressed in the presence of gL [34]. While not directly comparable to the ELISA, the combined reactivity against both antigens in the LIPS assay confirm that robust responses were generated to the vaccine independent of the epitope tags.

The vaccine appeared to induce antibody responses against gL that were higher than those seen in chronically infected elephants. Other members of the betaherpesvirus family, which include human hCMV and human herpesvirus 6 (HHV6), encode one or more glycoproteins that associate with gH/gL to facilitate receptor binding [20,43]. Structural studies within the betaherpesvirus family suggest that gL associates with glycoprotein O (gO) or other accessory factors, which may mask epitopes on gL [20,44]. Although EEHV has no apparent homologs to hCMV UL128/130/131 A or HHV6 gQ1 or gQ2, it does encode a truncated gO homolog [36,[45], [46], [47]]. In natural infections, gO, or possibly other unknown accessory EEHV glycoproteins, may mask gL, resulting in diminished responses. In contrast, in our vaccine formulation, gL may be exposed and available to direct antibody responses more readily.

Although the EEHV gH/gL heterodimer induced both gH and gL T cell responses, this response was not as robust as has been reported for similar vaccinations with EEHV gB [40]. In those studies, gB elicited polyfunctional CD4 and CD8 cells, whereas we found only bifunctional CD4 cells in this study. Moreover, gB-vaccinated mice produced more than four times as many TNFα/IL-2 cells compared to gH/gL-vaccinated mice [40]. Similar results were found in chronically infected adult elephants; gB was the most immunoprevalent T cell antigen, whereas there was no detectable response against gH or gL [39]. Therefore, it appears that neither gH nor gL are as effective in eliciting T cell responses as compared to gB, especially regarding CD8 responses [39].

EEHV has not been cultivated in the laboratory, thereby limiting our ability to evaluate viral immunity. Thus, a major question is whether the EEHV gH/gL dimer is sufficient to elicit a protective immune response against EEHV-associated disease. Data from all three herpesvirus subfamilies indicate that gH/gL induces effective neutralizing antibodies via vaccination or natural infection, in agreement with the observations that low antibody levels against gH/gL correlate with a risk of developing EEHV-associated disease [35,[48], [49], [50]]. Thus, these proteins are strong candidates for future vaccines. However, several studies have shown that additional glycoproteins complexed with gH/gL may be required for optimal receptor binding [20,43]. These include gO, which is conserved among betaherpesviruses and is critical for entry into a broad spectrum of cell types. Thus, further vaccination studies using protein complexes containing gH, gL, and gO in combination with gB are warranted. Finally, other vaccine platforms such as mRNA vaccines used in combination with subunit approaches described in this study may also induce broader responses than either approach alone.

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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.

CRediT authorship contribution statement

Jennifer L. Spencer Clinton: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tabitha E. Hoornweg:** Writing – review & editing, Validation, Resources, Formal analysis, Data curation. **Jie Tan:** Validation, Resources, Methodology, Formal analysis, Data curation. **Rongsheng Peng:** Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Willem Schaftenaar:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Victor P.M.G. Rutten:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Cornelis A.M. de Haan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paul D. Ling:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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Data availability

Data will be made available on request.

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