Asian elephant T cell responses to Elephant Endotheliotropic Herpesvirus (EEHV)

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\textbf{Running Head:} T cell responses to EEHV Proteins in Asian Elephants

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Abstract

Elephant Endotheliotropic Herpesvirus (EEHV) can cause lethal hemorrhagic disease in juvenile Asian elephants, an endangered species. One hypothesis to explain this vulnerability of some juvenile elephants is that they fail to mount an effective T cell response to the virus. To our knowledge, there have been no studies of Asian elephant T cell responses to EEHV. To address this deficiency, we validated the IFN-γ ELISpot assay for tracking antigen-directed T cell activity by monitoring rabies-specific responses in vaccinated elephants. Additionally, we generated monoclonal antibodies to Asian elephant CD4 and CD8 to facilitate phenotypic T cell profiling. Using these tools, we screened healthy elephants with a prior history of EEHV infection for reactivity against 9 EEHV proteins whose counterparts in other herpesviruses are known to induce T cell responses in their natural hosts. We identified glycoprotein B (gB) and the putative regulatory protein E40 as the most immunogenic T cell targets (IFN-γ responses in 5 of 7 elephants) followed by the major capsid protein (MCP) (IFN-γ responses in 3 of 7 elephants). We also observed that IFN-γ responses were largely from CD4+ T cells. We detected no activity against the predicted major immediate early (E44) and large tegument (E34) proteins- both immunodominant T cell targets in humans latently infected with cytomegalovirus. These studies have identified EEHV-specific T cells in Asian elephants for the first time, lending insight into the T cell priming that might be required to protect against EEHV disease and will guide the design of effective vaccine strategies.

Keywords:
IFN-γ, ELISpot, EEHV, Asian elephant, T cell

Importance

Endangered Asian elephants are facing many threats, including lethal hemorrhagic disease from elephant endotheliotropic herpesvirus (EEHV). EEHV usually establishes chronic, benign infections in mature Asian elephants but can be lethal to juvenile elephants in captivity and the wild. It is the leading cause of death in captive Asian elephants in North America and Europe. Despite availability of sensitive tests and
protocols for treating EEHV-associated illness, these measures are not always effective. The best line of defense would be a preventative vaccine. We interrogated normal healthy elephants previously infected with EEHV for T cell responses to 9 EEHV proteins predicted to induce cellular immune responses. Three proteins elicited IFN-γ responses, suggesting their potential usefulness as vaccine candidates. Our work is the first to describe T cell responses to a member of the proposed fourth subfamily of mammalian herpesviruses, the Deltaherpesvirinae, within a host species in the clade Afrotheria. An EEHV vaccine would greatly contribute to the healthcare of Asian and African elephants that are also susceptible to this disease.
Introduction

Elephant Endotheliotropic Herpesvirus (EEHV) can cause acute hemorrhagic disease in juvenile Asian elephants, with a high mortality rate. There are four species of EEHV that are endemic in Asian elephants (EEHV1A, 1B, 4 and 5), where the majority of adults are most likely latently infected with several or all of these species (1). The two chimeric variants of EEHV1, EEHV1A and 1B, cause the majority of lethal disease, in both captive and wild elephants (2). The greatest incidence of death from EEHV occurs in calves from 1-8 years of age and recent estimates indicate that it is the single largest cause of death in captive juvenile Asian elephants in North America and Europe (2). Hemorrhagic disease caused by EEHV is associated with a large viral burden, suggesting that uncontrolled infection plays a role in causing the disease. Why some elephants succumb to lethal infection remains unknown, but we hypothesize that one factor could be a failure to mount an effective cellular immune response. Insufficiencies in cell-mediated immunity are a major risk factor in other herpesvirus-associated infections and reactivation in humans [CMV (3-7), VZV (8, 9), EBV (10) and HHV-6 (11)]. Hence, there is a distinct need to understand the T cell response to EEHV in Asian elephants in order to find a solution to the devastating effects of this lethal virus.

To date, Asian elephant T cell responses at the cellular level have not been explored, in part because there are limited reagents to detect phenotypic (cell surface markers) and functional characteristics (Asian elephant cytokines). In the present study, we developed Asian elephant-specific reagents and methods, which we have used to elucidate the cellular immune response to EEHV. We have studied a herd of latently infected elephants with a normal pattern of reactivation and control (12, 13) in order to identify the specificity and functional profile of protective T cells. The sequenced genome of EEHV1A has identified approximately 115 open reading frames (ORFs), 37 of which are conserved core genes, common to all herpesviruses, and 15 of which are conserved within the beta-herpesvirus and gamma-herpesvirus sub-families (14, 15). Amongst these conserved proteins, several are similar to structural and regulatory proteins that have been identified as potent inducers of T cell responses in humans. Thus, we synthesized overlapping peptide libraries (15mers overlapping by 11 amino acids) spanning 9 EEHV
ORFs, which we used to interrogate the immune response in 7 latently infected adult and juvenile elephants. Now, using a panel of unique elephant-specific reagents, we report on the first T cell immune responses directed against EEHV, which should assist in the design of future vaccines or T cell therapies and the evaluation of their efficacy.

Results

Rabies-specific IFN-γ responses are detectable within the peripheral blood of Asian elephants following routine vaccination

Conditions for investigating T cell responses in any animal within the clade Afrotheria have not been established. Thus, to first validate the IFN-γ ELISPOT assay as a means to assess protective T cell immune responses in elephants we first focused on rabies as a model pathogen and specifically on measuring immunity to the rabies nucleocapsid protein (NC), which has been shown to induce cellular immune responses (16). Five of our elephants had a prior history of receiving a rabies vaccine as part of their regular health management and received a booster vaccination during the course of our study. To monitor T cell immunity in these animals we utilized a pepmix (library of consecutive 15mer peptides, overlapping by 11 amino acids) spanning Rabies NC as an immunogen as well as Asian elephant IFN-γ-specific antibody pairs originally developed for an ELISA (17) and adapted here for use in the ELISPOT assay. Blood was collected from each of the elephants prior to (day 0) and at days 14 and 28 following vaccination and the frequency of reactive cells assessed following stimulation with the NC pepmix. As shown in Figure 1, there was a significant increase in the frequency of IFN-γ secreting cells (Spot Forming Cells; SFCs) at both day 14 (*p=0.018) and day 28 (*p=0.035) post-vaccination when compared with the control (DMSO solvent) at the corresponding time points. Additionally, we found that unlike PHA or PMA/ionomycin, Staphylococcus Enterotoxin B (SEB) was able to non-specifically activate elephant cells to secrete IFN-γ, and hence we incorporated SEB into our subsequent assays for use as a positive control (data not shown).

Identification of EEHV proteins that elicit IFN-γ responses
Having established the IFN-γ ELISpot as an effective means to detect Asian elephant antigen-specific T cell responses, we applied this approach to detect immune responses to selected proteins of EEHV1A, which has been associated with the largest number of deaths caused by EEHV. Thus, we characterized responses to 9 predicted EEHV1A proteins, which are described in Table 1. These proteins were selected largely because they share characteristics with other herpesvirus proteins that have been shown to elicit robust T cell responses (Table 1). Based on the sequence information from EEHV1A strain Kimba, we synthesized individual 15mer peptides, overlapping by 11 amino acids and arranged them into ORF-specific mixes or for larger ORFs, into sub-ORF mixes of approximately 60-90 peptides and subsequently used these pepmixes to screen PBMCs isolated from 7 elephants (Table 2) by IFN-γ ELISpot. Whilst 9 ORFs were studied, we detected significant responses to 3-gB (5 elephants; Figure 2), E40 (5 elephants; Figure 3) and MCP (3 elephants; Figure 4). Each of these figures shows responses to sub-ORF mixes compared to the negative control DMSO. Survivin was used as an additional negative control in early studies (Figure 2), however responses to survivin were generally lower than DMSO and so we chose DMSO as a more conservative control for most of our studies (Figures 3 and 4). Table 3 summarizes all 9 ORFs and the responses induced in each elephant screened.

Sufficient PBMCs from one elephant (elephant 3) who responded significantly to MCP were available to deconvolve some of the larger peptide mixes. Thus, we generated mini-pools as described previously (18)(Figure 5A), which we used individually to stimulate elephant PBMCs. As shown in Figure 5B, we detected activity against multiple stimulating minipools suggesting activity against numerous MCP peptides. This was confirmed for MCP130 (AA 517-531, sequence KNEYQDLEFFKPSNK, present in minipools 6 and 14; Figure 5B and 5C), which induced strong IFN-γ-specific activity when used to stimulate PBMCs from elephant 3 (Figure 5D).

**Phenotype of EEHV-peptide specific T cell responses**

To determine the phenotype of EEHV-reactive T cells, we first identified a human CD3 monoclonal antibody that recognizes Asian elephant CD3 at a highly conserved site on
the epsilon chain of CD3. To further understand whether responding T cells were helper (CD4+) or cytotoxic (CD8+) we generated Asian elephant CD8- and CD4-directed monoclonal antibodies, by first creating mouse L cell lines expressing predicted African elephant CD8 and Asian elephant CD4 proteins (Figure 6A and D). Each L cell line was then used to inoculate mice to generate hybridomas, which were screened and characterized as described previously (19). Supernatants were tested on Asian elephant PBMCs to confirm their ability to detect actual protein expression and successful hybridoma clones were then sub-cloned prior to making purified antibody which, in combination with a CD3 monoclonal, enabled the discrimination of CD8+ and CD4+ T cells (Figure 6B and E). Furthermore, CD8+ and CD4+ IFN-γ secreting populations could be detected by intracellular cytokine staining (ICS) following activation of Asian elephant PBMCs with SEB (Figure 6C and F).

Finally, we combined the cross-reactive CD3 monoclonal antibody and our custom-made CD8 and CD4 monoclonal antibodies to determine the nature of the IFN-γ response following stimulation with EEHV peptides via flow cytometry. We initiated our studies using the peptide MCP130 which induced strong responses in Elephant 3 (Figure 5). As shown in Figure 7 we were able to detect both CD4+ (37.7%) and CD8+ populations (31.7%), in PBMCs (Figure 7A). However, following peptide stimulation only CD3+CD4+ cells produced IFN-γ (0.29%) while CD3+CD8+ cells were unresponsive (0.038%) indicating that MCP130 contains a CD4+ T cell epitope (Figure 7B). Similarly, in PBMCs from two elephants who had robust responses to gB and MCP as detected by IFN-γ ELISpot, we detected dominant responses in the CD4+ T cell compartment following antigen stimulation (Figure 7C).

Discussion

This study has for the first time demonstrated the detection of EEHV-specific T cells in the peripheral blood of latently infected Asian elephants using an IFN-γ ELISpot assay expressly established and validated (using rabies as a model pathogen) to facilitate immunologic profiling. In a total of 7 elephants screened for activity against 9 antigens predicted to be immunogenic based on studies of homologs from other herpesviruses we
identified glycoprotein B, E40 and the major capsid protein as being immunodominant, eliciting immune responses predominantly in the CD4+ T cell compartment. These data provide the first clues to the types of T cell responses that might be required to prevent EEHV hemorrhagic disease in juvenile elephants and preliminarily identify potential EEHV vaccine candidate proteins.

Following primary infection EEHV establishes a lifelong latency, with occasional reactivation as demonstrated by intermittent shedding from mucosal surfaces such as the trunk and in saliva (12, 13). Based on routine monitoring of trunk wash samples or prior detection of viremia, we confirmed that all but two juveniles in the Houston zoo herd were latently infected with EEHV1 (Table 2). Hence we were assured that a T cell response specific to EEHV1A proteins would be measurable in this herd if it existed and if appropriate tools were available. Not knowing the dynamics of EEHV-specific T cell immunity, we first established our assays using Rabies, a killed whole virus vaccine known to induce protective T cell immunity (20, 21). Indeed, consistent with published studies we were able to detect IFN-γ producing T cells following stimulation with Rabies NC, 1 of the 5 encoded proteins (22), which significantly increased in number post-vaccination. These results confirm the effectiveness of the IFN-γ monoclonal antibodies and the sensitivity of the IFN-γ ELISpot assay for detecting circulating memory T cell responses to EEHV proteins in the peripheral blood of Asian elephants.

By utilizing our optimized ELISpot assay to assess the immunogenicity of EEHV candidate proteins, we identified three antigens - gB, MCP and E40 - that elicited significant IFN-γ responses in adult and juvenile elephants who are latently infected with EEHV. Both gB and MCP, which have homologs in HHV-6 and CMV, have been shown to elicit significant IFN-γ responses in humans (23-26). As both are likely abundant structural proteins that are delivered upon infection to the host cell, it is not surprising that they both elicit significant T cell responses in this latently infected herd. E40, a putative regulatory protein, which elicited broad yet modest T cell responses in this herd, is a protein unique to EEHV, so we are unable to speculate as to the significance of these findings based on the expression of this protein in EEHV. However, it is not surprising
that a putative regulatory protein would induce T cell responses, as regulatory proteins of other herpesviruses are well documented in eliciting T cell responses (24, 27, 28). Hence, we remain interested in further understanding responses to this novel protein.

We were able to determine the nature of the T cell response to both gB and MCP in positive elephants where we found that responses were predominantly from CD4+ T cells. In previous studies of other herpesviruses, gB has elicited responses in both CD8+ (29, 30) and CD4+ T cells (31-33), although dominant gB epitopes seem to be predominantly MHC class II associated. Our results for MCP are consistent with its homolog U57 in HHV-6, responses to which are also dominated by CD4+ T cells (25-27, 34). The dominance of CD4+ responses to both gB and MCP potentially indicates their importance in forming quality antibody responses, but CD4+ T cells have also been implicated in assisting CD8+ T cell migration to sites of infection (35) and there is increasing evidence of their cytotoxic potential (31, 36-38). Having characterized a dominance of CD4+ T cell responses in these two positive proteins, we do not discount the potential for MHC class I epitopes in other EEHV proteins. Indeed, responses to CMV, another beta-herpesvirus, have been dominated by the CD8+ T cell subset (39) and there is the potential for any of the remaining 106 proteins of EEHV1A not yet studied to contain MHC I epitopes.

We saw no significant response to the six other proteins screened in this study. These proteins were selected based on the ability of similar proteins to induce robust T cell responses in studies of other herpesviruses. Most notably, we expected to see strong responses to the structural protein E34 which most closely resembles pp150 in CMV and the regulatory protein major immediate early protein E44, whose homolog UL123 (IE1) of CMV is amongst the most immunogenic of ORFs in CMV (24, 28). Whilst EEHV is broadly recognized as a beta-herpesvirus like CMV and HHV-6, it has been proposed that the genetic differences between EEHV1A, 1B and EEHV2 and the other beta-herpesviruses make them candidates for a potential new sub-family of delta-herpesviruses, as an intermediate branch between beta-herpesviruses and gammaherpesviruses (40). The difference in responses to proposed EEHV homologs...
might therefore be attributable to the divergence of EEHV1A from the broad
classification of a betaherpesvirus, and the ultimate uniqueness of this virus.

As this is the first study of T cell responses in Asian elephants using IFN-γ ELISpot as a
primary screening tool, we were initially careful to draw parallels between our data and
similar studies in other species to confirm the sensitivity of our system in Asian
elephants. We are confident in our positive responses as they are within the range of
similar studies in humans and non-human primates (41-43). One drawback in our
analysis is that we did not have access to appropriate uninfected negative control
elephants. While there are 2 juvenile elephants (ages 5 months and 3.5 years) in the
Houston Zoo herd which are potentially naïve to EEHV, obtaining sufficient blood from
them to conduct ELISpot experiments remains impractical at the current time. In addition
to developing the ELISpot assay for detection of antigen-specific T cells directly from
peripheral blood, we also attempted to increase the sensitivity of our assay by selectively
expanding EEHV-specific T cells in vitro using both recombinant human cytokines and
elephant cytokines made in-house to support cell growth. To date, our efforts to identify a
specific protocol to selectively enrich and expand reactive populations has been
unsuccessful, but this is likely to change as additional elephant-specific reagents become
available.

The response to gB in two juvenile elephants seems to indicate some level of antigen-
experience in these elephants, which may suggest that they have both already had
primary EEHV1A infection. Based on routine monitoring of the herd, we are aware that
one of these juveniles (Elephant 7) has had a primary EEHV1A infection, but to our
knowledge the susceptibility of the other one (Elephant 6) to EEHV1A infection was still
unknown, despite this elephant already having an EEHV1B viremia (44). The gB
sequence is 79% conserved between EEHV1A and EEHV1B so it is possible that this
activity is due to cross-reactive T cell recognition, especially since this elephant also has
a significant response to E40. The strength of responses to gB in these two juveniles, who
are likely to have been infected more recently, rather than to MCP, which did not induce
any responses in juveniles and E40, which induced a response in only one juvenile, may
indicate gB is presented with higher frequency to naïve T cells, due to its surface exposure. As such, gB may serve as a better vaccine candidate. Indeed, herpesvirus vaccine development has largely focused on gB as a candidate (45-48) owing to its surface exposure and the presence of gB-specific antibodies in CMV seropositive patients (49).

A significant limitation of this study is the small sample size and hence lack of MHC diversity. In studying only 7 elephants, some of which are related, it is likely that we are only detecting dominant peptide sequences that bind to a very small set of MHC molecules. Indeed, studies of human herpesviruses are far more diverse in their population sampling and many have characterized the precise MHC haplotypes associated with particular epitopes (25, 26, 28). At this stage we can only hope to diversify our study population by screening more elephant herds until appropriate technologies to determine the MHC haplotype of the elephants is developed. For now, gauging dominant T cell epitopes irrespective of MHC haplotype in our current herd is a major step forward in understanding the nature of T cell responses to EEHV, for which no prior study of this nature has existed. In addition, we have generated two monoclonal antibodies and pioneered two elephant specific T cell assays that will be essential in advancing the understanding of EEHV and what might be required for future EEHV vaccine development.

Materials and Methods

Study population

The elephants in this study belong to a single herd of 9 elephants consisting of 5 females (ages 5 months and 7, 27, 36 and 48 years old) and 4 males (3, 7, 12 and 52 years old). All elephants in the herd are routinely monitored for EEHV viremia and as such, blood is regularly obtained from them for the purposes of EEHV screening and further study (IACUC approval AN-5182). Two of the elephants (5 month old female and 3 year old male) were not studied because obtaining sufficient blood from them to conduct experiments was not feasible. Several of the juvenile elephants are related. In addition, all
elephants in this study have evidence of prior EEHV1 infection from shedding, viremia or serology (Table 2). Basic characteristics of the elephant herd used in these studies can be found in Table 2 and Figure 8.

PBMC preparation

Thirty to 40 mL of venous blood was collected into lithium heparin tubes (BD Vacutainer). Within 2 hours of sample collection, blood was diluted 1:1 in room temperature RPMI 1640 medium (Thermo Fisher) and centrifuged over a Ficoll-Hypaque gradient (Lymphoholyte, Cedarlane, Burlington, North Carolina) to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were cryopreserved in heat-inactivated fetal bovine serum (HI-FBS) and 7.5% DMSO and stored in liquid nitrogen until experiments were carried out. When ready to study, PBMC vials were thawed rapidly in a 37°C water bath and added to pre-warmed R-10 complete medium (RPMI+10%HI-FCS (Hyclone, location) + 1% antibiotic-antimycotic + 1% glutamax) and washed twice prior to counting and use in ELISpot or overnight stimulations.

Peptide libraries

Rabies nucleocapsid peptide library was synthesized by Genscript (Piscataway, New Jersey). MCP and MIE peptide libraries were synthesized by Genemed Synthesis (San Antonio, Texas). All other peptide libraries were synthesized by Mimotopes (Melbourne, Australia). With the exception of the Rabies nucleo-capsid peptide library, all peptides were re-constituted in DMSO at a concentration of 10mg/mL. Crude Rabies NC peptides were reconstituted to a concentration of 200-300μg/mL.

ELISpot

Ninety-six well PVDF membrane ELISpot plates (Merck Millipore, Billeria. USA) were coated overnight with anti-Asian elephant IFN-γ capture antibody (Podiceps, Netherlands) diluted in PBS at a concentration of 2.5μg/mL at 4°C. Prior to the addition of cells, plates were washed 3 times in sterile PBS and blocked in R-10 complete medium for several hours at 37°C. Cells were re-suspended in R-10 and added to plates at a density of 2x10^5 cells/well and stimulated in a final volume of 200μL per well at 37°C for
24 hours (Rabies nucleocapsid pepmix) or 96 hours (all other pepmixes). Pep-mixes were diluted in R-10 so that each peptide had a final concentration of 1μg/mL, except for Rabies NC, which had a final concentration of 0.2-0.4μg/mL. SEB (2.5μg/mL; List Biologicals, California) was used as a positive control while DMSO (Sigma-Aldrich, St Louis, Missouri) and survivin (1ng/mL; JPT, Adlershof, Germany) were used as negative controls. After stimulation, cells were removed from plates, and wells were washed 3X in PBS prior to washing 3X in PBS containing 0.05% Tween and 1% BSA at a concentration of 0.625μg/mL was added to plates for 2 hours at room temperature. Plates were then washed 4X in PBS containing 0.05% Tween prior to the addition of Streptavidin-conjugated alkaline phosphatase diluted 1:1000 in PBS containing 0.05% Tween and 1% BSA, for 1 hour at room temperature. Wells were then washed 3X in PBS containing 0.05% Tween and 3X in PBS prior to the addition of 1-step NBT/BCIP substrate solution (Thermo Fisher). Plates were watched for 5 minutes at room temperature until color development, before the reaction was stopped by adding water to the wells. Wells were washed once more in water, and then dried overnight.

**ELISpot counting**

Spot forming cells (SFCs) were counted on an Immunospot ELISpot reader (CTL, Ohio, USA) using Immunospot software version 5.1.36. Settings were identical for all plates. Counts were then expressed as IFN-γ positive cells per 1x10^6 PBMCs.

**Asian elephant anti-CD8 and anti-CD4 monoclonal antibodies**

CD8 sequence from *Loxodonta africana* was cloned with an HA tag, into a puc57 vector by GenScript (Piscataway, New Jersey) and expressed in 293T cells (ATCC® CRL-3216™). For CD4, the predicted transcript was obtained via a tblastn search of an *Elephas maximus* transcriptome assembly against a set of known mammalian CD4 amino acid sequences. Using BglII and EcoRI restriction enzymes, sequences were sub-cloned into mscvPURO expression vectors and transfected into 293T cells, in addition to Lentivirus VSVG and HIT-60, for the production of infectious transgenic lentivirus. After a period of 48 hours, virus was harvested and used to infect mouse L cells.
Transgenic lentivirus was combined with L cells in the presence of polybrene for enhanced infection efficiency. L cells were then grown in DMEM (+10% HI-FCS+1%antimycotic-antibiotic) containing 2-10μg/mL puromycin, where the concentration of puromycin was increased to 10μg/mL over a period of several weeks. Cells were plated at 200 cells/well in order to identify puromycin-resistant clones. Following a period of 3-4 weeks, puromycin resistant clones were isolated and plated into individual wells of a 24 well plate. Characterization of each clone by Western blot and flow cytometry enabled identification of those clones that expressed CD8 and CD4 protein, using the HA tag as a target.

The cell lines expressing the highest level of CD8 and CD4 were used to immunize mice, who were subsequently assessed for anti-CD8 and anti-CD4 antibody titres in serum. Mice with the greatest titres were sacrificed and their spleen cells fused with myeloma cells to generate potential hybridomas. Hybridoma supernatants were then screened for their ability to bind CD8- and CD4- expressing cell lines versus control cells via CELLISA.

**Intracellular Cytokine Staining (ICS)**

PBMCs (5x10⁵) were stimulated with peptides, DMSO and positive control SEB overnight at 37°C (5% humidity), where brefeldin A (Thermo Fisher, Waltham, Massachusetts) was added after the first 2 hours in culture. Following stimulation, viability was determined using a Ghost dye™ Red 780 (Tonbo Biosciences, San Diego, California). Cells were surface stained with anti Asian elephant CD8 and CD4 (custom made at the Monoclonal Antibodies core facility at MD Anderson Cancer Center (MDACC); PE and Pacific blue labeled, respectively), then lysed and permeabilised using a kit (Cytofix/Cytoperm; BD). Cells were then stained for CD3 (Abcam; clone CD3-12) and IFN-γ (Podiceps; clone AE10F4G11, labeled in-house with FITC). An APC-labeled anti-rat secondary was added following incubation with primary antibodies, in order to detect bound anti-CD3 primary antibody. Cells were then washed and fixed in stabilizing fixative (BD Biosciences). Cells were acquired on a BD FACSCanto II flow cytometer. At least 100,000 events were collected for each sample, determined by BD...
FACSDiva™ software. Following acquisition, analysis was performed using FlowJo V10 software (Treestar).

Statistics

The statistical comparisons for the continuous data between the experimental groups and the negative control DMSO were performed using two-sample T tests with unequal variance assumptions. ELISpot SFC count data were log-transformed for stabilizing the variance and approximating the normality assumptions. A p<0.05 was considered as statistical significance. No multiple comparison procedure was considered. All analyses were performed using GraphPad Prism Software.

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Five elephants were vaccinated with killed Rabies vaccine at day 0 and blood was obtained from 5 elephants (aged 9-49) at days 14 and 28. PBMCs were stimulated in IFN-γ coated ELISpot plates with DMSO control or Rabies nucleo-capsid (NC) pepmix. Each sample was tested in triplicate at each time point, in at least three separate experiments. The mean (± SEM) of SFCs per 1 million PBMCs is shown, where *(p<0.05) indicates a statistically significant difference as determined by two sample t tests on log-transformed values compared to the DMSO control at the same time post vaccination.

Figure 2: Glycoprotein B (gB) responses in 5 elephants
PBMCs obtained from each elephant at three separate time points were screened in triplicate in at least 3 separate experiments. The mean (± SEM) of SFCs per 1 million PBMCs is shown, where *(p<0.05) and **(p<0.01) indicate the statistically significant difference as determined by two sample t tests on log-transformed values compared to the DMSO control. Survivin is included as an additional negative control. The dashed line represents the mean of DMSO (background) for each elephant.

Figure 3: E40 responses in 5 elephants
PBMCs obtained from each elephant at three separate time points were screened in triplicate in at least 3 separate experiments. The mean (± SEM) of SFCs per 1 million PBMCs is shown, where *(p<0.05), ***(p<0.001) indicate the statistically significant difference as determined by two sample t tests on log-transformed values compared to the DMSO control. The dashed line represents the mean of DMSO (background) for each elephant.

Figure 4: Major Capsid Protein (MCP) responses in 3 elephants
PBMCs obtained from each elephant at three separate time points were screened in triplicate in at least 3 separate experiments. The mean (± SEM) of SFCs per 1 million PBMCs is shown, where *(p<0.05) and **(p<0.01) indicate the statistically significant difference as determined by two sample t tests on log-transformed values compared to the
Figure 5: De-convolution of MCP2 peptide pool
Elephant 3, who had a significant IFN-γ ELISpot response to MCP2, was selected to study further with the intention of identifying the peptide sequence within MCP2 that T cells were responding to. A) MCP2 matrix summary, where numbers on the top row and first column indicate minipool numbers and each number in the grid represents an individual peptide. B) MCP2 minipool summary showing the mean (±SEM) of SFCs per 1 million PBMCs, where $p(****)<0.001$, $p(**)<0.01$ and $p(*)<0.05$ indicate the statistically significant difference as determined by two sample t tests on log-transformed values compared to the DMSO control. Boxes were placed around minipools 6 and 14, which intersected at MCP130. C) The sequences of the 9 peptides in minipools 6 and 14 with boxes around the sequence of MCP130 to demonstrate its presence in both minipools. D) PBMCs from elephant 3 were further stimulated with MCP130 alone, where there was a significant response above the DMSO control ($***p<0.001$).

Figure 6: CD8 and CD4 protein expression in L cells and PBMCs.
CD8 and CD4 expression was identified in L cells with primary antibodies directed against the HA epitope tag. No CD8 or CD4 expression was seen in CD8- and CD4-negative L cell lines (A and D respectively) as compared to broad expression in CD8- and CD4-positive cell lines (A and D). Using purified monoclonals on elephant PBMCs in combination with a cross-reactive CD3 monoclonal antibody, we could detect CD3+CD8+ T cells (B) and CD3+CD4+ T cells (E). Both CD3+CD8+ and CD3+CD4+ subsets can be further quantified as IFN-γ producers using an IFN-γ monoclonal antibody, shown here in cells stimulated with SEB (C and F).

Figure 7: Determining T cell phenotype following peptide stimulation.
PBMCs were surface-stained with Asian elephant CD8 and CD4 specific monoclonal antibodies and stained for intracellular CD3 and IFN-γ expression following peptide stimulation. Following exclusion of dead cells and doublets, cells were gated on CD3
expression prior to CD4 and CD8 expression (A). The IFN-γ expression of each the CD3+CD8+ fraction and CD3+CD4+ fraction are shown in one elephant who had a specific response to MCP130 (B). The frequency of IFN-γ expression in CD3+CD8+ and CD3+CD4+ cells was assessed in 2 elephants by FACS, where **(p<0.01) (C).

**Figure 8: Houston Zoo elephant herd**

Squares represent males and circles represent females. One male (white square) is not a member of the Houston Zoo herd but is the father of Elephant 5. Features of each elephant are described in Table 2.
Table 1. Summary of EEHV1A ORFs selected for screening in IFN-γ ELISpot assays

| ORF     | Common name                      | Protein type            | Homologs                        | T cell reference |
|---------|----------------------------------|-------------------------|---------------------------------|------------------|
| U57     | Major Capsid Protein (MCP)       | Capsid/structural       | HHV6 U57, HCMV UL86, HSV UL19   | (25, 26)         |
| E44, EE1* | ORF-L, Major Immediate Early    | Regulatory              | HCMV UL123                      |                  |
| U39     | glycoprotein B (gB)              | glycoprotein            | HCMV UL55                       | (23, 24)         |
| U48     | glycoprotein H (gH)              | glycoprotein            | HHV6 U48, HCMV UL75, HSV UL22   | (26)             |
| U42     | ICP27, Mta                       | Regulatory              | HHV6 U42, HCMV UL69, HSV UL54   | (41)             |
| E34, U11* | ORF-C, pp150                    | Tegument/structural     | HCMV UL32                       |                  |
| U71     | TP Myrs Teg                      | Tegument/structural     | HHV6 U71, HCMV UL99, HSV UL11   | (42)             |
| E40, EE2* | ORF-K                           | nuclear protein         | ??                              |                  |
| E44A, EE1A* | ORF-S                          | putative regulatory     | different reading frame to ORF-L |                  |

*Alternative nomenclature used in Wilkie et al., 2013 (15).
Table 2. Characteristics of the Houston Zoo elephant herd

| Elephant | Age (years) | Sex | EEHV1 viremia* | EEHV1 shedding (trunk wash) |
|----------|-------------|-----|----------------|-----------------------------|
| 1        | 52          | M   | Yes (unpublished) | Yes (unpublished)           |
| 2        | 48          | F   | Yes (unpublished) | Yes (13)                   |
| 3        | 27          | F   | Yes (12)        | Yes (12, 13)               |
| 4        | 36          | F   | Yes (unpublished) | Yes (13)                   |
| 5        | 12          | M   | Yes (unpublished) | Yes (unpublished)          |
| 6        | 7           | M   | Yes (44)        | Yes (44)                   |
| 7        | 7           | F   | Yes (44)        | Yes (44)                   |
| 8        | 3           | M   | No              | No                          |
| 9        | 0           | F   | No              | No                          |

*Indicating the detection of EEHV1 at least once in whole blood samples screened weekly for EEHV from 2009-2017.
Table 3. Responses to EEHV1A ORFs studied

| ORF       | Elephant 1 | Elephant 2 | Elephant 3 | Elephant 4 | Elephant 5 | Elephant 6 | Elephant 7 |
|-----------|------------|------------|------------|------------|------------|------------|------------|
| U57       | +          | +          | +          | -          | -          | -          | -          |
| E44, EE1* | -          | -          | -          | -          | -          | -          | -          |
| U39       | +          | -          | +          | +          | -          | +          | +          |
| U48       | -          | -          | -          | -          | -          | -          | -          |
| U42       | -          | -          | -          | -          | -          | -          | -          |
| E34, U11* | -          | -          | -          | -          | -          | -          | -          |
| U71       | -          | -          | -          | -          | -          | -          | -          |
| E40, EE2* | -          | +          | +          | +          | +          | +          | -          |
| E44A, EE1A* | -        | -          | -          | -          | -          | -          | -          |

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