Mapping B lymphocytes as major reservoirs of naturally occurring latent equine herpesvirus 5 infection

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Abstract

Equine herpesvirus 5 (EHV5) is a commonly detected gammaherpesvirus, which, along with the closely related EHV2, constitute the only two known pericerviruses that infect horses. Apart from detection in horse populations worldwide and the recent publication of the whole genome, there is little known about the biology and pathogenesis of this virus, with many assumptions made by parallels with EHV2. The long-term survival of gammaherpesviruses within infected hosts involves the establishment and maintenance of latency in selected cell and tissues types, particularly lymphocytes. A latent gammaherpesvirus infection is characterized by a limited number of genes expressing in a particular cell or tissue type. In this study, we have used in vitro co-culturing to detect EHV5 in equine PBMCs and characterize the predominant cellular site for the establishment and maintenance of a latent infection. These experiments were conducted by isolating PBMCs from 10 horses and sorting subpopulations into two T lymphocyte (CD4 and CD8), B lymphocyte and macrophage enriched or depleted fractions. These lymphocyte and macrophage fractions were examined for the presence of latent EHV5 by in vitro co-culturing with equine foetal kidney cells. The lymphocyte fraction enriched with B lymphocytes had a significantly increased (P=0.005) number of plaques formed during co-culturing, whereas the B lymphocyte depleted fraction had a significant reduction in the number of plaques formed after co-culturing. Taken together, these results demonstrate that equine gammaherpesviruses establish latency in the equine PBMCs, with the predominant site for maintenance of latent virus being B lymphocytes.

INTRODUCTION

Gammaherpesviruses are characterized by a narrow host range, slow replication cycle and the establishment of latent infections in cells of the immune system [1, 2]. The preferred sites of latency are generally different from those of alphaherpesviruses, which use cells of the nervous system [3, 4]. The low level of MHC class I expression in nerve cells may assist the maintenance of alphaherpesviruses in latently infected cells by reducing exposure to the immune system, whereas gammaherpesviruses encode for arrays of immune modulating genes that circumvent host-mediated destruction of latently infected cells [4–7].

The hallmark of latent gammaherpesvirus infection is the transcription of a limited number of viral genes called latency-associated transcripts (LATs) [8–12]. These gene products are usually detected during establishment and maintenance of latent gammaherpesvirus infection, and become undetectable or cease to be expressed by the time lytic replication commences. However, global gene expression studies have demonstrated that this process is more complex than previously thought and have questioned the validity of defining the period of latency from detection of LATs alone [13–15]. Hence, the presence of viral genome in the absence of preformed infectious virus still remains a classic and essential measure of latent herpesvirus infection.

Although the mechanism of latency is yet to be fully elucidated, studies have confirmed the important role of LATs in human gammaherpesviruses, such as human herpesvirus 4 (HHV4) and human herpesvirus 8 (HHV8), and the prototype gammaherpesvirus murine herpesvirus 68 (MHV68). Expression of genes such as Epstein–Barr nuclear antigen (EBNA1–6) and latent membrane proteins (LMP-1, -2A and -2B) are associated with neoplastic transformation of latently infected lymphocytes during HHV4 infections [16–20].

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Abbreviations: CPE, cytopathic effect; EFK, equine foetal kidney; EHV5, equine herpesvirus 5; HHV, human herpesvirus; LAT, latency-associated transcript; MHV68, murine herpesvirus 68; EHV2, equine herpesvirus 2.

Four supplementary tables are available with the online Supplementary Material.
Our understanding of equine gammaherpesvirus latency is very limited, but it has been demonstrated that equine B lymphocytes are major cellular reservoirs for latent equine herpesvirus 2 (EHV2) infection. However, there are also reports that suggest the involvement of more than one type of cell and possibly other lymphoreticular tissues in establishment and maintenance of EHV2 latency [21, 22]. Since the publication of these studies, there have been some improvements in the availability of antibodies to equine cell markers. In this regard, there are no published reports describing whether EHV5 is also capable of maintaining latent genome in equine PBMCs. The poor availability of antibodies to equine cell markers. In this regard, there are no published reports describing whether EHV5 is also capable of maintaining latent genome in equine PBMCs. The poor in vitro growth potential of EHV5 and high incidence of co-infection with EHV2 might have limited such work. Despite these challenges, EHV5 has been isolated from equine PBMCs and a wide variety of other tissues such as nasal swabs, ocular swabs, lung tissues and uterine discharges [23–28]. Therefore, the major focus of this study is to examine the role of the PBMC subpopulations in naturally occurring latent EHV5 infections.

RESULTS

Positive selection and purity of selected PBMC subpopulations

Peripheral blood mononuclear cell populations were examined for their reactivity to six mAbs (Table S2, available in the online Supplementary Material). However, efficient separation of PBMC subpopulations using magnetic-activated cell sorting (MACS) first required assessment and titration of antibodies to optimize binding while minimizing the background and non-specific binding. The final set of four mAbs (and dilutions) used in this study were directed to CD4 (1/100), bovine CD5 (1/100), CD8 (1/50) and CD163 (1/160) based on the flow cytometry results. The mAbs to CD3 and CD21 reacted with a lower percentage of cells compared to mAbs to CD4 and CD8 used for T lymphocytes and CD5 for B lymphocytes. Due to this poor reactivity, the number of positively enriched cells was insufficient to carry out downstream experiments, and consequently these two mAbs were excluded from further use in this study. Using this optimized protocol for MAb labelling, a final volume of 80 ml of whole blood and a normalized concentration of 10^7 to 10^8 PBMCs to sort, the positively enriched fractions resulted in yields of between 3 and 7% (Table S3).

Cell fractions enriched for each of the four specific equine cell markers were assessed using flow cytometry analysis (Fig. 1). The average purity and reproducibility were highest for CD4 enriched fractions and lowest for CD8 enriched fractions (Table S4, Fig. 1). In addition, selected samples from the depleted populations were also stained with secondary antibody and assessed for efficiency in the enrichment procedures (Fig. 1). Taken together, these experiments demonstrate the basic parameters and reproducibility of the PBMC subpopulations used throughout the course of this study.

![Flow cytometry analysis](image)

**Fig. 1.** Flow cytometry analysis of cell populations that have been enriched (a to d) or depleted (e to h) using antibodies against CD4 T lymphocytes (a, e), B lymphocytes (b, f), CD8 T lymphocytes (c, g) and macrophages (d, h). After enrichment or depletion, cells were incubated with FITC-conjugated rabbit anti-mouse IgG and run through the FACS panels. Histograms showing FITC (x-axis) and cell count (y-axis) channels, where the events within the P3 region were considered positive for the cell markers that were used for purity analysis.
Lymphocyte co-culturing shows B lymphocytes as a major reservoir for equine gammaherpesviruses

To identify the site of latency in EHV5 infected individual horses, unsorted PBMCs and sorted subpopulations of PBMCs were co-cultured in the presence of equine foetal kidney (EFK) cell monolayers susceptible to EHV5 lytic infection [29, 30]. In this assay, subpopulations included the positively selected cells enriched for T lymphocytes (CD4 and CD8), B lymphocyte and macrophages, in addition to the associated flowthrough populations, which had been depleted of cells positive for these markers. During the 21 days of incubation, wells that were inoculated with frozen and thawed cells or the media controls did not show any plaques or signs of cytopathic effect (CPE). This result confirmed that any virus present in the PBMCs and the different cell subpopulations were likely to be from latently infected cells rather than from reactivated virus that may have existed in these samples or had been released during processing and sorting of the cells [21, 31].

Over the 10 trials, a total of 260 plaques were counted in all of the cultures containing enriched cell populations with a mean of 6.5 plaques per $10^6$ cells (Fig. 2) in each of the subgroups (B cell, CD4, CD8 and macrophages). Culture of the specific cell depleted populations resulted in a total of 320 plaques, with a mean of 7.8 plaques over the 10 trials (Fig. 2). A total of 69 plaques were counted from the unfractionated PBMC (Fig. 2). Cultures containing enriched B lymphocytes produced 17.4 plaques per $10^6$ cells, contributing more than half (174) of the total 260 plaques counted (Fig. 3a) and demonstrated that formation of plaques was most highly associated with co-culturing B lymphocytes ($P<0.005$). Further evidence that these B lymphocytes were the predominant cell type associated with latency was the observation that the population depleted of this cell type had significantly fewer plaques than other negatively selected populations (Fig. 3b). Overall, in these experiments, B lymphocyte depleted populations had a total of 24 plaques over the 10 experiments from a total of 320 plaques in all cultures containing specific cell depleted fractions ($P=0.005$). The strong association of latently infected cells with B lymphocytes and poor association with CD4 and CD8 T lymphocytes strongly suggest that
B lymphocytes are a major site for latent equine gammaherpesvirus infections.

In order to differentiate the EHV5 from EHV2 plaques formed in these assays, a total of 65 well-isolated plaques were collected from the co-culture of the positively enriched fractions (Fig. 4). A discriminatory PCR for EHV5 and EHV2 DNA was performed on these 65 plaques (Table 1), which identified 20 EHV5-positive plaques, with the remaining 45 identified as EHV2. No plaques were detected that were positive or negative to both the EHV5- and the EHV2-specific PCRs. The results (Table 1) show that 65% (13/20) of all EHV5-positive plaques and 44% (20/45) of all EHV2-positive plaques were from B lymphocyte enriched fractions. Overall, this experiment demonstrated a major role for B lymphocytes in harbouring latent equine gammaherpesvirus infection.

**Genome load estimation in latently infected cells**

To further examine whether specific cell types were preferentially associated with EHV5 and EHV2 latent infection, viral genome copies were directly quantified in four positively sorted subpopulations and normalized to detection of the b2m gene by qPCR. The mean EHV5 genome load was more abundant in B lymphocytes than any other cell type (Fig. 5a). The mean genome load of EHV5 in B lymphocyte was 10-fold higher than CD4 T lymphocytes, while the lowest EHV5 genome loads were detected in macrophages. While the trends of viral genome load in the different cell populations were similar to that described by the co-culture assay, there was no statistically significant difference in EHV5 genome load between the groups using qPCR assays. The more definitive result obtained from the co-culture experiment compared to the qPCR analysis may be due to factors that include the higher sensitivity of the co-culture method and the consequent limitation in the power of the qPCR analysis, which could only be improved by increasing the number of horses used in this study. Analysis of results from individual horses showed the levels of EHV5 genome detected in B lymphocyte enriched fractions were at least 10-fold higher than in T cells and in macrophages in 9 out of 10 individuals. The exception was horse 6, which had a higher EHV5 genome in CD4 and macrophages than B cells. A different horse, horse 1, had a higher EHV5 genome load in CD8 T cells than B lymphocytes. Compared with other investigated cell populations, there were higher quantities of EHV5 genome consistently (9 out of 10 cases) detected in B cell enriched populations. Similarly to EHV5, the EHV2 genome load was higher in B cells than in CD4 T cells in all 10 horses and higher in B cells than CD8 T cells in 9 horses. The single exception was again horse 1, which, like EHV5, had more EHV2 genome detected in CD8 enriched cells than B cells. However, in contrast to EHV5, the macrophage subpopulation was the cell subpopulation with the second highest level of latent EHV2 infection (Fig. 5b).

**Frequency of latently infected B lymphocytes**

In vitro limiting dilution reactivation assay

Gammaherpesvirus latency occurs in only a fraction of the relevant host cell populations, and the actual proportion of latently infected cells varies between viruses and between individuals. In well-studied human herpesviruses such as HHV4 and HHV8, end-point limiting dilution assays have been used to quantify the frequency of latently infected cell reactivations [1, 31, 32]. In this experiment, 10⁶ B lymphocytes were serially diluted before being co-cultured with established EFK monolayers in 96-well tissue culture plates. The numbers of gammaherpesvirus infected lymphocytes were then estimated by scoring individual wells for CPE and calculating the percentage of positive wells from the replicates. The numbers of infected lymphocytes varied from animal to animal, where at

<table>
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<th>Cell fractions</th>
<th>CD4 T lymphocytes</th>
<th>CD8 T lymphocytes</th>
<th>B lymphocytes</th>
<th>Macrophages</th>
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<tr>
<td>EHV5</td>
<td>3</td>
<td>1</td>
<td>13</td>
<td>3</td>
<td>20</td>
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<tr>
<td>EHV2</td>
<td>7</td>
<td>9</td>
<td>20</td>
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least 10^3 lymphocytes were harbouring EHV2 and EHV5 from a total of four independent trials (Fig. 6a). To specifically determine the number of cells containing EHV5 DNA, a limiting dilution nested qPCR (Table S1) showed that at least one in 10^4 to 10^5 B lymphocytes was positive for EHV5 DNA. Finally, the frequency of reactivation events for EHV5 and EHV2 was quantified using a 63.2 % Poisson distribution line, where one reaction event was likely to occur (Fig. 6b). These results suggest that the frequency was one reactivation event in 14 200 B lymphocytes (Fig. 6b).

**DISCUSSION**

This investigation of equine lymphocyte and macrophage sub-populations was conducted to identify the major cell type responsible for harbouring latent EHV5 infection. These studies showed that B cells are preferentially targeted by the two equine gammaherpesviruses as sites of latency. This is the first report to describe a major role for B lymphocytes in EHV5 latency. The work has also provided additional evidence to the previous finding that the EHV2 predominating site of latency is B lymphocytes. In that regard, sorting of B lymphocytes with 95–100 % purity was an improvement from the previous EHV2 report by Drummer et al. [21] where purity ranged from 60 to 80 %. This experiment has also presented an improvement from the earlier study with the use of a macrophage/monocyte marker for sorting and was also able to investigate the two CD4 and CD8 T-lymphocyte fractions within the equine PBMC.

This study has demonstrated B lymphocytes as major reservoirs of EHV5 and EHV2 latently infected cells, although

![Fig. 5. Evaluation of (a) EHV5 and (b) EHV2 genome load in positively sorted PBMC subpopulations by qPCR. DNA was extracted from positively sorted cells directly after sorting. Data were normalized using b2m gene qPCR.](image-url)

![Fig. 6. (a) Limiting dilution assay to determine the number of B lymphocytes latently infected with EHV5 and EHV2 in individual horses. (b) Frequency of B-lymphocyte reactivation from latency inferred using the data from (a), where the horizontal dotted line represents the 63.2 % Poisson distribution line at which frequency of reactivation was calculated. The vertical dotted line indicates the number of cells where one reactivation event has occurred.](image-url)
latently infected cells were detected to varying degrees in all subpopulations examined. Three of the four antibodies to equine cell markers used here have been used in previous studies to sort cell populations [33]. Whether these viruses are latent in more strictly defined cell populations will be better elucidated following the future expansion of the equine immunology tool kit. While T lymphocytes can express CD5, CD5 is also expressed in all murine B lymphocytes and has also been described as a significant B-cell marker in humans [34–36]. The B29A clone used in this study was prepared against bovine CD5, but reacts with equine B cells only when used as a marker of equine PBMC populations [37, 38] and is distinct from the other CD5 equine T-lymphocyte markers that are known (such as HT23A and HB19A). Similarly, in the absence of an adequate and reproducible method for sorting CD3-positive T cells, CD4 and CD8 were used as surrogate markers in this study for T-cell subpopulations, although these markers can also be found on NK cells (CD8), dendritic cells (CD8 and CD4) and some monocyte/macrophages (CD4). The poor association of EHV5 and EHV2 latency with CD4- and CD8-positive cells, combined with the strong association of EHV5 and EHV2 latency in B lymphocytes, is consistent with a role for B lymphocytes as a major reservoir of latency for EHV5 and EHV2.

When used in co-culture with EFKs, the numbers of plaques counted from the depleted population were higher than from enriched cell populations and plaques generally appeared more quickly from the depleted (day 5) compared to the enriched (at least 8 days) fractions. This may suggest depletion techniques could be a better alternative to facilitate the detection of reactivation in PBMCs. Past experiments have demonstrated that contact of latently infected lymphocytes with other permissive cells is a requirement for viral reactivation to occur [21]. Hence, it is worth noting that depleted cell fractions are free from surfaces coated with an antibody and microbead mixture, although deple- tion techniques may require more than one antibody and extended steps to achieve enrichment of a specific cell population. Reactivation of a latently infected cell involves a complex process of virus–host interaction with a molecular mechanism that is not fully understood [39, 40]. Successful in vitro reactivation models for HHV4- and HHV8-infected B cells were developed by physically stimulating B-cell antigen using cross-linking of surface immunoglobulins with anti-Ig antibodies [41]. In addition, several host physiological responses such as oxidative stress, concurrent viral infections and generalized immunosuppression have been associated with gammaherpesvirus reactivation [42].

In the wider Gammaherpesviridae family, a variety of different B-lymphocyte subpopulations have been described as sites of latent infection for different herpesviruses. Subsets of memory B cells have been identified as long-term HHV4 latency reservoirs [1, 32, 43–45], whereas CD19-positive B cells have been identified as the latency reservoir for HHV8 [46, 47]. MHV68 latency is established in transitional B cells [48]. Access to well-optimized and a wide range of antibodies to human and mouse cell surface markers has enabled the above identification of specialized B-cell populations. Similarly, future improvements to the equine specific antibody tool box could provide access to a wider range of subset-specific markers that will assist in identifying more accurately the phenotype of B-lymphocyte subsets that are involved in equine gammaherpesvirus latent infections.

The observation made in this study that the B lymphocyte was the major cell type for maintenance of latency is consistent with the observation in other human and animal gammaherpesvirus [2, 17, 31]. However, our results also showed the presence of EHV2 and EHV5 genome in other cell populations, and that a number of the reactivated viruses were from T-cell and macrophage enriched populations. Whether future improvements to the purity of enriched fractions might influence this result is not known, but these results may also suggest that B cells may not be the only source of cells latently infected with the equine gammaherpesviruses. The establishment of MHV68 latency in B-cell-deficient mice prompted the assumption that additional cell types may also support latent infections. Subsequent investigations have confirmed this assumption and demonstrated that peritoneal macrophages and dendritic cells can support latency in B-cell-deficient mice [48]. Similarly, previous studies on EHV2 have indicated the presence of low-quantity latent virus in T lymphocytes [21, 31, 48–50].

In contrast, HHV4 has evolved a conserved strategy where memory B cells are a strictly required site of latency [51]. In this virus, a latent genome is permanently committed only to mature memory B cells, while pre-B cells that are not yet specialized to memory B cells show regression of the latent HHV4 genome [52]. This indicates that the mechanism of latent genome maintenance is reliant on manipulating the host immune response rather than spreading to new B cells following reactivation events [32], which is an efficient strategy to avoid active replication and hide away from immune recognition. Additional evidence from MHV68 infection in B-cell-deficient mice showed that artificially induced inflammation does not facilitate reactivation of latent virus but increases the recruitment of latently infected macrophages from the bone marrow to the site of inflammation [31].

Identification and experimental detection of LATs is a logical step that follows the mapping of the major site of gammaherpesvirus latency. For equine gammaherpesviruses, there are no reports that have been able to confirm the expression of LATs, with unsuccessful attempts to amplify selected EHV2 genes using reverse transcription linked PCR by Hazilawati [53] and using a subtractive hybridization technique on latently infected B lymphocytes [54]. In the genomes of equine gammaherpesviruses, ORF73 is a homologue of HHV8 ORF73 (LANA) [55] and has been assigned as a putative LAT. However, most gammaherpesviruses encode more than one LAT, and these additional genes are usually unique to the particular species of the virus [56]. In that regard, the genome of equine gammaherpesviruses encodes for a number of...
unique genes and their role in latency needs investigation. However, the traditional view of latency, that lytic genes are inactive and not transcribed, has been successfully challenged with more recent work that uses a global gene profiling technique, demonstrating the complexity of the process [57–59]. As a result, the current notion of latency has been broadened to include differential gene expression, viral and cellular microRNAs (miRNAs) and epigenetic factors [47, 60, 61]. The role of miRNA in regulating host and viral gene expression is becoming a major area of herpesviral research [62–66]. Currently, more than 300 viral miRNAs have been discovered, and most of these are from human herpesviruses [62, 67–69]. Hence, future investigations regarding the latency strategies of equine gammaherpesviruses should involve the complementation of classical culture methods with recent advances in computational biology for profiling the global gene transcription patterns of EHV5 and EHV2. Examination of the associated viral and cellular transcriptomes and miRNAs complex will enable a better understanding of the cellular and molecular mechanisms in equine gammaherpesvirus latency.

METHODS

Sampling of horses and isolation of PBMCs

The experimental design and animal sampling methods were reviewed and approved by the animal ethics committee of The University of Melbourne, Australia (1312738.1). Initially, whole blood from 17 horses was screened for the presence of EHV5 and EHV2 DNA using virus species-specific qPCR (Table S1). Whole blood was collected from 10 of these 17 horses (80 ml of blood was collected per horse in a final concentration of 20 IU ml⁻¹ heparin) by jugular vein puncture. The collected whole blood was diluted one to one with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), then 20 ml of diluted whole blood was overlaid onto 20 ml of Ficoll-Paque PLUS (GE Healthcare) and centrifuged (Allegra X-12R; Beckman) at room temperature for 30 min without brake at 400 g. The white layer containing the mononuclear cells at the interface between plasma and the Ficoll layer was carefully aspirated using a Pasteur pipette. The mononuclear cell fractions from the same horse/sample were pooled and resuspended in a final volume of 40 ml with PBS. After pelleting cells at 1000 g for 5 min at 4 °C, cells were resuspended in PBS and washed in PBS prior to counting. Viable cells were counted using Trypan blue exclusion (0.4 % w/v Trypan blue; Sigma–Aldrich) in a haemocytometer (Brightline; Hauser Scientific).

mAb labelling of equine PBMCs

Antibodies to lymphocyte subtype markers used in this study are shown in Table S2. Although CD5 is usually considered to be present on T and B lymphocytes, the antibody used in this study (clone B29A) to bovine CD5 has been demonstrated to react with equine B lymphocyte only [37, 38]. For labelling of equine PBMCs for FACS analysis, 10⁶ to 10⁷ cells were incubated with 50 µl of CD4 (1/100) or CD8 (1/50) or CD5 (1/100) or CD163 (1/100) in a 96-well tissue culture round-bottomed plate (Sarstedt) and incubated for 15 min on ice. The plates were centrifuged at 1500 g for 5 min at 4 °C. After centrifugation, the supernatant was removed and cells were washed with 200 µl FACS buffer (1 mM EDTA, 25 mM HEPES pH 7.0, 1 % v/vFBS in PBS). After two washes, cells were stained with secondary antibody using FITC-conjugated rabbit antibody to mouse IgG (DAKO, 1/160) or PE-conjugated rabbit antibody to mouse IgG (DAKO, 1/500) by incubation for 15 min in the dark. Finally, the cells were washed twice and resuspended in 200 µl FACS buffer for flow cytometry analysis.

Flow cytometry analysis

Flow cytometric analyses were performed using FACS Calibur (Becton Dickinson) and FACSVerse (BD Biosciences) machines, where 10 000 events for FACS Calibur and 1000 events for FACSVerse were collected and analysis of the runs was performed using CellQuest or FACSuite software (Becton Dickinson). Exclusion of dead cells was based on forward and side scatter and autofluorescence signals. Lymphocytes were gated according to the density of events in side and forward scatter plots. These regions were confirmed by back gating after staining with lymphocyte marker-specific mAbs. The intensity of non-specific fluorescence in the gated population was defined by including cells stained only with secondary antibody.

Microbead labelling of lymphocyte subpopulations

In preparation for cell sorting, the mAbs to the four markers (CD4, CD8, bovine CD5, CD163) at the dilutions indicated above were used to label 10⁷ PBMCs as described. Positively labelled cells were resuspended in 80 µl of FACS buffer and incubated with 20 µl of anti-mouse IgG microbeads for 20 min at 4 °C according to the manufacturer’s recommendations (Miltenyi Biotec). After incubation, FACS buffer was added to a final volume of 1–2 ml and centrifuged at 300 g for 10 min at 4 °C. The supernatants were decanted, and the pellet was resuspended with a similar volume of FACS buffer prior to sorting the cells.

MACS of lymphocyte subpopulations

Sorting of positively labelled cells was conducted using MS columns of the OctoMACS set according to the manufacturer’s recommendation (Miltenyi Biotec). This technique was performed by placing MS columns in the magnetic field of the OctoMACS separator. The columns were rinsed using degassed FACS buffer before applying the antibody and microbead-labelled cell suspensions. The flowthrough was collected as a specific cell depleted fraction. The MS columns with bound cells were washed three times with FACS buffer before removal from the magnetic field and elution of the positively sorted (enriched) cells.

Lymphocyte co-culturing and infectious centre assays

A monolayer of EFK cells was prepared 2–3 days prior to collection of blood and sorting PBMCs. For each experiment from the 10 horses, three six-well plates of co-cultures were prepared. The first plate contained wells where EFK
monolayers were overlaid with 10^6 cells from each of the positively enriched fractions, a negative control (FACS buffer) and 10^6 total unsorted PBMCs. The second plate contained wells where EFK monolayers were overlaid with a negative control (FACS buffer) or the entire specific cell depleted fractions or 10^6 total unsorted PBMCs. The third plate contained wells where EFK monolayers were overlaid with a negative control (FACS buffer), 10^6 frozen (–80 °C) and thawed cells (FT) of the positively enriched cell fractions and 10^6 frozen (–80 °C) and thawed PBMCs. The third plates of co-culture using FT cells were included to demonstrate cells were infected with the latent virus at the time of processing these samples and that no intact, infectious virions were present in or with these cells prior to co-culture. The PBMC/EFK overlays were co-cultured for 2 h at 37 °C and 5 % v/v CO2 in the air with occasional rocking, and then overlaid with a 1% v/v methycellulose supplemented Dulbecco’s modified Eagle’s medium (DMEM) that contains 1% v/v FBS, 10 mM HEPES, pH 7.4 and 50 µg ml^-1 of gentamycin. The cultures were maintained for up to 3 weeks for the production of plaques. Monolayers were examined daily for CPE with an inverted Leica MPS60 microscope. Counting of total numbers of plaques at the end of the third week of the co-culture was carried out and well-isolated plaques were collected for EHV2 and EHV5 species-specific qPCR analysis.

**In vitro limiting dilution reactivation assay**

A limiting dilution assay was used to detect the number of cells latently infected by the two equine gammaherpesviruses and the frequencies of reactivation for these two viruses in B lymphocytes. Protocols for this assay were adopted from published works with minor modifications [1, 2, 31, 48, 70]. Briefly, half-log serial dilutions were prepared from 10^6 B lymphocyte enriched cells across 11 wells with the final (twelfth) well containing only negative controls (media only). Each of these dilutions was performed in eight replicates in 96-well tissue culture plates. The cells were then plated on established EFK monolayers in 96-well tissue culture plates and incubated with culture media (DMEM with 1% v/v FBS, 10 mM HEPES, pH 7.4 and 50 µg ml^-1 of gentamycin) at 37 °C in 5% v/v CO2 in air for the next 7–14 days before the wells were scored for CPE, and the percentage of positive wells was calculated from the total of eight replicates. The highest dilution with CPE was used to calculate the number of latently infected lymphocytes from each experiment. To determine the number of cells where a single reactivation was likely to occur, a standard curve was constructed from the number of cells in each dilution and the percentage of CPE-positive wells. A 63.2 % Poisson distribution line was used to interpolate the number of cells that supported a single EHV5 and EHV2 reactivation event.

**DNA preparation and qPCR analysis of the PBMC subpopulations**

Viral DNA or total nucleic acid was extracted from a 140 µl sample or approximately 10^6 cells using a QIAamp viral RNA extraction kit (QVREK; Qiagen) according to the manufacturer’s recommendations. Quantification of the viral genome in the PBMCs and the subpopulations was carried out by qPCR targeting viral gH of EHV5 and gB of EHV2 genes, respectively, and β-2-microglobulin (b2m) was used as host reference gene (Table S2). Amplification of DNA was in 25 µl total reaction volume containing GoTaq Flexi Buffer (10 mM KCl, 2 mM Tris/HCl pH 9.0, 0.02 % v/v Triton X-100), 2 mM MgCl2, 200 µM dNTP, 200 and 300 nM gene-specific primers, 1.25 U of GO Taq DNA polymerase (Promega) and 0.8 µM Syto9 double-stranded DNA binding dye (Thermo Fisher Scientific) and completed by adding 2 µl purified template DNA. Amplification was performed in 0.2 ml thin wall tubes in a thermal cycler (Stratagene Mx3000p). The reaction was initially denatured at 95 °C for 5 min and followed by 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C and 1 min extension at 72 °C. Fluorescence data acquisitions were at the end of each extension cycle. A final melt curve analysis included one cycle of each at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s with fluorescent data acquisition between 55 and 95 °C. Cycle threshold (Ct) values were generated from MxPro software. Absolute quantification of viral equivalent genome copies was carried out by constructing a standard curve using plasmid standard containing EHV5 gH, EHV2 gB and equine β2m genes.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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