Viral genes and cellular markers associated with neurological complications during herpesvirus infections

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Abstract
Despite the importance of neurological disorders associated with herpesviruses, the mechanism by which these viruses influence the central nervous system (CNS) has not been definitively established. Owing to the limitations of studying neuropathogenicity of human herpesviruses in their natural host, many aspects of their pathogenicity and immune response are studied in animal models. Here, we present an important model system that enables studying neuropathogenicity of herpesviruses in the natural host. Equine herpesvirus type 1 (EHV-1) is an alphaherpesvirus that causes a devastating neurological disease (EHV-1 myeloencephalopathy, EHM) in horses. Like other alphaherpesviruses, our understanding of virus neuropathogenicity in the natural host beyond the essential role of viraemia is limited. In particular, information on the role of different viral proteins for virus transfer to the spinal cord endothelium in vivo is lacking. In this study, the contribution of two viral proteins, DNA polymerase (ORF30) and glycoprotein D (gD), to the pathogenicity of EHM was addressed. Furthermore, different cellular immune markers, including alpha-interferon (IFN-α), gamma-interferon (IFN-γ), interleukin-10 (IL-10) and interleukin-1 beta (IL-1β), were identified to play a role during the course of the disease.

INTRODUCTION
Approximately 130 different herpesviruses have been identified in most mammals, reptiles and birds. These viruses share similar structural and biological characteristics, such as the unique ability to establish latency [1]. Many herpesviruses are neurotropic and can cause serious acute and chronic neurological disease of the peripheral nervous system and central nervous system (CNS). The neurological complications include meningitis, encephalitis, myelitis, vasculopathies, acute and chronic radiculoneuritis, and various inflammatory diseases of the eye [2]. Neurological disease either occurs during primary infection or during the course of virus reactivation or reactivation. Despite the importance of neurological disorders associated with herpesviruses, the mechanisms by which herpesviruses infect the CNS have not been definitively established.

In horses, equine herpesvirus type 1 (EHV-1) and equine herpesvirus type 4 (EHV-4) are the most clinically and economically important. Nucleic acid identity between both viruses genomes is above 80%, indicating that they are functionally closely related [3–5]. Despite their similarity, EHV-1 and EHV-4 pathogenesis is strikingly different. While both viruses infect via the respiratory route, only infection with EHV-1 regularly results in abortion, perinatal mortality and EHV-1 myeloencephalopathy (EHM) [6, 7]. EHM is the result of an inflammatory cascade that is associated with EHV-1 infection of vascular endothelial cells within the CNS. This infection results in microthrombus formation and extravasation of mononuclear cells, causing perivascular cuffing and local haemorrhage [8]. Horses infected with EHV-1 typically present mild to moderate respiratory disease and fever, with up to 10% of infected horses developing EHM. Clinical signs range from mild temporary ataxia to paralysis that can lead to recumbency...
and urinary incontinence, and often results in euthanasia [9]. Despite the importance and increasing occurrence of EHM, current understanding of EHM pathogenesis is limited, beyond the essential role of viraemia in transmitting the virus to the vasculature of the CNS. Cell-associated viraemia is detected 4–12 days after primary infection of the respiratory tract and its associated lymphoid tissue; EHM occurs at the end of this viraemic phase [10–13].

Allen et al. found that EHV-1 strains with high neuropathogenic potential are characterized by a longer duration and greater magnitude of viraemia when compared to EHV-1 strains with lower neuropathogenic potential [14]. This evidence supports the argument that prolonged exposure of the CNS vascular endothelium to high viral loads increases the risk of EHM. A single nucleotide polymorphism in the viral DNA polymerase (ORF30, N752 replaced by D752) was discovered to be significantly associated with a higher magnitude and longer duration of viraemia, and enhancement of the neurovirulence potential of naturally occurring EHV-1 strains [15]. The causal relationship between this single nucleotide polymorphism in EHV-1 polymerase and neuropathogenicity in the horse was confirmed using recombinant viruses with differing polymerase sequences [16, 17]. In addition, Vandekerckhove et al. showed that N752 strains are more limited in replication in epithelial cells, while D752 strains are faster and superior at infecting monocytic cells by using respiratory tissue explants [18].

Furthermore, supportive of the central role of viraemia for EHM pathogenesis is the fact that a leukocyte-associated viraemia is not a consistent feature of EHV-4 infections and, consequently, EHV-4 is only occasionally associated with abortion and extremely rarely with neurological disorders [3, 19–21]. Additional evidence supporting these observations has been provided in studies showing that only EHV-1 infects mononuclear cells below the basement membrane, while EHV-4 replication is limited to the respiratory epithelium and does not cross the basement membrane in a tissue explant model [22]. This clear difference in cellular tropism may well be associated with differences in the gene products involved in virus entry and/or spread from an infected to a neighbouring uninfected cell and several genes, in particular, envelope glycoproteins have been shown to be implicated in executing such functions [23].

Glycoprotein D (gD) has been shown to be an essential receptor-binding protein in many alphaherpesviruses [24–26], and it is suggested to be a main player in the viral entry process. Furthermore, it has been suggested that equine major histocompatibility complex class I is a functional EHV-1 gD receptor that plays a pivotal role in entry into equine cells [27]. Using mutant viruses where the gD genes from EHV-1 and EHV-4 were swapped, Azab and Osterrieder have shown that efficient EHV-1 and EHV-4 entry is dependent on gD [28]. The authors showed that gD binds multiple cell surface receptors and plays a defining role with respect to the cellular host range of EHV-1 and EHV-4. Clearly, much remains to be learned about the precise mechanism underlying the transmission of EHV-1 from the respiratory epithelium to monocytic cells and the CNS vasculature and the key viral and host molecules involved in these processes.

In an effort to examine key herpesviral proteins that facilitate spread of EHV-1 to the CNS vasculature, we used ORF30 gene variants with differing neuropathogenic potential (N752 and D752) based on the Ab4 strain of EHV-1 [17]. In addition, we used the close genetic relatedness between EHV-1 and EHV-4 to exchange essential genes between EHV-1 and EHV-4, and generated a gD EHV-1/EHV-4 replacement mutant (Ab4 gD4) [28]. The aim of this study was to use an equine model to determine the role of ORF30 and gD for EHM pathogenesis. This information may also be useful to better understand the mechanisms used by related herpesviruses with neuropathogenic outcomes. Furthermore, a secondary aim was to study the possible role of gD and ORF30 variants for immunoregulation. Horses were infected with the respective viruses, and differences in innate and adaptive immune responses and severity of clinical disease, nasal viral shedding and viraemia were determined.

RESULTS

Generation and characterization of mutant viruses

Two-step Red-mediated recombination was performed to restore gp2 in the Ab4 BAC clone (Aab4_gp2) before exchanging gD1 with gD4 (Ab4 gD4) (Fig. 1). PCR analysis showed that both constructs were of the correct size and position, and RFLP and nucleotide sequencing confirmed the constructs (data not shown). To determine proper expression of gp2 and gD4, equine dermal (ED) cells were infected with the viruses and cell lysates were analysed by Western blotting. Both viruses expressed gp2, >250 kDa, at comparable levels and Ab4 gD4 expressed gD4, approximately 55 kDa, properly in infected cells (Fig. 2a) [28].

In vitro growth kinetics were similar for Ab4 WT, Ab4 N752 and Ab4 gD4 viruses

In vitro growth curves were conducted for Ab4 WT, Ab4 N752 and Ab4 gD4 virus in ED cells. Inoculation of ED cells

Clinical disease differed between infection groups

Animals in the Ab4 WT group and the Ab4 N752 group showed clinical signs of respiratory disease including pyrexia, lethargy, ocular and nasal discharge, and coughing. Ab4 N752- and Ab4 WT-infected horses showed a more severe respiratory disease until day 11 post-infection (p.i.) (P<0.05) (Fig. 3a), and Ab4 N752-infected horses continued to exhibit significant clinical scores between days 12 and 21 p.i. (P<0.05), whereas Ab4 WT-infected animals showed no significant signs of respiratory disease after day 12. Ab4 gD4-infected horses presented only very mild respiratory disease
that was not significantly different from signs in the control animals. On day 9 p.i., three out of eight animals in the Ab4 WT-infected group presented with neurological disease. Two horses developed reduced tail tone, hind limb ataxia, urinary incontinence and recumbency, and were euthanized. The third horse presented with only mild hindlimb weakness and...
recovered fully by day 11 p.i. All three horses exhibiting clinical signs of EHM also presented xanthochromia in the cerebrospinal fluid (CSF; observed on day 9 p.i. for the two euthanized animals and on day 11 p.i. for the horse with mild neurological signs; data not shown).

Body temperatures are shown in Fig. 3(b). All infected groups showed significant primary fevers between days 1 and 3 p.i. (P<0.05), with the Ab4 WT-infected horses showing significantly higher fevers than any other group (P<0.01). Only Ab4 WT- and Ab4 gD4-infected horses showed significant secondary fevers (P<0.01 and P<0.05, respectively). The onset of this secondary fever was delayed in the Ab4 gD4-infected horses until 7 to 12 days p.i. (P<0.01). Ab4 N752-infected horses did not exhibit significant secondary fever responses. None of the control animals were febrile at any point throughout the study.

**Ab4 WT-infected horses showed higher levels of shedding and cell-associated viraemia**

Nasal virus shedding and cell-associated viraemia are shown in Fig. 3(c) and (d) respectively. All animals from the infection groups shed significant amounts of the virus with which they had been inoculated for multiple days p.i. (P<0.001). The Ab4 WT-infected horses presented significantly increased levels of virus shedding when compared to the Ab4 gD4-infected horses or control horses from days 1 to 7 p.i. (P<0.001), but no statistical difference was observed between Ab4 WT and Ab4 N752. Ab4 gD4-infected horses shed significantly lower levels of virus in nasal secretions than horses in the other two infection groups (P<0.001). None of the control animals shed virus.

As observed for nasal virus shedding, no viral DNA was detected in PBMCs from uninfected control animals. Ab4
WT-infected horses presented an overall magnitude of viraemia that was significantly higher than the levels observed in Ab4 N752-infected horses ($P<0.001$), and approached significance when compared to Ab4 gD4-infected horses ($P=0.0687$). Ab4 gD4-infected horses presented delayed onset of viraemia.

Horses in all infection groups showed significant increases in EHV-1 virus neutralization (VN) titres. VN titres are shown in Fig. 4. Following infection, EHV-1 VN titres increased significantly in all infection groups when compared to the controls ($P<0.00001$). Ab4 gD4-infected horses had significantly lower titres than either Ab4 WT- or Ab4 N752-infected horses ($P<0.00001$). No increases in VN titres were observed in uninfected control horses throughout the experiment.

Ab4 WT-infected horses exhibited decreased gamma-interferon (IFN-γ) and complete inhibition of interleukin-10 (IL-10) production in CSF. Average levels +/-SEM of IFN-γ and IL-10 in the CSF are shown in Fig. 5(a, b), respectively. Alpha-interferon (IFN-α) and interleukin-4 (IL-4) were not detected in the CSF of horses in this study, and no changes were observed for interleukin-17 (IL-17) following EHV-1 infection or in the uninfected control horses (data not shown). IFN-γ was significantly decreased ($P<0.001$) and IL-10 production was completely inhibited following Ab4 WT infection only. No significant changes in IFN-γ or IL-10 production were observed in horses from the other infection groups or in control horses. Furthermore, no significant differences were observed in any cytokines examined between horses exhibiting EHM and horses unaffected by EHM within the Ab4 WT group (data not shown).

**Induction of interferon responses in nasal secretions of infected horses**

Fig. 6(a–e) shows average levels +/-SEM of IFN-α (Fig. 6a–c) and IFN-γ (Fig. 6d, e) in nasal secretions. IFN-α significantly increased after infection in nasal secretions from all infected horses ($P<0.0001$). Furthermore, Ab4 WT-infected horses presented significantly increased levels of IFN-α compared to those in either of the other infection groups in nasal secretions ($P<0.0001$). Horses that were affected by
EHM showed overall lower IFN-α levels in nasal secretions on days 1 and 2 p.i. (P=0.07 and 0.1 on days 1 and 2 p.i., respectively) than horses in the same group that were not affected by EHM (Fig. 6b).

In contrast, IFN-γ was only significantly increased in Ab4 WT-infected horses (P<0.0001), while levels did not increase significantly in any of the other infection groups. Horses that presented signs of EHM showed a trend for lower IFN-γ responses compared to horses in the same group without signs of EHM, albeit the differences were not statistically significant (Fig. 6e). No significant changes were observed for IL-17, while IL-4 and IL-10 were undetectable in nasal secretions (data not shown).

**Interleukin-1 beta (IL-1β) was increased in the blood on days 1–3 after EHV-1 infection**

Fig. 7 shows the relative quantity (RQ) of IL-1β expression levels in blood samples from days 1 to 7 p.i. compared to pre-infection levels. Ab4 WT-, Ab4 N752- and Ab4 gD4-infected horses showed significantly increased levels of IL-1β on day 1 p.i. (P<0.0182) when compared with pre-infection IL-1β levels. Ab4 WT-infected horses also had significantly increased levels of IL-1β on days 2 and 3 p.i. (P<0.01 and P=0.0174, respectively) when compared to pre-infection levels. Further, Ab4 gD4-infected horses showed significantly lower IL-1β expression on day 5 p.i. when compared to Ab4 N752 infected horses (P=0.03682).

The remaining PBMC mRNA expression data showed a high degree of variability between individual horses of the same group (data not shown). Nevertheless, we could observe some trends that were similar to those reported previously [29]. Most notably, increases in pro-inflammatory cytokines were noted in all infection groups on days 1, 2 and/or 3 p.i., and T-helper 1 and regulatory cytokines were increased on days 6 and/or 7 p.i., coinciding with the onset of respiratory disease and viraemia, respectively (data not shown).

**DISCUSSION**

Several alphaherpesviruses have been shown to invade/infect nervous tissues inducing different degrees of neurological disorders. Although transmission/transport to reach nervous tissues varies depending on the virus, the final outcomes are usually devastating. Studying neuropathogenic alphaherpesviruses is hindered by the absence of a suitable *in vivo* model; some of studies of the immune response have been done in mouse model systems, which are often not representative. In contrast, natural host systems including pseudorabies virus (PRV) in pigs have shown to be good model systems to investigate alphaherpesvirus biology, including neuroinvasion and host immune response [30]. Here we present another model for studying neuropathogenicity and host immune responses of alphaherpesviruses in the natural host, EHV-1. We show that at least two viral proteins, DNA polymerase (ORF30) and gD, have a neuropathogenic potential and influence the host immune responses, particularly IFN-γ, IFN-α, IL-10 and IL-1β, resulting in changes in the severity of disease outcomes and possibly important cellular immune markers during nervous tissue infection.

Most notably, we observed differences in the severity of respiratory disease that did not coincide with the
neurological potential in horses infected experimentally with different mutant viruses. Contrary to what has been reported previously [17], we observed that horses infected with Ab4 N752 showed significantly more overall respiratory disease when compared to Ab4 WT-infected horses or Ab4 gD4-infected horses. In contrast to respiratory disease, only horses in the Ab4 WT-infected group developed EHM, while no horses in the other infection groups showed signs of neurological disease. Similarly, it has been reported that it is difficult to reliably induce abortions in experimental challenge infections unless Ab4 WT virus is used as the challenge virus [31]. A possible explanation is that N752 strains may be more prone to replicate in epithelial cells than D752 strains, which are more likely to transfer quickly to local monocytic cells below the basement membrane of the respiratory epithelium [18]. Consistent with the finding that the N752 variant (Ab4 N752) induced more respiratory disease, but appeared to have a reduced capacity to induce neurological signs and lower levels of viraemia, are studies suggesting that the D752 strains transfer to blood leukocytes in horses more efficiently, resulting in higher levels of viraemia [14, 16, 17, 32, 33]. These findings also suggest that

Fig. 5. IFN-γ and IL-10 levels in CSF pre-infection and 11 days p.i. (a) IFN-γ; (b) IL-10. The bottom and top of each boxplot indicate the first and third quartiles, and the band inside the box indicates the median. Upper (and lower) whiskers extend to the highest (and lowest) value. Asterisks indicate statistically significant differences in cytokine levels between pre-infection levels and day 11 p.i. levels (P<0.001).
early events at the respiratory tract may shape downstream responses and clinical outcome, which is one of the functions of the respiratory epithelium [34, 35]. However, it is important to note that horses infected with the N752 variant do develop EHM in the field. In addition, the only way to reliably induce EHM experimentally in a significant proportion of animals with any contemporary EHV-1 strain is to perform challenge infections in horses over 20 years of age [32, 36]. Multiple host and viral factors determine whether an individual horse develops EHM and being infected with a D752 variant simply increases the likelihood of developing EHM. As previously reported, the present study shows a positive correlation between the magnitude of viraemia and incidence of EHM [32], although only a percentage of viremic horses developed EHM (37.5% in the Ab4 WT group). While we were not able to find a positive correlation between the duration of viraemia and incidence of EHM, levels and onset of viraemia did correlate with the secondary fever response consistent with previous reports (Fig. 3d, b, respectively) [10–12]. In summary, our findings suggest that

![Graphs showing IFN-α and IFN-γ levels in nasal secretion](https://example.com/graph.png)

**Fig. 6.** IFN-α and IFN-γ levels in nasal secretion pre-infection and on days 1 and 2 p.i. (a) IFN-α levels in uninfected control horses and Ab4 WT-infected horses. (b) IFN-α levels in Ab4 WT-infected horses that did or did not present signs of EHM. (c) IFN-α levels in uninfected control horses, Ab4 N752-infected horses and Ab4 gD4-infected horses. (d) IFN-γ levels in all groups of horses. (e) IFN-γ levels in Ab4 WT-infected horses that did or did not show signs of EHM. The bottom and top of each boxplot indicate the first and third quartiles, and the band inside the box indicates the median. Upper (and lower) whiskers extend to the highest (and lowest) value. Asterisks indicate statistically significant differences in cytokine levels between pre-infection levels and day 1 or 2 p.i. levels (P<0.0001).
while differences in the EHV-1 DNA polymerase do not play a major role in EHV-1 replication in cell culture (Fig. 2b, c), they are relevant for EHM pathogenesis and in particular for the establishment of viraemia.

Differences in the neuropathogenic potential between EHV-1 and EHV-4 are not fully understood, although it is likely that the difference in cellular tropism is associated with differences in the gene products involved in virus entry and/or spread from an infected to a neighbouring uninfected cell. Previous data suggest that the differences are at least in part determined by the envelope gD. Interestingly, Ab4 gD4-infected horses did show significant bi-phasic fevers with a late onset of secondary fevers (Fig. 3b). As observed for the other infection group of horses, and in agreement with other studies [10–12], secondary fever spikes (Fig. 3b) coincided with or marked the onset of viraemia; although overall viraemia (Fig. 3d) was reduced when compared to infection with Ab4 WT. These results suggest that even if gD plays an important role in cell entry processes, gD is not the major or only determinant for infection of PBMCs. These findings are consistent with previous in vitro studies [28] and imply that other viral proteins likely contribute to viral transfer from the epithelium to the PBMCs and PBMCs to the CNS. Two further viral proteins (glycoprotein B – gB and pUS3) have recently been shown to play a critical role for virus transfer [37]. We surmise that the process of virus transfer between respiratory epithelium, PMBCs and the CNS is a complicated process that requires the concerted action of

![Graph showing IFN-γ levels](image-url)
different viral proteins to ensure efficient virus transfer to the CNS endothelium.

While replacing gD did not prevent establishment of viraemia (Fig. 3d), horses infected with the gD4 mutant (Ab4 gD4) showed significantly attenuated respiratory disease and a significant reduction in nasal viral shedding (Fig. 3c) when compared to infection with Ab4 WT or the Ab4 N752 mutant. This is consistent with several other studies with related alphaherpesviruses. An HSV-1 gD mutant demonstrated reduction in disease severity when compared with parental and rescued viruses in a murine flank model [38]. A live attenuated virus deleted in HSV-2 gD has shown to protect mice from vaginal, skin and neuronal disease [39]. Wild-type PRV-infected pigs showed clinical signs and histological and histopathological findings typical of PRV infection, but no signs of disease were observed after infection with PRV-gD(−) [40]. Finally, Newcastle disease virus recombinants, based on the LaSota vaccine strain and expressing gB and gD of laryngotracheitis virus, were attenuated in vivo [41]. Our results, together with those reported for other alphaherpesviruses, suggest that gD is involved in disease attenuation in vivo.

One of the major challenges in controlling spread of EHV-1 is in recognizing which horses are likely to be shedding virus and are contagious. In agreement with other studies [10, 29, 42], we observed that all infected horses shed significant amounts of virus starting on day 1 after experimental infection (Fig. 3c). Horses infected with the Ab4 WT virus shed throughout the onset of neurological disease, as has been described previously [43], and showed overall higher levels of shedding than horses infected with Ab4 N752 or Ab4 gD4. These results are in agreement with other studies [17] showing that horses infected with the D752 variant presented a more prolonged shedding of infectious virus than horses infected with the N752 variant. Finally, exchanging gD of EHV-1 with EHV-4 gD resulted in an attenuation of nasal viral shedding compared to levels of shedding in horses infected with either of the other viruses, indicating a role for gD for replication and spread at the respiratory epithelium and supporting the clinical disease findings.

Antibody responses in all four experimental groups were measured by a standard VN test. All infected horses showed increases in EHV-1 VN titres following infection, with significant increases observed as soon as day 7 p.i. and with peak levels observed between 21 and 28 days p.i., confirming successful challenge infection (Fig. 4). At termination of the study, by day 72 p.i. all infected horses continued exhibiting EHV-1 VN titres, which is consistent with other studies where high EHV-1 VN titres were observed for months following infection or vaccination [44]. Interestingly, Ab4 gD4-infected horses showed significantly lower VN titres than Ab4 WT- or Ab4 N752-infected horses. These results are consistent with a recent study showing that mice vaccinated with a HSV-2 gD mutant were protected from vaginal, skin and neuronal disease despite low levels of neutralizing antibody activity [39], suggesting that induction of neutralizing antibodies to gD does not play a major role in protection from herpesvirus infections. To determine whether animals are at risk for infection, more complete immunological profiling is necessary, combining humoral and cell-mediated immunity assessment.

Cytokines and chemokines are key intracellular mediators of inflammation and are crucial candidate mediators of cell migration from the bloodstream into the CNS [45]. The mechanism underlying CNS endothelial infection of horses naturally infected with EHV-1 is unknown, as is the cytokine profile in the CSF of horses exhibiting EHM compared with CSF of horses with no clinical evidence of neurological diseases. To further determine the role of ORF30 and EHV-1 gD for regulation of immune responses, we analysed cytokine responses at sites of EHV-1 pathogenesis including nasal secretions, PBMCs and the CSF. Interestingly, the expression patterns of cytokines differed depending on sites of EHV-1 infection in the body, and alteration of both ORF30 and gD led to modulation of immune responses. In the CSF of horses infected with the D752 variant (Ab4 WT), we observed a significant reduction in IFN-γ levels and a complete inhibition of IL-10 production on day 10 p.i. (Fig. 5a, b, respectively). This is consistent with a previous study showing that neutral tissues of horses euthanized due to clinical EHM did not express any IFN-γ and IL-10 [46]. IFN-γ has antiviral properties and helps to protect neurons from encephalitis during viral infection of the CNS [47]. Several studies conducted in mice have demonstrated an important role for IFN-γ in immune protection from HSV-1 and HSV-2 infections [48–51]. The anti-inflammatory cytokine IL-10 is known to be an inhibitor of proinflammatory responses in the CNS [52–54]. The fact that CSF from horses infected with the neuropathogenic EHV-1 strain did not express any IL-10 may be related to the acute nature of the disease or to the ability of EHV-1 to evade or alter the immune response of the host [55]. Other herpesviruses, such as equine herpesvirus type 2 and Epstein–Barr virus, have been shown to harbour IL-10-like genes [56, 57] and affect cellular immunity by inhibiting the production of the IFN-γ [57], although given that this effect is observed with both mutant viruses, it is likely to be non-specific and not attributed to ORF30 or gD. Taken together, the ability of the Ab4 WT isolate to down-regulate IFN-γ and IL-10 production in the CSF of horses points to increased local inflammation and could explain the higher risk for neurological manifestation of the disease after infection with this isolate. Also, because CNS endothelia are exquisitely capable of responding to changes in their environment, such as tissue damage and infection [58], inflammatory cytokines released in response to infection likely promote the recruitment of circulating leukocytes [59, 60], further promoting PBMC–CNS interactions and changes in inflammatory cytokines levels. Further studies will be needed to determine the exact mechanisms leading to PBMC–CNS interactions and downstream responses.
In nasal secretion collected from horses infected with EHV-1 mutants, we found increases in IFN-α compared to previous studies [29, 61]. Surprisingly, Ab4 WT-infected horses presented significantly increased levels of IFN-α compared to the other infection groups in nasal secretions (Fig. 6a, c). These data suggested that EHV-1 induces robust IFN-α secretion with major differences between viral strains, which may be related to the fact that viral replication overall was higher in the Ab4 WT-infected horses, as evidenced by nasal viral shedding. Interestingly, the Ab4 WT group, horses that were affected by EHM showed overall lower IFN-α levels in nasal secretions on days 1 and 2 p.i. than horses in the same group that were not affected by EHM (Fig. 6b), suggesting a possible protective effect of early IFN-α responses at the respiratory tract. IFN-α is part of the innate immune response. It is induced by interaction of viral components, such as double-stranded DNA, with host cell pattern recognition receptors [62], and plays a role in limiting virus replication and, most importantly, virus spread to surrounding cells [63]. In addition, and contrary to what was found in the CSF, IFN-γ was increased in Ab4 WT-infected horses following infection, but no significant differences were observed between pre- and post-infection samples in the other infection groups (Fig. 6d). Horses that presented signs of EHM showed lower IFN-γ responses compared to horses in the same group without signs of EHM, although differences were not statistically significant (Fig. 6e). It is likely that induction of strong interferon responses in the respiratory tract leads to activation of antiviral responses at this site that aid rapid clearance of the virus from the respiratory tract. On the flip side, induction of strong antiviral responses will also lead to chemoattraction of PBMCs and ultimately facilitate transfer of virus to underlying mononuclear cells. The balance between replication efficiency at the respiratory epithelium, the magnitude of the induced interferon response and efficiency of cell-to-cell interaction and viral transfer to underlying mononuclear cells are all likely factors determining viraemia and ultimately EHM.

Herpesviruses can induce altered and, possibly, overzealous inflammatory cytokine responses in host cells attempting to counter a viral attack [64]. One of the major pro-inflammatory cytokines responsible for early responses in the course of a herpesvirus infection is IL-1β. For bovine herpesvirus type 1, it has been shown that IL-1β enhances anti-bovine herpesvirus type 1 immunity when administered to infected calves [65]. Human cytomegalovirus infection is often associated with upregulated IL-1β [64], and human herpesvirus 6 induces a rapid and strong IL-1β synthesis in PBMC cultures [66]. In our study, we found that IL-1β was increased in PBMCs of EHV-1-infected horses during the first days following experimental infection (Fig. 7). IL-1β expression was significantly increased for only 1 day in Ab4 N752-infected horses and Ab4 gD4-infected horses, Ab4 WT-infected horses showed significantly increased IL-1β mRNA expression for 3 days p.i., suggesting that Ab4 WT infection provided stronger pro-inflammatory stimuli for a longer duration in PBMCs, likely coinciding with more viral transfer and subsequent higher levels of viraemia in this group.

Similar to what has been reported in a murine study of EHV-1 infection [67] and a recent pony study [29], overall increases in cytokine/chemokine responses in PBMCs coincided with the biphasic fever response and marked the onset of respiratory disease (seen in the first 48 h following infection) and viraemia (day 6 and/or 7 p.i.) [29]. Overall, there was high variability in samples of individual horses.
Nevertheless, most of our results are in agreement with previous studies [29], showing an increase in pro-inflammatory responses (IL-1β, TNF-α) early on, and T-helper 1 associated (INF-γ), and regulatory cytokines (TGF-β and IL-10) coinciding with the onset of viraemia (data not shown). Furthermore, we found that overall cytokine levels were lower in Ab4 gD4-infected horses (data not shown), corresponding to the overall attenuated clinical disease in this group.

Taken together, our results show differences in respiratory and neurological disease, as well as nasal shedding and viraemia in horses in response to mutation/modification of EHV-1 ORF30 and gD. These differences appear to be associated with modulations of host immune responses and induction of interferons (particularly INF-γ) and cytokines (IL-1β and IL-10). Finally, we have shown that gD is an important virulence factor in horses, and this information could be important in the development of future recombinant vaccines.

**METHODS**

**Animals**

Horses used in this study were clinically healthy yearling horses of both sexes. Five horses served in both the control group and the Ab4 WT group, as experiments for each group were performed successively. All animals were tested for prior exposure to EHV-1 and EHV-4 using VN tests, and all horses chosen exhibited titres <4 for EHV-1 and <40 for EHV-4. Animals were group-housed in a naturally ventilated barn with adjustable sidewall openings throughout the experiment, and were fed twice a day with a diet of hay and pelleted concentrate and ad libitum water. The maintenance and experimental protocols were reviewed and approved by Michigan State University Institutional Animal Care and Use Committee.

**Viruses**

In this study, a neuropathogenic strain of EHV-1, Ab4 (Ab4 WT), naturally containing D752 in ORF30, and previously isolated from a quadriplegic mare [68] was used. Additionally, an N752 mutant of the Ab4 WT resulting in a low neuropathogenic isolate of EHV-1 (Ab4 N752) was generated as previously described [17] and used for infection of horses in the Ab4 N752 group. A gD mutant was generated by using BAC mutagenesis as previously described [28, 69] to replace the gD of EHV-1 with the gD of EHV-4 (Ab4 gD4). For this, a self-excisable Ab4 BAC was constructed. A linear DNA fragment containing gp2, aphAI, an I-SceI site and two flanking arms (duplicated sequences into the target mini-F cassette) was released by I-CeuI digestion from the pEPMCS-in-Belo-gp2 vector (Fig. 1). The transfer fragment was then electroporated into GS1783 containing EHV-1 Ab4 BAC. Kanamycin-resistant colonies were selected and screened by PCR and RFLP. A second round of Red recombination was performed to obtain the final self-excisable construct, pAb4_gp2, after the loss of the aphAI-I-SceI cassette. The 2209 bp gD4Kan transfer fragment was amplified from pcDNAgD4Kan [28], digested with DpnI and electroporated into GS1783 harbouring pAb4_gp2. Electroporated cells were selected on agar plates containing chloramphenicol and kanamycin (25 μg ml⁻¹). Kanamycin-resistant colonies were screened by PCR and RFLP and positive clones were subjected to a second Red recombination to obtain the final construct, pAb4gD4, after excision of the aphAI-I-SceI cassette. The respective genotypes of the final constructs were confirmed by RFLP and nucleotide sequencing. Ab4_gp2 and Ab4 gD4 viruses were reconstituted after transfection of 2 μg purified BAC DNA into RK13 cells using Lipofectamine 2000 (Invitrogen). Three days later, the supernatant and cells were collected and used to infect confluent ED cells. Pellets of infected ED cells were resuspended in radiolabeled cell culture supernatant (50 mM Tris, pH 7.4, 1 % Triton X-100; 0.25 % Na-deoxycholate and 150 mM sodium chloride, 1 mM EDTA) with a protease inhibitor cocktail (Roche). Cell lysates were heated at 95 °C for 5 min and proteins were separated by 12 % SDS-PAGE as described previously [70]. Expression of gp2 and/or gD4 was detected with anti-EHV-1 gp2 and/or EHV-4 polyclonal anti-gD antibodies (1:500 dilution), respectively. Goat anti-mouse or anti-rabbit IgG coupled to peroxidase (Southern Biotech, Birmingham, USA) at 1:10 000 dilutions was used as secondary antibody. Reactive bands were visualized by enhanced chemoluminescence (ECL plus, Amersham).

**Growth kinetics of Ab4 WT, Ab4 N752 and Ab4 gD4 virus**

Growth kinetics of Ab4 WT, Ab4 N752 and Ab4 gD4 were evaluated in monolayers of ED cells as described previously [28, 71]. Briefly, the cells were infected in triplicate with Ab4 WT, Ab4 N752 and Ab4 gD4 virus at an m.o.i. of 3. After an adsorption period of 1 h, cells were washed, overlaid with growth medium and incubated at 37 °C, 5 % CO2. Cells and culture supernatant were harvested separately at successive time intervals between 0 and 48 h post-inoculation, and infectious virus was titrated in ED cells after fixing with absolute ethanol, staining with 0.3 % crystal violet, by counting plaques. Titres were expressed as p.f.u. ml⁻¹.

**Experimental design and infection**

Four experimental groups of horses were selected for this study and infections were performed several months apart. The same experimental design was used for each group and is represented in Table 1. Group 1 (n=8) consisted of uninfected control horses (control group); group 2 horses (n=8) were infected with Ab4 WT virus (Ab4 WT group). Horses in group 3 (n=9) were infected with Ab4 N752 (Ab4 N752 group); and group 4 (n=8) were infected with Ab4 gD4 (Ab4 gD4 group). All infection groups were infected on day 0 of the study by intranasal instillation of 5×10⁷ p.f.u. of the respective viruses in 10 ml of saline.

**Clinical data**

Physical examinations were performed prior to infection, daily from day 1 to 14 and every other day from day 14 to 21 p.i. (Table 1). For each day, a total clinical score was calculated by evaluating the presence of cough, nasal discharge,
ocular discharge and neurological signs as described previously [12]. Additionally, the body temperature was checked prior to infection and twice daily until day 10 p.i. (Table 1). A fever was defined as a rectal temperature >38.6 °C.

Sample collection

Experimental samples were taken from all animals in the infection groups at the time points indicated in Table 1. Nasal swab samples for virus isolation were collected using Dracon swabs (Baxter Healthcare) and were stored in 1 ml of virus transport media (PBS containing 5% glycerol, 800 U ml⁻¹ penicillin/streptomycin, 200 U ml⁻¹ gentamicin and 100 U ml⁻¹ nystatin) at −80 °C until further analysis. Blood was collected by jugular venipuncture into heparinized tubes for detection of cell-associated viraemia, into serum separator tubes (BD) for VN tests (serums were stored at −20 °C until processed) and into PAXgene RNA system (BD) tubes for cytokine mRNA measurement. Nasal secretions were collected for cytokine measurement by insertion of a tampon into the ventral nasal meatus for 20 min before samples were stored at −80 °C until further analysis, as described previously [72]. Finally, CSF was collected by ultrasound-guided cervical centesis between C1 and C2, as described previously [73], on day −5 prior experimental infection and day 11 p.i. Immediately after collection, aliquots of 1 ml CSF were snap frozen in liquid nitrogen and then stored at −80 °C until processed.

Analysis of viral DNA from nasal samples

Viral DNA was isolated from nasal swab samples using the QIAamp DNA Blood Mini Kit, according to the manufacturer’s instructions (Qiagen). The presence of EHV-1 viral DNA in nasal samples was determined using a previously described real-time PCR assay specific for the gB gene of EHV-1 virus [10]. To further confirm the specificity of the virus that was shed from each experimental group (Ab4 WT, Ab4 N752 and Ab4 gD4), nucleotide sequencing analysis of viral DNA was performed. Briefly, viral DNA isolated from days 1, 2 or 3 p.i. from all infected animals was analysed for the presence of ORF1 and gp2 genes to confirm the EHV-1 Ab4 strain specificity, while ORF30 and gD genes were analysed to confirm the mutation specificity in these groups. Following the manufacturer’s protocol, PCR reactions for ORF30, gD and gp2 genes were performed using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) and GoTaq G2 Hot Start Polymerase (Promega) for ORF1 gene PCRs using the following primer sets. ORF30: F5¢–GGATATACCAACCGTTAGTG–3¢, R5¢–CCCATATATTATAAAGG–3¢. gD: F5¢–GCTGCTTGTACTGTATGTTA–3¢, R5¢–ACATGCTCATATGTTCTCCG–3¢. ORF1: F5¢–GCCTGTTGACTGTATGTTA–3¢, R5¢–ACATGCTCATATGTTCTCG–3¢. ORF1: F5¢–ACATGCTCATATGTTCTCG–3¢. After analysing the PCR products with gel electrophoresis, the samples were gel-purified using Wizard SV Gel and PCR Clean-Up System (Promega) and sent to the Research Technology Support Facility Genomics Core at Michigan State University for Sanger sequencing. The sequenced files were analysed using

Table 1. Experimental design

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<tr>
<th>Day of study</th>
<th>Physical exams</th>
<th>Body temperatures</th>
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LaserGene’s SeqMan Pro (DNASTAR) and Vector NTI software (ThermoFisher Scientific).

**Cell-associated viraemia**

For detection of cell-associated viraemia, PBMCs were isolated from heparinized blood using density-gradient centrifugation over Histopaque-1077 (Sigma). Total DNA was isolated from PBMCs using the QIAamp DNA Blood Mini Kit, according to the manufacturer’s instructions (Qiagen). Viral DNA load was determined by real-time PCR with a specific probe recognizing gB of EHV-1, as described previously [10]. β-actin was used as the cellular housekeeping gene [74]. Viral load was expressed as the log of EHV-1 gB DNA copies/10^5 β-actin copies.

**Antibody responses to EHV-1 and EHV-4 and measurement of cytokines in nasal secretions and CSF**

VN tests were performed in serum samples as described previously [11]. Nasal secretions and CSF were tested for the presence of IL-4, IL-10, IL-17, IFN-γ with a fluorescent bead-based system (Luminex IS 100 instrument, Luminex) [75]. The data were reported as median fluorescent intensities. For standard curve fitting and subsequent calculation of concentrations in samples, the logistic 5p formula (\( y = a + b/(1 + (x/c)^d) \)) was used (Luminex 100 Integrated System 2.3).

**Measurement of cytokine mRNA expression in blood**

Total RNA from PAXgene tubes was extracted according to the manufacturer’s instructions. One microgram of RNA was reversed-transcribed into cDNA. Cytokine/chemokine-specific cdNA was quantified by real-time PCR using equine-specific intron-spanning primers for IL-1β, IL-8, IL-10, interleukin-12, IFN-γ, TNF-α, TGF-β, FoxP3, granulocyte-macrophage colony-stimulating factor, Tbet, and the housekeeping gene β-gus with an Applied Biosystems 7900 detection system. The primers and probes used are published at www.ca.uky.edu/GLuck/HorohovDW_EIR-ClonedCytokines.asp [76]. The RQ of each gene was determined with the 2^−ΔΔCt method [77] using the average day 5 values of all horses for each gene as calibrators.

**Statistical analysis**

All continuous data were evaluated for assumptions of normality and goodness-of-fit model using a chi-square test based on the residual deviance and degrees of freedom. Whenever the data did not fit a Poisson regression analysis well, negative binomial regression analysis was used. For analysis of body temperature, nasal viral shedding and viraemia, logistic regression analysis was performed to evaluate the probability of occurrence of fever, nasal viral shedding or viraemia with the three viral strains when compared to the controls. The repeated measurements on the horses were taken into account during the analysis. Whenever multiple comparisons were performed within one analysis, a Bonferroni adjustment of P-value was used to evaluate statistical significance. A P-value of <0.05 was considered statistically significant.

**Funding information**

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Acknowledgements**

We would like to thank Julie Dau and Dave O’Daniel, Michigan State University, USA, for helping with horses and cleaning. We also thank Michaela Zeitlow and Selena Taferner, Freie Universität Berlin, Germany, for helping with mutagenesis.

**Ethical statement**

All experimental protocols were reviewed and approved by Michigan State University Institutional Animal Care and Use Committee (approval number: 05/13-111-00; project: Development of alternative models to study EHM).

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