Characterizing viral distribution, viral load and cell tropism during early latent Equid Herpesvirus 1 (EHV-1) infection: novel insights into EHV-1 latency pathogenesis

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List of abbreviations

Ab	antibody
Ag	antigen
B2M	*equus caballus* beta-2-microglobulin
BHQ	black hole quencher
BoHV	Bovine Herpesvirus
bp	basepair(s)
BV	bloodvessel
CaHV	Canid Herpesvirus
CD	cluster of differentiation
cDNA	complementary DNA
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
CXCL	chemokine (C-X-C motif) ligand
D	aspartate
DAB	diaminobenzidin
dapB	*bacillus subtilis* dihydrodipicolinate reductase
DNA	deoxyribonucleic acid
dPCR	digital PCR
dpi	days post infection
DRG	dorsal root ganglion
E	early
EHM	equine herpesvirus myeloencephalopathy
EHV	Equid Herpesvirus

Ab4 WT	wildtype strain Ab4
EICP	EHV-1 infected cell protein
ETIF	EHV-1 alpha-trans-inducing factor
F	forward
FAM	5-(and-6)-Carboxyfluorescein mixed isomer
FFPE	formalin-fixed-paraffin-embedded
FeHV	Felid Herpesvirus
FoxP3	forkhead box P3
g	glycoprotein
GaHV	Gallid Herpesvirus
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GrzB	granzyme B
H&E	Hematoxylin and Eosin
H2O2	hydrogen peroxide
HDAC	histone de-acetylase
HEX	hexachloro-fluoresceine
HSPG	heparan sulfate proteoglycan
HSV	Herpes Simplex Virus
IBR	infectious bovine rhinotracheitis
ICP	infected cell protein
ICTV	International Committee on Taxonomy of Viruses
ID	identification
IE	immediate early
IEP	immediate early protein

IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IR	internal repeat
ISH	*in situ* hybridisation
kbp	kilobase pair(s)
L	late
LAT	latency-associated transcript
M	molar
MesLn	mesenterial lymphnode
MHC	equine major histocompatibility complex
miRNA	micro RNA
mRNA	messenger RNA
MSU	Michigan State University
N	asparagine
nM	nanomolar
NPC	nuclear pore complex
ORF	open reading frame
PBMC	periphereal blood mononuclear cell
PCR	polymerase chain reaction
PFPE	paraformaldehyde-fixed-paraffin-embedded
PFU	plaque forming unit
pi	post infection
PPIB	peptidylprolyl isomerase B

PsRV	Pseudorabies Virus
qPCR	quantitative polymerase chain reaction
R	reverse
RALT	respiratory tract-associated lymphoid tissue
RLn	retropharyngeal lymphnode
RNA	ribonucleid acid
ROX	carboxy-x-rhodamine
SGC	satellite glial cell
SN	serum neutralization
ST	sympathetic trunk
SuHV	Suid Herpesvirus
SVV	Simian Varicella Virus
TAMRA	carboxytetramethylrhodamine
T-cell	T-lymphocyte
TG	trigeminal ganglion
TNF	tumor necrosis factor
TR	terminal repeat
T_{RM}	tissue resident memory T-cells
UL	unique long
URT	upper respiratory tract
US	unique short
vs.	versus
VZV	Varicella Zoster Virus
W	watt

µL	microliter
µm	micrometer

I. INTRODUCTION

Herpesviruses are enveloped, double stranded DNA viruses, which are widely spread in nature infecting an immense variety of host species from bivalves to humans. More than 130 species have been discovered so far (BROWN & NEWCOMB, 2011), closely co-evolved with their host species over time and thus symbolizing an impressive example of coevolutionary success. This highly efficient host adaptation is clearly demonstrated by the key characteristic of all herpesviruses: the ability to establish a chronic persistent infection called latency, which symbolizes a perfect coexistence in balance with the host immune system (DAVISON, 2002). From a clinical and economical point of view, Equid Herpesvirus 1 (EHV-1) is the most relevant herpesvirus affecting horses worldwide as it can cause respiratory disease, equid herpesvirus myeloencephalopathy (EHM), abortions and neonatal foal death. Following initial EHV-1 infection, the virus replicates in the upper respiratory tract (URT) epithelium and subsequently enters the blood circulation by infecting mononuclear cells. The virus is then transported to secondary infection sites like central nervous system (CNS) or uterus endothelium during cell-associated viremia where it can cause secondary disease manifestations (EHM, abortion) (HUSSEY et al., 2006; GOEHRING et al., 2010b; WILSTERMAN et al., 2011; SLATER, 2014). As the currently available vaccines or treatment options can only possibly ameliorate but not fully prevent EHV-1 disease outbreaks, strict biosecurity measures including quarantine and travel restrictions represent the most effective prevention strategies, however, with significant economic impact (LUNN et al., 2009; GOEHRING et al., 2010c). Like its close human pathogen relatives, Herpes Simplex Virus 1 (HSV-1) and Varicella Zoster Virus (VZV), EHV-1 is highly prevalent in the host population with presumably > 60 % of horses being latently infected (EDINGTON et al., 1994; LUNN et al., 2009). Most importantly, latently infected horses, where the virus can reactivate at any given time, are the main source for new infections, possibly resulting in new outbreaks. Although EHV-1 has been studied for years, the details of latency establishment and reactivation are still not well understood (SLATER, 2014). The current understanding is that similar to its close relatives HSV-1, VZV and Bovine Herpesvirus 1 (BoHV-1), EHV-1 enters sensory nerve endings in the vicinity of the URT epithelium and reaches the trigeminal ganglia (TG) via neuronal transport for latency establishment (GILDEN et al., 1983; SLATER et al., 1994; EFSTATHIOU & PRESTON, 2005). Furthermore, latent EHV-1 has been repeatedly detected in respiratory tract-associated lymphoid tissue (RALT) and in circulating mononuclear cells (WELCH et

al., 1992; EDINGTON et al., 1994; CHESTERS et al., 1997; SMITH et al., 1998). During latency, the virus persists within the host cell nucleus in a non-infectious state with limited viral gene transcription. However, little is known about the molecular mechanisms that are involved in establishment and regulation of latency.

Our *main goal* is to limit further EHV-1 outbreaks. As it is unlikely to prevent initial EHV-1 infection, research approaches need to focus on finding potential strategies to control EHV-1 in its latent state. Therefore, the *short-term goal* of this dissertation is to further characterize EHV-1 latency pathogenesis following experimental infection of the natural host. The main study presented in this dissertation is based on previous findings of our research group (SAMOILOWA, 2019): Interestingly, at a time point beyond viremia (70 days post infection (dpi)), EHV-1 DNA was detected not only in TG and RALT but also in various other sensory, sympathetic and parasympathetic ganglia as well as in abdominal lymphoid tissue.

The present study in this dissertation follows up by aiming to answer remaining questions e.g. regarding the likely transportation route toward alternative latency locations and the predominant cell tropism during latency at these sites of persistence. Therefore, we compared EHV-1 viral load and distribution on 70 dpi to a time point closer to acute infection (30 dpi) and hypothesized that EHV-1 chronic persistent infection is influenced by the time passed after inoculation. Furthermore, we aimed to investigate the cellular localization of latent EHV-1 as this information is crucial for elucidating the molecular basis of EHV-1 latency.

Overall, reflecting upon the review of recent literature in the field of EHV-1 (and related herpesvirus) latency, the main study in this dissertation provides novel insights into EHV-1 latency pathogenesis.

II. LITERATURE REVIEW

1. Molecular biology of EHV-1

1.1. Taxonomy

The International Committee on Taxonomy of Viruses (ICTV) classifies EHV-1 as a member of the order *Herpesvirales* (DAVISON, 2010). Being identified in the past solely through their typical herpes virion architecture, novel nucleotide sequencing data allowed to further classify herpesviruses of mammals, birds and reptiles (the *Herpesviridae*), fish and amphibians (the *Alloherpesviridae*) and bivalves (the *Malacoherpesviridae*) (DAVISON et al., 2009). This assigns EHV-1 to the family of *Herpesviridae*, among which it shares certain biological properties with other family members. However, differences in host range, cell tropism and replication cycle subdivide the *Herpesviridae* in subfamilies of (A) *Alpha*-, (B) *Beta*- and (C) *Gammaherpesvirinae* ((DAVISON et al., 2009; PELLETT & ROIZMAN, 2013).

A variable host range, relatively short reproductive cycle, rapid cell spread, and efficient destruction of infected cells characterizes EHV-1 as a member of the *Alphaherpesvirinae*. Notably, while latency is a hallmark of all herpesviruses, the subfamilies differ in their cell tropism for latency establishment (ROIZMAN & BAINES, 1991; PELLETT & ROIZMAN, 2013). For example, while Alphaherpesviruses are generally thought to remain latent particularly in peripheral sensory nerves, Gammaherpesviruses choose lymphoid tissue as harbor during latency (PELLETT & ROIZMAN, 2013).

Important other members of the *Alphaherpesvirinae* include pathogens infecting humans (HSV-1, HSV-2, and VZV), avian species (Gallid Herpesvirus 1 (GaHV-1, Infectious laryngotracheitis virus), GaHV-2, Marek's Disease Virus), as well as mammals (Suid Herpesvirus 1 (SuHV-1, Pseudorabies virus (PsRV)), BoHV-1 (Infectious bovine rhinotracheitis virus), BoHV-5 (Bovine encephalitis herpesvirus), Canid Herpesvirus 1 (CaHV-1), Felid Herpesvirus 1 (FeHV-1). Besides to EHV-1, equids are the natural hosts to the Alphaherpesviruses EHV-3 (Equine coital exanthema virus), EHV-4 (Equine rhinopneumonitis virus), EHV-6 (Asinine Herpesvirus 1), EHV-8 (Asinine Herpesvirus 3) and EHV-9 (Gazelle Herpesvirus 1). Other known herpesviruses in equids include EHV-2, EHV-5, EHV-7 (Asinine Herpesvirus 2) and belong to the subfamily of Gammaherpesviruses (TELFORD et al., 1993; BORCHERS et al., 1999; MA et al., 2013).

Based on genome structure, - size and protein similarity, EHV-1 belongs to the genus

Varicellovirus along with the important animal pathogens SuHV-1, BoHV-1, BoHV-5, CaHV-1, and FeHV-1 (DAVISON, 2010). Notably, the human pathogen VZV is the prototype for *Varicellovirus* research and is often represented by its close relative Simian Varicella Zoster Virus (SVV) in natural host models (ZERBONI & ARVIN, 2016).

1.2. Viral structure and genomic organization

EHV-1 features the typical *Alphaherpesvirinae* virion structure: A single copy of double stranded (ds) DNA is surrounded by a protective icosahedral capsid, which comprises six proteins and is of approximately 100-110 nanometers in diameter (PERDUE et al., 1975; PELLETT & ROIZMAN, 2013). This nucleocapsid is encompassed by 12 proteins forming an interlayer, called tegument (MCLAUCHLAN & RIXON, 1992), and finally by an envelope derived from host cellular membranes. The envelope consists of lipids, polyamines, and a total of 12 viral glycoproteins which are projecting from the surface: Eleven glycoproteins have functional homologues present in HSV-1 (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, gN) and one glycoprotein (gp2) is unique for EHV-1 and its close relative EHV-4 (TURTINEN & ALLEN, 1982; PAILLOT et al., 2008).

The genome of EHV-1 was the third Alphaherpesvirus genome to be sequenced, following those of VZV and HSV-1 (TELFORD et al., 1992). It is 150,223 basepairs (bp) in length with a G + C base content of 56,7 %. At least 78 distinct genes are organized collinearly with those of their human Alphaherpesvirus counterparts within the following components: two unique segments (unique long (UL) and unique short (US)) that are flanked by two repeat regions (internal repeat (IR) and terminal repeat (TR)) (TELFORD et al., 1992; SHAKYA et al., 2017). While originally four identical Open Reading Frames (ORFs) were found to be copied in the IR/TR region (ORF 64, 65, 66, 67) (TELFORD et al., 1992), this number was updated by the characterization of two additional genes of regulatory function (ORF 77, 78) (HOLDEN et al., 1992; KIM et al., 2006; AHN et al., 2007). ORF 1- 63 are located in UL and ORF 68-76 in US (TELFORD et al., 1992).

While the most important encoded proteins associated with the virion, viral replication functions and gene regulation have been discovered so far (TELFORD et al., 1992; SLATER, 2014), the entire coding potential of the EHV-1 genome has yet to be elucidated. We can therefore expect to gain exciting new insights by novel Next Generation Sequencing techniques (e.g. total RNA Sequencing, microRNA Sequencing, target enriched RNA Sequencing) in the future.

1.3. Cell infection cycle

EHV-1 can infect a variety of cell types, ranging from respiratory tract epithelial cells during initial infection to mononuclear cells during viremia and to vascular endothelial cells of the central nervous system or the uterus (OSTERRIEDER & VAN DE WALLE, 2010). During the productive (acute) infection, the virus enters these target cells, replicates and propagates to high numbers of new virion particles. This infection cycle results in lysis of the infected cell and is therefore named lytic infection cycle (SLATER, 2014). Independently, all herpesviruses can also enter a latent cycle in certain cell types, resulting in a non-productive chronic persistent infection. The cell tropism for latency establishment is more restricted than for lytic infection and is specific for each herpesvirus subfamily (PENKERT & KALEJTA, 2011). Like all Alphaherpesviruses, EHV-1 typically establishes latency in sensory neurons of the trigeminal ganglia (TG) (SLATER et al., 1994; EFSTATHIOU & PRESTON, 2005; BLOOM, 2016). However, EHV-1 also seems to behave lymphotropic for its latency establishment as latent EHV-1 has been detected previously in respiratory tract-associated lymphoid tissue (RALT) and in circulating CD8+ T-lymphocytes (T-cells) (WELCH et al., 1992; EDINGTON et al., 1994; CHESTERS et al., 1997; SMITH et al., 1998). The entry into either infection cycle is orchestrated upon entry into the host cell by a still unknown control mechanism (SLATER, 2014).

Regardless of the target cell and the two different outcomes of the biphasic cycle, each infection starts with the coordinated process of viral entry (AZAB & OSTERRIEDER, 2017): Following direct contact with EHV-1 or contact with infected cells, viral envelope glycoproteins (mainly gC and gB) bind to cell surface heparan sulfate proteoglycan (HSPG) and chondroitin sulfate proteoglycans (CSPG) (OSTERRIEDER, 1999; AZAB & OSTERRIEDER, 2017). Once attached, the virus is transported along filopodia (a mechanism also referred to as “surfing”) towards the cell body, where gD associates with specific entry receptors (AZAB & OSTERRIEDER, 2017). Equine major histocompatibility complex I (MHC-I) serves as an entry receptor on fibroblasts, epithelial and endothelial cells (KURTZ et al., 2010; SASAKI et al., 2011). There is also evidence of a yet undiscovered unique entry receptor for EHV-1 which is involved in gD binding (FRAMPTON et al., 2005). Subsequently, the actual entry is performed either by fusion of the viral envelope with the host cell membrane or through endocytosis depending on the cell type (FRAMPTON et al., 2007; VAN DE WALLE et al., 2008; HASEBE et al., 2009) and the nucleocapsid and tegument proteins are released into the cell. While most of the tegument proteins separate from the capsid during this process, a few inner tegument proteins remain

bound and connect with dynein motor proteins linked to cell microtubules (FRAMPTON et al., 2010). This forms a transport complex along microtubules, which enables the viral capsid to be actively transported towards the nucleus. After docking to the nuclear pore complex (NPC), the capsid releases the viral genome into the nucleus while itself remains behind in the cytoplasm (OJALA et al., 2000; PAILLOT et al., 2008). The subsequent steps, taking place in the nucleus, are characterized by viral gene transcription and its regulation, which differs fundamentally depending on whether the virus enters a lytic or latent cycle (JONES, 2013; SLATER, 2014).

1.3.1. Viral gene expression during productive and latent infection

The lytic cycle is defined by viral DNA replication, protein expression and assembly of new virion particles. Once the viral DNA is released to the nucleus, the viral gene expression is activated, the core of which is a tightly regulated cascade of immediate early (IE), early (E) and late (L) genes (CAUGHMAN et al., 1985; GRAY et al., 1987b; SMITH et al., 1992). Viral regulatory gene products and host cell proteins orchestrate up- and down-regulation of the distinct phases of viral gene expression (see chapter 1.3.2). The first gene to be transcribed is the sole IE gene (ORF64), which is required for E and L gene expression. Early gene expression initiates viral DNA replication (GRAY et al., 1987b; GRAY et al., 1987a; SMITH et al., 1992) and L genes are eventually transcribed, encoding for mainly viral structural proteins, required for virion assembly. Once the cycle is complete, newly generated viral DNA is packed into capsids. Nucleocapsids then associate with some tegument proteins and leave the nucleus through the inner and outer nuclear membrane. Further tegument proteins are added in the cytoplasm and the virus acquires its final envelope, containing all viral glycoproteins by fusing with a vesicle originated from the Golgi complex. Finally, a new generation of infectious virions egresses from the cell which inevitably results in death of the latter (PAILLOT et al., 2008; CARTER & SAUNDERS, 2013).

During the latent cycle of infection, the entire round of transcription and translation of the gene cascade is not accomplished - precisely, lytic gene expression is repressed (PENKERT & KALEJTA, 2011). The cycle begins with establishment of latency, where the viral gene transcription cascade is rapidly silenced and gene expression limited to latency-associated transcripts (LATs) (see chapter 3). The virus enters a dormant state, is non-infectious and persists as a circular episome (EFSTATHIOU et al., 1986; MELLERICK & FRASER, 1987; EFSTATHIOU & PRESTON, 2005). This process is

initiated by a combination of viral and host factors, which build the basis of a careful balance during maintenance of latency (see chapter 3). Importantly, the silencing of viral genes during latency is not ultimate and whereas some late genes (strict-late) are strictly inhibited, the expression of some other late genes (leaky-late) still occurs on a low-level basis (NICOLL et al., 2012; ROIZMAN et al., 2013). Periodically, external stimuli (e.g. stress and immunosuppression) animate viral gene expression, which can result subsequently in re-entering the lytic cycle (reactivation event) (PENKERT & KALEJTA, 2011; JONES, 2013).

1.3.2. Gene transcription cascade

The gene regulation cascade is crucial for understanding the characteristics of a lytic and latent life cycle, their balance, as well as their diagnosis approach. The key role in the complex series plays an array of regulatory genes and their proteins (P): one IE gene (ORF64, IEP), four E genes (ORF65, EICP22; ORF5, EICP27; ORF63, EICP0; IR2, IRP) and one L gene (ORF12, ETIF).

The cascade is initiated by the ETIF Protein (HSV-1 VP16 homologue), a tegument protein, which is transported into the infected cell and activates IE gene expression (PUREWAL et al., 1994; LEWIS et al., 1997). The IE gene, represented twice in the inverted regions of the EHV-1 genome, has been shown to be essential for viral replication *in vitro* (GRUNDY et al., 1989; GARKO-BUCZYNSKI et al., 1998). The encoded IE protein is synthesized subsequently by host cell RNA polymerase and embodies the key regulatory factor (GRAY et al., 1987b; GRAY et al., 1987a). As such, it features autoregulation of its own expression, activation of other viral promoters and acts in cooperation with EICP22 and EICP27 proteins to initiate E and L gene expression (SMITH et al., 1992; ZHAO et al., 1992; HOLDEN et al., 1995). Within the IE gene maps the IR2 gene, which is expressed during E and L stages and seems to have a downregulating function for the IE promoter (HARTY & O'CALLAGHAN, 1991; SMITH et al., 1994). Transcription of E genes and subsequent E protein synthesis induces viral DNA replication (MUYLAERT et al., 2011). While EICP22 and EICP27 proteins act in concert with IE protein, EICP0 protein can activate all classes of EHV-1 promoters independently and seems to be particularly important for L gene activation (BOWLES et al., 1997; BOWLES et al., 2000). Once viral replication has started, L genes are expressed at maximum levels (PELLETT & ROIZMAN, 2013). They encode structural proteins forming the virion, the assembly of which marks the end of a lytic cycle.

1.3.3. Diagnosis of viral infection state in the present study

One part of the present study focuses on the differentiation of EHV-1 lytic or latent infections in tissue samples. Basis for this diagnosis is the presence or absence of L gene mRNA and virion proteins as those gene products indicate a complete productive round of replication – hence they define a lytic cycle. Therefore, a sample can be defined as latently infected when viral genomic DNA is present (via qPCR) while L mRNA and L protein is not detectable (PUSTERLA et al., 2010). More specifically, the transmembrane gB mRNA, encoded by L gene ORF33 and gB, is generally used as indicator for lytic infection using reverse transcription qPCR (RT qPCR) and Immunohistochemistry (IHC) respectively (PUSTERLA et al., 2010). The transmembrane protein gB is highly conserved among all herpesviruses (MCGEOCH & COOK, 1994), is essential for viral growth *in vitro* and functions in target cell attachment, penetration, and cell-to-cell fusion (WELLINGTON et al., 1996; NEUBAUER et al., 1997; SPIESSCHAERT et al., 2015).

Notably, there is a caveat concerning this definition: While it is generally accepted that infectious virus cannot be detected during latency (JONES, 2003), viral gene expression is frequently animated during latency without resulting in re-entering the lytic cycle. Thus, there is the possibility that low abundance of gB mRNA might be present during latency as the gB gene is a leaky-late gene (ROIZMAN et al., 2013). Overall, although low levels of gB mRNA might be expressed occasionally during latency, it is unlikely that they reach high enough levels to be detected by the RT qPCR approach unless there is lytic gene expression including viral replication and virion assembly.

2. EHV-1 Transmission and Pathogenesis

2.1. Transmission, pathogenesis and disease manifestation

EHV-1 is a ubiquitous viral pathogen and causes respiratory disease, abortion, and neurologic disease (equine herpesvirus myeloencephalopathy (EHM)) in horses worldwide. Horses usually get exposed to EHV-1 and to its close relative EHV-4 very early in life, probably as young foals while having contact with their nursing dam (GILKERSON et al., 1999b; GILKERSON et al., 1999a). Interestingly, whilst EHV-4 is genetically strikingly similar to EHV-1, infections with EHV-4 usually manifest only in mild respiratory disease but abortion and neuropathogenicity is scarce (PATEL & HELDENS, 2005). Following primary infection, both viruses establish latency in a high percentage of horses, the latter of which then become an important virus reservoir. Devastating EHV-1 disease outbreaks often start with a single horse (carrier animal), where latent EHV-1 reactivates following

stress events (transport, hospitalization, change in horse population) or due to an immunocompromised state. Subsequent transition to lytic infection leads to rapid transmission to susceptible herd mates and potentially to disease manifestation.

Transmission of EHV-1 typically occurs through inhalation/ingestion of virus or direct contact with infectious materials (LUNN et al., 2009). Subsequently, EHV-1 targets epithelial cells of the URT mucosa, where entering the lytic infection cycle results rapidly in primary replication followed by epithelial cell destruction and nasal viral shedding (KYDD et al., 1994a). In young or immunological naïve horses, respiratory symptoms like nasal and/or ocular discharge, occasional coughing and primary fever predominate, whereas respiratory disease is rather mild or subclinical in the adult horse (PAILLOT et al., 2008; WILSTERMAN et al., 2011; GIESSLER et al., 2020b). Furthermore, simultaneously to the active replication in the URT, EHV-1 infects adjacent sensory nerve endings and is transported to the TG, where it establishes life-long latency by manipulating the host cell environment and is creating a balance with the immune system (see chapter 3.3). Furthermore, EHV-1 features several additional immunomodulatory properties: During the initial phase of replication, EHV-1 is able to increase its virulence by modulating the innate immune response at the respiratory epithelium; for example by interfering with chemotaxis of leukocytes and/or expression of interferons (SOBOLL HUSSEY et al., 2011; SOBOLL HUSSEY et al., 2014). Another important strategy of EHV-1 that aims to evade the elimination through the immune system involves invading the latter by targeting and hiding in immune cells (POELAERT et al., 2019). Specifically, EHV-1 infects and uses monocytes in the URT to be transported through the connective tissue to vascular endothelium and respiratory tract-associated lymphoid tissue (RALT) (KYDD et al., 1994b; GRYSPEERDT et al., 2010; VANDEKERCKHOVE et al., 2010; BAGHI & NAUWYNCK, 2014), which is also a known latency site for EHV-1 (CHESTERS et al., 1997). The virus is then transferred to PBMCs and subsequently distributed throughout the body in a cell-associated viremia, which can be typically detected until around day 14 post infection (GIBSON et al., 1992; HUSSEY et al., 2006; ALLEN, 2008; LUNN et al., 2009; PUSTERLA et al., 2009; GOEHRING et al., 2010b) and a secondary fever usually peaks during this period (HUSSEY et al., 2006; GOEHRING et al., 2010a; GOEHRING et al., 2010b). Viremia is therefore essential for reaching the vascular endothelium of secondary infection sites such as CNS, uterus, gonads or the eye (DUTTA & MYRUP, 1983; EDINGTON et al., 1986; KYDD et al., 1994b; TEARLE et al., 1996; ALLEN & BREATHNACH, 2006; WALTER et al., 2012; HUSSEY et al., 2013), where endothelial cell infection of arterioles may cause

vasculitis and thrombosis (EDINGTON et al., 1986; GOEHRING & VAN OLDRUITENBORGH-OOSTERBAAN, 2001; PUSTERLA & HUSSEY, 2014). In spinal cord and brain, the adjacent affected neural tissue becomes necrotic following hypoxia and ischaemia, referred to as “equine stroke” (EDINGTON et al., 1986), which can cause myeloencephalopathy (EHM). Furthermore, there is growing evidence that activation of the coagulation cascade is linked to EHV-1 viremia and thus may be a contributing factor involved in thrombosis formation (GOEHRING et al., 2013; YEO et al., 2013; STOKOL et al., 2015; WILSON et al., 2019). Similarly, arteriolar thrombosis in the endometrium of the pregnant uterus causes late term abortion (GOEHRING & VAN OLDRUITENBORGH-OOSTERBAAN, 2001; SMITH & BORCHERS, 2001; GARDINER et al., 2012) with neonatal foal death being a consequence of intrauterine EHV-1 infection (GOEHRING & VAN OLDRUITENBORGH-OOSTERBAAN, 2001). Infections with EHV-4 on the other hand, typically do not cause neurologic disease or abortion like EHV-1, albeit their close relatedness and similarity in initial infection and replication in the URT. Although the differences in pathogenesis is not completely understood, it has been shown that EHV-4 usually does not cause cell-associated viremia (MA et al., 2013).

Therefore, it seems likely that viremia represents a key element in EHV-1 infections and is prerequisite for secondary EHV-1 disease manifestations (ALLEN, 2008; GOEHRING et al., 2010b; OSTERRIEDER & VAN DE WALLE, 2010). However, neurologic disease is usually developed only in a small percentage of EHV-1 infected and viremic horses and viral and host factors contributing to EHM pathogenesis are still poorly understood (GOEHRING et al., 2006; LUNN et al., 2009; HOLZ et al., 2017). It has been shown that EHV-1 neuropathogenicity is strongly associated with the virulence of naturally occurring DNA polymerase gene (ORF30) strain variants: Specifically, a nucleotide polymorphism resulting in a single amino acid variation of the polymerase (asparagine (N) changes to aspartic acid (D) at amino acid position 752) increases the neuropathogenic capacity (NUGENT et al., 2006). It has been shown in infection experiments, that neuropathogenic D752 strains are associated with higher levels and a prolonged period of viremia than their non-neuropathogenic counterparts (N752 strains); the higher load of circulating virus and the prolonged exposure of the vascular endothelium supposedly increases the risk for EHM development (GOODMAN et al., 2007; ALLEN, 2008; LUNN et al., 2009; HOLZ et al., 2017).

This theory is also supported by the dramatic increase of EHM incidence in old horses

(> 20 years of age), as a likewise significantly higher viral load is detectable during viremia in the elderly compared to younger individuals (HENNINGER et al., 2007; ALLEN, 2008; GIESSLER et al., 2020b). Age-related changes in the immune response most likely drive the severity of EHV-1 pathogenesis (HENNINGER et al., 2007), however, host factors contributing to EHM development are largely unknown and currently under investigation by our research group.

2.2. Peripheral blood mononuclear cell tropism during viremia

EHV-1 is capable to infect monocytes, T- an B-lymphocytes *in vitro* and *in vivo*, but the main subpopulation within PBMCs that EHV-1 targets during primary infection has been controversially discussed in literature (VAN DER MEULEN et al., 2000; GOODMAN et al., 2007; GRYSPEERDT et al., 2010; WILSTERMAN et al., 2011; POELAERT et al., 2019). It has been shown in nasal mucosal explant studies that EHV-1 efficiently 'hi-jacks' monocytes for the initial transport upon replication in the respiratory mucosa (VANDEKERCKHOVE et al., 2010), which was also reported *in vivo* (GRYSPEERDT et al., 2010). Furthermore, T-lymphocytes (T-cells) seem to be important carrier cells during viremia (WILSTERMAN et al., 2011; POELAERT et al., 2019). However, whether EHV-1 is more prone to infect CD4+- or CD8+ T-cells remains inconclusive (SLATER et al., 1994; CHESTERS et al., 1997; HENNINGER et al., 2007; WILSTERMAN et al., 2011; POELAERT et al., 2019). Another important point of discussion concerns the state of the virus within mononuclear cells during viremia, which has yet to be defined. There is evidence that EHV-1 gene expression is silenced in monocytes, including reduced expression of viral proteins (VAN DER MEULEN et al., 2000; LAVAL et al., 2015). This strategy may provide an essential advantage for the virus to hide within PBMCs during the period of viremia. Interestingly, Poelaert et al. (2019) showed that EHV-1 is able to efficiently replicate in T-cells, but opposed to what is known for lytic infections, virion assembly is not completed. Viral capsids are formed but retained in the nucleus of the infected cell until it binds to a secondary target cell, where the virus then can be transferred through the formation of a virological synapse (POELAERT et al., 2019). This is important to further understand the transfer of the virus to the endothelium of the CNS and the pregnant uterus and hence for EHV-1 pathogenesis. Interestingly, the same phenomenon of nuclear accumulation of viral capsids and cell-to cell fusion has also been described in neurons and non-neuronal cells infected with VZV, the very closely related human Varicellovirus (REICHELT et al., 2008; REICHELT et al., 2011). It is tempting to assume that this strategy may also be used by EHV-1 to infect neurons potentially during latency establishment.

3. Aspects of latency

3.1. Latency sites of relevant Alphaherpesviruses

Alphaherpesviruses establish latency predominantly in neurons of sensory ganglia (SLATER et al., 1994; JONES, 2003; BLOOM, 2016; DEPLEDGE et al., 2018). Typically, trigeminal ganglia (TG) represent preferred sites for latency establishment as their sensory nerve endings are readily accessible for newly synthesized virions during productive (primary) infection in the respiratory epithelium (BARINGER & SWOVELAND, 1973; MEIER et al., 1993; SLATER et al., 1994; BAXI et al., 1995; RAMAKRISHNAN et al., 1996; JIANG et al., 1998). However, latency pathogenesis and latency locations of different Alphaherpesviruses coevolved with their natural hosts and often mirror the specific pathogenesis of the initial infection (MAHALINGAM et al., 1992). Below there is a brief review on latency pathogenesis and locations of different EHV-1 relatives. Within the subfamily of Alphaherpesviruses, HSV-1 belongs to the genus *Simplexvirus*, VZV and BoHV-1 both belong like EHV-1 to the genus *Varicellovirus*.

3.1.1. Herpes Simplex Virus 1

HSV-1 is characterized by a very high seroprevalence in the human population and infections typically occur early in life by oral transmission (BLOOM & DHUMMAKUPT, 2016). Primary infection is generally subclinical and allows the virus to establish a persistent infection mainly in TG for the life of the host (see chapter 3.2). Periodically, mostly stress-induced reactivation may cause either asymptomatic shedding or a localized ulcerated lesion (cold sores, *herpes labialis*) on a distinct area of the lip, which is innervated from the infected neuron (BLOOM & DHUMMAKUPT, 2016). A recent review revealed that asymptomatic shedding occurs in at least 70 % of the population at least once a month (MILLER & DANAHER, 2008), which indicates frequent low-level reactivation and reflects the high seroprevalence of HSV-1.

Consistent with HSV-1 infections being predominantly localized to the oropharyngeal region, HSV-1 latency is usually established mainly in TG and spinal cord dorsal root ganglia (DRG) (BARINGER & SWOVELAND, 1973; MAHALINGAM et al., 1992; OBARA et al., 1997) but also in other sensory (pterygopalatine, ciliary, otic, submandibular, geniculate) and autonomic (superior cervical and nodose) ganglia of the head and neck (RICHTER et al., 2009). For example, viral transmission to the eye can result in local replication and subsequent latency establishment in ganglia pteryopallatine and superior cervical ganglia (RICHTER et al., 2009; BLOOM & DHUMMAKUPT, 2016). In severe cases

of primary ocular infections and reactivation, HSV-1 causes recurrent corneal lesions, which can often result in blindness of the individual (LIESEGANG, 2001). Further studies discussed the involvement of HSV-1 in esophageal and gastroduodenal lesions following ingestion of HSV-1 during primary infection and suggested subsequent latency establishment in nodose ganglia as part of the vagus nerve (WARREN et al., 1978; LÖHR et al., 1990; GESSER & KOO, 1997; NAGEL et al., 2014). Furthermore, there is evidence of latent infection of thoracic sympathetic trunk ganglia, however only in rare cases (GALLOWAY et al., 1979; MAHALINGAM et al., 1992; NAGEL et al., 2014).

While overall not common for HSV-1, infections may result in cell-associated viremia in rare cases of immunocompromised patients or neonates (SMITH, 2012). During this severe, disseminated HSV-1 disease, the virus is distributed to various organs (liver, spleen, lung, lymph nodes), and results often in fatal encephalitis (WHITLEY et al., 1982; LUKER et al., 2003; BLOOM & DHUMMAKUPT, 2016). The travel route towards the brain may be via blood during disseminated disease and/or by neuronal transport from infected ganglia (WHITLEY et al., 1982; KENNEDY & CHAUDHURI, 2002). As latent HSV-1 can be detected frequently in normal brain tissue without history of severe HSV-1 disease, hence without history of HSV-1 viremia, neuronal HSV-1 transfer towards CNS seems to occur on a regular basis (GORDON et al., 1996; KENNEDY & CHAUDHURI, 2002).

Taken together, HSV-1 latency seems to be generally limited to cranial nerve ganglia, which are accessible during primary infection of the oropharynx.

3.1.2. Varicella Zoster Virus

The human Alphaherpesvirus VZV causes varicella (chickenpox) during primary infection, which is a generalized vesicular skin rash. Unlike HSV-1, VZV is transported via cell-associated viremia throughout the body resulting in widespread infection of the skin. Subsequently, latency is established in sensory and sympathetic neurons, where it can be reactivated to cause a second, more localized form of disease, herpes zoster (shingles) (DEPLEDGE et al., 2018). This is characterized by a vesicular rash which is localized to the specific skin area innervated by the respective infected latency site. The main sites of VZV latency are thought to be sensory neurons of TG (GILDEN et al., 1983; KENNEDY et al., 1998; LEVIN et al., 2003) and DRG (KENNEDY et al., 1999; REICHELT et al., 2008) where VZV gains access to the neurons in proximity of infected skin lesions during primary infection (chickenpox) (FURUTA et al., 1997). However, various other sensory ganglia (geniculate, vestibular, spiral) and autonomic (thoracic sympathetic trunk ganglia,

nodose, enteric) ganglia may be potential additional latency sites in which VZV DNA has been readily detected (GILDEN et al., 1987; FURUTA et al., 1992; MAHALINGAM et al., 1992; FURUTA et al., 1997; GILDEN et al., 2001; CHEN et al., 2011; DEPLEDGE et al., 2018). Interestingly, potential latency sites like vestibular and spiral ganglia are not directly linked by neurons to the epidermis of the skin (FURUTA et al., 1997), and therefore not easily accessible via viral neuronal transport. Furthermore, VZV DNA can be detected in sensory ganglia before the primary vesicular skin infection occurs (ZERBONI et al., 2005; OUWENDIJK et al., 2013). Therefore, there is growing evidence that cell-associated viremia serves as an alternative, hematogenous route towards VZV latency sites (ZERBONI et al., 2005; OUWENDIJK et al., 2013). Indeed, besides its characteristic neurotropism, VZV is also known to be lymphotropic (KU et al., 2002; SCHAAP et al., 2005). It has been shown that T-cells, which transport the virus to skin regions during viremia, also transferred VZV into DRG in a xenograft mouse model (ZERBONI et al., 2005). However, studying VZV latency pathogenesis is challenging due to its immense human-cell tropism and a lack of effective animal models (BLOOM, 2016). Detailed studies to identify the pathogenesis of Simian Varicella Virus (SVV, a close relative to VZV) were conducted by Ouwendijk et al. (2013 and 2016) in the natural host (African green monkeys). The authors showed that SVV infects T-cells during primary infection, which transport the virus throughout the body during viremia. Infected T-cells could then be identified adjacent to neurons up to 20 dpi. This supports the results of VZV in *in vitro* studies (GRIGORYAN et al., 2015) and xenograft *in vivo* mouse models (ZERBONI et al., 2005) and suggests that VZV lymphotropism and subsequent hematogenous spreading enhances the frequency of latency establishment in various neurons (ZERBONI & ARVIN, 2016).

3.1.3. Bovine Herpesvirus 1

BoHV-1 is an important pathogen of cattle causing infectious bovine rhinotracheitis (IBR), genital disorders (infectious pustular vulvovaginitis, balanopostitis), abortions, fatal systemic infections in calves and in rare cases meningoencephalitis (BRYAN et al., 1994; ROELS et al., 2000; JONES, 2016). Depending on whether initial infection occurs on upper respiratory tract or genital tract mucosa, latency is established via neuronal transport in sensory neurons of TG or sacral ganglia, respectively (JONES, 2016). Additional neural latency sites may exist but have not been further investigated so far (JONES, 2016). Importantly, BoHV-1 suppresses the immune system during primary infection: By targeting and destroying CD4+ T-cells and impairing cell-mediated immunity, BoHV-1 facilitates manifestation of secondary bacterial infections that may result in live-threatening

bronchopneumonia, also referred to as bovine respiratory disease complex (shipping fever) (ESKRA & SPLITTER, 1997; WINKLER et al., 1999; JONES & CHOWDHURY, 2008). Lymphotropism also enables BoHV-1 to be distributed to secondary infection sites in a cell-associated viremia, and BoHV-1 was detected in PBMCs and lymphoid tissue (tonsils, lymphnodes, spleen) during acute infection (BAGUST & CLARK, 1972; NYAGA & MCKERCHER, 1979; CASTRUCCI et al., 1992; BRYAN et al., 1994; KAASHOEK et al., 1996; FUCHS et al., 1999; WINKLER et al., 1999). However, some authors indicate that viremia seems to occur mainly in very young calves resulting in systemic infection (KAASHOEK et al., 1996; MWEENE et al., 1997). Consistent with its lymphotropism, BoHV-1 can persist in lymphocytes after acute infection (FUCHS et al., 1999) and establishes latency in non-neural sites like PBMCs (FUCHS et al., 1999; WANG et al., 2001), tonsils (WINKLER et al., 2000), lymphnodes, and spleen (MWEENE et al., 1997).

A great advantage of BoHV-1 latency research is the fact that it can be studied in calves as the natural host. In addition, latent BoHV-1 can be reliably reactivated by treating latently infected calves with the synthetic corticosteroid dexamethasone. This model therefore provides valuable research possibilities to further investigate the switch from latency to reactivation (JONES, 2016).

3.2. Latency establishment and reactivation in neurons

With the onset of viral replication in the respiratory epithelium and infection of mononuclear cells, viral particles also start to enter axonal nerve endings by fusion with the neuronal plasma membrane (JONES, 2013; SLATER, 2014; BLOOM, 2016). The subsequent active transport to the nucleus along microtubules is critical in neuronal cell infection considering the long distance between axonal nerve ending and neuronal nucleus located in ganglia (DIEFENBACH et al., 2008). Furthermore, the consistent polar orientation of microtubules in axons requires an efficient retrograde transport from plus end (axon termini) to minus end (nucleus) (SMITH, 2012). At least three inner tegument proteins have been described to build a retrograde transport complex with dynein motor proteins on microtubules for efficient capsid delivery to the neuronal nucleus (LUXTON et al., 2005; ANTINONE & SMITH, 2010; SMITH, 2012; RICHARDS et al., 2017). The importance of a well-functioning transport complex for latency establishment in neurons has been recently highlighted by HSV-1 and PsRV studies, where mutated versions of a specific complex protein (pUL37) led to abort of capsid delivery to the nucleus and therefore inhibit latency establishment *in vitro* and *in vivo* (RICHARDS et al., 2017; PICKARD et al.,

2020). Upon arrival to the neuronal cell body, viral genome is then released into the nucleus and circularizes. Subsequently, latency is established either promptly or following a short replication cycle (COEN et al., 1989; EFSTATHIOU et al., 1989; KATZ et al., 1990; STEINER et al., 1990). While replication is not necessary for latency establishment, efficient replication and subsequent spreading can result downstream in higher numbers of latently infected neurons (THOMPSON & SAWTELL, 2000). The axonal architecture of neurons possibly favors prompt latency establishment as the virus has to overcome long travel distances: It is discussed for HSV-1 that sufficient amounts of the viral gene cascade transactivator, VP16 (ETIF homologue), may not arrive at the distant neuronal nucleus resulting in a failure of initiating replication and therefore promoting immediate latency establishment (EFSTATHIOU & PRESTON, 2005; ANTINONE & SMITH, 2010; HAFEZI et al., 2012; SMITH, 2012)

Notably, while latently infected neurons are present from day one post infection (BLOOM, 2016), the term latency generally reflects the stable dormant viral state in the whole organism after the acute infection declines 14 – 21 dpi (GIBSON et al., 1992; HUSSEY et al., 2006; ALLEN, 2008; LUNN et al., 2009; PUSTERLA et al., 2009; GOEHRING et al., 2010b) and therefore is agreed to begin around 28 dpi (BLOOM, 2016).

The latent state in its complexity of involved molecular and cellular mechanisms is still poorly understood (PENKERT & KALEJTA, 2011; ZERBONI & ARVIN, 2016). It is assumed that after abort of the viral gene transcription cascade, the viral genome associates tightly with cellular histones (DESHMANE & FRASER, 1989; KNIPE & CLIFFE, 2008) and persists in a quiescent form as a circular episome (EFSTATHIOU et al., 1986; MELLERICK & FRASER, 1987). A precisely regulated balance between viral factors, neuronal environment and host immunity is now established (EGAN et al., 2013) (see chapter 3.3).

Periodically, this balance is challenged by certain stimuli like stress, fever, tissue damage, or immune suppression (NICOLL et al., 2012). This can initiate unregulated transcription of multiple viral genes, an event which is referred to as animation (PENKERT & KALEJTA, 2011) and can ultimately result in reactivation (PENKERT & KALEJTA, 2011; KENNEDY et al., 2015). However, animation including low-level lytic gene expression probably occurs frequently without culminating in virion assembly, but instead the virus re-enters the dormant state (PENKERT & KALEJTA, 2011; KIM et al., 2012; KENNEDY et al., 2015). There is still a lack of knowledge which criteria lead to the final

shift towards exit from latency, where animation is followed by organized IE, E, L gene transcription, viral DNA replication and virion assembly (KIM et al., 2012; KENNEDY et al., 2015). Upon reactivation, virions can egress directly from the neuronal cell body or travel anterogradely along axons towards the primary infection site (DIEFENBACH et al., 2008; SMITH, 2012). Anterograde transport is likely promoted by kinesin molecules but overall not fully understood (SMITH, 2012): Whether viral particles (naked capsid and glycoproteins) are transported separately or as assembled enveloped virion in vesicles remains inconclusive (DIEFENBACH et al., 2008; SMITH, 2012). Finally, the virus exits the axonal termini supposedly by exocytosis (SAKSENA et al., 2006), may then infect epithelial cells and either cause asymptomatic shedding or recurrent lesions. The axonal architecture of sensory neurons therefore allows the latent virus to retain a direct loophole to peripheral tissue sites and to new susceptible hosts eventually in case of reactivation.

3.3. Maintenance of latency: balance between viral and host factors

3.3.1. Host cell chromatin and gene regulation

Upon release of the viral genome into the host cell nucleus, viral DNA is exposed to the nuclear environment (NICOLL et al., 2012). Unlike the bare viral DNA in virions, eukaryotic DNA is bound to histones and other proteins which pack up the long DNA molecule in a complex called chromatin (KNIPE & CLIFFE, 2008). Active and repressive forms of histones can regulate gene transcription, as they influence the density of chromatin upon genes. The activity state of histones as such is associated with amino acid tail modifications, most importantly acetylation and methylation (EFSTATHIOU & PRESTON, 2005; KNIPE & CLIFFE, 2008). For example, acetylation results in active chromatin and gene transcription. De-acetylation by histone de-acetylases (HDACs) therefore leads to chromatin condensation and gene silencing. These host cell chromatin structures become associated with viral DNA (NICOLL et al., 2012; TALLMADGE et al., 2018). While this appears to be a host cell defense mechanism in order to silence viral gene expression via condensed chromatin, the virus itself is presumably actively involved in this process and may be able to manipulate the chromatin structure for its own gene regulation (WANG et al., 2005b; NICOLL et al., 2012; LAVAL et al., 2015). Indeed, during lytic HSV-1 infection, viral proteins like VP16 (ETIF homologue) and/or IE protein may reduce overall chromatin assembly upon viral lytic genes and promote active (open) chromatin possibly by inhibiting HDACs (HOBBS & DELUCA, 1999; KENT et al., 2004; KNIPE & CLIFFE, 2008). For EHV-1, histone methylation (TALLMADGE et al., 2018) and acetylation (LAVAL et al.,

2015) has been shown to regulate viral gene transcription during lytic infection.

During latency, however, the viral episome is tightly associated with condensed forms of chromatin particularly upon viral lytic genes. It has been suggested for HSV-1 that this may be linked to the failure of VP16 delivery into the nucleus during latency establishment (KNIPE & CLIFFE, 2008; HAFEZI et al., 2012). While repressive chromatin is presumably involved in lytic gene silencing, acetylated (open) chromatin has been detected upon the sole gene region, which is active during latency: the LAT gene (NICOLL et al., 2012). In addition, LAT itself supposedly reduces active chromatin during latency (WANG et al., 2005b; CLIFFE et al., 2009), implying one of the multiple potential mechanisms of LAT in its function as a latency regulator.

3.3.2. The role of latency-associated transcripts

Presumably all Alphaherpesviruses express LATs that contribute to the network of regulation during latency (JONES, 2013; BLOOM, 2016; DEPLEDGE et al., 2018). However, their presence is overall not required for latency establishment (NICOLL et al., 2012). Despite years of research since their discovery in 1987 (SPIVACK & FRASER, 1987; STEVENS et al., 1987), the exact role and mechanism of action is still poorly understood (NICOLL et al., 2012) and best characterized in HSV-1 research. Supposedly, LATs are capable of reducing lytic gene expression (GARBER et al., 1997) by still unknown mechanisms. One possibility could be an anti-sense, inhibiting binding of LAT to IE mRNA (STEVENS et al., 1987), which aborts further translation processes. As already mentioned, another possible way could involve LAT dependent manipulation of host cell chromatin structure that associates with viral genes. For example, tightly condensed chromatin on viral lytic-gene promoters could be appreciated in the presence of LAT, but not in LAT negative mutants (WANG et al., 2005b). In addition, LATs may also be capable of supporting neuronal survival through an anti-apoptotic mechanism (PERNG et al., 2000; INMAN et al., 2001; JONES, 2003; TORMANEN et al., 2019). Typically, LATs are encoded to a genomic region antisense to the IE gene (HSV-1 ICP0) and persist after splicing as a 2.0 kbp stable intron (JONES, 2003; EFSTATHIOU & PRESTON, 2005). While the LAT gene locus seems to be conserved among well-studied Alphaherpesviruses like HSV-1, VZV, and BoHV-1, the precise gene region encoding EHV-1 LAT is controversially discussed in literature (HOLDEN et al., 1992; BAXI et al., 1995; CHESTERS et al., 1997; BORCHERS, 1999; PUSTERLA et al., 2009; ABDELGAWAD et al., 2016; VARGAS-BERMUDEZ et al., 2018). During HSV-1 and BoHV-1 latency, LATs are

abundantly expressed, which is why they are established markers used in HSV-1 and BoHV-1 latency diagnosis (JONES, 2003). Both HSV-1 and BoHV-1 LATs express micro-RNAs (miRNAs) during latency (UMBACH et al., 2008; JABER et al., 2010; JURAK et al., 2010). Mi-RNAs are small (~22-nucleotide), non-coding RNAs, associated with viral gene regulation by targeting and binding to mRNAs (GREY, 2015). It has been shown that they may be able to suppress lytic gene transcription and support survival of latently infected cells (NICOLL et al., 2012; GREY, 2015). BoHV-1 LAT additionally has coding potential for a protein with anti-apoptotic activity, which is a unique feature among known LATs of Alphaherpesviruses (JIANG et al., 1998; CIACCI-ZANELLA et al., 1999; MOTT et al., 2003; MEYER et al., 2007). Until recently, VZV was assumed to be the only Alphaherpesvirus not known to express LAT (BLOOM, 2016; ZERBONI & ARVIN, 2016). Depledge et al. (2018) revised this theory and succeeded in a breakthrough in VZV research by detecting a complex organized LAT using target enriched RNA Sequencing. Like HSV-1 and BoHV-1, the VZV LAT is encoded antisense to the corresponding VZV IE gene location but seems to be expressed at very low-levels and does not encode miRNA. Furthermore, the authors detected an additional IE transcript during VZV latency which is possibly involved in the process of reactivation (DEPLEDGE et al., 2018). Hence, the known functions of LATs, their size and level of transcription is not uniform among Alphaherpesviruses (EFSTATHIOU & PRESTON, 2005; DEPLEDGE et al., 2018).

Several studies in the past detected transcripts during EHV-1 latency using in-situ hybridization (BAXI et al., 1995; CHESTERS et al., 1997), conventional nested PCR (CHESTERS et al., 1997; BORCHERS, 1999; ABDELGAWAD et al., 2016; VARGAS-BERMUDEZ et al., 2018) or nothern blot analysis (HOLDEN et al., 1992). According to this data there is evidence that potential LATs are encoded to regions antisense to the ICP0 homologue (ORF 63) (BAXI et al., 1995; BORCHERS, 1999; ABDELGAWAD et al., 2016; VARGAS-BERMUDEZ et al., 2018) and/or antisense to a region overlapping the 3’ end (CHESTERS et al., 1997) or 5’ end (HOLDEN et al., 1992) of the sole IE gene (ORF 64). But whether these are transcripts periodically expressed during low-level gene expression during latency or true LATs that are structurally and functionally comparable to those of HSV-1, BoHV-1 or VZV, remains to be elucidated. One can speculate that the putative EHV-1 LAT is not as abundantly expressed as in HSV-1 or BoHV-1 latency and similar to VZV LAT research, methods with higher sensitivity like RNA Sequencing are crucial to repeatedly define and characterize the molecular structure of EHV-1 LAT.

3.3.3. The role of different host cell types in ganglia

3.3.3.1. Neuron-satellite glial cell complex

Neurons are stable, long living cells that do not divide and normally express only low amounts of T-cell receptors (MHC class 1 and 2 molecules) (EGAN et al., 2013; SLATER, 2014). Therefore, neurons serve as ideal latency sites, where the virus can persist lifelong in symbiosis with the host (LIU et al., 2001). Within ganglia, each sensory neuron is tightly wrapped in a sheath of laminar cells, representing the glial cells of the peripheral nervous system. These satellite glial cells (SGCs) are multifunctional nursing cells that regulate the neurons' microenvironment and serve as physical support by forming a protective envelope around the neuron (HANANI, 2005). It has been shown that SGCs synthesize growth factors and proliferate following nerve trauma and malnutrition which presumably preserves the enclosed neuron (LEE et al., 1998; ELSON et al., 2003; VAN VELZEN et al., 2009). Similarly, SGC proliferation also has been detected in response to acute herpesvirus infections, which possibly prevents random virus spreading throughout the ganglia in a shield like manner (LAVAIL et al., 1997; ELSON et al., 2003). However, SGCs are very versatile in function and their roles during viral infection have just started to become elucidated. There is growing evidence that SGCs act as tissue-resident immune cells with phenotypic and functional similarities to antigen presenting cells (HANANI, 2005). As such, SGCs likely are involved in the innate immune response to herpesvirus infections in neurons: It has been shown that SGCs are activated during primary viral infection and can express cytokines/chemokines (IFN-α, IL-6, TNF-α, CXCL10) to orchestrate T-cell infiltration into the ganglia (HANANI, 2005; VAN VELZEN et al., 2009; OUWENDIJK et al., 2016; DEPLEDGE et al., 2018). This may highlight the importance of SGCs in clearing virus during acute infection/reactivation by them compensating the neurons insufficient ability to express viral antigens to the immune system (VERJANS et al., 2007; VAN VELZEN et al., 2009).

Whether SGCs become infected productively during acute and/or serve as safe harbor during latent infection is broadly (and somewhat controversially) discussed for HSV-1 and VZV (CROEN et al., 1988; WROBLEWSKA et al., 1993; LAVAIL et al., 1997; WILKINSON et al., 1999; CAI et al., 2002; LEVIN et al., 2003; HANANI, 2005; REICHELT et al., 2008; BERTKE et al., 2011; ZERBONI & ARVIN, 2016). While VZV infects and replicates efficiently in satellite cells using human DRG xenotransplantation models (REICHELT et al., 2008), satellite cells seem to be less susceptible for productive

HSV-1 infection using the same *in vivo* model (ZERBONI et al., 2013). Restrictive HSV-1 viral replication in satellite cells has also been reported in other studies (WILKINSON et al., 1999; ELSON et al., 2003). Moreover, VZV can induce plasma membrane fusion between infected neuronal cell bodies and the surrounding satellite cells, resulting in rapid viral transfer from neuron to satellite cells and subsequent spreading to nearby neuron-satellite cell complexes (REICHELT et al., 2008). During VZV reactivation, the rapid ganglionic spreading of reactivated virions increases the chances for anterograde axonal transport towards an entire area of the skin (dermatome), resulting in the typical dermatomal skin rash (WILKINSON et al., 1999; ZERBONI et al., 2013). Furthermore, the neuronal damage post ganglionic spreading and signaling processes between neuron-satellite complexes have been associated with the characteristic neuropathic pain during VZV reactivation, which is not common in HSV-1 infections (ZERBONI et al., 2013). Indeed, the phenomenon of ganglionic spreading could not be detected in HSV-1 reactivation and recurrent HSV-1 lesions are typically more focal and restricted to a single anatomical spot (SMITH, 2012; ZERBONI et al., 2013; BLOOM & DHUMMAKUPT, 2016).

Numerous studies tried to define the precise cell location during HSV-1 and VZV latency within the neuron satellite complex of ganglia obtained during autopsy: While latent HSV-1 was consistently localized to neurons (CROEN et al., 1988; CAI et al., 2002; WANG et al., 2005a), some early VZV studies using *in situ* hybridization described VZV transcripts (CROEN et al., 1988; WROBLEWSKA et al., 1993) or DNA (LUNGU et al., 1995) to be present in satellite glial cells during latency. However, this has been re-evaluated later by laser capture microdissection (WANG et al., 2005a) and by cell separation of neurons and satellite cells prior to PCR evaluation (LEVIN et al., 2003). Both studies concluded that the predominant cell type for VZV latency establishment is the neuron. Notably, the low amounts of viral DNA and/or transcripts present during latency as well as the close proximity and interaction between satellite cells and neurons makes it quite challenging to consistently define the cellular localization of latent virus in ganglia (MAHALINGAM et al., 1999; LEVIN et al., 2003).

3.3.3.2. T-lymphocyte immunity

The virus-host coexistence during chronic persistent infection requires a precise balance between the dormant virus and the host immune system. In addition, this balance during latency must benefit not only the virus but also the host in order to be an evolutionary successful concept (SIMMONS & TSCHARKE, 1992; LEGER & HENDRICKS, 2011).

Within neurons, taming the virus in a non-lytic state is obviously an advantage for the host, as each reactivation leads to neuronal death which cannot be regenerated (LEGER & HENDRICKS, 2011; EGAN et al., 2013). It has been shown that CD8+ T-cells are involved in maintaining the viral latent state by acting in a non-lytic way. They are therefore important host factors preserving the neuron and the (latent) virus (SIMMONS & TSCHARKE, 1992; LIU et al., 1996; LIU et al., 2000; KHANNA et al., 2003; VERJANS et al., 2007). Viral antigen during lytic- and low-level gene expression during latent infection may attract CD8+ T-cells via cytokines/chemokines that are most likely expressed by SGCs and to a lesser extent by neurons themselves (SIMMONS & TSCHARKE, 1992; HANANI, 2005; VAN VELZEN et al., 2009; OUWENDIJK et al., 2016; DEPLEDGE et al., 2018). Following antigen exposure, T-cells can differentiate to memory T-cells that remain permanently as virus specific memory T-cell and are ready to react with a fast response in case of re-exposure. Memory T-cells can further be divided into (a) central memory T-cells, homing to lymphoid tissue; (b) effector memory T-cells, which are distributed to tissues in the periphery while constantly screening the environment for their specific antigen; and (c) tissue resident memory T-cells (T_{RM}), a subset that typically remains permanently guarding the primary infection site (EGAN et al., 2013). It has been speculated that following infiltration in to the ganglia during primary infection, CD8+ T-cells remain in close proximity to neurons during latency probably for a life-time, forming a subset of CD8+ T_{RM} (LIU et al., 1996; LIU et al., 2000; EGAN et al., 2013). Interestingly, CD8+ T_{RM} do not provoke apoptosis of the infected neuron but suppress lytic gene expression via interferon-γ and granzyme B and therefore prevent reactivation (LIU et al., 2001; KHANNA et al., 2003; EGAN et al., 2013). Viral LATs and/or miRNA may stimulate the anti-apoptotic effect and therefore complete the balance of latency from the viral side (EGAN et al., 2013).

An increased level of glucocorticoids during stress, hormonal imbalances or immunosuppression can disrupt this fine-regulated interplay from the host side by inhibition of CD8+ T_{RM} function (EGAN et al., 2013; YU et al., 2018). Moreover, the immune regulation in the context of latency is likely more complex as previously thought and may also involve subsets of regulatory T-cells that control the cell count and function of CD8+ T_{RM} (YU et al., 2018). However, the exact mechanisms of interaction between CD8+ T_{RM} and latent virus is still poorly understood, and some authors even question their importance during latency (MOTT et al., 2016). Most of these studies mentioned above are based on HSV-1 research in non-natural host (mice) models or in *in vitro* explant models. While these

models offer valuable research possibilities, they cannot mirror the highly coevolved adaption between the virus and the natural host immune system. To truly understand the complex balance of latency, it is essential to conduct studies in the natural host. Studying EHV-1 latency in the natural host therefore provides an excellent research model.

Taken together, the virus is not able to truly "hide" lifelong from the host immune system during latency (KHANNA et al., 2004). The concept of latency rather involves host immunity and viral factors to establish a complex control mechanism which is then advantageous for both participants (Figure 1) (LEGER & HENDRICKS, 2011).

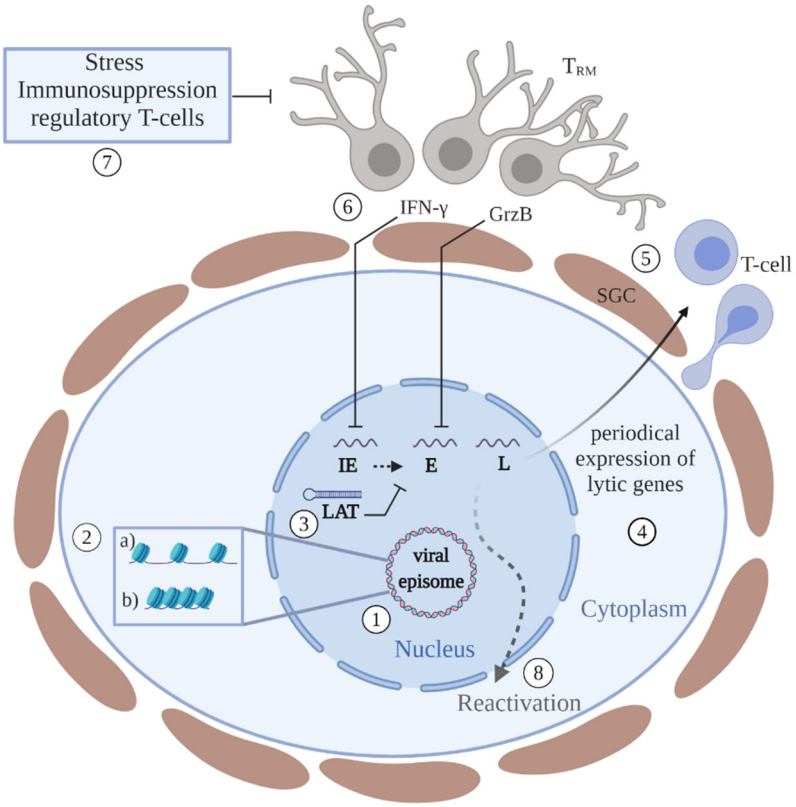

Figure 1.: The fragile balance between viral and host factors during latency (modified from EGAN et al., 2013). Figure created with BioRender.com.

Following entry into the neuronal nucleus, the viral genome circularizes (1) and associates tightly with cellular histones (2). While condensed forms of chromatin (2b) are involved in repressing lytic genes, active chromatin (2a) allows transcription of latency-associated transcript (LAT) promoter. LATs (3) supposedly promote silencing of lytic genes and support neuronal survival. Periodically, low-level lytic gene transcription (4) attracts T-cells possibly through signals produced by satellite glial cells (SGCs). T-cells come in close contact with neuron and SGC (5) and can differentiate into tissue resident memory T-cells (T_{RM}). T_{RM} inhibit viral lytic gene transcription through interferon-γ (IFN-γ) and granzyme B (GrzB) (6). External stimuli (stress events or immunosuppression) or regulation by regulatory T-cells can inhibit T_{RM} activity (7) which may result in higher transcription levels of lytic genes and a shift from latency to reactivation (8).

3.4. EHV-1 latency – status quo and statement of research purpose

Aspects of EHV-1 latency are still not fully understood in detail and the predominant sites for latency establishment have been controversially discussed in literature (WELCH et al., 1992; EDINGTON et al., 1994; SLATER et al., 1994; BAXI et al., 1995; CHESTERS et al., 1997; SMITH et al., 1998). Like all known Alphaherpesviruses, EHV-1 supposedly establishes latency in the TG during primary infection, where its persistence has been repeatedly confirmed (SLATER et al., 1994; BAXI et al., 1995; PUSTERLA et al., 2012). In addition, latent EHV-1 has been shown to persist in RALT and in circulating PBMCs (EDINGTON et al., 1986; WELCH et al., 1992; BAXI et al., 1995; CHESTERS et al., 1997; SMITH et al., 1998). This is consistent with the lymphotropism of EHV-1 during acute infection resulting in viremia – an essential key feature for its pathogenesis which EHV-1 shares with its close relatives BoHV-1 and VZV. In early EHV-1 latency prevalence studies, presumably latent EHV-1 DNA was also detected in the spleen (WELCH et al., 1992; EDINGTON et al., 1994). This also has been reported for BoHV-1 latency (MWEENE et al., 1997) and further supports lymphotropism as an additional strategy for EHV-1 latency establishment.

Our research group therefore hypothesized in a previous study that EHV-1 latency is not limited to TG and RALT and additional latency sites may exist (SAMOILOWA, 2019). Our aim was to analyze EHV-1 persistent infection 70 dpi in various lymphatic and neural tissues of three groups of horses: Group 1 was infected with neuropathogenic EHV-1 strain Ab4 (wildtype (WT)); group 2 with an EHV-1 polymerase mutant at position 752 (D > N; (Ab4 N742)); and group 3 with an EHV-1 glycoprotein D (gD), where gD of EHV-1 was replaced with gD of EHV-4 (Ab4 gD4). Horses of all groups were studied for 70 dpi, followed by euthanasia and clinical data, nasal viral shedding and viremia are described in detail elsewhere (HOLZ et al., 2017; HOLZ et al., 2019).

Consistent with VZV latency research, we detected EHV-1 DNA (via qPCR) not only in TG, but also in various sympathetic/parasympathetic ganglia of head, neck, thorax, abdomen, and in DRG (SAMOILOWA, 2019). This wide neural distribution of EHV-1 DNA beyond the period of viremia was confirmed in all three infection groups and was not reported before. However, the important question whether EHV-1 is transported via neuronal or hematogenous route to these potential sites of latency remained unanswered.

In addition, we could confirm that EHV-1 establishes latency in lymphatic tissue: EHV-1 DNA was frequently detected in RALT and most importantly in abdominal mesenteric

lymph node and spleen. However, lymphotropism during latency was different among groups: While Ab4 WT and Ab4 gD4 established latency in respiratory (pharyngeal roof, mandibular, retropharyngeal lymph nodes) and in abdominal (spleen, mesenteric) associated lymph nodes, Ab4 N752 was significantly less lymphotropic for its latency establishment (SAMOILOWA, 2019). Interestingly, horses in the Ab4 N752 group also showed significantly lower levels of viremia (HOLZ et al., 2017), which is consistent with *in vitro* studies, where N752 mutants are significantly less capable to infect monocytes following primary infection in the respiratory epithelium (VANDEKERCKHOVE et al., 2010). In contrast, Ab4 WT infected horses outnumbered the other groups in the magnitude of viremia and Ab4 WT was more prone to establish latency in abdominal lymphatic tissue compared to the other strain variants (HOLZ et al., 2017; SAMOILOWA, 2019).

The consistent detection of chronic persistent EHV-1 infection in various neural and (abdominal) lymphatic tissue samples together with differences in viremia and lymphotropism in latency establishment among the three infection groups suggested further investigation of the potential involvement of viremia in EHV-1 latency pathogenesis.

III. HYPOTHESIS AND OBJECTIVES

The present study aims to further understand how EHV-1 is transported to abdominal neural tissue samples and which cells are predominantly latently infected at these locations. This information is crucial to understand the regulation and further define molecular aspects of EHV-1 latency pathogenesis.

We hypothesize that EHV-1 latency is a dynamic state and time passed after primary infection influences viral distribution and persistence.

To address this hypothesis, we compare EHV-1 latency establishment between two groups of experimentally infected horses (EHV-1 strain Ab4, wild type) that were euthanized 30 dpi (group 1) and 70 dpi (group 2). The main objectives of the present study are:

i) Compare EHV-1 viral load and state of infection in neural and lymphatic tissue collected at day 70 pi with a timepoint closer to acute infection and viremia (30 dpi).

ii) Determine the cellular localization of persistent EHV-1 within neural tissue by *in situ* hybridization (ISH) to further understand the molecular basis of EHV-1 latency pathogenesis.

IV. RESULTS

1. Publication: Viral load and cell tropism during early latent Equid Herpesvirus 1 infection differ over time in lymphoid and neural tissue samples from experimentally infected horses

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Viral Load and Cell Tropism During Early Latent Equid Herpesvirus 1 Infection Differ Over Time in Lymphoid and Neural Tissue Samples From Experimentally Infected Horses

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Upper respiratory tract infections with Equid Herpesvirus 1 (EHV-1) typically result in a peripheral blood mononuclear cell-associated viremia, which can lead to vasculopathy in the central nervous system. Primary EHV-1 infection also likely establishes latency in trigeminal ganglia (TG) via retrograde axonal transport and in respiratory tract-associated lymphatic tissue. However, latency establishment and reactivation are poorly understood. To characterize the pathogenesis of EHV-1 latency establishment and maintenance, two separate groups of yearling horses were experimentally infected intranasally with EHV-1, strain Ab4, and euthanized 30 days post infection (dpi), (n = 9) and 70 dpi (n = 6). During necropsy, TG, sympathetic trunk (ST), retropharyngeal and mesenteric lymph nodes (RLn, MesLn) and kidney samples were collected. Viral DNA was detected by quantitative PCR (qPCR) in TG, ST, RLn, and MesLn samples in horses 30 and 70 dpi. The number of positive TG, RLn and MesLn samples was reduced when comparing horses 30 and 70 dpi and the viral copy number in TG and RLn significantly declined from 30 to 70 dpi. EHV-1 late gene glycoprotein B reverse transcriptase PCR and IHC results for viral protein were consistently negative, thus lytic replication was excluded in the present study. Mild inflammation could be detected in all neural tissue samples and inflammatory infiltrates mainly consisted of CD3+ T-lymphocytes (T-cells), frequently localized in close proximity to neuronal cell bodies. To identify latently infected cell types, *in situ* hybridization (ISH, RNAScope®) detecting viral DNA was used on selected qPCR- positive neural tissue sections. In ganglia 30 dpi, EHV-1 ISH signal was located in the neurons of TG and ST, but also in non-neuronal support or interstitial cells surrounding the neuron. In contrast, distinct EHV-1 signal could only be observed in neurons of TG 70 dpi. Overall, detection of latent EHV-1 in abdominal tissue samples and non-neuronal cell

localization suggests, that EHV-1 uses T-cells during viremia as alternative route toward latency locations in addition to retrograde neuronal transport. We therefore hypothesize that EHV-1 follows the same latency pathways as its close relative human pathogen Varicella Zoster Virus.

Keywords: EHV-1, horses, latency, Alphaherpesviruses, pathogenesis, trigeminal ganglia, lymphocytes

INTRODUCTION

Equid Herpesvirus 1 (EHV-1) belongs to the family of Alphaherpesvirinae and is a clinically important herpesvirus in horses that causes respiratory disease, myeloencephalopathy and abortions worldwide. EHV-1 is typically spread by direct horse-to-horse contact through respiratory tract secretions and replicates in the upper respiratory tract epithelium following initial infection. Subsequently, EHV-1 infects mononuclear cells, enters the blood circulation and is thereby disseminated widely to secondary infection sites, such as the central nervous system (CNS) or the uterus (1–3). The main cell type infected during cell-associated viremia are monocytes and T-lymphocytes (T-cells), followed by B-lymphocytes (B-cells) (4–7).

During or shortly after acute lytic infection and replication, the virus starts to establish a life-long chronic persistent infection in the host. This latent infection is an evolutionary advantage found in all alphaherpesviruses, as it allows the virus to remain in the host undetected from the immune system while maintaining its capability for reactivation (8–10). A successful latent infection is described to be strongly dependent on a well-balanced host-virus interaction (11, 12), and neurons are thought to be excellent locations for latency establishment, as they represent a stable, long-living cell population. Furthermore, neurons are not known to express viral antigen, which is an important advantage for the virus allowing it to evade the immune system (3). During latency, viral DNA is present in the nucleus of infected cells in a non-integrated form, without lytic transcription and translation processes (13–15). In addition, there is growing evidence, that T-cells and satellite cells surrounding neurons are playing key roles in maintaining Alphaherpesvirus latency supposedly by interacting directly with viral transcription (12, 16).

EHV-1 has been shown to establish latency in the trigeminal ganglia (TG), like its relatives Herpes Simplex Virus 1 (HSV-1), Bovine Herpesvirus 1 (BoHV-1) and Varicella Zoster Virus (VZV) (8, 10, 12, 17) but also in respiratory associated lymphoid tissue (RALT) and circulating CD8+ T-cells (18–22). Furthermore, our research group has recently shown that 70 days post infection (dpi), chronic persistent EHV-1 infection is not limited to TG and RALT, but EHV-1 DNA can also be detected (via qPCR) in various other sensory- (spinal cord dorsal root ganglia), sympathetic and parasympathetic- ganglia as well as in abdominal lymphoid tissue (mesenteric lymph node, spleen) (23). We reported wide distribution of chronic persistent EHV-1 infection beyond viremia. Important remaining questions are how the virus gets to these sites of presumed latency, and which cells predominantly harbor the virus at these locations; e.g., which cells are preferred for latency establishment.

Most Alphaherpesviruses gain access to the sensory nerve endings in the vicinity of the primary infection sites and use retrograde axonal transport to reach the neuronal cell bodies. For VZV, which, like EHV-1, belongs to the subfamily of Varicellovirinae, it is thought that VZV is transported to the sensory ganglia during viremia and in VZV-infected T-cells, as well as by axonal transportation (24, 25). EHV-1 tropism for T-cells is also well described and it is proposed to be important for viral dissemination throughout the host and for bypassing viral clearance by the immune system (7, 22, 26). Interestingly, while EHV-1 gene transcription and protein expression is active during viral replication in epithelial cells, during viremia, the virus seems to be carried in a non-replicative state, until secondary locations, such as CNS or uterine endothelium, are reached and virus transfer occurs (7). However, it is unclear if EHV-1 is also transported to ganglia in the same manner and uses this route for successful latency establishment.

In the present study, we compared EHV-1 viral load and state of infection in neural and lymphatic tissue (based on previously identified locations at 70 dpi) (23) with a timepoint closer to acute infection and viremia (30 dpi). We hypothesized that EHV-1 chronic persistent infection is influenced by time passed after inoculation. Furthermore, we wanted to investigate latency establishment and maintenance on a cellular level by using *in situ* hybridization (ISH) to localize viral DNA within neural tissue.

MATERIALS AND METHODS

Animals

Tissue samples used in this study were obtained from a total of fifteen yearling horses of both sexes (six females and nine males). All horses were clinically healthy and screened for EHV-1 and EHV-4 serum neutralizing antibodies using serum neutralization (SN) tests prior to infection. Only horses with a titer <4 for EHV-1 and <40 for EHV-4 were selected for the study. Animals were fed twice a day and had *ad libitum* access to water. Horses were housed together in a naturally ventilated barn throughout the experiment. The maintenance and experimental protocols were reviewed and approved by Michigan State University Institutional Animal Care and Use Committee.

Experimental Design

Two separate experimental groups of horses were established (group 1: $n = 9$, group 2: $n = 6$) and infected by intranasal instillation of 5×10^7 plaque forming units (PFU) of EHV-1 in 10 ml of saline of the neuropathogenic EHV-1 strain Ab4 (D752 variant). This virus was previously isolated from a quadriplegic

mare (27). Horses in group 1 were euthanized 30 dpi, while horses in group 2 were euthanized 70 dpi.

Clinical Data, Nasal Virus Shedding, and Viremia

Collection of clinical data, nasal viral shedding and viremia are described in detail elsewhere (28). Briefly, clinical examinations were performed and nasal swabs for viral DNA isolation were collected prior to infection (at day −5 and −3) and daily from day 1 to 14, and every other day from day 14 to 21 dpi. Blood samples were taken prior to infection (at day −5) and daily from 1 to 10 dpi for detection of cell-associated viremia. Nasal viral shedding and viremia were analyzed by real-time PCR (qPCR) using specific probes and primers for EHV-1 glycoprotein B (1).

Necropsy and Tissue Collection

All horses were euthanized by sedation with detomidine (0.012 mg/kg, i.v.) followed by administration of pentobarbital (380 mg/kg, i.v.) and immediately (within 10–15 min) transported to the Veterinary Diagnostic Laboratory at MSU for necropsy and sample collection. Abdominal sympathetic trunk ganglia (ST), TG, retropharyngeal lymph nodes (RLn) and mesenteric lymph nodes (MesLn) were collected from all animals. For two horses (one in each infection group), RLn samples were not available for the study. Furthermore, kidneys were collected from all animals as negative tissue controls, hypothesizing, that this sample type represents no target for EHV-1. Time from euthanasia to completed tissue collection was <70 min. All tissues were fixed in 5% paraformaldehyde for 24 h, followed by routine tissue processing and paraffin embedding.

DNA/RNA Extraction

Viral DNA and total RNA from paraformaldehyde-fixed-paraffin-embedded (PFPE) tissue was extracted using AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany). Briefly, five serial sections of 4 µm (20 µm in total) were used and processed according to the manufacturer's protocol. Extraction from DNA-free and RNA-free water was included as quality control during extraction. For neural tissue, Hematoxylin and Eosin (H&E) slides were screened for the presence of ganglia cell bodies before DNA/RNA isolation. All RNA extraction samples were tested with qPCR for absence of genomic DNA using primers and probe for equine glyceraldehyde-3-phosphate dehydrogenase (eqGAPDH) as previously published (29). Genomic DNA positive RNA samples were treated with the RQ1 Rnase-free Dnase Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and re-tested afterwards, as described above. Only samples negative for genomic DNA were converted to complementary DNA (cDNA) using the Quantinova Reverse Transcription Kit (Qiagen, Hilden, Germany) with random hexamer primers according to the manufacturer's protocol.

Real-Time PCR

For detection of EHV-1 genomic DNA and late gene mRNA, a qPCR assay targeting a 106 bp long region of the glycoprotein B gene (open reading frame 33) was performed as previously published (1). Forward and reverse primers were used in a final concentration of 450 nM and the probe in a final concentration of 100 nM. Sequences for primers and probes used in this study are listed in **Table 1**.

TABLE 1 | Primers and probes for qPCR used in this study.

eGAPDH	
egapdh (F)	5′- GCCATCACTGCCACCCAG-3′
egapdh (R)	5′- TGGCAGCACCAGTAGAAGCA-3′
egapdh (probe)	5′[FAM]- AGGGGCTGCCCAGAACATCATCC - [TAMRA]3′
B2M	
B2M (F)	5′-ATGGAAAGCCAAATTTCCTG-3′
B2M (R)	5′-ACCGGTCGACTTTCATCTTC-3′
B2M (probe)	5′[HEX]-TGGGTTCCATCCGCCTGAGA –[BHQ1]3′
gB	
gB (F)	5′-CATACGTCCCTGTCCGACAGAT-3′
gB (R)	5′-GGTACTCGGCCTTTGACGAA-3′
gB (probe)	5′[FAM]- GGTACTCGGCCTTTGACGAA -[BHQ1]3′

All qPCR reactions were performed in a total reaction volume of 20 µl using 10 µl 1 × SensiFAST™ Probe Lo-ROX Kit (Bioline, Luckenwalde, Germany) and 5 µl of the template. All samples were analyzed in duplicates and amplified with the following thermal profile: initial 95°C step for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s (and hold 60°C for 60 s).

For absolute quantification, DNA and mRNA results were compared to a standard curve generated with cloned EHV-1 oligonucleotides that were kindly provided by W. Azab and N. Osterrieder. Viral copy numbers were then normalized to a standard curve generated with oligonucleotides specific to the housekeeping gene equine B2M, as previously described (30). Viral DNA and mRNA concentrations were expressed as copies per 10^6 cells, considering that each diploid eukaryotic cell contains two copies of B2M gene (30). Positive EHV-1 DNA (extracted from lung of an EHV-1 aborted fetus) and horse DNA (extracted from equine liver) were included as positive controls for EHV-1 gB and B2M qPCR assay. DNA-free and RNA-free water was used as negative control.

Hematoxylin and Eosin Staining & Histology Grading

For Histological evaluation, 4 µm thick sections of the PFPE blocks were routinely stained with Hematoxylin and Eosin (H&E). Ganglion cells, satellite cells and nerve fibers were evaluated for the presence of histopathological changes and inflammatory infiltrations were assessed and scored as mild, moderate or severe, if present.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on samples positive for EHV-1 genomic DNA using EHV-1/EHV-4 polyclonal caprine antiserum (VMRD, Pullman, USA) cross-reactive with EHV-1 and EHV-4 antigen (Ag). Serial section of PFPE blocks were deparaffinized and antigen retrieval was performed by incubation in citrate buffer (0.1 M, pH6.0) and

heating in a microwave oven (700 W) for 20 min. Endogenous peroxidase was quenched through incubation with H_2O_2 (1%, 15 min) and subsequently nonspecific binding of proteins was blocked with blocking buffer containing rabbit serum (1:10, 30 min), followed by incubation with the primary antibody (Ab) against EHV-1/EHV-4 Ag (VMRD, Pullman, USA, polyclonal, goat, 1:1,600) for 1 h at room temperature. Subsequently, samples were incubated with rabbit anti-goat biotinylated Ab (Vector laboratories LTD, Burlingame, USA), then incubated with avidin-biotin-complex (1:100, 30 min) and visualized by 3,3'-diaminobenzidine (DAB) (Vector laboratories LTD, Burlingame, USA). Counterstaining was performed with Mayers Hemalum. Lung tissue from an EHV-1 aborted fetus was included as positive control. As a negative control, the primary Ab was omitted and replaced by the corresponding antibody dilutent (blocking buffer containing rabbit serum). Non-neuronal cells were characterized on selected neural samples using antibodies directed against CD3 (DAKO, Hamburg, Germany, polyclonal, rabbit, 1:500), CD20 (Thermo Scientific Labvision, Fremont CA, USA, polyclonal, rabbit, 1:1,000) and S-100 (DAKO, Hamburg, Germany, polyclonal, rabbit, 1:6,000) using routine methods. Briefly, pre-treatment was performed as described for EHV-1/EHV-4 with blocking buffer containing goat serum, followed by incubation with the correspondent primary Ab overnight at 4°C. Incubation with goat anti-rabbit biotinylated Ab, detection and counterstaining was performed as described for EHV-1/EHV-4 Ab.

In situ Hybridization

In situ hybridization detecting viral DNA was performed by RNAScope® technology (Advanced Cell Diagnostics, Inc., USA) as previously described (31–33) using the RNAScope® 2.5 Detection Kit (Red) and the EHV-1 probe (V-EHV-1-ORF33, Cat.no.552651) targeting the region between nucleotides 61485-62416 of AY665713.1. Briefly, 4 µm serial sections were cut from PFPE blocks of selected EHV-1 qPCR positive TG (group 1, $n = 3$, group 2, $n = 4$) and ST (group 1, $n = 4$; group 2, $n = 3$). Subsequently, deparaffinization, pretreatment and hybridization were performed according to the manufacturer's protocol using the provided pretreatment solutions and wash buffer. All incubation steps were performed in a humidity control tray and a HybEZTM oven (Advanced Cell Diagnostics, Inc., USA). Following hybridization the signal was detected using Fast Red as chromogen provided by the manufacturer (RedB:RedA, 1:60 ratio). Counterstaining was performed using 50% Gill's Hematoxylin 1 (American MasterTech, Lodi, CA), followed by bluing with tap water and 0.02% ammonium hydroxide water. Slides were air dried and mounted with Xylene and EcoMount (EcoMount, Biocare Medical, Concord, CA).

EHV-1 qPCR positive (lung of an EHV-1 aborted fetus) and qPCR negative (TG) tissues were used as controls and an EHV-1-scrambled probe was designed targeting the same region as the target probe but with minor sequence alteration. For technical assay control, the housekeeping gene Peptidylprolyl isomerase B (PPIB) was used as positive control and the bacterial gene dihydrodipicolinate reductase (dapB) was included as negative control according to the manufacturer's protocol.

TABLE 2 | Distribution of EHV-1 genomic DNA 30 and 70 days post infection (dpi) (EHV-1 gDNA copies/10^6 cells).

dpi	Horse ID	TG	ST	RLn	MesLn
30	1,598	–	–	1.43×10^4	3.81×10^2
	1,619	1.42×10^4	–	2.19×10^3	6.35×10^1
	1,620	3.13×10^3	–	7.72×10^1	7.89×10^2
	1,651	5.41×10^3	1.98×10^5	n.d.	6.64×10^3
	1,621	2.58×10^4	–	1.40×10^3	6.85×10^2
	1,636	1.10×10^4	–	7.30×10^2	1.43×10^2
	1,638	2.03×10^3	1.80×10^4	7.53×10^2	2.81×10^2
	1,628	3.05×10^3	3.27×10^4	3.63×10^3	6.45×10^1
	1,629	4.03×10^3	5.07×10^3	1.97×10^2	–
70	905	4.19×10^1	3.82×10^4	–	–
	909	–	n.d.	n.d.	–
	913	–	–	–	1.72×10^3
	900	9.93×10^1	n.d.	–	–
	914	2.11×10^1	1.57×10^4	2.62×10^2	1.47×10^3
	908	3.51×10^1	2.55×10^3	–	7.48×10^2

–, negative; n.d., not determined; TG, trigeminal ganglia; ST, sympathetic trunk ganglia; RLn, retrophrayngeal lymph node; MesLn, mesenteric lymph node.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences in the number of EHV-1 qPCR positive samples between the two infection groups were evaluated using Fisher's Exact Test. Mann-Whitney U test was used to compare significant differences in viral load between the two infection groups. P-values < 0.05 were considered statistically significant.

RESULTS

Clinical Data, Viral Nasal Shedding, and Viremia

Animals in both infection groups showed respiratory symptoms and a classical bi-phasic fever post infection. Viral DNA amounts in nasal shedding and cell-associated viremia were detected in both groups for multiple days post infection. Overall, no significant differences were observed when comparing the two groups (data not shown) (28).

Detection of EHV-1 gB DNA and Characterization of Viral Activity

EHV-1 DNA was detected at both timepoints post infection in TG, ST, RLn, and MesLn samples. Viral distribution and -load for both groups are shown in **Table 2**. Kidney samples were consistently negative for EHV-1 DNA in both groups (data not shown). Lytic replication of EHV-1 was excluded for each EHV-1 DNA positive sample using reverse transcription PCR (RT-PCR) for gB mRNA and IHC for viral antigen. Specific gB mRNA amplification signal or viral antigen could not be detected in EHV-1 DNA positive tissue samples. Therefore, we concluded that there was no lytic EHV-1 infection in any of the

examined tissue samples. Two out of 15 retropharyngeal lymph nodes were not available for this study and for horses 70 dpi, 3/6 sympathetic trunk ganglia samples could not be included in further analysis due to the absence of neural cell bodies in the collected tissue sample.

Comparison of Viral Distribution and Copy Number at 30 and 70 dpi

When comparing samples from horses 30 dpi with samples from horses 70 dpi (**Table 2**), a reduction of the percentage of positive tissue samples could be detected for TG, RLn, and MesLn samples, but this difference was only statistically significant for RLn samples. All of the available RLn samples at day 30 pi were positive (8/8), while only 1/5 was positive at day 70 pi. There were 8/9 positive TG and MesLn samples 30 dpi compared to 4/6 and 3/6 positive samples by 70 dpi, but the difference in percentage of positive samples for this group was not statistically significant. For ST, 4/9 in the 30 dpi group were positive for EHV-1 DNA and 3/4 samples were positive at day 70 pi, but this difference was not statistically significant. The viral copy number in both TG and RLn samples was significantly higher at 30 dpi when compared to samples collected at 70 dpi (**Figures 1A,B**). No statistically significant difference in viral copy number could be observed in ST or MesLn samples (**Figures 1C,D**).

Histopathological Evaluation and Cellular Characterization

Neural tissue sections were analyzed for histopathological changes at 30 and 70 dpi. All TG and 7/12 ST had mild inflammatory infiltrations and mild satellitosis (**Figure 2A**) at both 30 and 70 dpi. In addition, Nageotte's bodies, which are a sign for neuronal degeneration (34) were detected in TG and in ST occasionally in both groups but no differences in numbers were observed when comparing time points (**Figure 2B**). No differences in severity of inflammation could be observed when comparing the two groups. Further characterization of ganglia at

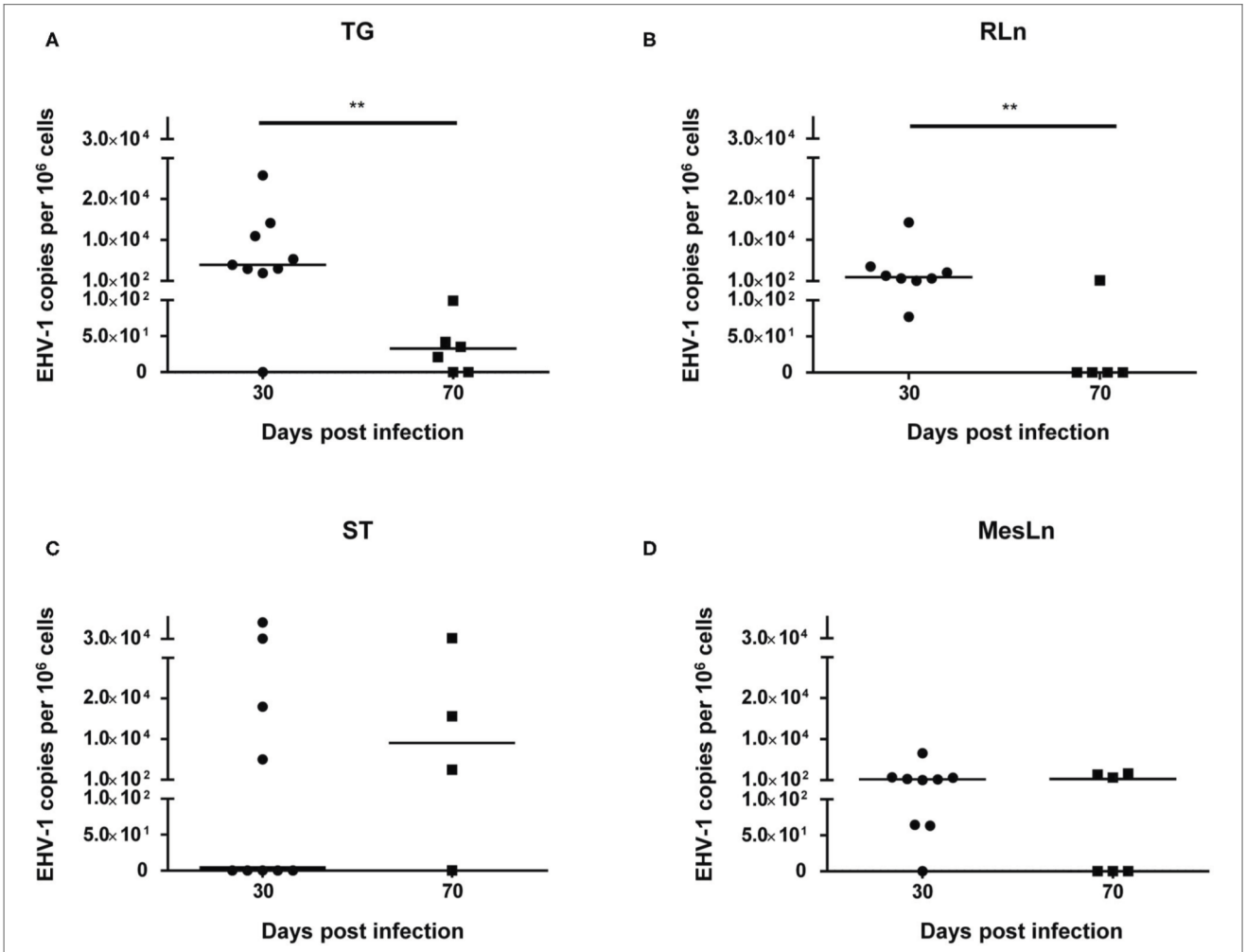

FIGURE 1 | Significant differences (**$P < 0.01$) in viral copy numbers in **(A)** trigeminal ganglia and **(B)** retropharyngeal lymph nodes at 30 dpi compared to 70 dpi. Viral copy numbers in **(C)** sympathetic trunk and **(D)** mesenteric lymph nodes at 30 dpi compared to 70 dpi. Line indicates the median.

30 dpi by IHC revealed that inflammatory infiltrates consisted of mainly CD3+ T-cells (**Figures 3A,B**). Few CD20+ B-cells were detected (**Figure 3C**).

Detection of EHV-1 gB DNA by *in situ* Hybridization

The EHV-1 gB probe detected large number of positive cells in the known EHV-1 positive control (**Figure 4A**), while the negative control probe (a.k.a. EHV-1 scrambled probe), showed no signal (**Figure 4B**). In addition, no signal was detected with the EHV-1 gB probe in a trigeminal ganglion from an EHV-1 negative horse (as determined by qPCR), which was euthanized for unrelated reasons (**Figure 4C**).

A positive signal could be detected in 6/7 TG samples and 4/7 ST samples that had tested positive for EHV-1 by qPCR. Results are shown in **Table 3** and **Figures 4D–F**. At 30 dpi, all tested TG (3/3) had a positive ISH signal in ganglion cells as well as strong concurrent labeling in non-neuronal cells surrounding ganglia (**Figures 4D,E**). Within neurons, the ISH signal was located in the nucleus and the cytoplasm. For ST at 30 dpi, 3/4 were positive by ISH with signal being detected in the cytoplasm of neuronal cell bodies and in 2/4 samples also in non-neuronal cells. At 70 dpi, an EHV-1 hybridization signal was detected in the nuclei of ganglia cell bodies from 3/4 TG, but not in non-neuronal cells (**Figure 4F**). For ST, only 1/3 samples from horses euthanized at 70 dpi had a positive signal in the cytoplasm of the neurons but was also positive in non-neuronal cells.

DISCUSSION

This study compares neuropathogenic EHV-1 strain Ab4 distribution and persistence in selected lymphoid and neural tissues collected at different time points (30 and 70 days) following experimental infection. We detected EHV-1 in non-neuronal support or interstitial cells of trigeminal and sympathetic trunk ganglia. These findings provide new insight in EHV-1 latency establishment as we propose that in addition to retrograde neuronal transport, EHV-1 uses mononuclear cells during viremia as alternative route toward latency locations.

The current understanding is that EHV-1 latency is established in the trigeminal ganglia and respiratory associated lymphoid tissue during primary respiratory tract infection and/or during cell-associated viremia (8, 9, 21). Both of our sample collection timepoints were chosen after active viral replication and viremia had ceased in all horses. Typically, this occurs around 14 days after experimental infection (1, 35–38), which was also the case in all horses sampled for this study. Furthermore, at both collection time points, neither gB (late gene) mRNA, nor viral protein could be detected, favoring the idea that EHV-1 was present in a quiescent state in both EHV-1 DNA positive tissue sets, as defined previously (15). Thirty days post infection, EHV-1 (gB) DNA was detected at high copy number in TG, ST, RLn and in MesLn samples. While TG and RLn are considered the most common latency locations described in previous studies, we also show latent EHV-1 to be present in abdominal neuronal and lymphoid tissues. This also confirms our previous results, where we showed viral distribution in various neuronal and lymphoid tissues, and not just in the vicinity of primary infection at day 70 post infection (23). These findings raise the question of how the virus is transported to abdominal locations, as a thorough understanding of EHV-1 pathogenesis is crucial to further define the balance between latency and reactivation.

The pathogenesis of EHV-1 latency establishment is currently best studied for the trigeminal ganglia but overall poorly understood. EHV-1 is thought to follow the strategy of other Alphaherpesviruses and to reach neuronal cell bodies via retrograde axonal transport (3, 8, 9, 39), following primary infection of the upper respiratory tract. Alphaherpesviruses are known to enter sensory nerve endings and are transported actively by using cellular molecular motor proteins to the ganglion cell body (40). Here, the virus translocates to the nucleus of the neuron and becomes latent after a short initial lytic replication cycle (12). While this pathway seems to be likely for EHV-1 latency establishment in neuronal tissue in the vicinity of the primary infection sites, retrograde axonal transport to abdominal sympathetic trunk ganglia would be challenging as the virus would have to travel very long distances and potentially overcome multiple synapses. VZV, a close relative to EHV-1, establishes latency in TG and dorsal root ganglia (DRG) but also infects various sensory and autonomic ganglia (41–46). Recently, there is growing evidence that VZV most likely uses infected lymphocytes as alternative route to reach ganglion neurons during cell-associated viremia (24, 25, 47).

In order to get a better understanding of the pathways and cellular interplays of EHV-1 latency establishment, we used *in situ* hybridization to determine the cellular localization of latent EHV-1 DNA in TG and ST samples. While in samples collected 30 dpi a positive EHV-1 ISH signal was noticed in the neurons of TG and ST, a strong signal was also detected in the (non-neuronal) support and interstitial cells. These findings suggest that there exists an alternative pathway for EHV-1 latency establishment, which might be similar to VZV. Numerous studies described neurons as main infected cell types in ganglia during latency for HSV-1, BoHV-1 and VZV (48–51). However, only few studies tried to determine latent EHV-1 localization in ganglia. Baxi et al. claimed latent EHV-1 in trigeminal neurons by using RNA *in situ* hybridization to detect potential latency associated transcripts (9). Neurons represent a stable cell population where the virus is able to hide lifelong in the host (52, 53) without being detected by the immune system. The viral DNA translocates to the nucleus of the infected neuron and persists in a circular episome, not integrated into the host DNA. In addition to the nuclear localization of viral DNA, we could also detect ISH signal frequently in the cytoplasm of TG and ST neurons. This cytoplasmatic signal could not be detected in samples with the EHV-1 scrambled (negative) probe or with the technical assay control probe provided by the manufacturer. Therefore, unspecific probe binding seems unlikely. Similarly, cytoplasmatic signal of VZV DNA could be found in neurons, where the virus reactivates, and lytic infection occurs (54). IHC for lytic protein and for late gene (gB) mRNA could not be detected in the

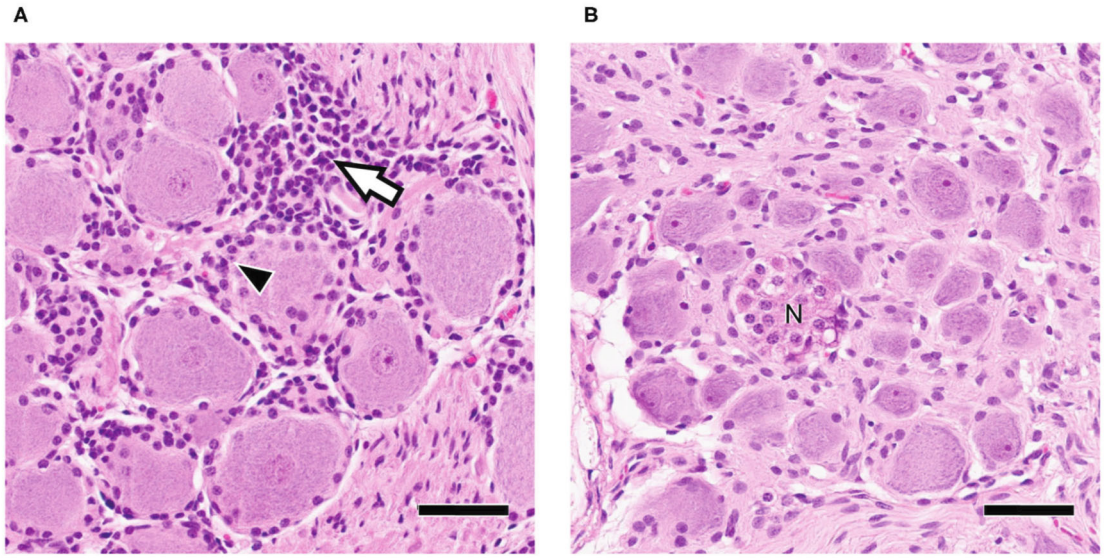

FIGURE 2 | (A) Trigeminal Ganglion 30 dpi: Moderate infiltration of inflammatory cells (white arrow) and satellitosis (arrowhead); **(B)** Sympathetic trunk ganglion 30 dpi: Nageotte's body (N) as sign of neuronal degeneration; Bars = 50 μm.

TABLE 3 | Cellular visualization of EHV-1 DNA in trigeminal ganglia and sympathetic trunk ganglia using *in situ* hybridization.

	30 dpi				70 dpi			
			Viral localization (ISH)				Viral localization (ISH)	
Tissue	Horse ID	Viral load gDNA gB	Neuron	Non-neuronal cells	Horse ID	Viral load gDNA gB	Neuron	Non-neuronal cells
TG	1,651	5.41×10^3	+	+	905	4.19×10^1	+	–
	1,621	2.58×10^4	+	+	900	9.93×10^1	+	–
	1,628	3.05×10^3	+	+	914	2.11×10^1	+	–
					908	3.51×10^1	–	–
ST	1,651	1.98×10^5	+	+	905	3.82×10^4	+	+
	1,638	1.80×10^4	–	–	914	1.57×10^4	–	–
	1,628	3.27×10^4	+	–	908	2.55×10^3	–	–
	1,629	5.07×10^3	+	+				

Viral load expressed in EHV-1 gDNA copies/10^6 cells based on qPCR.
dpi, days post infection; ISH, in situ hybridization; TG, trigeminal ganglia; ST, sympathetic trunk ganglia; +, positive ISH signal; –, no ISH signal.

present study, and thus we exclude an overall active infection. However, latent infection is not exclusively silent and occasional transcription of lytic genes is described for HSV-1 and VZV latent infection at low levels (55). Therefore, we might have detected low levels of viral DNA or gB mRNA in the cytoplasm in the present study. This phenomenon needs further investigation, as we did not detect viral gB mRNA via RTqPCR and assume an overall higher sensitivity for the RTqPCR assay. However, RNAScope® assay has been described to be highly sensitive due to the unique selective amplification scheme in combination with a serial target probe design (31, 56).

To the best of our knowledge, EHV-1 has not been detected in non-neuronal (support or interstitial) cells within ganglia previously. Non-neuronal cells are mainly composed of glial cells of the peripheral nervous system (satellite glial cells) and mononuclear cells. Each neuronal cell body is enveloped by several satellite cells forming a functional unit. This is important for the regulation of extracellular chemical conditions of the neuron and providing physical support (34, 57). Furthermore, satellite cells act as antigen presenting cells during infections, attract T-cells and can interact with the neuron by signaling processes (57, 58). In acute human herpesvirus infections, the barrier formation of satellite cells around the neurons seems to protect the neuron from virus spread (57). However, it has been shown that this barrier is a flexible wall, where macrophages still can penetrate (59) and infiltrating T-cells tend to come in close contact to the neuron-satellite sheath (16). Furthermore, satellite cells are infected during productive VZV infection and

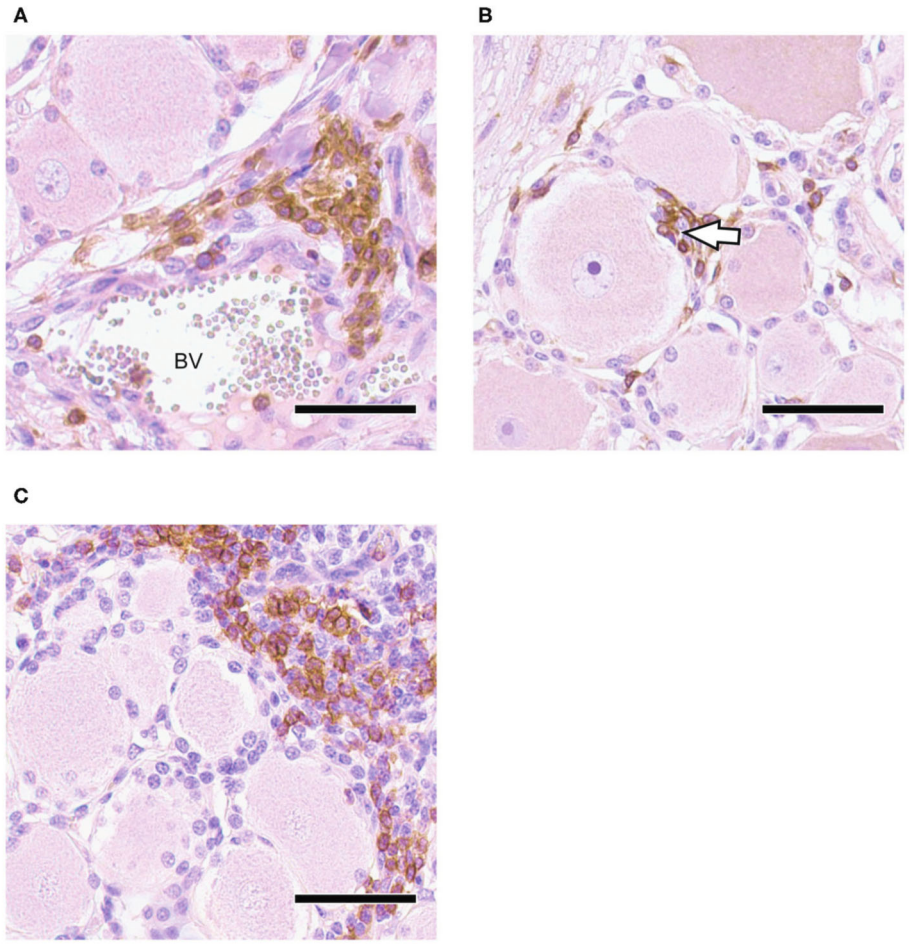

FIGURE 3 | Trigeminal Ganglion 30 dpi, IHC for CD3 and CD20, DAB with Mayer's hemalum counterstaining: **(A)** CD3+ T-cell infiltration in vicinity of blood vessel (BV); **(B)** CD3+ T-cells penetrate neuron-satellite sheet (arrowhead); **(C)** localized infiltrates of CD20+ B-cells associated with non-labeling T-cells; bars = 50 μm.

seem to transfer virus to neurons by cell-to-cell fusions *in vivo* mouse models (60). To further differentiate lymphocytes from satellite cells in the present study, we used CD3+, CD20+ and S-100 IHC staining. We could show that besides local CD3+ and CD20+ lymphocytic inflammatory infiltrates, CD3+ T-cells also associate with satellite cells and tend to squeeze into the satellite sheath around neurons. This phenomenon was not detected for CD20+ B-cells. These results suggest that the EHV-1 ISH signal from non-neuronal cells surrounding neurons in the current study, might mainly attributed to T-cells and satellite cells.

T-cell tropism is well described for EHV-1 and suggested to be a key strategy for immune evasion and dissemination in the host (4, 7, 19, 21, 61, 62). During primary viral infection, EHV-1 seems to initially infect monocytes (4, 5, 63), while T-cells are the preferred cell type during viremia (6, 7, 61) to transport the virus to secondary target organs like the CNS or uterus. Among the circulating EHV-1 strains, the neurovirulent strain Ab4 has been shown to infect immune cells efficiently and rapidly following replication in the respiratory epithelium and subsequently result in a longer viremia with higher viral loads compared to non-neuropathogenic strain counterparts (5, 36, 64). It has been suggested that viral replication is not productive or restricted in carrier cells (65) or viral capsids are accumulated in the nucleus of the carrier cell, but viral egress is hampered until the endothelium of a secondary target organ is reached (7). This has also been described for VZV (11) and it has been shown that migration markers on T-cells are still intact following VZV infection which enables the virus to be transported to target sites (66). Interestingly, while there was still distinct EHV-1 ISH signal in the nucleus of the trigeminal ganglia at 70 dpi, no signal could be detected in non-neuronal cells at this time point. Moreover, there was a significant decrease in viral load when comparing TG 30 and 70 dpi. For HSV-1 infections, it has been shown that the number of T-cells in ganglia declines continuously between days 21 and 92 p.i. (16). Apoptosis of infected lymphocytes and degeneration of some neurons could be reason for the decrease of viral load in ganglia in the present study. However, it might

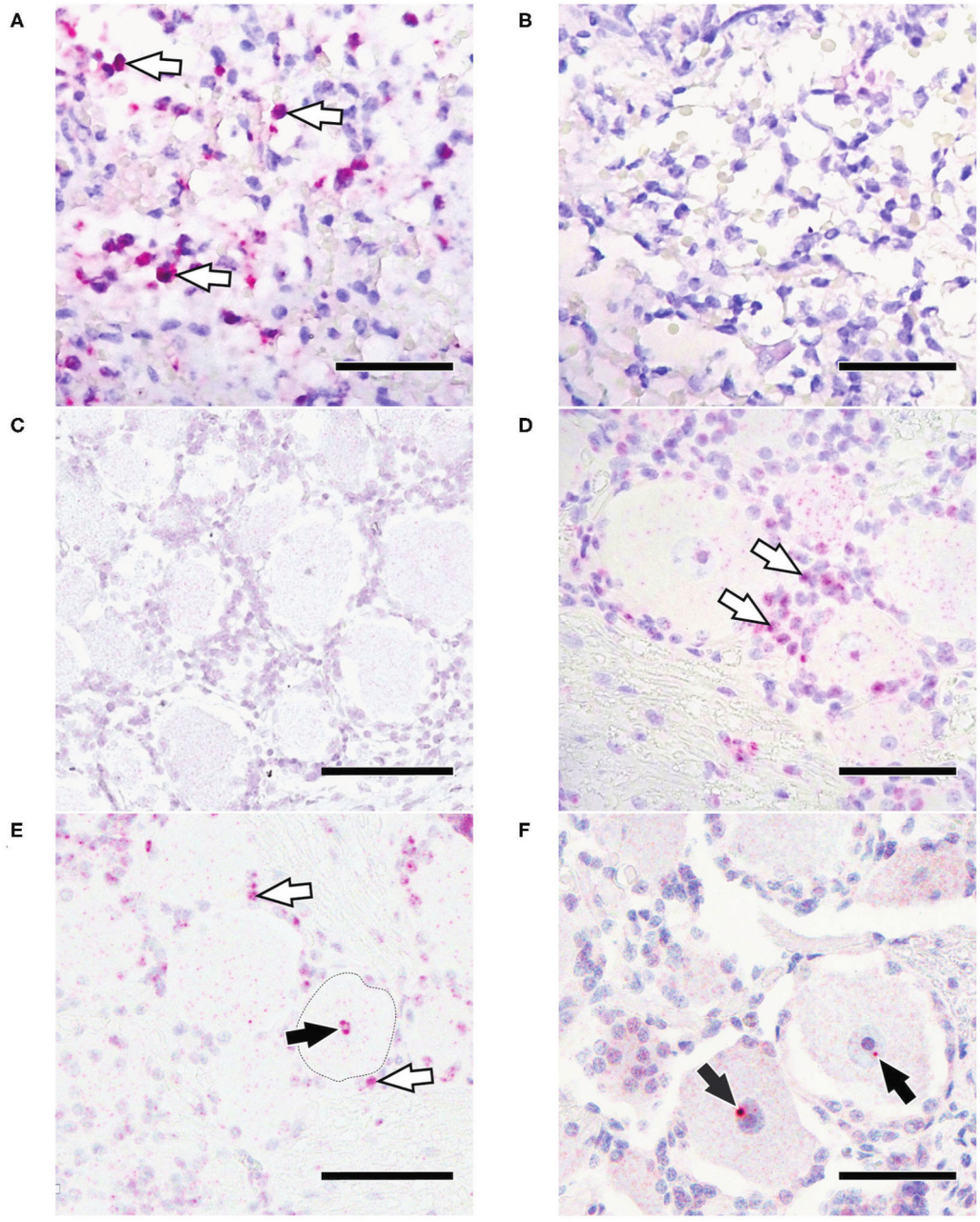

FIGURE 4 | *In situ* hybridization for EHV-1 gB. **(A)** Positive control with positive labeling (arrowheads); **(B)** Positive control tissue with no signal using an EHV-1 scrambled probe; **(C)** TG from a EHV-1 qPCR negative horse lacks positive ISH signal; **(D)** EHV-1 qPCR positive TG 30 dpi with strong signal in non-neuronal cells (arrowheads); **(E)** EHV-1 qPCR positive TG 30 dpi with strong in non-neuronal cells (white arrowheads) and nucleus of the neuron (black arrowhead); **(F)** EHV-1 positive TG 70 dpi with positive signal in the nucleus of the neuron (arrowhead). Bars = 50 μm.

also be evidence for the existence of a persistently infected EHV-1 memory T-cell pool, where T-cells are retrieved from ganglia, (re-)circulate in the blood and home to lymphoid tissue, where the virus remains stationary latent and/or starts new re-circulation. However, in our previous study no or only very low amounts of EHV-1 DNA was present in peripheral blood mononuclear cells (PBMC) at day 70 pi (unpublished data). This may imply that the majority of latently infected mononuclear cells are in tissue sites and it is challenging to detect very low amounts of silent virus in the circulating PBMCs using our methods as the collection time point represents a moment in time in the circulating latent infected cell pool. Further studies involving methods with higher

sensitivity, e.g., Next Generation Sequencing, are needed to finally define the role and type of mononuclear cells in EHV-1 latency establishment.

Furthermore, latency in lymphatic tissue has been described as an advantage for the virus, as infected T-cells can enter rapidly the blood circulation to be transported to primary infection sites for replication, once reactivation occurs (19). In the present study, we report EHV-1 DNA in RLn and MesLn at 30 and 70 dpi. Respiratory associated lymphoid tissue has been previously described as latency location for EHV-1 (19–21, 67) and EHV-1 DNA could also be detected in MesLn and in the spleen (19, 23, 67). When comparing results 30 and 70 dpi, it was somewhat surprising, that the number of positive retropharyngeal lymph node samples decreased by 80% over time. While apoptosis of some EHV-1 infected T-cells is likely contributing to this decrease, positive T-cells could also migrate from lymph nodes toward neurons and/or other lymphatic tissue and therefore deplete positive signal in lymph nodes over time. A neurotropic re-circulation could also explain the findings in sympathetic trunk ganglia 70 dpi, where positive EHV-1 non-neuronal cells could still be detected.

In addition, while EHV-1 strain Ab4 is known to strongly dysregulate the host immune response by increasing inflammation and resulting in a high quantity of circulating infected cells, other EHV-1 strains may act differently in their immune evasive strategies (7, 68). Therefore, latency establishment and reactivation also may be strain dependent and more research is necessary to reveal the latently infected host pool in the populations worldwide.

Taken together, the present study confirms previous findings, identifying EHV-1 as both neurotropic and lymphotropic. EHV-1 most likely uses retrograde axonal transport as a direct pathway from the upper respiratory epithelium toward the TG, where latency is established in the nucleus of the neuron after a short replication cycle. Furthermore, we hypothesize, that T-cell tropism assists EHV-1 in its neurotropism and additionally provides latency establishment in lymphatic tissues other than respiratory associated lymphoid tissue. The navigation to neuronal structures throughout the body may enable the virus to establish latency in numerous ganglia, but further studies in random horse populations are required to elucidate if other neuronal structures than the TG can be repeatedly confirmed as alternative latency locations.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Michigan State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KG and SS carried out the experiments and data analysis. KG wrote the manuscript with support from GSH and LG. GSH developed the experimental design of the horse infection experiment and conducted the clinical study. MK and DS conducted the necropsy and sample collection and GSH, KG, and LG contributed to sample collection. KM contributed to interpretation of histopathological and *in situ* hybridization results and performed figure formatting. FL and JS helped with the design and performance of the *in situ* hybridization experiment. FL, JS, and RF contributed to the interpretation of the results. LG supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

V. CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation aims to further the understanding of EHV-1 latency pathogenesis in the natural host. In two different study set-ups we detected presumably latent EHV-1 in a variety of lymphatic and neural tissue samples at day 70 post experimental infection (pre-study) (SAMOILOWA, 2019), and found differences in viral distribution, load and cellular localization when compared to a time-point closer to viremia (30 dpi) (GIESSLER et al., 2020a). We overall conclude that EHV-1 latency is dynamic over time in its maintenance. Therefore, analyzing post-mortem tissue samples can only provide a "snapshot" of this complex state (DEPLEDGE et al., 2018). Most importantly, we could show that EHV-1 is present in non-neuronal cells within ganglia during latency, which is most likely attributed to infected T-cells and/or satellite glial cells. This work therefore refines the current knowledge of EHV-1 latency establishment in ganglia by hypothesizing that EHV-1 can use mononuclear cells during viremia as an additional hematogenous route towards neural latency locations.

A more detailed characterization of the infected non-neuronal cell pool is now required to further investigate this hypothesis. For SVV, colocalization of virus and cell marker in a dual-immunofluorescence assay could prove the presence of infected T-cells adjacent to neurons (OUWENDIJK et al., 2013). Importantly, in this study the authors investigated acute infection and therefore used SVV nucleocapsid protein staining together with T-cell antibody staining for visualization (OUWENDIJK et al., 2013). During latency, where viral protein is usually not detectable, successful colocalization requires a combination of ISH (detection of viral genome or LAT) and IHC (detection of cell-type antigen). For HSV-1, this technique revealed that HSV-1 targets specific subsets of neurons for latency establishment by showing that LAT positive neurons (ISH) are predominantly characterized by a specific neuronal cell marker (IHC) (YANG et al., 2000). This method benefits from the fact that LAT is abundantly expressed during HSV-1 latency and readily detectable by ISH or RT-PCR (STEVENS et al., 1987; FRASER et al., 1992). Currently, there is a lack of a reliable marker (e.g. LAT) for EHV-1 latency and diagnostic approaches are somewhat limited as viral DNA is present in very low quantities during latency (MAHALINGAM et al., 1999; DUNOWSKA et al., 2015; KENNEDY et al., 2015; DEPLEDGE et al., 2018). This challenges diagnostic sensitivity (MAHALINGAM et al., 1999; DUNOWSKA et al., 2015) and was probably the reason why first attempts to identify EHV-1 infected non-

neuronal cells through a combined ISH (EHV-1 DNA)-IHC (CD3+ T-cells) double-staining assay failed in our laboratory.

Future approaches that aim to further define EHV-1 latency may therefore consider including high sensitivity methodology like Deep Whole Genome Sequencing and/or RNA sequencing. RNA sequencing is a powerful tool that finally allowed the discovery of the VZV LAT (DEPLEDGE et al., 2018). However, the direct sequencing of whole clinical or post-mortem samples results in mixed host and viral nucleic acid sequences, with a significantly lower proportion of the latter (DEPLEDGE et al., 2011). The attempt to determine low abundant viral DNA/RNA within mixed host/virus deep sequencing results can be metaphorically speaking like searching for a needle in a haystack. It was not until the introduction of "target enrichment" followed by deep sequencing, before the VZV LAT could be detected (DEPLEDGE et al., 2018). In this approach, RNA "bait" probes were designed to hybridize with the VZV genome and separate viral nucleic acids prior to sequencing (DEPLEDGE et al., 2018). In a follow-up study of our recent findings we aim to further analyze EHV-1 transcripts during latency in neural (TG, ST) and lymphatic (RLn, MesLn) tissue samples at day 30 pi, using target (EHV-1) enriched RNA sequencing as previously described for VZV (DEPLEDGE et al., 2018).

Defining transcripts that are consistently present during EHV-1 latency would provide us with a latency marker and allow to ultimately confirm abdominal neural and lymphatic latency locations for EHV-1. Furthermore, it would be interesting to investigate potential differences in the EHV-1 transcriptome during latency depending on the infected cell type (neuron vs. PBMC). It might be possible that viral gene expression and depth of latency differs between neurons and PBMCs as a result of the adaptation to the host cell environment. The capacity of EHV-1 to strongly adapt to PBMCs has already been described during acute EHV-1 infection (LAVAL et al., 2015; POELAERT et al., 2019). In contrast to the lytic infection in epithelial cells, PBMCs are infected in an arrested manner, where replication is hampered (in monocytes) (LAVAL et al., 2015) or viral capsids are accumulated (in T-cells) but "on standby" and not released until the target cells are reached (POELAERT et al., 2019). These strategies enable the virus to be transported effectively throughout the host body during viremia (LAVAL et al., 2015; POELAERT et al., 2019). The viral state in PBMCs may therefore hold an exceptional position during both acute and latent infection.

On the host side of the balance of latency, the immune response is an important factor that

influences the depth of viral transcriptional silence and therefore needs to be further investigated in future studies. We can speculate that similar to what is known in HSV-1 and VZV latency, T_{RM} and SGC may inhibit viral gene transcription by expressing cytokines (VAN VELZEN et al., 2009; EGAN et al., 2013; OUWENDIJK et al., 2016), but this has yet to be confirmed for EHV-1 latency. Interestingly, virus reactivation occurs more frequently in older individuals, which is probably linked to age-related changes in the immune system (GRINDE, 2013). For example, it has been shown in humans that the proportion of regulatory T-cells increase in the elderly (LAGES et al., 2008; VUKMANOVIC-STEJIC et al., 2015), which was correlated to the reactivation of chronic persistent infectious disease (LAGES et al., 2008). The higher counts of regulatory T-cells lead to a decrease and functional inhibition of CD8+ T-cells in ganglia and therefore promote viral lytic gene transcription and reactivation (YU et al., 2018). Our research group is currently investigating the immune response of old and young horses following EHV-1 infection with the aim to determine host factors contributing to the pathogenesis of neurological disease. Using samples of this study setting, it would be interesting to characterize and compare the T-cell population in ganglia of old horses and young horses. IHC staining for regulatory T-cell (FoxP3+), CD8+ T-cell and CD4+ T-cell marker may provide valuable information on T-cell type/counts in correlation to viral load and transcriptional activity.

While latency studies in the natural host are inevitable to involve the interaction of the host immune system, future studies may additionally consider *in vitro* approaches. An interesting model has been recently described for HSV-1 and PsRV latency establishment in ganglia explants (RICHARDS et al., 2017). In this study, viral mutants were established with an impaired ability for retrograde axonal transport through a mutation of a viral transport complex protein. This eliminated latency establishment via neuronal pathway *in vitro* and *in vivo* (RICHARDS et al., 2017; PICKARD et al., 2020). As primary infection and replication remained potent the authors suggest that these mutants may be promising vaccine candidates for HSV-1, PsRV and other Alphaherpesviruses like EHV-1 (RICHARDS et al., 2017; PICKARD et al., 2020). Theoretically, similar EHV-1 mutants with impaired ability for infecting ganglia via neuronal pathway may be an interesting proof of concept approach for investigating neuronal infection via circulating PBMCs, using ganglia explant models as previously described (RICHARDS et al., 2017).

Notably, all the conclusions of this dissertation are based on experimental infection studies with the neuropathogenic strain EHV-1 Ab4. Infections with EHV-1 Ab4 are known to result in a high magnitude of infected PBMCs and prolonged duration of viremia (ALLEN,

2008; HOLZ et al., 2017). Therefore, it is important to determine whether T-cell tropism during latency is a consistent strategy among other circulating EHV-1 strains and whether abdominal latency locations can be confirmed in random horse populations. For example, this could be investigated by tissue sample collection at slaughter and subsequent latency diagnosis. As we speculate that the EHV-1 DNA burden during latency might be below the detection limits of the recently applied methodology of qPCR, it might be worthwhile to consider the potential advantages of technological advancements in the field of PCR. Digital PCR (dPCR) is a new emerging PCR technology in clinical virology diagnostics (SALIPANTE & JEROME, 2019). Like qPCR, dPCR is based on primer/probe binding on target sequences followed by amplification and detection by fluorescence. However, for dPCR, the sample is not assayed in a homogenous solution but divided into many separate sub-reactions, for example by oil microdroplets (droplet digital PCR) (SEDLAK et al., 2014). This guarantees an isolation of few target sequences in a single micro-reaction which results in higher target concentration. Therefore, dPCR is thought to outperform qPCR in precision and is less affected by PCR inhibitors that are present in certain (tissue) samples (QUAN et al., 2018; SALIPANTE & JEROME, 2019). Following analysis of the micro-reactions the absolute quantification of target concentration can be calculated without extrapolation to a standard curve which simplifies quantification especially for rare target sequences. One limitation of dPCR is the smaller total reaction volume compared to qPCR, resulting consequently in a smaller input volume of sample (QUAN et al., 2018). This might lead in some assays to a lower sensitivity of dPCR (QUAN et al., 2018). However, the detection sensitivity of dPCR is controversially discussed in literature (KOEPFLI et al., 2016; VERHAEGEN et al., 2016; SALIPANTE & JEROME, 2019) but seems overall to be comparable to qPCR (KELLEY et al., 2013). As dPCR has been used in HSV-1 research to detect latent HSV-1 genome in ganglia (AUBERT et al., 2014; AUBERT et al., 2016; BAIRD et al., 2018), it might be a promising approach for EHV-1 latency diagnosis. After all, it is critical to evaluate the potential advantages of dPCR for each specific assay (and sample type), as it is currently an expensive alternative to qPCR.

Overall, this dissertation provides novel insights in EHV-1 latency pathogenesis, draws conclusions on EHV-1 latency regulation in ganglia and opens up interesting directions for future studies. It still must be determined whether EHV-1 uses its lymphotropism during viremia for reaching as many safe neural latency harbors as possible or whether this happens “by accident” while travelling to the CNS/uterus endothelium. However, from an evolutionary point of view, creating multiple locations for hiding its genome may be of greater

benefit for EHV-1 than causing disease manifestation and potential death of the host. From this perspective, one might speculate that EHV-1 causes fatal disease manifestation following viremia “by (dreadful) accident” while paving its way for latency establishment and actually aiming for its persistence in the host and thus in the equine population.

VI. SUMMARY

Characterizing viral distribution, viral load and cell tropism during early latent Equid Herpesvirus 1 (EHV-1) infection: novel insights into EHV-1 latency pathogenesis

Equid Herpesvirus 1 (EHV-1) is an important ubiquitous Alphaherpesvirus that infects horses worldwide. It causes respiratory disease, equine herpesvirus myeloencephalopathy (EHM), abortion and neonatal foal death. Primary EHV-1 infections of the upper respiratory tract epithelium (URT) typically result in a peripheral blood mononuclear cell-associated viremia. This transports the virus through the body toward the vascular endothelium of the central nervous system (CNS) or the pregnant uterus, where it can lead to severe disease manifestations (EHM, abortion). During or shortly after primary infection, EHV-1 also establishes a chronic persistent infection in the host. This important strategy of all herpesviruses is called latency and symbolizes a non-productive state of infection which is characterized by repression of viral lytic gene expression. Latency ensures the viral persistence in the host population, as periodical reactivation events can lead to viral spread and ultimately to new outbreaks. As there is currently a lack of fully protective EHV-1 vaccines, understanding the pathogenesis and maintenance of EHV-1 latency becomes of utmost importance for the development of potential defense strategies. As the respiratory tract is the primary site of infection, it is thought that EHV-1 infects sensory nerve endings during initial infection and subsequently establishes latency in the trigeminal ganglia (TG). Moreover, respiratory tract-associated lymphoid tissue (RALT) and peripheral blood mononuclear cells (PBMCs) have been described as additional latency locations. Beyond that, our research group recently proposed that EHV-1 can persist additionally in a variety of sensory, sympathetic and parasympathetic ganglia of the head, neck and abdomen as well as in abdominal lymphoid tissue. Therefore, the present study aims to follow-up and to further characterize EHV-1 transportation toward latency locations and its cell tropism during latency *in vivo.*

Two separate groups of yearling horses were experimentally infected intranasally with EHV-1, strain Ab4, and euthanized 30 days post infection (dpi), (n = 9) or 70 dpi (n = 6), respectively. During necropsy, TG, sympathetic trunk (ST), retropharyngeal and mesenteric lymph nodes (RLn, MesLn) and kidney samples (control) were collected. Viral DNA load was determined by quantitative PCR (qPCR) in the respective tissue samples and results were compared between the two infection groups (30 dpi, 70 dpi). Furthermore, a

given sample was defined as latently infected when EHV-1 DNA was detected (via qPCR), while the EHV-1 late gene glycoprotein B reverse transcriptase PCR (RT-PCR) and immunohistochemistry (IHC) results for viral protein were consistently negative. Neural tissue samples were further evaluated for the presence of histopathological changes and inflammatory infiltrates were characterized by IHC staining against CD3 (T-lymphocytes, (T-cell)) and CD20 (B-lymphocytes, (B-cell) antigen and differentiated from satellite glial cells by S-100 IHC staining. Finally, selected EHV-1 DNA positive neural tissue samples were further analyzed by *in situ* hybridization (ISH, RNAScope®) to determine the cellular localization of persistent EHV-1.

EHV-1 DNA was detected in TG, ST, RLn, and MesLn samples in horses 30 and 70 dpi. Lytic infection was excluded in the present study as neither of the samples were positive for EHV-1 late gene gB mRNA or viral protein. When comparing horses 30 and 70 dpi, a decrease in the number of positive TG, RLn and MesLn samples was observed. In addition, the viral copy number in TG and RLn declined significantly from 30 to 70 dpi. Histopathological evaluation revealed mild inflammation in all neural tissue samples and IHC identified CD3+ T-cells as the main proportion of inflammatory infiltrates adjacent to neuronal cell bodies. Notably, EHV-1 ISH signal in ganglia 30 dpi was not only located in the neurons of TG and ST, but also in non-neuronal support/interstitial cells surrounding the neuron. In contrast, the neuron was the sole cell type showing distinct EHV-1 signal in TG 70 dpi. For ST samples 70 dpi, EHV-1 ISH signal was sporadically detected in the cytoplasm of the neurons but also in adjacent non-neuronal cells.

Overall, this dissertation reports consistent detection of latent EHV-1 DNA in a variety of lymphatic and neural tissue samples and differences in viral distribution, viral load and cellular localization when evaluated at two different time-points during early EHV-1 latency. The differences in findings over time highlight that latency is a dynamic state and the analysis of post-mortem tissue samples may only allow us to make conclusions for this particular “snapshot” of latency. Most importantly, the distinct non-neuronal cellular localization proposes that EHV-1 may use T-cells as cellular vehicle during viremia toward potential latency locations. This may symbolize an additional, hematogenous route for latency establishment besides the well described neuronal pathway and highlights a striking similarity between EHV-1 and its close relative human pathogen Varicella Zoster Virus.

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VII. ZUSAMMENFASSUNG

Virusverteilung, Viruslast und zelluläre Lokalisation im Pferd während der frühen Latenzphase einer Equiden Herpesvirus 1 (EHV-1) Infektion: neue Erkenntnisse zur Pathogenese der EHV-1 Latenz

Infektionen mit dem Equiden Herpesvirus 1 (EHV-1) führen zu respiratorischen Erkrankungen, Rückenmarkserkrankungen (Equide Herpesvirusmyeloenzephalopathie (EHM)) und Aborten und stellen durch die hohe Virus Prävalenz innerhalb der Familie der *Equidae* weltweit eine ernsthafte Bedrohung für domestizierte sowie wildlebende Einhufer dar. Während der primären Infektion der oberen Atemwege wird das Virus nach einer initialen Vermehrung in lokale Lymphknoten transportiert und gelangt von dort anschließend in die Blutbahn. Auf diesem Weg der Virämie kann sich das Virus im gesamten Körper ausbreiten und Gefäßendothelien von Rückenmark oder Uterus erreichen. Neben diesem akuten Infektionsverlauf verfügt EHV-1 über die Fähigkeit zur Ausbildung einer chronisch persistierenden Infektion. Daher stehen am Anfang oftmals verheerender EHV-1 Ausbrüche meist reaktivierende „Reservoir-Tiere“, die das Virus nach überstandener Infektion in sich beherbergen. Diese Schlüsselstrategie aller Herpesviren, nach initialer Infektion in bestimmten Geweben (üblicherweise Zellen des Immun- oder des Nervensystems) in einen Ruhezustand zu fallen und weitgehend unentdeckt vom Immunsystem des Wirtes auf unbestimmte Zeit zu überdauern, wird als Latenz bezeichnet. Während dieser „Schlummerphase“ persistiert das virale Genom im Kern infizierter Zellen, ohne sich zu replizieren und ohne sogenannte lytische Gene zu exprimieren, die für die Produktion eines vollwertigen, infektiösen Virions notwendig sind.

Es ist wenig über die Umstände und Faktoren bekannt, die den Balanceakt der Latenz beeinflussen und in Richtung der lytischen Genexpression und damit der Reaktivierung verschieben. Da aktuell verfügbare Impfstoffe eine Infektion und Erkrankung nur mäßig effektiv zu verhindern vermögen, ist es essenziell, die Pathogenese der Latenz besser verstehen zu lernen, um künftig Abwehrstrategien entwickeln zu können. Basierend auf den Erkenntnissen früher Studien in den 1990er Jahren, wird bis heute vermutet, dass EHV-1 im Zuge der initialen Infektion die Axone des *Nervus trigeminus* infiziert und Neurone des Ganglion trigeminale (TG) sowie zusätzlich regionale Lymphknoten der oberen Atemwege als Rückzugsgebiete für die Phase der Latenz heranzieht. Unsere Forschungsgruppe konnte vor Kurzem ergänzend aufweisen, dass EHV-1 vermutlich in weitaus mehr

neuralen und lymphatischen Geweben persistiert als bisher angenommen (SAMOILOWA, 2019): Zu einem Zeitpunkt von 70 Tagen nach experimenteller Infektion, konnte EHV-1 DNA in einer Vielzahl von sensorischen, sympathischen und parasympathischen Ganglien sowie in respiratorischen und abdominalen lymphatischen Geweben nachgewiesen werden.

Um die Pathogenese der latenten EHV-1 Infektion besser verstehen zu können, war das Ziel der vorliegenden Dissertation, das virale Verteilungsmuster, die Viruslast und die zelluläre Lokalisation zu zwei verschiedenen Infektionszeitpunkten zu vergleichen. Hierfür wurden zwei Gruppen von Pferden mit EHV-1 Wildtyp Stamm Ab4 intranasal infiziert und 30 (n = 9) respektive 70 (n = 6) Tage nach Infektion euthanasiert. Während der Sektion wurden TG, *Truncus sympathicus* (Grenzstrang) (ST), *Lymphonodi (Lnn.) retropharyngeales* (RLn) und *Lnn. mesenteriales* (MesLn) und Niere (als Kontrollorgan) entnommen. Anschließend wurden die Gewebe beider Infektionsgruppen mittels quantitativer PCR (qPCR) auf EHV-1 DNA untersucht. Eine Probe wurde als latent infiziert definiert, wenn EHV-1 DNA nachgewiesen, jedoch gleichzeitig das Auftreten von sowohl lytischer (Glykoprotein B (gB)) mRNA (mittels Reverse Transkriptase PCR) als auch lytischem Protein (gB) (mittels Immunhistochemie (IHC)) ausgeschlossen wurde. Des Weiteren wurden Nervengewebeproben auf histopathologische Veränderungen untersucht und entzündliche Infiltrate mithilfe von IHC Färbung gegen CD3-Antigen (T-Lymphozyten (T-Zellen)) und CD20-Antigen (B-Lymphozyten (B-Zellen)) weiter charakterisiert und von neuronalen Stützzellen (Satellitenzellen) (IHC gegen S-100 Antigen) abgegrenzt. Um schließlich die zelluläre Lokalisation von persistierendem EHV-1 zu bestimmen, wurden ausgewählte EHV-1 DNA positive Nervengewebeproben zusätzlich mittels *In-Situ*-Hybridisierung (ISH, RNAScope ®) analysiert.

Sowohl 30 als auch 70 Tage nach Infektion konnte EHV-1 DNA in TG-, ST-, RLn- und MesLn- Proben nachgewiesen werden. Zudem wurde eine lytische Infektion nach gegebener Definition konsistent ausgeschlossen. Die Anzahl EHV-1 positiver TG-, RLn- und MesLn- Proben nahm über den Zeitraum von 30 auf 70 Tage nach Infektion ab. Zusätzlich wurde ein signifikanter Rückgang der Viruslast in TG- und RLn-Proben von 30 auf 70 Tage nach Infektion beobachtet. Die histopathologische Auswertung ergab milde entzündliche Veränderungen in allen Nervengewebeproben, wobei CD3+ T-Zellen den Hauptanteil der entzündlichen Infiltrate ausmachten und häufig unmittelbar angrenzend zu neuronalen Zellkörpern zu finden waren. Insbesondere wurde virale DNA zum Zeitpunkt 30 Tage nach Infektion mittels ISH nicht nur in den Nervenzellkörpern eines Ganglions

(TG und ST) nachgewiesen, sondern auch in den umliegenden nicht-neuronalen Zellen (Satellitenzellen und/oder Lymphozyten). In den TG-Proben 70 Tage nach Infektion war das EHV-1 Signal dagegen nur im neuronalen Zellkörper nachzuweisen. Bei ST-Proben zu diesem Zeitpunkt konnte nur vereinzelt positives Signal im neuronalen Zytoplasma sowie in den angrenzenden nicht-neuronalen Zellen festgestellt werden.

Zusammenfassend beschreibt die vorliegende Dissertation einen konsistenten Nachweis latenter EHV-1 DNA in einer Vielzahl von neuralen und lymphatischen Geweben, einen Unterschied in Viruslast und Verteilung zwischen beiden Infektionsgruppen sowie ein eindeutiges EHV-1 Signal in Satellitenzellen/Lymphozyten angrenzend zu neuronalen Zellkörpern. Diese Ergebnisse lassen schlussfolgern, dass die Latenz ein durchaus dynamisches Stadium darstellt und die post-mortem Analyse von Gewebeproben daher nur eine Momentaufnahme ermöglichen kann. Gleichwohl deutet der hier dargebotene Einblick in die EHV-1 Latenz darauf hin, dass EHV-1 möglicherweise neben dem neuronalen Transport auch den lymphozytären Transport während der Virämie nutzt, um weitere Latenz-Lokalisationen zu erreichen. Damit würde die Pathogenese der latenten EHV-1 Infektion weniger, wie bisher angenommen, derjenigen des Herpes Simplex Virus 1 entsprechen, sondern vielmehr derjenigen des Varizella Zoster Virus.

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