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Novel insights into the roles of glycoprotein B and pUS3 during equine herpesvirus pathogenesis

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Abbreviations

AAV2	adeno-associated virus type 2
ACM	astrocyte-conditioned medium
BAC	bacterial artificial chromosome
BHV-1	bovine herpesvirus type 1
CCD	charge-coupled-device
Chlo	chloropormazine
CHO	Chinese hamster ovary
CNS	central nervous system
CrFK	Crandell feline kidney
CrFK	Crandell feline kidney
DIP	defective interfering particles
DMEM	Dulbecco's modified Eagle's medium
dUTPase	deoxyuridine triphosphatase
Dyn	dynasore
E	early
EBV	Epstein–Barr virus
ED	equine dermal
EGFP	enhanced green fluorescent protein
EHM	equine herpesvirus myeloencephalopathy
EHV-1	equine herpesvirus type 1
EHV-4	equine herpesvirus type 4
EICPO	EHV-1 Infected Cell Polypeptide 0
Endo H	endoglycosidase H
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinases
ETIF	equine α-trans-inducing factor
FHK	fetal horse kidney
gB	glycoprotein B
gB1	EHV-1 gB
gB4	EHV-4 gB
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
Gen	genistein
gG	glycoprotein G
gH	glycoprotein H
gI	glycoprotein I
gJ	glycoprotein J
gM	glycoprotein M
gN	glycoprotein N
gp2	glycoprotein 2
HCMV	human cytomegalovirus
HeLa	Henrietta Lacks
HSV-1	herpes simplex virus type 1

IE	immediate early
Ig	Immunoglobulin
IMDM	Iscove's modified Dulbecco's medium
INM	inner nuclear membrane
IRs	inverted repeats
L	late
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAC1	macrophage receptor 1
MAG	myelin-associated glycoprotein
MDCK	Madin-Darby canine kidney
MDV	Marek's disease virus
MHC-I	major histocompatibility complex I
MOI	multiplicity of infection
mRFP1	monomeric red fluorescent protein
MS	mass spectrometry
mTORC1	mammalian target of rapamycin complex 1
MuHV-4	murid herpesvirus 4
Nedd4	neural precursor cell expressed developmentally down-regulated protein 4
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMMHCIIA	non-muscle myosin heavy chain IIA
ORFs	open reading frames
p.i.	post infection
PACE	Paired basic Amino acid Cleaving Enzyme
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PI	propidium iodide
PILRα	paired immunoglobulin-like type 2 receptor
PNGase	with peptide-N-glycosidase
PRV	pseudorabies virus
PSGL-1	P-selectin glycoprotein ligand-1
RFLP	restriction fragment length polymorphism
RIPA	radioimmunoprecipitation assay
RK13	Rabbit kidney
RL2	repeats long 2
RNA-Seq	ribonucleic acid sequencing
RS1	repeats short 1
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAGE	serial analysis of gene expression
SaHV-1	Saimiriine Herpesvirus 1
SDS	sodium dodecyl sulfate
TEER	transendothelial electrical resistance
TFA	trifluoroacetic acid
TGN	Trans-Golgi Network
TRs	terminal repeats
UL	unique long

US	unique short
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4
VSV	vesicular stomatitis virus
VZV	varicella zoster virus

Chapter 1

Introduction

1.1 History

During the early nineteen-hundreds, equine respiratory illnesses were grouped under the unspecific term of "equine influenza". Due to the limited resources for veterinary research at that time, it was impossible for many years to individually characterize the pathogens classified under this undifferentiated influenza complex. Only after combining the observations of numerous investigators, a respiratory disease, caused by a pathogen referred to as equine abortion virus,was classified as equine rhinopneumonitis. This virus would later become known as equine herpesvirus type 1 (EHV-1) [4, 5]. The virus responsible for this pathology was finally isolated by W.W. Dimock and P.R. Edwards during the necropsy of an aborted fetus in 1932 [6-8]. This disease was characterized by abortions or paresis preceded by respiratory symptoms. When a fetus was inoculated with this virus, isolated from respiratory infections, it resulted in classical herpetic abortion. High titers against the abortion herpesvirus could also be shown in horses after experiencing an episode of respiratory disease [4, 5, 10]. A worldwide spread of this virus, closely resembling the morphology of herpes simplex virus type 1 (HSV-1), was reported after serological surveys of horses were conducted in 17 countries [4, 12]. Two subtypes of EHV-1 were differentiated with only one subtype showing the ability to cause post-respiratory symptoms such as abortion and paralysis. The second subtype was very limited with regards to its systemic pathogenicity, and although similar, also had distinct genetic characteristics. It was therefore later reclassified as the separate virus species equine herpesvirus type 4 (EHV-4) [13, 14].

1.2 Taxonomy

Herpesviruses have evolved with their hosts over long periods of time and are found in a wide variety of vertebrates and invertebrates [15]. These herpesviruses have been grouped in the order *Herpesvirales* which contains three families. Viruses infecting mammals, birds and reptiles are grouped in the family *Herpesviridae*, while the *Alloherpesviridae* family incorporates the fish and amphibian viruses. The *Malacoherpesviridae* family, so far, only contains an Ostreid herpesvirus [1, 16]. The *Herpesviridae* family is classified into three subfamilies, the *Alpha*-, *Beta*- and *Gammaherpesvirinae*. Members of the *Alphaherpesvirinae* diverged from the other subfamilies around 200 to 220 million years ago and are widespread among both humans and animals, including HSV-1 and 2, varicella zoster virus (VZV) and pseudorabies virus (PRV) [1, 17]. Also six viruses of equids are classified within this subfamily, all belonging to the genus *Varicellovirus [1, 7, 18]*. Two of these viruses, EHV-6 and EHV-8, are naturally found in donkeys [19], while EHV-9 was isolated from gazelles [20]. The remaining three have the horse as a natural host, including EHV-1 and EHV-4, which are the topic of this dissertation (Fig. 1.1).

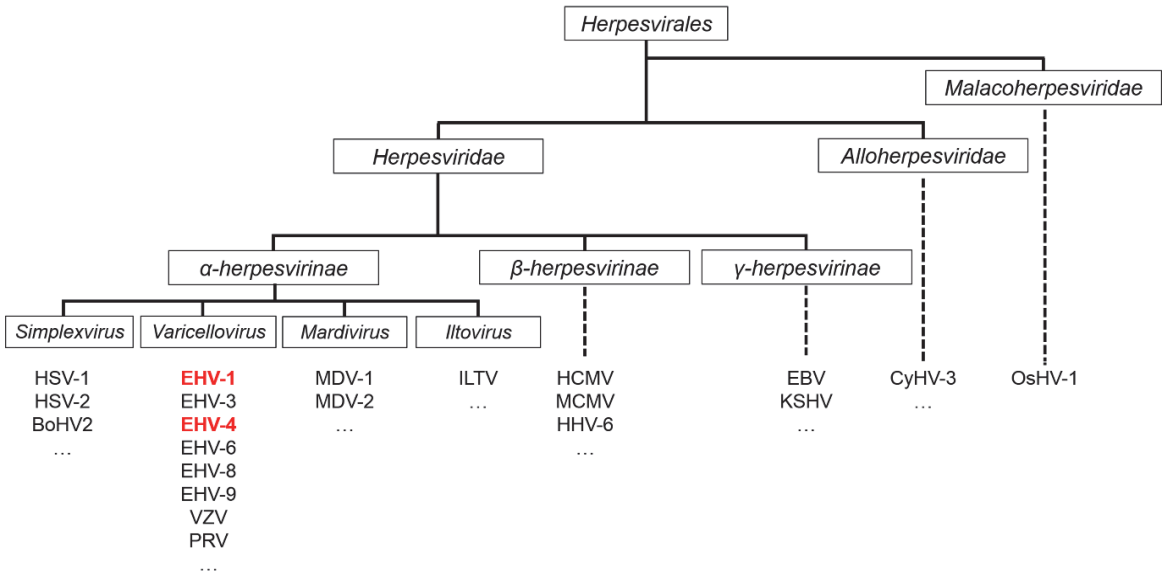

Figure 1.1. The Order *Herpesvirales*. Phylogenetic tree of the *Herpesvirales* order with emphasis on the alphaherpesviruses, and EHV-1 and EHV-4 (red) [1].

1.3 Structure

Mature virus particles of herpesviruses have a complex structure, containing over 30 different viral as well as cellular proteins [21]. The linear double-stranded DNA genome is organized in a unique long (UL) and a unique short (US) region. As characteristic for the D class herpesvirus genome structure, the US region is flanked by the terminal repeats sequence (TRs) which is also repeated in an inverted orientation internally (IRs) between the US region and the UL region [22]. Contrary to other herpesviruses with a D class genome, such as VZV and PRV, the EHV-1 UL region is only present in a single orientation within the virions [23-25]. The viral genomes of EHV-4 (145 kbp) and EHV-1 (150 kbp) both contain 76 unique and highly similar genes that have the potential to code for 77 proteins due to the occurrence of splicing. However, 80 or 79 open reading frames (ORFs) are present in the genomes of EHV-1 or EHV-4, respectively, since four (ORF 64-67) or three (ORF 64-66) genes are duplications. Furthermore, amino acid identities between EHV-1 and EHV-4 proteins range from 55 % to 96 %, indicating that they are functionally closely related [23, 26, 27]. In a mature virion, the viral genome is contained in an icosahedral capsid. This nucleocapsid is surrounded by a proteinaceous tegument layer and a lipid membrane envelope containing membrane-associated viral proteins (Fig. 1.2) [1, 28].

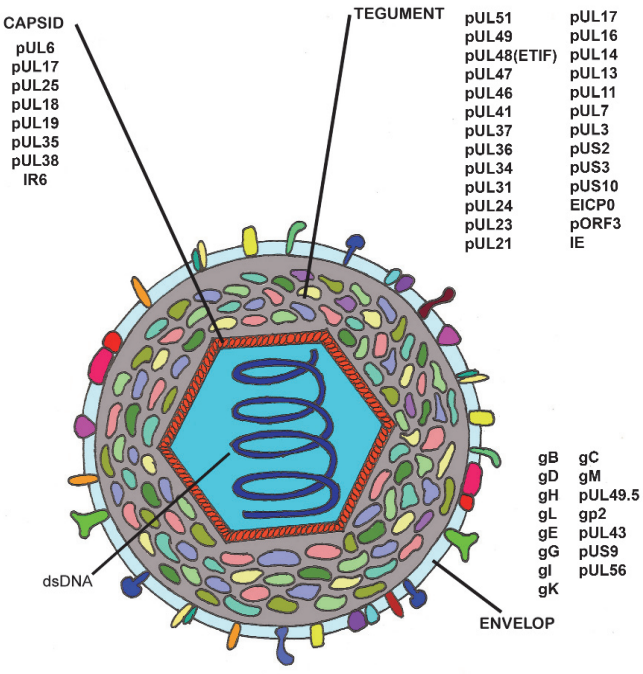

Figure 1.2. Structure of an EHV-1 virion (adapted from [2]). Summary of structural proteins included and grouped according to the respective structural compartments.

1.4 Viral proteins

The genomes of EHV-1, EHV-4 and other closely related alphaherpesviruses such as PRV have been annotated in accordance with those of HSV-1 (prototype virus of genus *Simplexvirus*) and VZV (prototype virus of genus *Varicellovirus*) [23, 29-32]. The role of EHV-1 and EHV-4 proteins can often be deduced based on its HSV-1, VZV or PRV counterparts, and gene function can be extrapolated from the orthologues that are conserved within the viral genus, subfamily or even family [33]. Based on this premise, the functions of EHV-1 and EHV-4 gene products will be discussed. This will be achieved by either referring to studies done directly on the equine herpesvirus gene products or by extrapolating the functions found for the gene product orthologues of closely related alphaherpesviruses. Nevertheless, several genes and/or gene functions are unique to EHV-1 and EHV-4 and will be addressed accordingly.

1.4.1 Capsid proteins

Herpesvirus capsids are characterized by their size of approximately 100-125 nm and their icosahedral shape and triangulation number of 16. The capsid is formed out of 150 hexons (hexavalent capsomers) and 12 pentons (pentavalent capsomers) [1, 28, 34]. The pentons are located at the peaks and the hexons form the faces and edges of the capsid. The capsomers are connected by triplexes in groups of three [28]. In analogy with HSV-1 and VZV, hexons of EHV-1 and EHV-4 capsids are believed to be formed by six copies of pUL35 and six copies of pUL19. The triplexes are formed by one product of pUL38 and two copies of pUL18. Almost all pentons are believed to be formed by pentamers of the product of pUL19. However, one pentamer should be formed by twelve products of pUL6 and form a portal through which the

viral genome enters the capsid [32, 35-37]. Initially, these viral capsids are assembled around a scaffold protein core consisting of the proteins encoded by UL26 and UL26.5. After the construction of the capsids, these proteins cleave themselves and are removed from the capsid before DNA packaging. However, the protease subunit of pUL26 is reported to remain in the capsid even after DNA packaging [38].

1.4.2 Tegument proteins

The tegument is formed by structures in the space between the nucleocapsid and the envelope. The thickness of the tegument layer differs between the different stages of viral replication as additional layers of tegument are added during the course of cell egress [28]. EHV-1 and EHV-4 encode homologues to most [pUL51, pUL49, pUL48 (equine α-trans-inducing factor (ETIF)), pUL47, pUL46, pUL41, pUL37, pUL36, pUL34, pUL31, pUL24, pUL23, pUL21, pUL17, pUL16, pUL14, pUL13, pUL11, pUL7, pUL3, pUS2, pUS3, pUS10, EHV-1-Infected Cell Polypeptide 0 (EICP0), IE], but not all [pUS11, pUS12 and ICP34.5], alphaherpesvirus tegument proteins known to date, of which the functionality in several cases has been confirmed for EHV-1 and/or EHV-4 as well [17, 32, 39-54]. In analogy with HSV-1, it is predicted that some of these components of the tegument (EICP0, IE, pUL34 and pUL31) are added to capsids in order to facilitate nuclear egress. Some of these proteins are subsequently lost again during nuclear egress (e.g. pUL31 and pUL34), followed by the formation of the final tegument during the cytoplasmic stage and re-envelopment [55]. Tegument proteins are reported to play important roles during herpesvirus replication by shutting down host protein synthesis, stimulating viral gene expression and inhibiting cellular defenses [28]. Structurally, tegument proteins can be divided into two groups. Inner tegument proteins are characterized by interacting with the capsid. Outer tegument proteins, on the other hand, interact with the envelope. Apart from interacting with the capsid or envelope, tegument proteins also interact with each other [56]. For the purpose of this dissertation the tegument protein pUS3 will be discussed more in detail.

The pUS3 serine/threonine protein kinase is encoded by every alphaherpesvirus discovered so far. It can phosphorylate serine (S) or threonine (T) residues present in a distinct target site $R_nX(S/T)xx$ (n is equal to or larger than 2; X= arginine, alanine, valine, proline or serine; x can be any non-acidic residue) in analogy with the cellular protein kinase A and B [57-60]. Not much is known about the US3 homologues of equine herpesviruses, but much of its potential can be deduced from the enormous body of research done on US3 of other alphaherpesviruses [45, 61]. Just as all other alphaherpesvirus pUS3 orthologues, pUS3 of EHV-1 and EHV-4 possess a kinase domain with an ATP binding domain that contains a highly conserved lysine essential for ATP binding and a catalytic site with a highly conserved aspartic acid. Tempering with either of these residues renders pUS3 inactive [61]. The US3 gene products of HSV-1 and PRV, in particular, have been well characterized. The performed studies depict a multifunctional protein that phosphorylates a number of viral and cellular substrates, thereby influencing various steps of the viral replication [61-63]. For HSV-1, bovine herpesvirus type 1 (BHV-1) and PRV, an anti-apoptotic role, blocking apoptosis upstream and/or downstream of the mitochondrial apoptotic stage, has been described [64-70]. Another important function of pUS3 is facilitating nuclear egress by phosphorylating the viral nuclear egress factors pUL31 and pUL34, emerin (the inner nuclear membrane component), and also lamin A/C (the scaffolding components of the nuclear envelope) as shown in detail for HSV-1 and also Marek's disease virus (MDV) [71-79]. In order to improve viral gene expression, HSV-1 pUS3 phosphorylates histone deacetylases, thereby blocking histone deacetylation [80-82]. Furthermore, pUS3 influences cell-to-cell spread by cytoskeletal reorganization leading to stress fiber breakdown and cell projection formation that

interconnects cells (described for HSV-1, PRV, BHV-1 and MDV) [65, 71, 83-85]. pUS3 can also phosphorylate a specific motif in the cytoplasmic tail of gB, thereby downregulating the cell surface expression and regulating gB's intracellular transport [86, 87]. Another viral protein that is regulated through pUS3-mediated phosphorylation is the tegument protein pUL47. pUS3 facilitates the nuclear localization of pUL47, which in turn enables pUS3 to also localize in the nucleus [88]. Viral replication and mRNA translation is additionally stimulated when pUS3 mimics protein kinase B and activates mammalian target of rapamycin complex 1 (mTORC1) which in turn inhibits the translational repressor 4E-binding protein 1 [89]. The wide variety of functions associated with pUS3 emphasizes the importance of this viral protein kinase for alphaherpesvirus replication.

1.4.3 Envelope proteins involved in cell entry

The lipid membrane envelope surrounding the nucleocapsid and tegument of EHV-1 and EHV-4, contains 12 glycoproteins on its surface, of which all [EHV homologues of gB, glycoprotein C (gC), gD, gH, glycoprotein L (gL), gE, glycoprotein G (gG), glycoprotein I (gI), gK, gM and glycoprotein N (gN)], apart from glycoprotein 2 (gp2), are conserved amongst alphaherpesviruses [23, 32, 90, 91].

The initial binding with heparan sulfate is facilitated by EHV-1 and EHV-4 gC [92, 93]. gC also is believed to play an important role during virion release [93]. In addition, gC also interferes with the innate immune response by binding complement [92, 94]. After initial attachment, gD binds with its receptor on the cell surface. For EHV-1 and EHV-4 it has been shown that gD can bind with major histocompatibility complex I (MHC-I), cell surface glycosaminoglycans and αV integrins. When interaction with MHC-I is possible, EHV-1 seems to facilitate membrane fusion at the cell surface, while binding with glycosaminoglycans or αV integrins seems to correlate with membrane fusion after endocytosis [95-100]. The ability of gD to bind with a receptor on the cell surface determines the cell tropism of the virus, which differs significantly between EHV-1 and EHV-4 [96]. The binding of gD with its cognate receptor enables it to bind and alter the conformation of the gH/gL complex [101]. The gH/gL heterodimer complex plays an essential role in regulating the membrane fusion event of herpesviruses. The underlying mechanism, however, is not fully understood [101]. For EHV-1 gH, the ability to interact with α4β1, contrary to EHV-4 gH, is reported to regulate the route of cell entry [102]. It is proposed that, after interacting with gD, it undergoes a conformational change allowing it to interact with gB. gB is a type-1 transmembrane protein with a signal sequence at its N terminus [103]. The subsequent membrane fusion mainly facilitated by gB [3, 104], and will be discussed more in detail.

Viruses with an envelope, like herpesviruses, enter cells through fusion of the viral and cellular membranes [3]. Fusion proteins, such as gB, facilitate this process by overcoming the high kinetic barriers involved in membrane fusion [105]. Viral fusion proteins achieve this by utilizing the energy released during their conformational change [106]. Most viral fusion proteins belong to one of three structural classes. Class I fusion proteins are characterized by a perpendicular orientation, formation of functional trimers, and are predominately α-helical. The virus families *Retroviridae, Orthomyxoviridae, Paramyxoviridae, Coronaviridae, Filoviridae, Arenaviridae* have fusion proteins belonging to this class. Class II proteins, on the other hand, are orientated parallel to the membrane and consist predominantly of β-sheets. These fusion proteins need to form dimers in order to be functional. The virus families *Togaviridae, Flaviviridae and Bunyaviridae* belong to this class [3, 106]. Class III proteins are trimeric with three central α-helices and β-sheets containing fusion loops. Fusion proteins of the viral families

Rhabdoviridae, Herpesviridae and Baculoviridae belong to this class [107]. Together with protein G of the vesicular stomatitis virus (the prototype protein of class III), gB is a member of the class III fusion proteins [11, 101, 108-111].
Independently of their structure, conformational changes of these fusion proteins are triggered in very distinct ways. There are members of all three structural classes where low pH alone triggers the conformational change [3]. Avian α-retroviruses, on the other hand, require binding with a cell receptor first in order to render it sensitive to low pH [112]. Other fusion proteins of class I and III, including gB, require binding with additional viral surface proteins [3]. More specifically, gB needs to associate itself with gH/gL in order to undergo a conformational change [101]. These three proteins form the core fusion machinery, which in some cases is complemented by additional receptor binding glycoproteins [113]. It is proposed that binding of the gH/gL complex brings the two membranes together and allows gB to undergo a loop-to-helix transition, thereby enabling the insertion of the fusion loops into the opposing cell membrane. This would initiate fusion of the two membranes and entry of the capsid into the cell [101]. A model for how gB facilitates fusion has been suggested based on that of VSV protein G (Fig. 1.3), where the C-terminal end of the elongated gB folds back along the outside of the trimer fusing the two membranes together [3, 11, 108, 111]. However, unlike VSV protein G, gB only functions when partnered with gH/gL. Herpesvirus gB might also undergo some pH-dependent conformational changes in certain situations, but these are relatively minor compared to those of VSV protein G [11, 114-116]. It therefore remains to be seen to what extent the VSV "foldback" model can be extrapolated to gB. Especially when considering that the gBs of numerous herpesviruses, including EHV-1 and EHV-4, do not require pH changes for cell entry [102, 117-119].

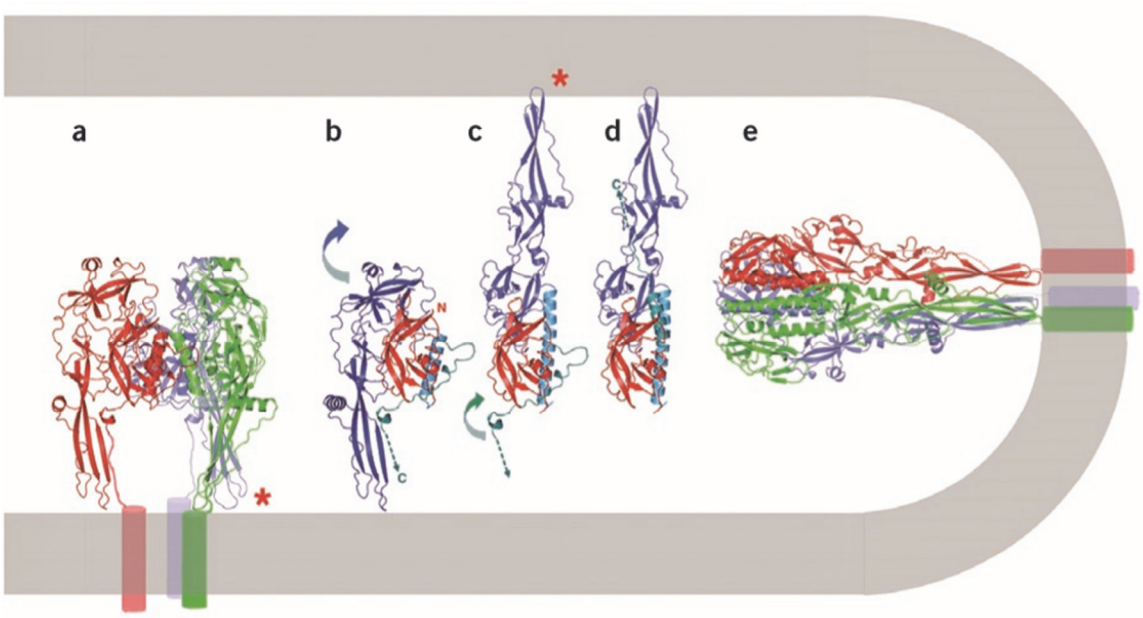

Figure 1.3. Predicted structural conformational changes of gB based on VSV-G (copied from [3]). (a) Pre-fusion trimer. The three subunits are in red, blue and green. The fusion loops (*) are orientated away from the target membrane. (b) Pre-fusion monomer [in the orientation of the red subunit in (a)]. Dashed line, the part of the C-terminal segment that is missing from the crystal structure [9]; "N": the N terminus. (c) Suggested extended intermediate conformation of one monomer. The fusion domains have reoriented [curved arrow in (b)], with the fusion loops (*) now in contact with the target membrane; the reorientation seems to be driven in part by a loop-to-helix transition that elongates the helix at the trimer contact. The C-terminal segment (dashed arrow) must fold back along the outside of the trimer (curved arrow) to complete the transition to the post-fusion conformation. (d) Post-fusion conformation of one monomer. The C-terminal segment has folded back, and it now projects toward the fusion loops. (e) Postfusion conformation of the trimer [11], with colors as in (a).

The body of research specifically done on gB of EHV-1 and EHV-4 is still very limited. EHV-1 gB (gB1), formerly known as gp14, undergoes a similar processing as gB of other alphaherpesviruses where a glycosylated precursor is cleaved into two subunits that subsequently form disulfide-bonded dimers (discussed more in detail below) [120, 121]. gB1 plays an essential role in penetration, direct cell-to-cell spread and cell-cell fusion. The role of gB1 as a fusion protein was further confirmed when its function could be partly compensated by a chemical fusogen [104, 122].

Several functional motifs have been identified in gB which contribute to gB regulation through the different stages of infection. This is especially the case for the cytoplasmic tail of gB where three endocytosis motifs, two tyrosine-based motifs and one di-leucine motif, are conserved throughout the members of the alphaherpesvirus subfamily including EHV-1 (^{925}YMSM^{928}; ^{965}YTRL^{968}) and EHV-4 (^{920}YMSM^{923}; ^{960}YTRL^{963}) [123, 124]. These motifs have been analyzed for HSV-1, VZV and PRV, and have been implicated in gB-cell-surface retrieval, TGN localization and gB incorporation into the virion envelope [123, 125, 126]. For VZV, an immunoreceptor tyorsine-based inhibition motif was identified that is involved in cell–cell fusion

[111]. Another factor with possible importance for gB-mediated cell entry is the putative integrin-binding motif tyrosine-glycine-leucine (EHV-1: ^{326}YGL^{328}; EHV-4: ^{323}YGL^{325}) of gB. YGL is an amino acid motif that is present in gB of PRV, MDV, EHV-1 and EHV-4 and has been shown to bind to certain integrins when presented by cellular osteopontin [127-129]. Furthermore, it has also been shown to play an important role in cell entry for several rotaviruses [130]. It was determined by prediction (I-TASSER, Center for Computational Medicine and Bioinformatics, University of Michigan) that the YGL motif is located in the extracellular domain of the gB protein. Its extracellular location in a glycoprotein that is a member of the conserved cell entry complex found in all alphaherpesviruses, combined with its importance in rotaviruses for cell entry, indicated that it might also have a similar role in EHV-1 and EHV-4. Another motif conserved amongst many herpesviruses is a furin cleavage site that plays a pivotal role in gB processing.

Furin, formerly known as Paired basic Amino acid Cleaving Enzyme (PACE), is an calcium-dependent serine endoprotease of the subtilisin-like proprotein convertase family that plays an important role in homeostasis, cancer, dementia, both bacterial and viral diseases, and many more pathologies [131-139]. It activates numerous proproteins through cleavage within secretory pathway compartments, more specifically the TGN and the endosomal system [140, 141]. Furin activates these cellular, but also viral and bacterial proteins, by cleaving at an arginine rich consensus site (Arginine–X–Lysine/Arginine–Arginine; where X is any amino acid)[142-145]. Furin plays an important role in the pathogenesis of many viruses. The envelope proteins of HIV, influenza and dengue virus must be cleaved by furin or similar proteases to become fully functional [142, 146, 147]. The role of furin cleavage has also been evaluated for gB of several herpesviruses. Mitigating furin cleavage of gB has been proven to be dispensable for the replication of human cytomegalovirus (HCMV), BHV-1, Epstein–Barr virus (EBV), VZV, murid herpesvirus 4 (MuHV-4) and PRV *in vitro* [109, 148-152]. However, changes in phenotype were seen for several of these viruses harbouring a furin cleavage site in gB. For example, uncleaved BHV-1 gB showed no differences with regards to penetration and showed only slightly delayed one-step growth kinetics compared to parental BHV-1 with wild-type gB. However, plaque sizes were significantly reduced, indicating a role for furin cleavage in cell-to-cell spread of BHV-1 [149]. Penetration kinetics revealed that also mutant PRV with uncleaved gB could penetrate cells at the same rate as wild-type gB. However, syncytia formation was inhibited for mutant PRV with uncleaved gB as well as mutant VZV with uncleaved gB. In addition, VZV with uncleaved gB showed attenuated replication in human skin xenografts [109, 151]. Reduced syncytia formation was also observed for EBV, indicating a conserved function for furin-mediated cleavage among gB homologues from different herpesvirus subfamilies [150]. Mitigating furin cleavage of MuHV-4 gB had no effect on cell entry of fibroblasts and epithelial cells, but showed a significant entry deficit in myeloid cells such as macrophages and bone marrow-derived dendritic cells [148]. gB of HSV-1 on the other hand, is not cleaved at all [153]. Both gB1 (^{518}RRRR^{521}) and EHV-4 gB (gB4) (^{513}RTRR^{516}) also have furin cleavage sites, but have not yet been evaluated as of date. It would therefore be very interesting to see what their impact is on the phenotype of EHV-1 and EHV-4.

1.5 Replication cycle

The replication cycle of EHV-1 and EHV-4 (Fig. 1.4) begins with the attachment of the virion to the cell surface membrane through the interacation of gC with cellular surface heparan sulfate [93]. This is followed by binding of gD with one of its cellular receptors [95-100], which induces subsequent cell entry trough membrane fusion. This membrane fusion is facilitated by gB and

the gH/gL complex [3, 101, 104]. Once the virus particle is in the cytosol, the viral capsid, together with several inner tegument proteins, is transported by the cellular motor protein dynein along microtubules to the nucleus [154]. In analogy with HSV-1, EHV-1 and EHV-4 are presumed to dock with a nuclear pore and release their DNA into the nucleus [155, 156].
After viral DNA enters the nucleus, the immediate early (IE) gene [homologue of HSV-1 ICP4/ repeats short 1 (RS1)] is transcribed. The promotor of the IE gene is transactivated by ETIF, the EHV-1 and EHV-4 homologue of pUL48, which is brought into the cell as a structural component of the tegument. ETIF is also essential for secondary envelopment and virus egress [40, 157-159]. The product of the IE gene is major trans-activator of early (E) and certain late (L) genes and also functions as a repressor of its own transcription [48, 160-162]. In analogy with HSV-1, the products of E genes are believed to play a role in nucleotide metabolism and DNA synthesis [163]. Specifically for EHV-1 and EHV-4, some of these early gene products, such as pUS1 and pUL54, are believed to take part, together with IE, in activating early and late EHV-1 gene promoters [164-167]. Upon HSV-1 infection, pUS1 also alters directly or indirectly the expression of key cell cycle regulatory proteins [168]. Also additional roles for HSV-1 pUL54 are reported where it is involved in inhibiting host cell splicing and inhibiting host cell mRNA levels during infection [169, 170]. In addition, the EICP0 tegument protein of EHV-1 (homologue of HSV-1 RL2) can independently activate the promotors of IE, E and L genes [47, 171]. EHV-1 gene expression is kept in check by the product of the early gene IR2 (a truncated version of the IE) and the IR3 gene [172-175]. The IR3 gene lies antisense to the IE transcript and is unique to EHV-1. It does not produce a translated protein, but still downregulates IE gene expression [172, 176, 177].
Next, L genes are transcribed which mainly encode for structural proteins enabling the assembly of capsids that subsequently occurs in the nucleus. The viral genome is also amplified and inserted in preformed capsids [178-181].
Conserved genes in the herpesvirus family facilitating viral DNA replication, which proceeds by a rolling circle mechanism, are in the first place UL5, UL8 and UL52. The proteins encoded by these genes form a helicase/primase complex that is essential for replication of viral DNA by synthesizing short RNA primers to which new nucleotides can be added by the heterodimeric DNA polymerase encoded by UL30 (catalytic subunit) and UL42 (processivity subunit) [28, 182-185].
In analogy with HSV-1, six conserved herpesvirus proteins are believed to associate with the pUL6-formed capsid portal in order to facilitate DNA packaging. More specifically, it is believed that the products of UL15, UL28, and UL33 form the terminase that cleaves the concatemeric viral DNA and supplies energy to the packaging process via ATP hydrolysis [178, 180, 181].
Three possible modes of egress have been proposed for the newly formed nucleocapsids of alphaherpesviruses. Firstly, virus particles bud with the inner nuclear membrane (INM) and undergo primary envelopment. These particles would then be transported through the endoplasmic reticulum and TGN. However, this does not account for the fact that major components of enveloped herpesvirus particles in the perinuclear lumen are absent from mature virions. A second proposed route, is the partial transportation of non-enveloped particles to the cytoplasm through enlarged nuclear pores. However, nuclear pores maintain their integrity until late events in herpesvirus infection which argues against this proposed route. Most probably, nuclear egress occurs through primary envelopment at the INM and release into the cytoplasm after de-envelopment at the outer nuclear membrane [21].

The newly formed herpesvirus nucleocapsids first acquire an initial layer of tegument containing the conserved proteins, pUL31 and pUL34, that are essential for nuclear egress. Both proteins mediate efficient nuclear egress together with the tegument proteins pUS3 and pUL47, and in association with the envelope glycoproteins gB and gH [186-188]. The nucleocapsids, together with certain tegument proteins, are then released in the cytoplasm when the primary envelope fuses with the outer nuclear membrane [189]. This is followed by microtubule-facilitated transport to the TGN where the particles are modified and reach their mature form. During this process of egress, additional tegument proteins are recruited, followed by secondary envelopment at the TGN. These fully formed infectious virions are then released through the normal route of exocytosis [28].

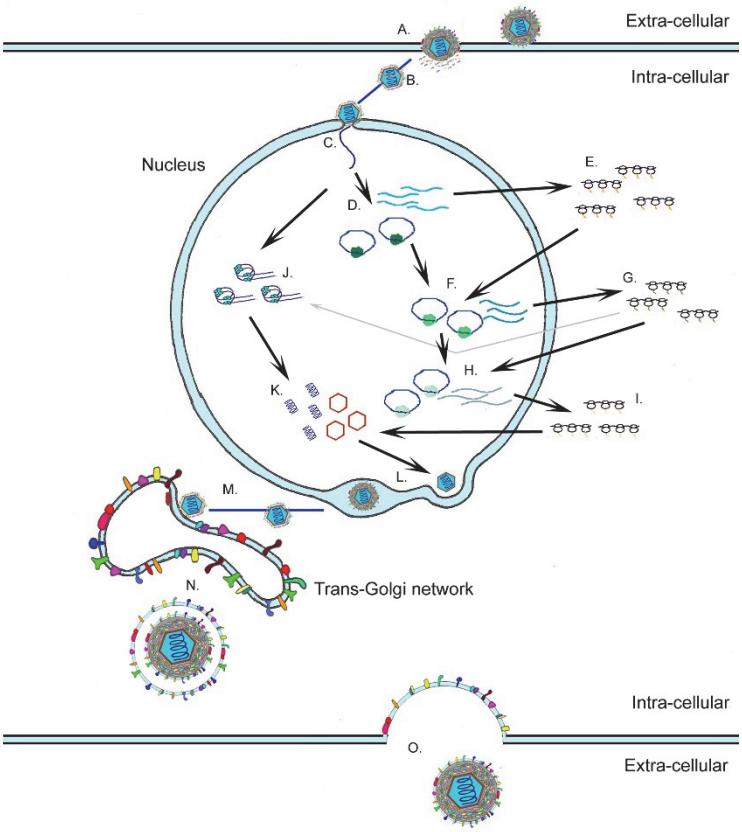

Figure 1.4. The EHV-1 and EHV-4 replication cycle (adapted from [2]). (A)The initial attachment of the virion to the cell surface membrane is mediated through interactions of gC and gB with heparan sulfate and glycosaminoglycans. This is followed by cell entry by means of membrane fusion facilitated by subsequent conformational changes in gD, gH/gL and gB. (B) Once the nucleocapsid, together with several inner tegument proteins is released into the cytoplasm, it is transported along microtubules to the cell nucleus. (C) In analogy with HSV-1, the nucleocapsid are presumed to dock with a nuclear pore and release their DNA into the nucleus. (D) Initially the IE gene is transcribed and (E) translated into gene product that activates (F) the transcription of early genes, (G) followed by translation resulting in proteins involved in virus replication. (H) L genes are transcribed, (I) which mainly encode for structural proteins. (J) The viral genome is also amplified. (K) Virus genomes are then incorporated into the newly formed capsid. (L) These nucleocapsids together with certain tegument proteins bud through the INM and undergo primary envelopment. (M) The virus particles subsequently lose their primary envelope and are released into the cytoplasm. This is followed by microtubule-facilitated transport to the TGN, (N) where the particles are modified and undergo secondary envelopment. (O) The virus particles are then transported to the cell surface by vesicle-mediated transport and released by exocytosis.

1.6 Pathogenesis

1.6.1 Overview of the equine herpesvirus pathogenesis

EHV-1 and EHV-4 can transfer by direct contact, mainly through infected nasal discharge. However, EHV-1 can also occasionally transmit by aerosol or contaminated feed, water or equipment [4, 27]. The primary site of infection for EHV-1 and EHV-4 is the upper respiratory tract where it can cause rhinopneumonitis [4, 27, 190]. In order to pass the basement membrane, EHV-1 can infect individual immune cells, particularly of the monocyte lineage, possibly mucosal dendritic cells [191]. After passing the basement membrane, infected immune cells can

migrate to the blood stream or lymphatic system. After migrating to parts of the lymphoreticular system, EHV-1 is spread among peripheral blood mononuclear cells (PBMC), which cause cell-associated viremia [7]. As a result, EHV-1 is able to spread throughout the body and is preferentially transferred to EC within certain tissues, which is accompanied by a strong immune response. This causes vascular lesions and secondary hypoxic degeneration of affected tissues [27, 190]. The physiological interactions between PBMC and EC that facilitate this viral transfer will be discussed more in detail below (see chapter 1.6.2). EHV-1 replication occurs mainly in EC of the blood vessels of the pregnant uterus or the central nervous system (CNS) and will ultimately lead to abortion or neurological symptoms, respectively (Fig. 1.5) [192, 193].

Moreover, EHV-1 and EHV-4 can establish latency within lymphoid tissues and neurons of the trigeminal ganglion, enabling the virus to persist within the horse population [194, 195]. It was hypothesized that EC infection is initiated upon close contact following adhesion of PBMC to EC and direct cell-to-cell transfer of virus. This mechanism does not require virus egress from PBMC [196]. A recent study reported on virus transfer from PBMC to EC, demonstrating the potential importance of this mode of infection in the presence of neutralizing antibodies [197]. In contrast, EHV-4 replication remains restricted to the primary site of infection in the vast majority of cases (upper respiratory tract), and cell-associated viremia, as described for EHV-1, is very rare [27, 198]. The genetic differences that are responsible for this variation in pathogenicity are still not fully elucidated.

One parameter that seems to be linked to pathogenicity is cellular host range. Contrary to EHV-4, EHV-1 can replicate in a wide variety of cell lines from different species [4, 199-201]. This is likely the case because EHV-1 can interact with a larger set of cellular receptors than EHV-4 in order to gain entry into target cells [44, 95, 98, 100, 104, 202, 203]. These multiple entryways may also be responsible for the higher pathogenicity of EHV-1. In contrast, EHV-4 can only successfully infect equine cells and African green monkey epithelial kidney (Vero) cells [96, 204].

The differences in pathogenesis were originally ascribed to EHV-4 infection being limited to epithelial cells [205]. However, *in vitro* and *in vivo* studies have shown that both EHV-4 and EHV-1 can also infect endothelial cells [27, 206, 207]. In the case of PBMC, however, conflicting results have been reported. Recent studies in mucosal explants showed that EHV-4 has only a very limited ability to enter leukocytes, whereas EHV-1 readily infects leukocytes, especially those of the monocytic lineage [208]. Furthermore, significantly lower viral DNA loads were detected for only a short period of time in the case of EHV-4 viremia *in vivo* [209], compared to EHV-1 [210]. These results closely correlate with reported *in vitro* results [207]. In that study, a significantly lower number of infected PBMC were seen during EHV-4 infection compared to infection with EHV-1.

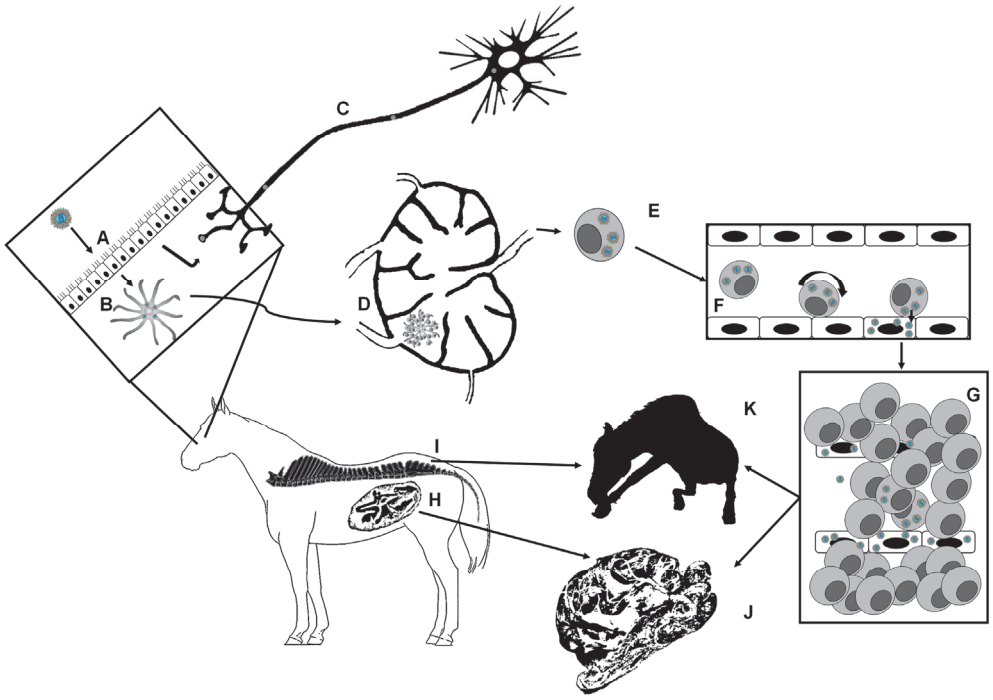

Figure 1.5. EHV-1 pathogenesis. (A) After initial replication in the upper respiratory tract, (B) EHV-1 infects leukocytes and migrates past the epithelial basement membrane. (C) EHV-1 can also infect sensory neurons and establish a latent infection in the trigeminal ganglion. (D) The infected immune cells migrate by means of the lymphatic system and bloodstream to the lymphoreticular system where the virus is transferred to PBMC, and latency is also established. (E) The newly infected PBMC then enter the bloodstream and cause EHV-1 viremia. (F) These infected PBMC interact with EC lining of the blood vessels trough the physiological mechanisms of tethering and rolling. Once firmly attached to the EC, viral transfer occurs between the infected PBMC and the underlying EC. (G) Productive EHV-1 infection in these EC induces inflammation and secondary hypoxic degeneration of the affected tissues. EHV-1 replication occurs mainly in blood vessels of the pregnant uterus (H) or the central nervous system (I) which can ultimately lead to abortion (J) or EHM (K) respectively.

Similar numbers of infected PBMC, as reported for EHV-4, were documented when an RSD motif of EHV-1 gD that can bind to cell surface integrins was mutated. The results implied that the absence of the RSD motif in EHV-4 gD might be a critical determinant of cellular tropism and explain the differences between EHV-1 and EHV-4 [207]. However, it was shown later that EHV-4 can infect PBMC *in vitro* with similar efficiency as EHV-1 when using an EHV-4 strain that contains the entire repertoire of viral glycoproteins [96]. A new EHV-4 strain was used in this latter study, which is different from the strains used in previous studies. The difference of infection rate of the two studies may, thus, be explained by the fact that an EHV-4 strain deficient for gM was used in the earlier report [207, 211]. This mutation was shown to cause a significant defect in virus egress and cell-to-cell spread [211], giving an unrepresentative comparison between EHV-1 and EHV-4. However, this does not exclude the possibility that the

presence of the RSD motif influences events downstream of cell entry. These contradictory results concerning PBMC tropism have thus far not been elucidated.

1.6.2 The interaction between PBMC and EC.

Viral transfer from infected PBMC to the EC lining of the blood vessel is essential for the occurrence of EHV-1-related abortion and equine herpesvirus myeloencephalopathy (EHM) [212]. Therefore, comprehending the molecular mechanisms of the interactions between PBMC and EC is critical to our understanding of EHV-1 pathogenesis. The molecular mechanisms that are involved in leukocyte rolling and transmigration at the EC lining of the blood vessels has been a subject of intensive research for decades [213-216]. Considerable progress has been made in understanding this complex process [217].

Initial capture and rolling of leukocytes is mediated by selectins, present on the apical surface of EC, that interact with their ligands, present on the leukocyte surface [218]. These selectins belong to a family of structurally similar transmembrane glycoproteins, and are named after the cell types they originally were discovered on (E-endothelial cells; P-platelets) [219, 220]. This selectin-mediated rolling interaction triggers a signaling cascade that induces a conformational change of the integrins on the surface of the rolling leukocyte. This in turn results in a reduction of the rolling velocity [217]. Integrins are a widely expressed family of heterodimeric cell adhesion receptors consisting of an α and a β subunit [221, 222]. Post-adhesion strengthening of the leukocyte/EC interaction is characterized by an accumulation of integrins at the leukocyte/EC interface. This is then followed by transmigration of the leukocyte into the underlying tissues [223]. This process has been evaluated most thoroughly for neutrophils [217]. The importance of integrins in PBMC rolling is less well studied. Research performed *in vitro* suggests an important role of Very late antigen-4 (VLA-4; α4β1-integrin) for monocyte rolling [224, 225]. Other studies identified P&E-selectin/P-selectin glycoprotein ligand-1 (PSGL-1) interaction to be responsible for tethering and rolling while macrophage receptor 1 (MAC1; also known as CD11b–CD18 and $\alpha_M\beta_2$-integrin) predominantly mediated firm adhesion of monocytes to EC [226]. Also initial tethering and rolling of T-lymphocytes is mediated by interactions with P-and E-selectin [227], while rolling interaction for firm adhesion were mainly achieved through VLA-4-mediated interactions [228].

Much of our understanding of leukocytes, interacting with EC, comes from experiments performed in *in vitro* systems. PBMC-EC interactions were investgated under either static conditions or by means of flow chamber setups [229]. Shear forces exerted on PBMC in the latter setup were shown to contribute significantly to the interaction process by enhancing selectin-mediated adhesion [230]. Despite these *in vitro* systems being relatively simple in their setup, remarkable accurate results were obtained on PBMC recruitment which were later confirmed by *in vivo* experiments [229].

Under flow conditions, it was shown that the upregulation of adhesion molecules on the surface of EC of the equine reproductive tract during pregnancy could therefore explain the preference of EHV-1 for such cells [196]. Also an increased adhesion of T-lymphocytes to the endothelium was seen when the EC were cultured in the presence of astrocyte-conditioned medium thereby simulating the CNS environment [231]. This, in turn, could explain the preference of EHV-1 for the CNS. Contrary to the role of adhesion molecule expression on EC, very little is known about their up-or down regulation on the surface of infected PBMC. Differences in adhesion molecule down regulation were already reported for ED cells infected with either EHV-1 or EHV4 [232], and were recently also evaluated for PBMC. VLA-4, which can interact with vascular cell adhesion molecule 1 (VCAM-1) on EC, is only downregulated by EHV-4 and not EHV-1 (Azab

and Osterrieder; unpublished data). These differences could be partly responsible for the lack of viral transfer between EHV-4 infected PBMC and EC. These stationary and dynamic *in vitro* systems could therefore also be a useful tool to evaluate effects of viral PBMC modulation on the the interactions of EHV-1 infected PBMC and EC, and the viral transfer between them.

1.7 Aims of the study

Previous studies on other members of the viral protein complex (gD-gB-gH-gL), involved in cell entry, showed striking functional differences between these genetically highly conserved proteins of EHV-1 and EHV-4 [96, 102, 233]. More specifically, the exchange of gD between EHV-1 and EHV-4 identified gD as being solely responsible for the cellular tropism of the virus [96]. The principle of exchanging essential proteins between EHV-1 and EHV-4 also opened possibilities for other essential proteins such as gB. For this thesis, I therefore aimed to further characterize the functional differences of glycoprotein B of EHV-1 and EHV-4 and the subsequent roles of these differences in the pathogenesis of the respective viruses. It was also attempted for the first time to thoroughly characterize the role of putative functional motifs and the structural aspects of gB1 and gB4.

Despite many years of research, no fully protective vaccines have been developed [27, 234]. Clearly much still has to be learned about EHV-1 disease. Our research can significantly contribute to this by further unraveling the molecular mechanisms of the cell-associated viremia which is arguably the most important aspect of the EHV-1 pathogenesis. My research specifically focused on the roles of gB and pUS3 during this particular stage of pathogenesis [212]. Due to the conserved nature of cell-associated viremia among numerous herpesviruses, our results may also be very relevant for viruses such as VZV, PRV and others. In addition, if the mutant and recombinant viruses, constructed for our studies, exhibit potent *in vitro* replication, but significant defects in certain stages of the disease course, they could be promising candidates for future live vaccines.

1.8 References

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Chapter 2

Comparative Analysis of Glycoprotein B (gB) of Equine Herpesvirus Type 1 and Type 4 (EHV-1 and EHV-4) in Cellular Tropism and Cell-to-Cell Transmission

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2.1 Abstract

Glycoprotein B (gB) plays an important role in alphaherpesvirus cellular entry and acts in concert with gD and the gH/gL complex. To evaluate whether functional differences exist between gB1 and gB4, the corresponding genes were exchanged between the two viruses. The gB4-containing-EHV-1 (EHV-1_gB4) recombinant virus was analyzed for growth in culture, cell tropism, and cell entry rivaling no significant differences when compared to parental virus. We also disrupted a potential integrin-binding motif, which did not affect the function of gB in culture. In contrast, a significant reduction of plaque sizes and growth kinetics of gB1-containing-EHV-4 (EHV-4_gB1) was evident when compared to parental EHV-4 and revertant viruses. The reduction in virus growth may be attributable to the loss of functional interaction between gB and the other envelope proteins involved in virus entry, including gD and gH/gL. Alternatively, gB4 might have an additional function, required for EHV-4 replication, which is not fulfilled by gB1. In conclusion, our results show that the exchange of gB between EHV-1 and EHV-4 is possible, but results in a significant attenuation of virus growth in the case of EHV-4_gB1. The generation of stable recombinant viruses is a valuable tool to address viral entry in a comparative fashion and investigate this aspect of virus replication further.

2.2 Introduction

Glycoprotein B (gB) is a type 1 transmembrane protein and represents a highly conserved class III fusion protein present in members of the *Herpesviridae* family [1]. In members of the *Alphaherpesvirinae*, it is thought that gB mediates the virus entry process through membrane fusion after initial attachment of the virion via gC to cell surface glycosaminoglycans, binding of gD to its cognate receptor and activation of the heterodimeric gH-gL complex, which in turn primes gB for fusion [2–9]. In Herpes simplex virus type 1 (HSV-1), gB has been shown to bind several cellular receptors to facilitate viral entry; however, there is no data available on the role of equine herpesvirus type 1 (EHV-1) gB in receptor binding [1]. HSV-1 gB can bind to the paired immunoglobulin-like type 2 receptor (PILRα) to trigger viral fusion in the presence of gD [10]. In addition to PILRα, non-muscle myosin heavy chain IIA (NMMHCIIA; also known as myosin 9) [11] and myelin-associated glycoprotein (MAG) [12], a protein expressed in neuronal tissues, were also shown to interact with gB and facilitate HSV-1 entry although it has remained unclear whether specific gB-receptor interactions are critical for entry.

Crystal structures of HSV-1 [13] and Epstein–Barr virus (EBV) [14] demonstrated that gB has structural similarity with other viral fusion proteins, such as the G protein of vesicular stomatitis virus (VSV) [15] and gp64 of baculovirus [16], and likely acts as the key herpesviral fusion protein that requires gH/gL as a fusion regulator [17–20]. However, it is not fully understood how gB and gH/gL interact during viral fusion.

EHV-1 gB was first described in 1985 as antigenically and structurally similar to HSV-1 gB [21]. Later, it was shown that, like gB of other alphaherpesviruses, EHV-1 gB is essential for viral growth and direct cell-to-cell spread. This was deduced from the fact that only single infected cells, but no viral plaques, were observed when EHV-1ΔgB was used to infect non-complementing cell lines. A strong indication for its role as a fusogen was also reported; when viral titers of a gB-deficient EHV-1 virus could partly be restored by adding polyethylene glycol to induce fusion [3]. However, there is no data available on the function of EHV-4 gB, the close relative of EHV-1.

EHV-4 (145 kbp) and EHV-1 (150 kbp) both contain 76 unique and highly similar genes. Nucleic acid identity between EHV-4 and EHV-1 genes in general is above 80%, indicating that they are functionally closely related [22–24]. However, despite this high genetic similarity, the disease outcomes after infection differ substantially. EHV-4 only induces mild symptoms that are usually limited to upper respiratory tract infections associated with fever and general malaise [25,26]. While also causing respiratory disease, EHV-1 induces more severe clinical disease that includes abortion in pregnant mares [27–29] and the so-called EHV-1 myeloencephalopathy (EHM) [30,31]. On the cellular level, numerous differences in viral-cell interaction have been described. For instance, both viruses enter cells trough different pathways. More specifically, EHV-1 enters equine epithelial cells via direct fusion at the plasma membrane, while EHV-4 does so via an endocytic pathway [32].

The close genetic relatedness allowed us to exchange essential genes between EHV-1 and EHV-4 and to evaluate the effects both *in vitro* and *in vivo* in a way not possible for other members of the subfamily since EHV-1 and EHV-4 naturally infect the same host. We have been interested in exchanging glycoproteins that are part of the cell entry complex between EHV-1 and EHV-4 to further elucidate the process of virus entry [32–35]. So far, gD was found to play an essential role in determining the cellular tropism of EHV-1 and EHV-4 in culture [33]. gH on the other

hand was shown to be responsible for differences in the entry route taken by EHV-1 and EHV-4 [32]. We were interested in exchanging gB to uncover possible functional differences between the two viruses, thereby further elucidating the role of gB in tropism and pathogenicity. gB is highly similar between EHV-1 and EHV-4 and the proteins share an amino acid identity of 81.1% (Figure 2.1).

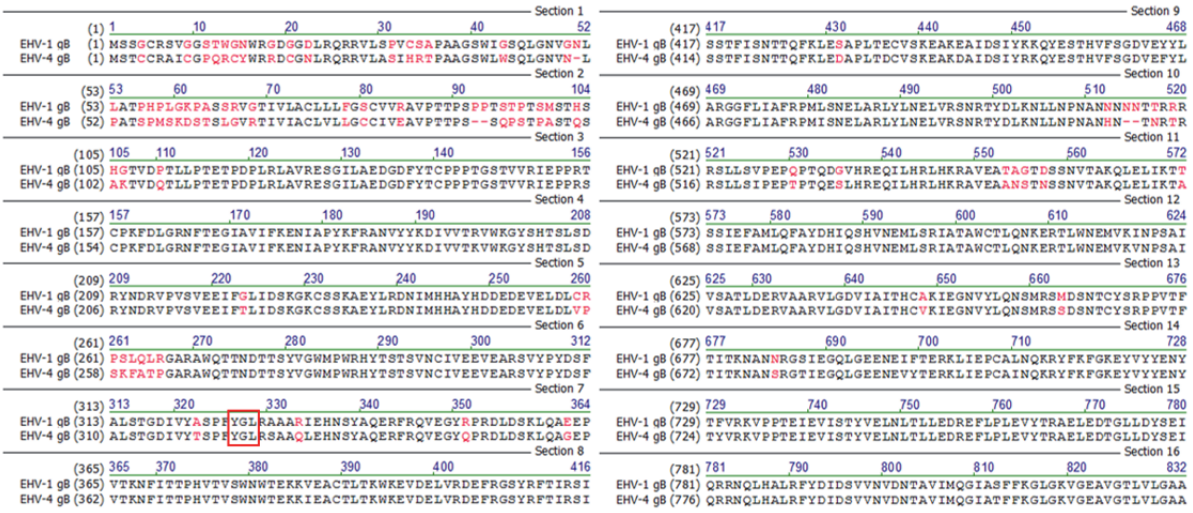

Figure 2.1. Amino acid sequence alignment of Equine Herpesvirus Type 1 and Type 4 (EHV-1 and EHV-4) glycoprotein B (gB). The putative integrin-binding motif tyrosine-glycine-leucine (YGL) present in the extracellular domains of both gB1 and gB4 (red frame). gB1 and gB4 exhibit 81.1% sequence identity. Sequences were aligned using Vector NTI software (version 9, Invitrogen, Carlsbad, CA, USA).

gB also contains a putative integrin-binding motif, tyrosine-glycine-leucine (YGL), which is conserved in both EHV-1 and EHV-4, and can potentially interact with α4β7, α4β1, and α9β1 integrins [36]. YGL is also present in the VP4 spike protein of rotaviruses where it mediates cell entry [36]. In a recent study, a similar integrin binding motif, leucine-aspartic acid-isoleucine (LDI), present in EHV-1 gH and interacting with cellular α4β1 integrins, has been implicated in determining the entry pathway taken by EHV-1 in equine cells [32]. Since integrin-binding motifs were shown to have significant roles during viral infection, we addressed the role of YGL-motif during EHV-1 and EHV-4 entry.

Here we show that exchanging gB between EHV-1 and EHV-4 resulted in the generation of stable recombinant viruses; however, a significant attenuation in the case of EHV-4_gB1 was evident.

2.3 Materials and Methods

2.3.1 Viruses

EHV-1 strain Ab4 [isolated from a quadriplegic mare [37] was cloned as a bacterial artificial chromosome (BAC) by replacing the nonessential *gp2 gene* with a mini-F plasmid, containing a *chloramphenicol resistance gene* and the *eGFP gene*, (Ab4Δgp2) [38–40]. The EHV-4 infectious BAC clone was generated by the insertion of a *lox*P-flanked BAC vector into the intergenic region between genes *58* and *59* [41]. Viruses were reconstituted after transfecting BAC DNA into human embryonic kidney (293T) cells, as described earlier [41–43]. Supernatant and cells were collected 48 h post-transfection, and high titer stocks of each virus were produced by passaging the transfection product on equine dermal (ED) cells.

2.3.2 Plasmids

Transfer plasmids encoding either EHV-1 or EHV-4 gB with a kanamycin resistance (*KanR*) gene were constructed. EHV-1 and EHV-4 gB genes were amplified by PCR using primers P1 and P2 or P3 and P4 (Table 1). The PCR products were digested with the restriction enzymes XhoI and XbaI (New England Biolabs, NEB, Schwalbach, Germany) and inserted into the vector pBluescript II KS+ (pKS), resulting in recombinant plasmids pKSgB1 and pKSgB4. To construct pKSgB1-KanR and pKSgB4-KanR, the *KanR gene* was amplified by PCR from plasmid pEPkan-S using primers P5, P6, P7, and P8 (Table 2.1), digested with the appropriate restriction enzymes, and inserted into pKSgB1 and pKSgB4. Correct amplification and insertion were confirmed by Sanger sequencing (LGC Genomics, Berlin, Germany).

Table 2.1. Oligonucleotide primers used in this study.

Primer	Product	Sequence
P1	gB1	*aat***ctcgag**atgtcctctggttgccgttc
P2	gB1	*aac***tctaga**ttaaaccatttttttcatttt
P3	gB4	*aat***ctcgag**atgtccacttgttgccgtgc
P4	gB4	*aca***tctaga**ttaaaccatttttttcgcttt
P5	KanR 1	*acc***ggatcc**accgtcgtacgcatcgaaccaggatgacgacgataagtaggg
P6	KanR 1	*ggt***ggatcc**ggtaggcggtgggcaggtgtcaaccaattaaccaattctgattag
P7	KanR 4	*act***ggatcc**acagttgtacgcattgaaccaggatgacgacgataagtaggg
P8	KanR 4	*tgt***ggatcc**agtaggcggcgggcaggtgtcaaccaattaaccaattctgattag
P9	gB1 deletion	agcgctgcgtgagcggcatttacataacctacgaggcgtcacatgtttaataaatattataggatgacgacgataagtaggg
P10	gB1 deletion	tcacactttgagtacgtgtcataatatttattaaacatgtgacgcctcgtaggttatgtacaaccaattaaccaattctgattag
P11	gB4 deletion	agcgctgcgctagcggcatttacataacatacgagacgtcaaatgttaaataaatatttaggatgacgacgataagtaggg
P12	gB4 deletion	tcaacccacaagtacgtgtcaaaatatttatttaacatttgacgtctcgtatgttatgtacaaccaattaaccaattctgattag
P13	gB4 KanR	agcggcgcacagcgctgcgtgagcggcatttacataacctacgaggcgtcatgtccacttgttgccgtgc
P14	gB4 KanR	aaatatgaggtcacactttgagtacgtgtcataatatttattaaacatgtttaaaccatttttttcgcttt
P15	gB1 KanR	aacggcgcacagcgctgcgctagcggcatttacataacatacgagacgtcatgtcctctggttgccgttc
P16	gB1 KanR	caaatatgagtcaacccacaagtacgtgtcaaaatatttatttaacatttttaaaccatttttttcatttt
P17	gB^{Y336A}	ctgtccaccggtgatattgtgtacgcgtctccgttt**GC**cggcctgagggctgccgctcgcaggatgacgacgataagtaggg
P18	gB^{Y336A}	gtagctattgtgctctatgcgagcggcagccctcaggccg**GC**aaacggagacgcgtacaccaaccaattaaccaattctgattag
P19	Sequencing	ctcggttttccactgtggag
P20	Sequencing	ggtgaatgaggatgaaacct
P21	Sequencing	cgaccacgccaagccccccaac
P22	Sequencing	cggcctcccccactttacccag
P23	Sequencing	atcgaaccacctagaacttg
P24	Sequencing	gtcagctggaactggac
P25	Sequencing	gggcgggagtagcacgtgtt
P26	Sequencing	agccccccaaatgggttgt
P27	Sequencing	ccacggtcatgtcccaagtt
P28	Sequencing	ttctcttcggttttccactg
P29	Sequencing	ttggcaaaaatactaggctt

Restriction enzyme sites are given in lower case bold letters; sequences in italics indicate additional bases which are not present in the EHV-1 or -4 sequence; Underlined sequences indicate the template binding region of the primers for PCR amplification with pEPkan-S; Upper case bold letters indicate the nucleotides that were mutated.

2.3.3 Cells

293T, Rabbit kidney (RK13), Henrietta Lacks (HeLa), African green monkey kidney (Vero), Crandell feline kidney (CrFK) and Madin-Darby canine kidney (MDCK) cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom), and 1% penicillin-streptomycin.

ED, Chinese hamster ovary (CHO)-K1, and CHO cells expressing HevA, HevB, and HevC (CHO-A, CHO-B and CHO-C cells, respectively; a kind gift from P. Spear, Northwestern University, Chicago, IL, USA) were grown in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Paisley, UK) supplemented with 20% FBS, 1% nonessential amino acids (Biochrom) and 1% 100 mM sodium pyruvate (Biochrom).
For generation of Vero cells, which transiently express EHV-4 gB (Vero/gB4), cells were transfected with the vector pcDNA3 containing gB4 (Invitrogen) using Lipofectamine 2000 (Invitrogen).
Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood collected by density gradient centrifugation over Histopaque 1077 (Sigma, St. Louis, MO, USA), following the manufacturer's instructions. After two washing steps, cells were suspended in RPMI 1640 supplemented with 10% FBS, 0.3 mg/mL glutamine, non-essential amino acids (Biochrom AG, Berlin, Germany), and 1% penicillin-streptomycin.

2.3.4 BAC Mutagenesis

The generated BACs were maintained in *Escherichia coli* (*E. coli*) GS1783 (a kind gift from Greg Smith, Northwestern University, Chicago, IL, USA), which harbor the recombination system of phage λ under the control of a temperature-sensitive repressor [44]. Deletion of gB in EHV-1 and EHV-4 was done by a two-step recombination method as described before [45]. Briefly, primers P9, P10, P11, and P12 (Table 1) were used to generate homology arms (50 nucleotides) by PCR enabling the substitution of *gB* by *KanR*. PCR products were digested with DpnI in order to remove residual template DNA. The fragments were then transformed into GS1783 cells containing the BACs by electroporation. Kanamycin-resistant colonies were purified and screened by PCR and restriction fragment length polymorphism (RFLP) to detect correctly recombinant clones. Positive clones were subjected to a second round of Red recombination, excising the *KanR gene*, to obtain the intermediate constructs pEHV-1ΔgB and pEHV-4ΔgB.
The transfer gB1Kan and gB4Kan sequences were amplified by PCR using pKSgB1-KanR and pKSgB4-KanR as templates and primers P13, P14, P15, and P16 (Table 1). PCR products were then electroporated into GS1783 harboring pEHV-1ΔgB or pEHV-4ΔgB. After selection on LB agar plates containing 34 μg/mL chloramphenicol and 50 μg/mL kanamycin, resistant colonies were purified and screened by PCR and RFLP to detect *E. coli* harboring recombinant pEHV-1_gB4Kan and pEHV-4_gB1Kan. Positive clones were subjected to a second round of Red recombination to obtain the final constructs pEHV-1_gB4 and pEHV-4_gB1.

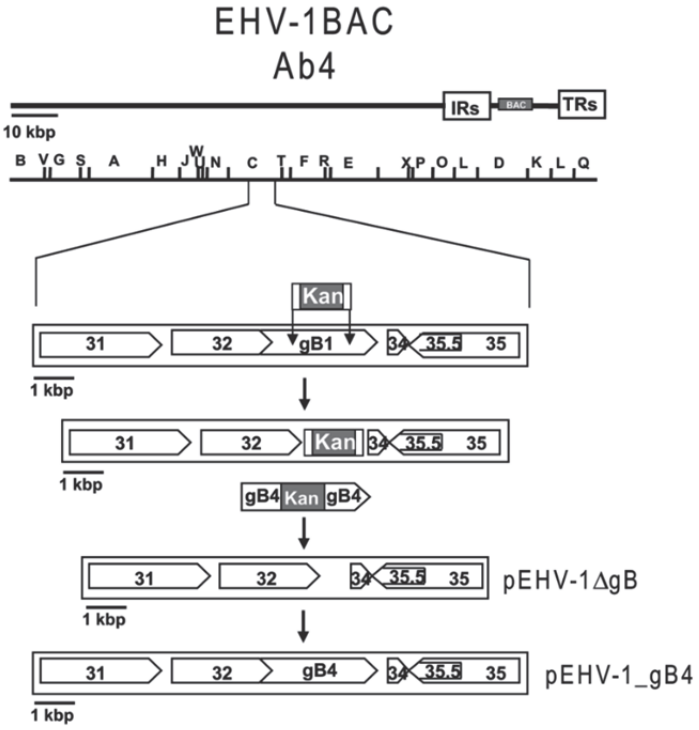

Figure 2.2. Schematic diagram of the procedures used to construct mutant genomes (EHV-1_gB4 as model). The two unique regions (UL and US) of the EHV-1 genome as well as the terminal and internal repeat sequences (TRS and IRS) are shown, as is the inserted mini-F cassette. Kanamycin resistance (*KanR)* PCR products were transformed into GS1783 containing the EHV-1 BACs by electroporation and the first step of the Red recombination method ensued. In the second step of Red recombination, the *KanR gene* was excised, thereby obtaining the pEHV-1ΔgB deletion construct. The PCR-amplified gB4Kan was then electroporated into GS1783 harboring pEHV-1ΔgB, and, again, two-step Red recombination was performed to obtain the final construct pEHV-1_gB4.

A point mutation targeting the YGL motif present in EHV-1 gB was generated by introducing alanine into the tyrosine position (EHV-1_gB^{Y336A}). Mutants were generated through two-step red recombination using primers P17 and P18 (Table 1) as described above.
The respective genotypes of all the mutants and revertants were confirmed by PCR, RFLP, and Sanger sequencing (primers P19-P29; Table 1; data not shown).

2.3.5 Western Blot Analysis

For Western blot analyses, pellets of infected ED cells were suspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris (pH 7.4), 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM sodium chloride, 1 mM EDTA) with the complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Sample buffer (1 M Tris-HCl (pH 6.8), 0.8% sodium dodecyl sulfate (SDS), 0.4% glycerol, 0.15% β-mercaptoethanol, 0.004% bromophenol blue) was added to lysates, the mixture was heated at 95 °C for 5 min, and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) as described before [46]. Expression of gB was detected with anti-EHV-1 gB Mab 3F6, which is also cross-reactive with EHV-4 gB (1:1000) [47]. Rabbit anti-β-actin antibody (1:2000) (Cell signaling Technologies, Danvers, MA, USA) was included as a loading control. Goat anti-mouse or goat

anti-rabbit (1:10,000) IgG peroxidase conjugates (Southern Biotech, Birmingham, AL, USA) were used as secondary antibodies. Reactive bands were visualized by enhanced chemiluminescence
(ECL Plus; Amersham, GE Healthcare, Piscataway, NJ).

2.3.6 Virus Growth Assays

To determine differences in viral replication in culture, single step growth kinetics for EHV-1 and multi-step growth kinetics for EHV-4 were conducted as described before [34]. Briefly, confluent ED cells were infected with a multiplicity of infection (MOI) of 1 in case of EHV-1 parental virus, EHV-1_gB4 and EHV-1 revertant. An MOI of 0.1 was used for EHV-1_ gB^{Y336A} in comparisons with parental EHV-1. In case of parental EHV-4, EHV-4_gB1, and revertant EHV-4, an MOI of 0.01 was used. After 1 h, cells were washed and treated with citrate buffer (pH = 3). Infected cells and supernatant were collected separately for EHV-1 and combined for EHV-4 at the indicated times post infection (p.i.), and stored at −80 °C. Viral titers were determined on ED cells.

Plaque size measurements were conducted by infecting ED cells for 1 h at 37 °C with an MOI of 0.01, followed by removal of the virus suspension and overlay with DMEM containing 0.5% methylcellulose (Sigma, St. Louis, MO, USA). After 72 h.p.i, the diameters of 100 fluorescent plaques for each virus were measured using ImageJ software vl.32j (vl.32j, National Institutes of Health, Bethesda, MD., USA, Year) (http://rsb.info.nih.gov/ij/). The obtained values were normalized and compared to the values of parental viruses, which were set to 100%. Three independent experiments were used to calculate average plaque sizes and standard deviations.

2.3.7 Virus Infection Assay

For evaluating efficiency of replication and cell tropism, confluent monolayers of different cell types were infected with an MOI of 0.1 of the respective viruses. Cells were then washed and overlaid with the appropriate medium. After 48 h p.i, cells were inspected with immunofluorescence microscope (Zeiss Axiovert, Jena, Germany) and pictures were taken with an Axiocam CCD camera (Zeiss, Jena, Germany).

2.3.8 Flow Cytometry

One million PBMC were incubated with EHV-1 and the engineered recombinant viruses at an MOI of 1 for 48 h at 37 °C. After incubation, the percentage of infected cells was determined by measuring eGFP expression with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.3.9 Pharmacological Inhibitors

Cells were pretreated with different drugs for 60 min as described before [48], and infected with parental and recombinant viruses using an MOI of 0.05 for 24 h in the presence of the drugs. Cells were then trypsinized and washed twice with PBS. After centrifugation, cells were resuspended in PBS, and 10,000 cells were analyzed for eGFP expression with a FACSCalibur flow cytometer. The drug concentrations used were 100 µg/mL genistein (Sigma, St. Louis, MO, USA) dissolved in DMSO, 10 µg/mL chlorpromazine (Sigma, St. Louis, MO, USA) in PBS, and 80 µM dynasore (Sigma, St. Louis, MO, USA) in DMSO.

2.3.10 Statistical Analysis

Statistical analyses (described in context) were performed using GraphPad PRISM (Version 5, GraphPad Software Inc., La Jolla, CA, USA). Normally distributed datasets, determined with the Shapiro–Wilks test, were analyzed with one-way ANOVA. Datasets that were not normally

distributed were analyzed with Kruskal–Wallis one-way analysis of variance for two or more samples that are independent or the Friedman test for repeated measures.

2.4 Results

2.4.1 gB Expression by the Recombinant Viruses

To determine whether gB was properly expressed by the recombinant viruses, ED cells were infected with parental, recombinant and revertant viruses. Cell lysates were then collected and subjected to Western blot analysis. For parental EHV-1, EHV-4 and related recombinant viruses, proteins with molecular weights of approximately 138 and 76 kD were detected that were not present in mock-infected cells (Figure 2.3). This is in accordance with previous reports where a partially glycosylated precursor of approximately 138 kD and a fully glycosylated subunit of gB of 75–77 kD were reported to be specifically recognized by Mab 3F6 [49]. These experiments confirmed that all generated recombinant and revertant viruses expressed gB as expected.

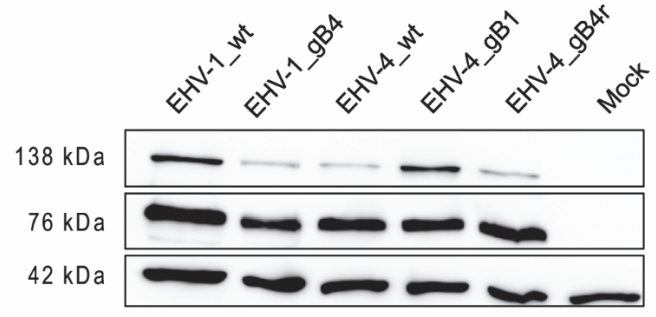

Figure 2.3. Expression of gB in virus-infected cells. Cell lysates were prepared from infected equine dermal (ED) cells and proteins were separated under reducing conditions by SDS-10%-PAGE. The blots were incubated with anti-gB MAb 3F6 and bound antibody detected with anti-mouse IgG peroxidase conjugate. EHV-1, EHV-4 and related recombinant virus proteins with an apparent molecular weight of approx. 130 and 76 kD were detected that are not present in mock-infected cells. Rabbit anti-β-actin antibody was used as a loading control (molecular weight of approx. 42 kDa).

2.4.2 EHV-4 gB Is Essential for Viral Replication

pEHV-4ΔgB DNA was transfected into Vero cells and the cells were monitored for 48 h. Only single infected cells (eGFP-positive/fluorescent cells) could be detected of which the number did not increase over time (Figure 2.4a). In contrast, Vero cells transfected with parental EHV-4 DNA showed syncytium formation after 48 h (Figure 2.4c). When Vero cells, which were transfected with gB4 as described previously for gH [35], were transfected with EHV-4ΔgB (Figure 2.4b), syncytium formation with a morphology similar to that induced by parental EHV-4 (Figure 2.4d) could be seen.

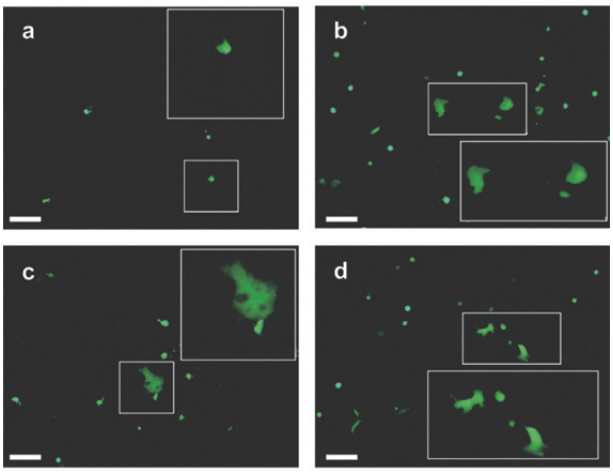

Figure 2.4. Transfection of Vero cells with EHV-4ΔgB. EHV-4ΔgB DNA was transfected in Vero cells (a) and Vero/gB4 cells (b). Also, parental EHV-4 DNA was transfected in both Vero (c) and Vero/gB4 cells (d). After 48 h of incubation, cells were inspected with an epifluorescent microscope (Zeiss Axiovert, Jena, Germany) and images were taken with a CCD camera (Zeiss Axiocam, Jena, Germany). The bar represents 100 µm and the white frames contain magnified inserts of the selected areas. Plaque formation of the gB-negative EHV-4 mutant was only evident on Vero/gB4 but not parental Vero cells.

2.4.3 Virus Growth in Culture

No significant differences were observed for any of the EHV-1 recombinants when compared to the parental virus (Figure 2.5a,c,d). We therefore concluded that the growth properties of recombinant EHV-1 in culture were not significantly affected by the exchange with EHV-4 gB.

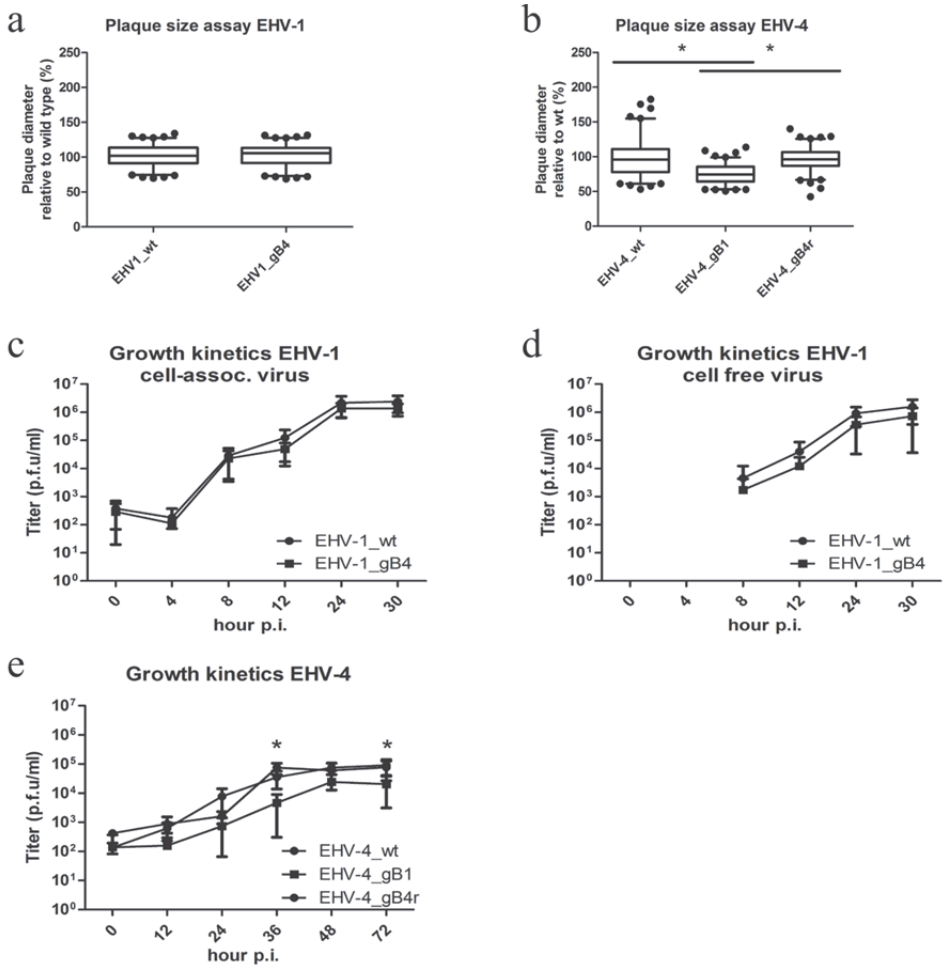

Figure 2.5. Growth characteristics of parental and recombinant EHV-1 and EHV-4 in cell culture. ED cells were infected with the respective viruses at an multiplicity of infection (MOI) of 0.01. Means ± SD of diameters of 100 plaques measured for each virus are shown. The plaque diameter of parental viruses was set to 100%. No significant differences (one-way ANOVA; $p > 0.05$) between parental EHV-1 and EHV-1_gB4 were obvious (a). A significant reduction (one-way ANOVA; $p < 0.05$) of plaque size for EHV-4_gB1 was evident when compared to parental and revertant virus. Means ± SD of diameters of 100 plaques measured for each virus are shown. The plaque diameter of parental viruses was set to 100% (b). For single step growth kinetics of EHV-1 recombinant viruses, ED cells were infected at an MOI of 1 (c,d), followed by citrate treatment (pH = 3) to remove remaining extra-cellular virions. Infected cells (c) and supernatants (d) were separately collected and virus titers were determined at the indicated times p.i. The data presented are means ± SD of three independent measurements. No significant differences were measured for the EHV-1 recombinant viruses when compared to the parental viruses (Friedman test-Dunn's multiple comparison test; $p > 0.05$). (e) For multi-step growth kinetics of EHV-4_gB1, ED cells were infected at MOI of 0.01, followed by washing. Infected cells and supernatants were collected and virus titers were determined at the indicated times p.i. The data presented are means ± SD of three independent measurements. A significant decrease was measured for EHV-4_gB1 at several time points (*) when compared to the parental and revertant viruses (Friedman test–Dunn's multiple comparison test; *p*

In the case of EHV-4_gB1, however, significantly reduced plaque sizes were seen compared to parental and revertant viruses (Figure 2.5b). Similarly, EHV-4_gB1 also showed a significantly reduced growth rate at several time points as evidenced by the growth kinetics (Figure 2.5e).

2.4.4 gB Has No Role in Determining the Host Range of EHV-1 and EHV-4 in Culture

EHV-1 can replicate and spread in many cell lines from equine and other origin [50]. In contrast, EHV-4 appears to be restricted mainly to equine cells and replicates poorly in only few cell lines, such as Vero cells [43]. In order to investigate whether gB plays a role in determining host range, as already shown for gD [33], several cell lines that are only permissible for either EHV-1 or EHV-4 were infected. Contrary to the exchange of gD between the two viruses, no changes in permissiveness were seen after exchanging gB between EHV-1 and EHV-4 (Figure 2.6; Table 2).

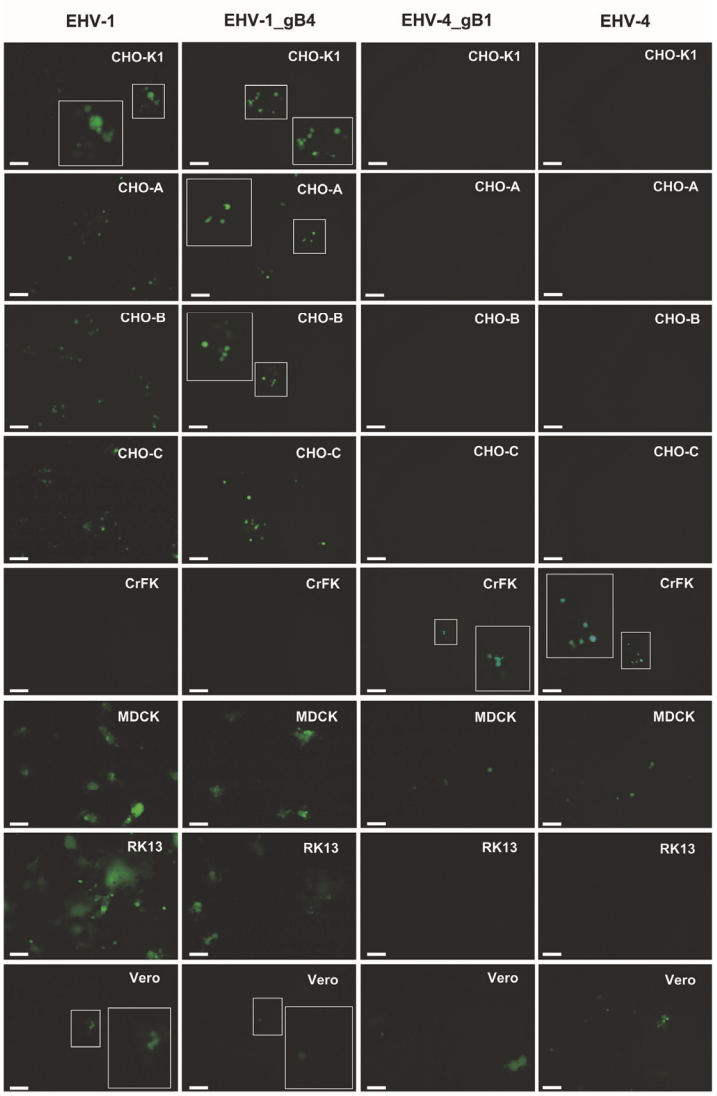

Figure 2.6. The role of gB) in EHV-1 cellular tropism. Chinese hamster ovary (CHO)-K1, CHO-A, CHO-B, CHO-C, Crandell feline kidney (CrFK), Madin-Darby canine kidney (MDCK), Rabbit kidney (RK13) and Vero cells were infected at an MOI of 0.1 with the parental EHV-1 and EHV-1_gB4, all of which express eGFP. At 24 h p.i., cells were inspected with a fluorescent microscope (Zeiss Axiovert, Jena, Germany) and images were taken with a CCD camera (Zeiss Axiocam, Jena, Germany). The bar represents 100 µm and the white frames contain magnified inserts of the selected areas. CHO-K1, CHO-A, CHO-B and CHO-C, and RK13 cells were highly resistant and MDCK virtually resistant to parental and recombinant EHV-4 infection. In addition, CrFK were highly resistant and Vero cells virtually resistant to parental and recombinant EHV-1 infection.

Table 2.2. The role of gB in EHV-1 cellular tropism.

Cell line	EHV-1	EHV-1 gB4	EHV-4	EHV-4 gB1
CHO-K1	+	+	−	−
CHO-A	+	+	−	−
CHO-B	+	+	−	−
CHO-C	+	+	−	−
GrFK	−	−	+	+
MDCK	+	+	+	+
RK13	+	+	−	−
Vero	+	+	+	+

Equine Herpesvirus (EHV), Chinese hamster ovary (CHO), Crandell feline kidney (CrFK), Madin-Darby canine kidney (MDCK), Rabbit kidney (RK13)

2.4.5 The Integrin-Binding Motif YGL Is Not Involved in EHV-1 Entry

The integrin-binding motif YGL was predicted with the I-TASSER server and is present in the extracellular domain of gB (http://zhang.bioinformatics.ku.edu/I-TASSER/). The motif is conserved in both EHV-1 and EHV-4. The YGL motif was mutated into AGL in EHV-1 (EHV-1_gB^{Y336A}) to evaluate its importance for cell entry and determining the cell entry pathway.

In previous studies, function-blocking MAbs α4β1 and α4β7 were used to investigate the role of integrins during entry into PBMC, ED and fetal horse kidney (FHK) cells. It was shown that blocking these potential receptors for EHV-1 and EHV-4 had no effect on their growth in culture or their ability to infect PBMC [35]. However, this does not exclude the possibility that the YGL motif could have an effect on viral growth and infection rates, since other unknown binding partners could be involved. However, no changes could be seen for either viral growth in culture (Figure 2.7a,c,d) or infection rates in PBMC (Figure 2.7b) between parental EHV-1 and EHV-1_gB^{Y336A}.

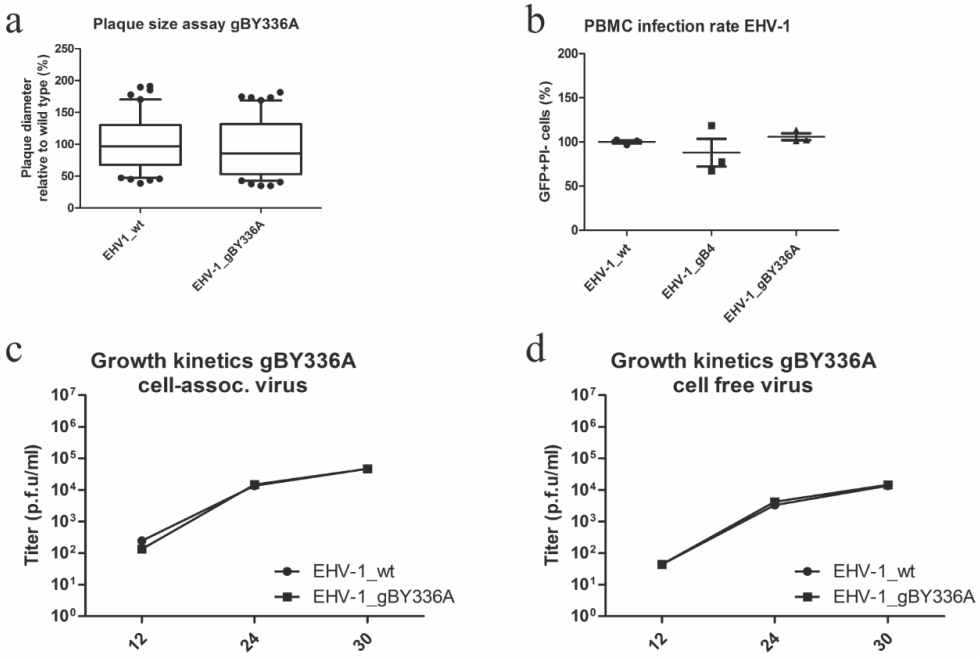

Figure 2.7. Characterization of EHV-1_gBY336A. (a) ED cells were infected with the respective viruses at an MOI of 0.01. Means ± SD of diameters of 100 plaques measured for each virus are shown. The plaque diameter of parental viruses was set to 100%. No significant differences (one-way ANOVA; $p > 0.05$) between parental EHV-1 and EHV-1_gBY336A were evident; (b) One million peripheral blood mononuclear cells (PBMC) were incubated with parental and recombinant EHV-1 viruses at an MOI of 1 for 24 h at 37 °C. After incubation, the percentage of infected cells was determined by flow cytometry. The rate of infection of parental virus was set to 100%. All data represent the means ± SD of three independent experiments (Kruskal–Wallis one-way analysis of variance; $p > 0.05$). For single-step growth kinetics of EHV-1 recombinant viruses, ED cells were infected at an MOI of 0.1 (c,d), followed by citrate treatment (pH = 3) to remove remaining extracellular virions. Infected cells (c) and supernatants (d) were separately collected and virus titers were determined at the indicated times p.i. The data presented are means ± SD of three independent measurements. No significant differences were detectable for the EHV-1 recombinant viruses when compared to the parental viruses (Friedman test–Dunn's multiple comparison test; $p > 0.05$).

2.4.6 gB and YGL Do Not Play a Role in Determining the Cell Entry Pathway

To investigate whether gB or the YGL motif have an effect on the viral entry pathways used by EHV-1, we conducted inhibitor studies using drugs that target various cellular functions associated with viral cell entry as described previously for gH [32].

Caveolae are plasma membrane invaginations that play an important role in cellular uptake of EHV-4 or EHV-1 after replacing authentic gH1 with EHV-4 gH [32]. Caveolae-mediated uptake depends on dynamin II [51], which can be inhibited by dynasore [52], and tyrosine kinases, which can be inhibited by genistein [53]. Chlorpromazine affects Clathrin-mediated endocytosis through the inhibition of Clathrin adaptor protein 2 assembly [54]. Since this drug does not affect the uptake of EHV-1 and EHV-4 [32], it was used as a negative control.

Contrary to the differences in cell entry pathway seen when exchanging gH, no significant differences could be seen between parental EHV-1 and EHV-1_gB4 or EHV-1_gB^{Y336A} after

using different inhibitors. In the case of EHV-4_gB1 as well as parental EHV-4, the number of infected cells was significantly reduced when treated with either genistein or dynasore. These data clearly indicated that gB has no role in routing the entry pathway of EHV-1 (Figure 2.8), but is rather required for entry steps after receptor binding.

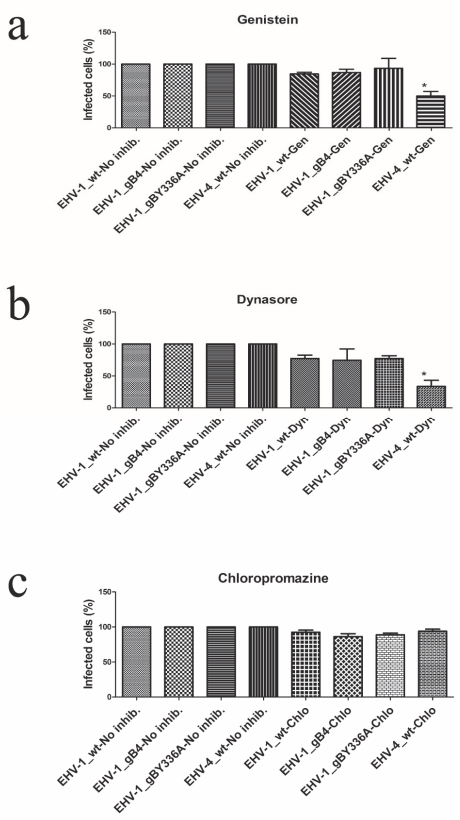

Figure 2.8. Inhibition of cell entry. ED cells were treated with the inhibitors genistein (Gen; (**a**)), dynasore (Dyn; (**b**)) and chloropormazine (Chlo; (**c**)), as indicated, before infection with parental or recombinant viruses at an MOI of 0.05. At 24 h p.i., cells were washed and the percentage of infected cells was determined by flow cytometry. The percentage of infection of the parental virus was set to 100%. The percentage of infection ($eGFP^+$) in the absence of inhibitors was set to 100%. All data represent the means ± SD of three independent experiments. Significant decreases (*****) were only seen for EHV-4_wt in the presence of dynasore and genestein (Kruskal–Wallis one-way analysis of variance; $p < 0.05$). However, no significant differences in infection rate were seen between parental and recombinant viruses in the different settings (Kruskal–Wallis one-way analysis of variance; $p > 0.05$).

2.5 Discussion

For several alphaherpesviruses, including EHV-1, it has been shown that gB is essential for infection [7,55–59]. More specifically, gB plays an important role in the cell-to-cell spread of alphaherpesviruses [2–6]. In the present study, we have addressed the importance of gB for EHV-4 replication. We have shown that the number of infected cells after transfection with pEHV-4ΔgB did not increase over time. This suggests that EHV-4 is not able to either be released from infected cells or enter uninfected cells without the help of gB. The gB-deleted virus was only able to induce syncytium formation when grown in pcDNAgB4-transfected Vero cells, in other words when the glycoprotein was provided *in trans*. It has been shown that gB is

involved in virus maturation and egress from the infected cells for different herpesviruses including HSV-1 and Kaposi's sarcoma-associated herpesvirus [60,61]. EHV-4 gB may also play a role in viral egress, including first or secondary envelopment or virion release into the extracellular space. However, we view an involvement of EHV-4 gB in egress as unlikely as those of other varicelloviruses are not required in the process either. Rather, we presume an essential role in virus entry and cell-to-cell spread but the exact role and the mechanism are not known at present and need to be further investigated. Taken together, the essentiality of gB functions during different virus replication steps is not unique to EHV-4 as all of the gB homologues across all subfamilies of the *Herpesviridae* studied until now have been shown to play similar roles.

The replacement of gB1 by gB4 in EHV-1 did not lead to significant changes in viral growth in culture. However, the EHV-4_gB1 recombinant exhibited a markedly reduced growth defect as evidenced by impaired viral cell-to-cell spread and reduced growth kinetics. This was particularly surprising since no apparent growth defect was seen in culture during previously conducted experiments where gB were exchanged between alphaherpesviruses of different natural hosts [62]. The growth defect of EHV-4_gB1 could be caused by a structural incompatibility of gB1 that EHV-4 was unable to compensate for. Alternatively, gB4 might have an additional role required for EHV-4 replication but not fulfilled by gB1. In a previous study, where gB of HSV-1 was replaced with gB of Saimiriine Herpesvirus 1 (SaHV-1), it was shown that HSV-1 gB possessed and additional function lacking for SaHV-1 gB. Loss of functional interaction with PIRLα was reported for SaHV-1 despite sequence alignment suggesting that the interaction site is conserved [63].

Therefore, it would be interesting to further elucidate the structural differences between gB1 and gB4 and identify putative viral interaction partners of gB. After binding of gD to cellular receptors it activates gH-gL complex, which in turn primes gB for fusion. Perhaps, the authentic EHV-4 gH-gL complex could not prime gB1 as efficiently as the native gB4 for fusion. It will, therefore, be interesting to examine whether a triple mutant, *i.e.*, EHV-4 harboring gH1-gL1-gB1 and *vice versa*, would lead to any changes of the virus' ability to effectively replicate.

Recently it was shown that EHV-1 gD, besides its primary interaction partner MHC class I, can bind to a wide array of receptors and that it is responsible for determining the host range of the virus. The case is less clear for EHV-4 gD, which also uses MHC class I as an entry receptor and likely another molecule to enter, e.g. Vero cells [33]. In the current study, we showed that exchanging gB has no effect on the entry of the recombinant viruses into the selected cell lines, suggesting that gB is not important in determining cellular host range. It seems likely that, in contrast to HSV-1 [10–12], gB of EHV-1 or EHV-4 does not need to bind to different cellular receptors to facilitate fusion, that gB binding is not important for cellular tropism, or that gB and receptor interaction are not absolutely required for entry. However, this conclusion does not exclude the role of gB, together with gC, to bind cell surface 52eparin sulfate and help the attachment of virions to cells during the initial events of infection [34].

Disrupting the integrin-binding motif YGL in gB1 did not have any effect on virus growth in culture. Furthermore, YGL apparently does not play a decisive role in determining the cell entry pathway of EHV-1 or EHV-4. These results are in accordance with previous work where binding between the integrin-binding motif YGL and selected known ligands α4β1 or α4β7 was mitigated using blocking antibodies. These experiments revealed that YGL does not need to interact with its known binding partners for infection [64]. From these results, we concluded that gB-integrin interaction does not play an important role in cell entry or determining the cell entry

pathway; however, it may have a role in signaling transduction that might be needed during other steps of virus replication. Furthermore, these data do not necessarily mean that no interaction occurs between EHV-1 and the respective integrins. The integrins may serve as a receptor and/or co-receptor for viral entry and their blockade may not have a measurable effect on virus infection, especially, if alternative receptors exist.

Due to its importance in cell-to-cell spread [6], gB would be an interesting target for future research on the spread of EHV-1 between infected PBMC and endothelial cells (EC). This process enables infection of EC even in the presences of neutralizing antibodies, causing vascular lesions and secondary hypoxic degeneration of affected tissues [24,65].

In summary, the replacement of gB1 by gB4 in EHV-1 did not lead to any significant changes in viral growth in culture compared to EHV-1. However, EHV-4 seems to be unable to fully compensate the structural changes introduced by the replacement of gB4 with gB1 with respect to replication in culture. Nonetheless, the generation of a stable EHV-1_gB4 recombinant virus gives us the tools to address viral entry and spread of EHV-1 and EHV-4 in other cell types in the future.

2.6 Acknowledgments

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2.7 Author Contributions

Bart Spiesschaert contributed to this work by designing, carrying out all the experiments, analyzing and interpreting the data. Nikolaus Osterrieder contributed to the drafting of the manuscript, revising it critically, and giving final approval of the version to be published. Walid Azab contributed to designing and interpreting the data as well as drafting of the manuscript and revising it critically. All authors read and approved the final manuscript.

2.8 Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3

Furin is solely responsible for cleavage of equine herpesvirus type 1 (EHV-1) glycoprotein B but cleavage is dispensable for virus replication

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3.1 Abstract

Glycoprotein B (gB) of both equine herpesvirus type 1 (EHV-1) and EHV-4 is predicted to be cleaved by furin in a similar manner to this of related herpesviruses. To investigate the contribution of furin-mediated gB cleavage to EHV-1 and EHV-4 growth, the cleavage sites were mutated. While mitigating furin recognition motif did not affect in vitro growth of EHV-1, reconstitution of the mutant EHV-4 was not successful, which confirms previous results indicating different properties of EHV-4 gB when compared to EHV-1. Western blot and mass spectrometry analysis of mutated EHV-1 gB suggest that mutating the furin cleavage site indeed prevented gB cleavage and resulted in a partial misfolding. In addition, a novel signal peptide cleavage site was identified for EHV-1 gB between residues 98 and 99, which is different from that previously published. We conclude that furin cleavage is solely responsible for gB cleavage and involved in the protein folding.

3.2 Introduction

Furin, formerly known as Paired basic Amino acid Cleaving Enzyme (PACE), is an endoprotease of the subtilisin superfamily that plays an important role in homeostasis, cancer, dementia, bacterial and viral diseases and many more pathologies [1, 2]. It activates numerous proproteins through cleavage within secretory pathway compartments, more specifically the trans-Golgi network (TGN) and endosomal vesicles [3, 4]. Furin activates cellular, but also viral and bacterial proteins, by cleaving at an arginine rich consensus site (Arg–X–Lys/Arg–Arg; where X is any amino acid) [5-8]. The function of the furin cleavage motif of glycoprotein B (gB) was evaluated for several herpesviruses. Abolishing furin cleavage is dispensable for *in vitro* replication of human cytomegalovirus (HCMV), bovine herpesvirus 1 (BHV-1), Epstein–Barr virus (EBV), Varicella zoster virus (VZV), murid herpesvirus 4 (MuHV-4) and pseudorabies virus (PRV) [9-14]. However, changes in phenotype were seen for several of these viruses. For example, smaller plaque sizes were reported for BHV-1 suggesting a role for proteolytic cleavage of gB in cell-to-cell spread [10]. Furthermore, reduced syncytium formation and attenuated replication in human skin xenografts were seen for PRV and VZV, respectively [12, 13]. Reduced cell fusion was also observed for EBV in epithelial and B-cells but not in fibroblasts, indicating a conserved function of furin-mediated cleavage among gB homologues from different herpesvirus subfamilies [11, 14]. Mitigating furin cleavage of MuHV-4 gB had no effect on cell entry of fibroblasts and epithelial cells, but showed a significant entry deficit in myeloid cells such as macrophages and bone marrow-derived dendritic cells [9]. gB of herpes simplex virus type 1 (HSV-1), on the other hand, is not cleaved [15]. It was clear from these studies that the role of furin cleavage of gB differs significantly from one virus to the other, but, when present, furin cleavage seemed to be critical for full gB functionality.

Equine herpesvirus (EHV)-1 is a member of the *Varicellovirus* genus within the *Alphaherpesvirinae* subfamily. The virus induces several severe clinical syndromes with infection of peripheral blood mononuclear cells (PBMC) being a key aspect in the pathogenesis, as viremia contributes to the systemic distribution of virus. The subsequent infection of different organs can result in abortion and neurological syndromes as well as respiratory disease [16-20]. Similar to other alphaherpesviruses, EHV-1 also requires gB for cell entry and cell-to-cell spread [21]. It was shown that gB mediates the virus entry process through direct fusion of the virion envelope with the cellular membranes. Alphaherpesviral gBs achieve fusion together with the heterodimeric gH-gL complex after gD has bound to its cognate receptor [21-25]. Information about EHV-1 gB is limited, but it appears to be a 138 kDa protein that, when fully glycosylated, is proteolytically cleaved into two subunits (77 kDa and 55 kDa). These subunits are linked by a disulfide bond(s) to form a 145 kDa complex [26]. As for other type-1 transmembrane proteins, herpesvirus gB has a signal sequence at its N-terminus involved in translocation of gB to the endoplasmic reticulum (ER) [27]. The signal peptide is subsequently cleaved, allowing structural modifications to gB. A previous report suggested signal peptide cleavage of EHV-1 gB between residues 85 and 86 (^{85}AV^{86}) [28]. Although the site responsible for cleaving EHV-1 gB into two subunits is believed to be a cleavage motif (^{518}RRRR^{521}), which is recognized by furin, a second endoproteolytic cleavage site was identified for EHV-1 gB (^{548}RA^{549}), suggesting the removal of the 28 amino acids between both cleavage sites [28]. In case of EHV-4, sequence analysis of gB also identified a putative furin cleavage site (^{513}RTRR^{516}). As there was no previous studies addressing the role of furin cleavage in EHV-1 replication, we addressed the question whether abolishing furin cleavage of EHV-1 gB has an attenuating effect on virus infection, particularly in PBMC infection, as has been shown before for MuHV-4 [9]. The experimental setup used for

the present study is very similar to the experiments performed by Oliver and colleagues on VZV [13]. Our studies identified a novel signal peptide cleavage site of EHV-1 gB and we report on the importance of furin-mediated cleavage of EHV-1 and EHV-4 gB by analyzing gB expression with western blot and mass spectrometry analysis.

3.3 Materials and Methods

3.3.1 Viruses

All viruses used in the study were recovered from infectious bacterial artificial chromosome (BAC) clones. Those were BACs of EHV-1 strain Ab4 [29] and EHV-4 strain TH20p [30]. Viruses were reconstituted after transfecting BAC DNA into human embryonic kidney (293T) cells, as described earlier [30-32]. Supernatants and infected cells were collected 48 h after transfection, and high titer stocks of each virus were produced by passaging the transfection product on equine dermal (ED) cells.

3.3.2 Cells

ED were grown in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 20% FBS, 1% of nonessential amino acids (Biochrom) and 1% of 100 mM sodium pyruvate (Biochrom). PBMC were isolated from heparinized blood collected from healthy horses by density gradient centrifugation over Histopaque 1077 (Sigma), following the manufacturer's instructions. After two washing steps, cells were suspended in RPMI 1640 supplemented with 10% FBS, 0.3 mg/ml glutamine, non-essential amino acids (Biochrom), and 1% penicillin-streptomycin.

3.3.3 BAC mutagenesis

The generated BACs were maintained in *Escherichia coli* (*E. coli*) GS1783 (a kind gift from Dr. Greg Smith, Northwestern University, Chicago, IL), which harbor the recombination system of phage λ under the control of a temperature-sensitive repressor [33]. Deletion or replacement of the furin cleavage motif was facilitated by two-step recombination as described before [34]. Briefly, primers P1, P2, P3, P4, P5, P6, P7 and P8 (Table 3.1) were used to generate homology arms (50 nucleotides) by PCR enabling the deletion or substitution of the furin motif and insertion of the Kanamycin resistance gene (Kan^R). PCR products were digested with *Dpn*I in order to remove residual template DNA. The fragments were then transformed into GS1783 cells containing the BACs by electroporation. Kanamycin-resistant colonies were purified and screened by PCR and restriction fragment length polymorphism (RFLP) to detect correct recombinant clones. Positive clones were subjected to a second round of Red recombination, which resulted in removal of the Kan^R gene, to obtain the final constructs pEHV-1Δ^{518}RRRR^{521}, pEHV-1_^{518}AAAA^{521}, pEHV-4Δ^{513}RTRR^{516} and pEHV-4_^{513}AAAA^{516}. pEHV-1_^{518}AAAA^{521} was subjected to a second round of two-step recombination in order to mutate the putative cleavage site "^{495}RSNR^{498}" (primers P9 and P10; Table 3.1). The respective genotypes of all the mutants were confirmed by PCR, RFLP, and Sanger sequencing (primers P11-P20; Table 1; data not shown).

Table 3.1. Oligonucleotide primers used in this study.

Primer	Product	Sequence
P1	Δ^{518}RRRR^{521}	aaccccaatgcaaacaataacaataacaccacgtctctcctgtcagtaccagaacctcagaggatgacgacgataagtaggg
P2	Δ^{518}RRRR^{521}	ctgaggttctggtactgacaggagagacgtggtgttattgttattgtttgcattggggttcaaccaattaaccaattctgattag
P3	^{518}AAAA^{521}	aacaataacaataacaccacg**GCCGC**a**GC**c**GC**gtctctcctgtcagtaccagaacctcagaggatgacgacgataagtaggg
P4	^{518}AAAA^{521}	ctgaggttctggtactgacaggagagac**GC**g**GC**t**GCGGC**cgtggtgttattgttattgttcaaccaattaaccaattctgattag
P5	Δ^{513}RTRR^{516}	accccaacgcaaaccataataccaattcgctactatcaataccagaacctactccaacccaggatgacgacgataagtaggg
P6	Δ^{513}RTRR^{516}	gggttggagtaggttctggtattgatagtagcgaattggtattatggtttgcgttggggtcaaccaattaaccaattctgattag
P7	^{513}AAAA^{516}	accccaacgcaaaccataataccaat**GCCG**ca**GC**c**GC**gtcgctactatcaataccagaacaggatgacgacgataagtaggg
P8	^{513}AAAA^{516}	gttctggtattgatagtagcgac**GC**g**GC**tg**CGGC**attggtattatggtttgcgttggggtcaaccaattaaccaattctgattag
P9	^{495}AAAA^{498}	gctgtacctgaacgagcttgtg**GCCG**c**CGC**c**GC**cacctacgacctaaaaaatctattgaaaggatgacgacgataagtaggg
P10	^{495}AAAA^{498}	ttcaatagatttttttaggtcgtaggtg**GC**g**GCG**g**CGGC**cacaagctcgttcaggtacagccaaccaattaaccaattctgattag
P11	Sequencing	ctcggttttccactgtggag
P12	Sequencing	ggtgaatgaggatgaaacct
P13	Sequencing	cgaccacgccaagccccccaac
P14	Sequencing	cggcctcccccactttacccag
P15	Sequencing	atcgaaccacctagaacttg
P16	Sequencing	gtcagctggaactggac
P17	Sequencing	gggcgggagtagcacgtgtt
P18	Sequencing	agccccccaaatgggttgt
P19	Sequencing	ccacggtcatgtcccaagtt
P20	Sequencing	ttctcttcggttttccactg

Underlined sequences indicate the template binding region of the primers for PCR amplification with pEPkan-S; Upper case bold letters indicate the nucleotides that were mutated.

3.3.4 Western blot analysis

For western blot analyses, pellets of infected ED cells were suspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.4], 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM sodium chloride, 1 mM EDTA) with the Complete EDTA-free protease inhibitor cocktail (Roche). Sample buffer (1 M Tris-HCl [pH 6.8], 0.8% sodium dodecyl sulfate [SDS], 0.4% glycerol, 0.15% β-mercaptoethanol, 0.004% bromophenol blue) was added, the mixture was heated at 95°C for 5 min, and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) as described before [35]. Expression of gB was detected with anti-EHV-1 gB MAb 3F6 (1:1,000) which recognizes a linear epitope located between aa 107 and 171 of EHV-1 gB, thereby only targeting the N-terminal cleavage fragment and uncleaved gB [36]. Bound 3F6 was detected with anti-mouse IgG peroxidase conjugate. Reactive bands were visualized by enhanced chemiluminescence (ECL Plus; Amersham).

3.3.5 Virus growth assays

To determine differences in viral replication *in vitro*, single-step growth kinetics were conducted as described before [37]. Briefly, confluent ED cells were infected with a multiplicity of infection (MOI) of 1. After 1 h of adsorption at 4 °C and 1 h of incubation at 37°C, cells were washed and treated with citrate buffer (pH= 3). Infected cells and supernatant were collected separately at the indicated times post infection (p.i.) and stored at -80°C. Viral titers were determined by titration on ED cells.

Plaque size measurements were conducted by infecting ED cells for 1 h at 37°C at an MOI of 0.01, followed by removal of the virus suspension and overlay with DMEM containing 0.5% methylcellulose (Sigma). After 72 h p.i, the diameters of 100 fluorescent plaques for each virus were measured using ImageJ software vl.32j (http://rsb.info.nih.gov/ij/). The obtained values were normalized and compared to the values of parental viruses which were set at 100%. Three independent experiments were used to calculate average plaque sizes and standard deviations.

3.3.6 Glycosylation analysis of gB

For deglycosylation, cell lysates were digested with peptide-N-glycosidase (PNGase) F or endoglycosidase H (Endo H) according to the manufacturer's instructions (New England BioLabs, Germany). After deglycosylation, lysates were subjected to SDS-PAGE and Western blot analysis as described above.

3.3.7 Infection assay

For evaluating the infection rates of the different PBMC subpopulations, $1X10^5$ PBMC were infected at an MOI of 1 with parental and mutant EHV-1 at 37°C for 1 h. PBMC were citrate-treated and washed twice as described above, followed by incubation at 37°C. The infected PBMC were stained 24 h p.i. with primary mouse antibodies against equine CD14 (monocytes), CD3 (T-lymphocytes) or IgM (B-lymphocytes) [29]. After washing, cells were labeled with a secondary Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (Invitrogen). The labeled PBMC were then analyzed by means of flow cytometric analyses of 10,000 cells. A FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Treestar) were used to detect GFP expression (encoded by the used viruses under HCMV IE promotor control) and Alexa Fluor 647 staining in infected PBMC.

3.3.8 Immunoprecipitation assay

Infected ED cells were suspended in RIPA buffer with the Complete EDTA-free protease inhibitor cocktail and incubated for 1 h on ice with occasional mixing. For immunoprecipitation, the cell lysates were pre-cleared for 30 min at 4 °C with 100 µl of protein G-Sepharose beads (Pierce). Anti-EHV-1 gB MAb 3F6 was then added to the samples and incubated overnight at 4°C, followed by 2 h of incubation with 200 µl of protein G-sepharose beads at 4°C. Beads were washed three times with PBS containing 0.2% Tween-20 (Carl Roth). The immunoprecipitates were subjected to SDS-PAGE as described above.

3.3.9 Protein sample preparation and mass spectrometry

After SDS-PAGE separation, excised protein bands were washed with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. Disulfide bonds were reduced by incubation in 60 µL of 10 mM DTT in 50 mM ammonium bicarbonate for 45 min at 56°C. Alkylation was performed by replacing the DTT solution with 55 mM iodoacetamide in 50 mM ammonium bicarbonate. In-gel digestion with trypsin, chymotrypsin, AspN, and elastase was performed overnight as described before [38]. Peptides were extracted using 20 mL of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile and the separated liquid was dried under vacuum. The samples were reconstituted in 6 mL of 0.1% (v/v) TFA and 5% (v/v) acetonitrile in water.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed using an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher) equipped with an UltiMate 3000 LC (Dionex) as recently described [39]. In brief, LC separations were performed on a capillary column (Acclaim PepMap100, C18, 2 µm, 150 mm x 75 µm i.d., Dionex) at an eluent flow rate of 200 nL/min using an acetonitrile 0.1% formic acid in water gradient. Mass spectra were acquired in a data-dependent mode with one MS survey scan with a resolution of 60,000 (Orbitrap Elite) and MS/MS scans of the 15 most intense precursor ions in the linear trap quadrupole.

For data processing, MS and MS/MS spectra were used to search against a custom made database containing all proteins of the SwissProt 2010_7 database (521,024 sequences; 183,901,752 residues) including the full-length EHV-1 gB sequence. For all searches, the following variable modifications were considered: carboxyamidomethyl for cysteine, oxidation

of methionine, and Asn/Asp amino acid exchanges. For identification of gB peptides, the processed MS/MS spectra were compared with the theoretical fragment ions of gB peptides using the MASCOT server version 2.2.2 (Matrix Science Ltd., London, UK). The maximum of two missed cleavages was allowed for tryptic and chymotryptic cleavages and up to six missed cleavages were allowed for the digestion with AspN and elastase. The mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively.

3.3.10 Statistical analysis

Statistical analyses (described in context) were performed using GraphPad PRISM 5 (Intuitive Software for Science). Normally distributed datasets, determined with the Shapiro-Wilks test, were analyzed with one-way ANOVA. Datasets that were not normally distributed were analyzed with Kruskal–Wallis one-way analysis of variance for two or more samples that are independent or Friedman test for repeated measures.

3.4 Results

3.4.1 Virus growth in vitro

No significant differences (n=3; Friedman test-Dunn's multiple comparison test; P>0.05) were observed during single-step growth kinetics in ED for either EHV-1_^{518}AAAA^{521} or EHV-1Δ^{518}RRRR^{521} when compared to the parental virus (Fig. 3.1a-b). We did not observe any significant differences either when evaluating the plaque sizes (n=100; one-way ANOVA; P>0.05; Fig. 3.1c). We, therefore, concluded that the growth properties of mutant EHV-1 *in vitro* were not significantly affected by the mitigation of the furin cleavage site. The reconstitution of EHV-4Δ^{513}RTRR^{516} and EHV-4_^{513}AAAA^{516} was not successful in a number of different attempts and no further evaluation was possible.

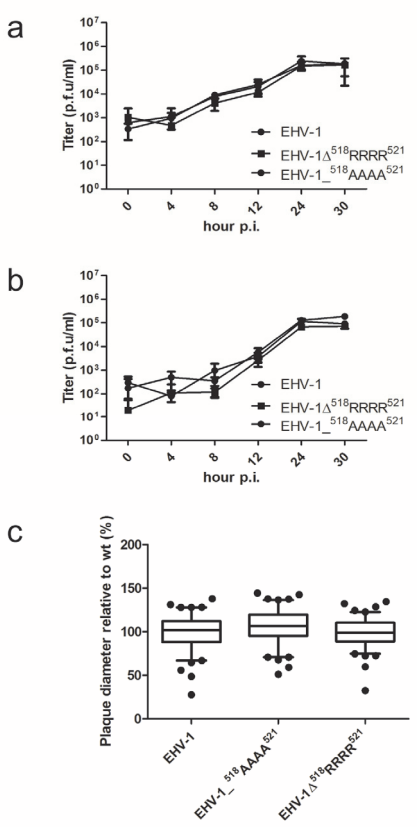

Figure 3.1. Growth and infection characterization. (a and b) For growth kinetics, infected cells (a) and supernatants (b) were separately collected and virus titers were determined at the indicated times p.i. The data presented are means ± SD of triplicate measurements. (c) ED cells were infected with the respective viruses at an MOI of 0.01. The central line in the box plot indicates the median of the data, while the edges of the box indicate the 25th and 75th percentiles. Extending from the box are whiskers, the top whisker expands to the 95th percentile and the bottom whisker to the 5th percentile. The dots represent the top and bottom 5th percentiles. The average plaque diameter of parental viruses was set to 100%.

3.4.2 Infection rates of different PBMC subpopulations by parental and mutant EHV-1

Monocytes were the most highly infected subpopulation by both parental [69.02±6.62% (EHV-1)] and mutant EHV-1 [70.17±5.98% (EHV-1_^{518}AAAA^{521}); 61.72±16.07% (EHV-1Δ^{518}RRRR^{521})], followed by the B-lymphocytes [24.28±15.27% (EHV-1); 33.75±13.33% (EHV-1_^{518}AAAA^{521}); 12.67±2.53% (EHV-1Δ^{518}RRRR^{521})] and T-lymphocytes [4.98±4.92% (EHV-1); 7.48±3.65% (EHV-1_^{518}AAAA^{521}); 4.12±1.15% (EHV-1Δ^{518}RRRR^{521})]. Contrary to what was reported for MuHV-4, no significant differences in infection rate between parental and mutant EHV-1 were seen (n=3; Kruskal–Wallis one-way analysis of variance; P>0.05) for monocytes, B-lymphoytes or T-lymphocytes (Fig. 3.2).

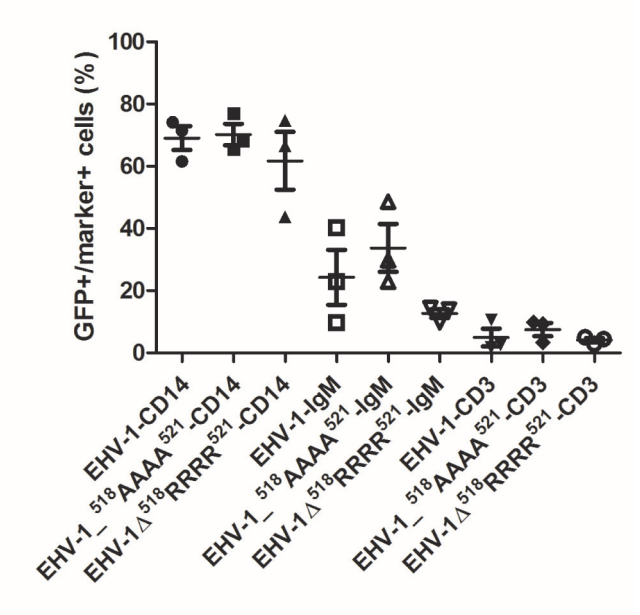

Figure 3.2. Infection rate of PBMC subpopulations. The percentage of infected cells for each subpopulation [Monocytes (CD14+), B-lymphocytes (IgM+) and T-lymphocytes (CD3+)] was determined relative to the total number of cells within the subpopulation by flow cytometry.

3.4.3 gB expression and cleavage in parental or mutant virus infected cells

To determine what effect the mutation of the furin cleavage site has on gB expression, ED cells were infected with parental or mutant viruses. Cell lysates were then collected and subjected to western blot analysis (Fig. 3.3 and 3.4). An apparent molecular weight of approximately 138 kDa (parental and mutant EHV-1) and 75 kDa (parental EHV-1) were detected. EHV-1Δ^{518}RRRR^{521} and EHV-1_^{518}AAAA^{521} showed an extra band with an apparent molecular weight of approximately 145 kDa and either approximately 90 kDa (EHV-1_^{518}AAAA^{521}) or 85 kDa (EHV-1Δ^{518}RRRR^{521}; Fig. 3.3a).

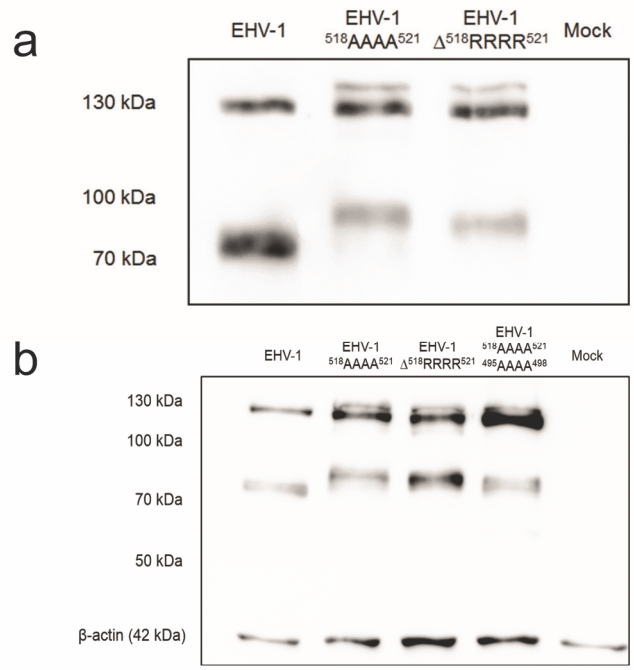

Figure 3.3. Expression of gB in virus-infected cells. (a and b) Western blot analysis performed under reducing conditions using a monoclonal anti-gB antibody. Rabbit anti-β-actin antibody was used as a loading control (molecular weight of approximately 42 kDa).

For deglycosylation analyses, PNGase-treatment resulted in proteins of approximately 110 kDa (parental and mutant EHV-1), 55 kDa (parental EHV-1), 65 kDa (EHV-1_^{518}AAAA^{521}) and 60 kDa (EHV-1Δ^{518}RRRR^{521}; Fig. 3.4a). The molecular weight of the extra 145 kDa in case of EHV-1_^{518}AAAA^{521} and EHV-1Δ^{518}RRRR^{521} was reduced to 120 kDa after PNGase-treatment (Fig. 3.4a). Deglycosylation with EndoH resulted in less pronounced decreases of molecular size for the proteins resulting in a molecular weight of approximately 120 kDa (parental and mutant EHV-1), 70 kDa (parental EHV-1), 80 kDa (EHV-1_^{518}AAAA^{521}), 80 kDa (EHV-1Δ^{518}RRRR^{521}) and 130 kDa for the extra band (mutant viruses; Fig. 3.4b). Overall, there was no difference between parental and mutant viruses in the reduction of the molecular weights of gB after deglycosylation. Taken together, this indicates that both mutant and parental gB glycosylated to same extent with a mixture of high-mannose and complex carbohydrate chains.
In order to explain the increase of apparent weight from 75 kDa to 90 kDa, the possibility of existence of alternative cleavage sites of the furin recognition motif was explored. Only one motif (^{495}RSNR^{498}) fulfilled the requirements of Arg-X-X-Arg↓ as described previously [1]. Therefore, the ^{495}RSNR^{498} was replaced by alanine amino acids, and gB of EHV-1 containing both mutations (EHV-1_^{518}AAAA^{521}/^{495}AAAA^{498}) was evaluated using Western blot. However, when the gB expression was compared, no difference was seen in molecular weight between EHV-1_^{518}AAAA^{521}/^{495}AAAA^{498} and its parental EHV-1_^{518}AAAA^{521} (Fig. 3.3b).

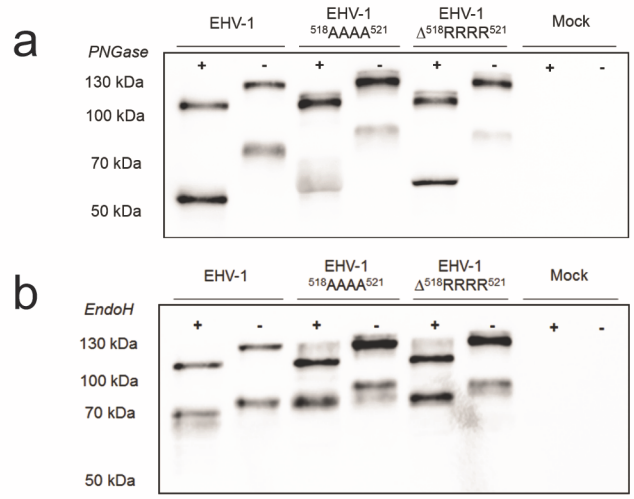

Figure 3.4. Glycosylation analysis of parental and mutant gB. SDS-PAGE analysis of gB expression after treatment with (a) PNGase or (b) EndoH. Controls (−) were treated similar to the corresponding enzyme-treated (+) samples, but without adding the enzymes.

3.4.4 Analyzing EHV-1 gB properties after furin cleavage mitigation by mass spectrometry

The observed changes in the migration pattern of gB are a clear indication of changes of the properties of gB, such as additional cleavage sites, protein folding or glycosylation. Since these changes cannot be attributed to differences in glycosylation (Fig. 3.4), it could still be caused by cleavage at an alternate, still to be determined, site or by misfolding of the protein. In order to determine the apparent upward shift of the lower gB band from 75 kDa to approximately 90 kDa, this subunit was immunoprecipitated from cell lysates of EHV-1 or EHV-1_^{518}AAAA^{521}-infected ED cells, separated by SDS-PAGE, and stained with Coomassie blue. The band, corresponding to approximately 75 kDa (EHV-1) or 90 kDa (EHV-1_^{518}AAAA^{521}), previously identified by western blot (Fig. 3.3a), was excised from the gel (Fig. 3.5a). The protein corresponding to 75 kDa was digested with trypsin, while the protein corresponding to 90 kDa was digested with either trypsin, elastase, AspN or chymotrypsin. The resulting peptides were eluted from the gel slices and subsequently subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). EHV-1 gB was unambiguously identified with high confidence in all samples. In the samples subjected to the trypsin digest, two predicted fragments [residues 28-66 and residues 67-84; predicted by ExPASy PeptideCutter (http://www.expasy.org/tools/peptidecutter/)] at the N-terminal end of gB were missing. The absence of this sequence indicates that this sequence is the signal peptide sequence, which was removed in an earlier stage of gB processing (Fig 3.5b). Contrary to the previously reported signal cleavage site (^{85}AV^{86}) [28], MS analysis after elastase digestion identified a new putative signal cleavage site between residues ^{98}TS^{99}.

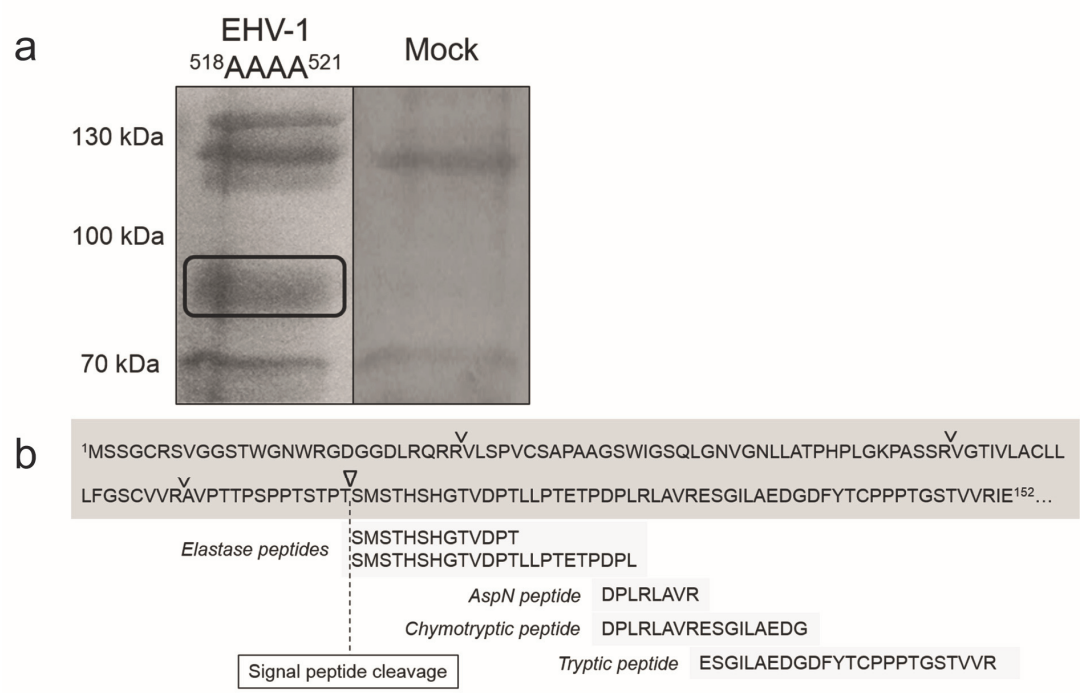

Figure 3.5. Identification of the 90 kDa gB protein of EHV-1_^{518}AAAA^{521}. (a) Commasie blue stained SDS-PAGE gel of immunoprecipitate obtained from cell lysates of EHV-1_^{518}AAAA^{521} and mock infected cells. The band corresponding to the approximately 90 kDa protein of interest was cut out of the gel, digested with elastase, AspN, chymotrypsin or trypsin and analyzed by LC-MS/MS. (b) Representative results from mass spectrometry of the approximately 90 kDa gB protein. Peptides that were identified are represented in separate boxes for each digest below the corresponding N-terminal sequence of gB. The pattern of peptides is evidence for signal peptide cleavage between residues 98 and 99 (▽ with broken line). No peptides corresponding to the signal peptide region (1–98) were identified, despite the fact that two large tryptic peptides are predicted within the signal peptide sequence with cleavage sites between residues 27/28, 66/67 and 84/85 (∨).

By MS analysis of the band corresponding to the approximately 90 kDa gB of EHV-1_^{518}AAAA^{521}, it was possible to detect peptides of the C-terminal end of gB. A high coverage of the gB sequence between the respective putative N-terminal signal cleavage sites and the gB C-terminal end was obtained, especially after trypsin and elastase digestion (Fig. 3.6a). In contrast, the C-terminal end of gB was never identified in the EHV-1 parental 75 kDa band (Fig. 3.6b).

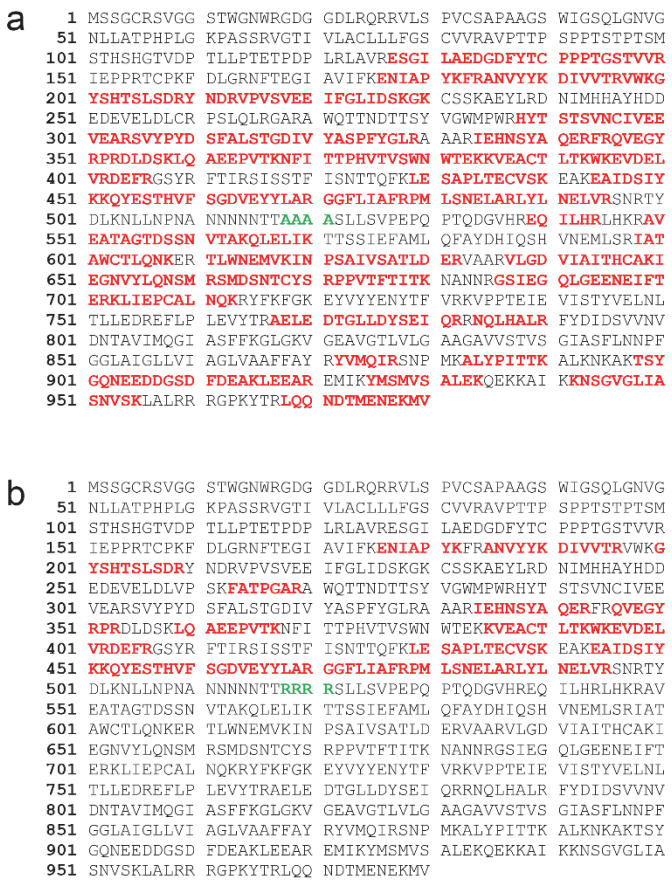

Figure 3.6. Mass spectrometry analysis of gB after trypsin digestion. The identified peptides are marked in red. The mutated/wild-type furin cleavage site is marked in green. (a) MS analysis of the band corresponding to the approximately 90 kDa gB of EHV-1_^{518}AAAA^{521}. (b) MS analysis of the band corresponding to the approximately 75 kDa gB of parental EHV-1.

3.5 Discussion

EHV-1 gB has been shown to be essential for replication [21], which is in accordance with this propensity of gB of other herpesviruses [40-43]. The results seen for furin-mediated cleavage of EHV-1 gB are also in line with reports on furin cleavage of gB from other herpesviruses, as cleavage also was dispensable for EHV-1 replication [9-14]. In contrast, reconstituting the mutant EHV-4 viruses was not successful, which we interpret as being in line with the reduced growth properties of EHV-4_gB1, an EHV-4 mutant expressing EHV-1 gB [44]. These previous data together with the current findings strongly suggest that EHV-4 is more sensitive to changes in gB than that of EHV-1, or that gB4 has additional functions, which are obstructed by making furin cleavage impossible. However, further studies are needed to confirm the role of furin cleavage of gB4 in virus replication.

Previous research done on furin cleavage of gB homologues of other herpesviruses has made evident varying differences in mutant phenotypes when evaluated for virus growth *in vitro* [9-14]. Just as previously reported for VZV, EBV and MuHV-4 [9, 13, 14], no differences in growth *in vitro* were seen for EHV-1Δ^{518}RRRR^{521} and EHV-1_^{518}AAAA^{521} when evaluated in ED cells. Contrary to the results for MuHV-4, however, no reduction in EHV-1-infection rates could be detected in myeloid cells [9]. This was of particular interest for EHV-1, since the cell-

associated viremic phase allows EHV-1 to spread throughout the host without being exposed to the immune system, thereby facilitating more severe pathologies such as abortion and neurological symptoms [45]. Taken together, our results suggest that furin cleavage of gB is of no or little importance for *in vitro* virus infection and replication. Of the herpesviruses tested, this result is most in line with what was reported for VZV, the prototype virus of genus *Varicellovirus*. Another similarity with VZV was what appears to be a partial cleavage of gB despite the mutation of the furin cleavage recognition site. Previously, two alternative cleavage sites near to the furin recognition site were proposed to be responsible for the partial gB cleavage but the functionality of these motifs were not tested [13]. With this in mind, the motif (^{495}RSNR^{498}) found upstream of ^{518}RRRR^{521} was a promising candidate, since it fulfilled the minimal requirements for furin recognition (Arg-X-X-Arg↓). Especially the fact that no lysine or arginine was present in the P2 position of the motif was interesting, because such absence of lysine or arginine can result in a lower furin cleavage efficiency [46], which could explain the apparent partial cleavage seen for EHV-1_^{518}AAAA^{521} (Fig. 3.3a). However, after a comparative analysis of the gB expression of EHV-1_^{518}AAAA^{521}/^{495}AAAA^{498} and the parental EHV-1_^{518}AAAA^{521} no differences were evident (Fig. 3.3b). This eliminated the possibility that the change in molecular weight was caused by alternate cleavage of furin, or any other member of the subtilisin superfamily since no other motifs fitting the motif requirements were present. Since glycosylation was also not causing the differences in size (Fig. 3.4), mass spectrometry analyses were conducted in order to determine whether cleavage by another endoprotease or misfolding of the protein were responsible. Mass spectrometry of peptides derived from the approximately 75 kDa and 90 kDa gB protein of parental EHV-1 and EHV-1_^{518}AAAA^{521}, respectively, clearly demonstrated that the signal peptide is cleaved from the gB protein, since no peptides corresponding to the signal peptide region were identified. The most N-terminal peptides found after AspN and chemotrypsin digestion were located more downstream compared to the N-terminal peptides found after elastase digestion. This may result from difficulties in the identification of AspN or chymotryptic peptides in the region of residues 99-117. Due to the unspecific nature of elastase cleavage, more detailed results could be obtained from the subsequent MS results. Taken together, the MS data indicate a signal cleavage site in position ^{98}TS^{99}. This is in contrast to the published signal cleavage site (^{85}AV^{86}), previously determined by Edman degradation [28].

After evaluating the approximately 90 kDa gB protein with mass spectrometry, using different enzymatic digestions as preparation, the entire protein was covered ranging from the newly identified signal cleavage sites to the C-terminal end of gB. This argues against partial cleavage of gB at an alternate cleavage site by an unknown endoprotease. Furthermore, an additional gB protein band, that fitted the predicted molecular weight (approximately 145 kDa) of the fully glycosylated uncleaved form of gB [26], appeared for the mutant viruses that was not present for parental EHV-1 (Fig. 3.3a). The presence of the full-length gB sequence in the gB protein of approximately 90 kDa, together with the presence of mature uncleaved gB of approximately 145 kDa suggests that the changes in apparent molecular mass are caused by (partial) misfolding of the fully glycosylated and full-length gB when furin cleavage is absent. This argues against the previously proposed model where gB was predicted to be cleaved at the ^{544}RLHKR^{548} motif and possibly also at the furin cleavage site [28]. This was expected since the proposed additional motif did not fulfill the minimal requirements of the general motif of subtilisin/kexin-type serine proteinase family [(of which furin is a member) Arg-$(X)_n$-Arg where n=0,2,4 or 6 and X is any amino acid except cysteine or proline] [47].

We conclude that furin is solely responsible for the cleavage of EHV-1 gB and that altering or deleting the canonical motif appears to result in partial misfolding of mature, full-length gB. This, however, does not have an effect on *in vitro* replication of EHV-1, which is in accordance with the previous reports on furin-mediated cleavage of gB in other herpesviruses. Furthermore, it would be very interesting to see if furin cleavage has an impact on the pathogenesis of EHV-1 in the natural host, experiments that are planned in the future.

3.6 Acknowledgements

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3.7 Author Contributions

B.S. contributed to this work by designing, carrying out all the experiments (apart from mass spectrometry analyses), analyzing and interpreting the data. EK and HS performed the mass spectrometry analyses. N.O. contributed to the drafting of the manuscript, revising it critically, and giving final approval of the version to be published. W.A. contributed to designing and interpreting the data as well as drafting of the manuscript and revising it critically. All authors read and approved the final manuscript.

3.8 Conflicts of Interest

The authors declare no conflict of interest.

3.9 Reference

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Chapter 4

Role of gB and pUS3 in EHV-1 transfer between PBMC and Endothelial cells: a dynamic *in vitro* model.

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4.1 Abstract

Infected peripheral blood mononuclear cells (PBMC) effectively transport equine herpesvirus type 1 (EHV-1), but not EHV-4, to endothelial cells (EC) lining the blood vessels of the pregnant uterus or central nervous system, a process that can result in abortion or myeloencephalopathy. We examined, using a dynamic in vitro model, the differences between EHV-1 and EHV-4 infection of PBMC and PBMC-EC interactions. Infection assays revealed that EHV-1 infected B-lymphocytes and monocytes more efficiently than EHV-4. In order to evaluate viral transfer between infected PBMC and EC, co-cultivation assays were performed. Only EHV-1 was transferred from PBMC to EC and viral glycoprotein B (gB) was shown to be mainly responsible for this form of cell-to-cell transfer. For addressing the more dynamic aspects of PBMC-EC interaction, infected PBMC were perfused through a flow channel containing EC in the presence of neutralizing antibodies. By simulating capillary blood flow and analyzing the behavior of infected PBMC through live fluorescence imaging and automated cell tracking, we observed that EHV-1 was able to maintain tethering and rolling of infected PBMC on EC more effectively than EHV-4. Deletion of US3 reduced the ability of infected PBMC to tether and roll compared to parental virus, which resulted in a significant reduction in virus transfer from PBMC to EC. Taken together, we conclude that systemic spread and EC infection of EHV-1, but not EHV-4, is caused by its ability to infect and/or reprogram mononuclear cells with respect to their tethering and rolling behavior on EC and consequent virus transfer.

4.2 Introduction

Equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) are members of the *Herpesviridae* family and *Alphaherpesvirinae* subfamily, which includes herpes simplex virus type 1 (HSV-1), varicella zoster virus (VZV) and pseudorabies virus (PRV) [1-4]. After initial replication in the upper respiratory tract, EHV-1 infects immune cells and migrates past the epithelial basement membrane to the lymph nodes and blood stream [1, 2, 5, 6]. This results in high levels of PBMC-associated EHV-1 viremia for prolonged periods of time, although onset and duration of viremia differs between EHV-1 strains [7, 8]. As a result, EHV-1 is able to spread throughout the body

where it infects endothelial cells (EC) causing vascular lesions and secondary hypoxic degeneration of the affected tissues [5, 9, 10]. EHV-1 replication occurs mainly in the endothelial lining of blood vessels of the pregnant uterus and the central nervous system (CNS), which can ultimately lead to abortion or equine herpesvirus myeloencephalopathy (EHM), respectively [9]. EHV-4 on occasion also has a viremic phase, which is, however, of much lower magnitude and shorter duration than that of EHV-1 and causes no vasculitis [11]. Given the strikingly different outcomes of infection with these two closely related viruses, it was originally surmised that EHV-4 replication is restricted to epithelial cells [12]. However, it was shown that the virus can in fact efficiently replicate in EC *in vitro* and *in vivo* in young animals [13, 14].

Infection of the PBMC is a key aspect of viral spread and pathogenesis [15]. Besides EHV-1, other alphaherpesviruses, such as VZV and PRV, have been shown to cause viremia, which contributes to the widespread distribution of virus and infection of organs [16, 17]. EHV-1 can replicate in PBMC in a restrictive fashion and apparently fails to establish a productive infection with production of free infectious virus [18-20]. Earlier *in vivo* studies done in ponies identified T-lymphocytes to be the most susceptible of the PBMC subpopulations [19]. In contrast, *in vitro* studies indicated monocytes to be the primary target of EHV-1 [18], which is in accordance with PRV where monocytes are important for virus transport throughout the body [21, 22]. VZV viremia, however, is mainly due to the infection of CD4+ T cells [23].

Another important aspect of EHV-1 pathogenesis is the viral transfer from infected PBMC to EC in the capillaries of the CNS and gravid uterus [15]. It was hypothesized that EC infection is initiated upon close contact following adhesion of PBMC to EC and direct cell-to-cell transfer of virus, a mechanism that would not require viral egress from PBMC [15]. The results are consistent with previous studies [reviewed by van der Meulen et al. [5]], which showed that EHV-1-infected PBMC did not produce free infectious virus. Possibly, EHV-1 and EHV-4 replication in circulating PBMC is restricted until mainly EHV-1-induced activation signals trigger later events as described for human cytomegalovirus (HCMV) [24, 25]. Based on the body of published data, we hypothesized that the difference in pathogenic potential between EHV-1 and EHV-4 is due to the differing abilities of EHV-1 and EHV-4 to infect and/or reprogram mononuclear cells.

A recent study reported on virus transfer from PBMC to EC, where EHV-1 infected PBMC were co-cultured on top of an EC monolayer in the presence of neutralizing antibodies. The reported viral transfer to the underlying EC demonstrated the potential importance of this mode of transfer during EHV-1 infection [26]. Unfortunately, the stationary experimental setup does not allow the evaluation of initial PBMC-EC interactions important for PBMC tethering and rolling. A dynamic flow set-up could serve as a useful tool to analyze these initial PBMC-EC interactions. Under flow conditions, it was shown that the upregulation of adhesion molecules on the surface of EC of the equine reproductive tract during pregnancy could therefore explain the preference of EHV-1 for such cells [15]. Also an increased adhesion of T-lymphocytes to the endothelium was seen when the EC were cultured in the presence of astrocyte-conditioned medium thereby simulating the CNS environment [27]. This, in turn, could explain the preference of EHV-1 for the CNS. Contrary to the role of adhesion molecule expression on EC, very little is known about their up-or down regulation on the surface of infected PBMC. Differences in adhesion molecule expression on infected PBMC with either EHV-1 or EHV-4 were reported recently (Azab and Osterrieder; unpublished data). It would be interesting to evaluate whether these differences in adhesion molecule expression translate into changes in the ability of infected PBMC to roll on EC under flow conditions.

Glycoprotein B (gB) is a fusion protein that is highly conserved among all herpesviruses. gB is known to play an essential role in entry but also cell-cell fusion and direct cell-to-cell spread of EHV-1 and EHV-4 [28-31]. This makes gB an interesting candidate for virus transfer between PBMC and EC. Exchanging essential genes, like gB, between EHV-1 and EHV-4, and evaluating the phenotype of these recombinant viruses, can give a unique insight in the role of these exchanged genes.

Protein kinase encoded in the unique-short region (pUS3) has been implicated in inhibiting apoptosis and modulating the actin skeleton [32-34]. Anchoring of adhesion molecules to the actin cytoskeleton is involved in the regulation and formation of cell-cell contacts [35], possible implicating pUS3 in regulating adhesion molecule expression on the surface of infected PBMC. In addition, the anti-apoptotic effects of pUS3 could also permit infected PBMC to continue functioning normally, allowing the PBMC to reach the target tissues with subsequent virus spread to uninfected endothelial cells.

In order to assess the behavior of infected PBMC tethering and rolling over EC, we developed an *in vitro* flow system that allowed us to monitor rolling PBMC through live imaging. To the best of our knowledge, this is the first report describing the kinetics of infected PBMC and showing virus transfer from infected PBMC to EC under flow condition. EHV-1, EHV-4 and EHV-1 deficient for US3 (EHV-1ΔUS3) were evaluated in this system in order to uncover the different factors involved in viral spread between infected PBMC and EC.

4.3 Materials and Methods

4.3.1 Viruses

All viruses used in the study were recovered from infectious bacterial artificial chromosome (BAC) clones. Those were BACs of EHV-1 strain Ab4 [36], EHV-4 strain TH20p [37], as well as modified BACs EHV-1_gB4, EHV-4_gB1, revertant EHV-1_gB1r [30], EHV-1_gD4, EHV-4_gD1 [38], EHV-1ΔUS3 and EHV-1 that contained US3 of EHV-4 (EHV-1_US3_4) fully rescuing the parental EHV-1 phenotype and functioning as revertant for this study (Proft and Azab, unpublished data). mRFP1-labeled EHV-1 was previously constructed by inserting the monomeric red fluorescent protein (mRFP1) into VP26 of EHV-1 strain RacL11 [39]. Viruses were grown on equine dermal (ED) cells. All viruses express enhanced green fluorescent protein (EGFP) for ready identification of infected cells.

4.3.2 Cells

Primary carotid artery endothelial cells (a kind gift from Ilka Slosarek, Freie Universität Berlin, Institut für Veterinär-Anatomie) were propagated in Dulbecco's modified Eagle's medium (DMEM; Biochrom) supplemented with 20% fetal bovine serum (FBS; Biochrom), 1% of nonessential amino acids (Biochrom), and 1% penicillin-streptomycin. ED cells were grown in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 20% FBS, 1% of nonessential amino acids (Biochrom) and 1% of 100 mM sodium pyruvate (Biochrom).

PBMC were isolated from heparinized blood collected by density gradient centrifugation over Histopaque 1077 (Sigma), following the manufacturer's instructions. After two washing steps, cells were suspended in RPMI 1640 (Biochrom) supplemented with 10% FBS, 0.3 mg/ml glutamine, non-essential amino acids, and 1% penicillin-streptomycin.

4.3.3 Virus growth assays

To evaluate virus replication in PBMC *in vitro*, single-step growth kinetics were done as described before [40]. Briefly, PBMC were infected with a multiplicity of infection (MOI) of 1. After 1 h of incubation, cells were washed and treated with citrate buffer (pH=3). Infected cells

and supernatant were collected at the indicated time points post infection (p.i.), and stored at -80°C. Viral titers were determined by titration on ED cells [41].

4.3.4 Infection assay.

For evaluating the infection rate of PBMC over time, $1x10^5$ PBMC were infected at an MOI of either 0.1 or 1 with EHV-1 and EHV-4 at 37°C for 1 h. Afterwards, PBMC were incubated in citrate buffer and washed twice. After 24 h of incubation at 37°C, infection of PBMC with EHV-1 and EHV-4 was evaluated at different time points (p.i.) by means of flow cytometry. A FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Treestar) were used to detect and analyze GFP expression in the infected PBMC. Dead cells were excluded by staining of the infected PBMC with propidium iodide (PI) at a final concentration of 10 μg/ml.

For evaluating the infection rates of the different PBMC subpopulations, $1x10^5$ infected PBMC were stained at 24 h p.i. with primary mouse antibodies against equine CD14 (monocytic lineage), CD3 (T lymphocytic lineage) or IgM (B lymphocytic lineage) [36]. Antibodies were kindly provided by Bettina Wagner, Cornell University, NY, USA. After washing, cells were labeled with a secondary Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (Invitrogen) and analyzed by flow cytometry.

4.3.5 Aggregation assay.

Twenty-thousand PBMC were infected with either EHV-1 or EHV-4 at an MOI of 1 at 37°C for 1 h in a flat-bottom 96-well plate. The number and size of cell aggregates formed by each virus was evaluated at different time points after infection using an inverted fluorescence microscope (Zeiss Axiovert 100) and photographed with an Axiocam charge-coupled-device (CCD) camera (Zeiss). The size of the aggregates was measured using ImageJ software vl.32j (vl.32j, National Institutes of Health, Bethesda, MD, USA) (http://rsb.info.nih.gov/ij/).

4.3.6 Co-cultivation assay.

EC were grown to confluence in 24-well plates (Sarstedt). PBMC were infected with either parental or recombinant viruses at an MOI of 0.1 for 1 h. After incubation, PBMC were treated with citrate buffer, washed twice and a total of $1x10^5$ PBMC were overlaid directly on the EC monolayer ("contact"), or, alternatively, placed into a Transwell insert (Corning Transwell support system) without direct contact between EC and PBMC ("no contact"). In the "no contact" situation, PBMC shared the same environment as EC through 0.4 μm pores in the insert, but PBMC were physically separated from the monolayer. The "no contact" situation was used to evaluate the efficacy of the citrate treatment of PBMC to inactivate non-penetrated virus. In the "contact" situation, experiments were done in the presence of an 1:100 dilution of an EHV-neutralizing antiserum (VNA) with a titer of 1:2048 [42]. All cells were incubated for 24 h prior to counting GFP-positive plaques on the EC monolayer using an inverted fluorescence microscope (Zeiss Axiovert 100).

4.3.7 Confocal microscopy

For detection of EHV-1 replication within infected PBMC, cells were infected with mRFP1-labeled EHV-1 virus at an MOI of 5 for at 37°C for 1 h. After washing, PBMC were incubated at 37°C for 24 h and then fixed with 3.5% paraformaldehyde and inspected with a Zeiss LSM 510 confocal microscope using a 63x oil immersion objective.

4.3.8 Flow chamber assay

Ten-million PBMC were infected with either parental (EHV-1 or EHV-4), mutant (EHV-1ΔUS3) or recombinant (EHV-1_US3_4) viruses as described above [43] and incubated for 24 h. GFP-positive cells were then isolated by FACS using a FACSAria (BD Biosciences) from the gated PBMC population. Sorted PBMC were then fluorescently labeled with CellVue Jade Dye

(Polysciences Inc.) for 5 min according to manufacturer's instructions. After staining, PBMC were washed 3 times and resuspended in medium containing VNA with a titer of 1:2048 at a dilution of 1:100.
EC were grown to confluence in µ-Slide 0.4 VI collagen IV-coated cell flow chambers (Ibidi Integrated BioDiagnostics) that were connected to a perfusion system by Luer locks (Ibidi Integrated BioDiagnostics). The flow chamber was mounted within an ibidi Heating System, Universal Fit (Ibidi Integrated BioDiagnostics), maintaining a temperature of 37°C. This allowed the introduction of $5x10^4$ PBMC to study leukocyte-endothelial interactions at a flow rate of 0.5 mm/sec, which is within the physiological mammalian range of 0.34-3.15 mm/sec [44]. The velocity was calculated according to the size of the chamber and the velocity in mammalian brain capillaries, and generated by a NE-4000 Double Syringe Pump (New Era Pump System).
PBMC-EC interactions were visualized with an Olympus IX-81 inverted fully motorized fluorescence microscope (Olympus) with a 10x/0.25 Zeiss Achrostigmat (Carl Zeiss) objective and a mercury arc lamp (HBO). Tiff stacks of 500 images were acquired at a 3.42 frames/sec with a monochrome CCD camera (Clara, ANDOR Technology) and MetaView imaging acquisition and analysis software (Molecular Devices). Image processing and automated tracking of rolling PBMC was accomplished in the FIJI package of ImageJ (www.fiji.sc) [45]. The processed images were assembled as a stack of tiff images and analyzed for rolling speed and number of rolling cells per video using the TrackMate plugin. TrackMate first identified fluorescent cells in each image of the stack, given an estimated diameter of the cells (15 pixels with a lateral pixel resolution of 323 nm x 323 nm) and a threshold value for the minimum fluorescence intensity. Next, the plugin linked the fluorescent cells between frames using a frame-to-frame max linking distance of 90 pixels. To obtain the actual rolling speed in µm/sec the resulting mean velocities in pixel/frame were corrected for the frame rate 3.42 frames/sec and the pixel distance of 0.645µm/pixel [$v_{(\mu m/sec)} = v_{(pixel/frame)}$ x 3.42 frames/sec x 0.645µm/pixel]. Passing debris or floating cells, that were falsely included in the analysis, were manually removed from the tracks using the plugin.
After 24 h of incubation, GFP-positive plaques on the EC monolayer were counted (excluding the inlet/outlet areas) using an inverted fluorescence microscope (IX-81, Olympus).

4.3.9 Statistical analysis

Statistical analyses (described in context) were performed using GraphPad PRISM 5 (Intuitive Software for Science). Normally distributed datasets, determined with the Shapiro-Wilk test, were analyzed with one-way ANOVA. Datasets that were not normally distributed were analyzed with Mann–Whitney U test for two-way or Kruskal–Wallis for one-way analysis of variance for more independent samples. Normally distributed grouped samples were analyzed with two-way ANOVA. In order to compensate for the large differences in dataset sizes of the mean velocities of rolling infected PBMC, 100 randomly equally sized subsets (their size being limited by the smallest dataset) were selected of each dataset using randomization loop, R software and were then analyzed with Kruskal–Wallis one-way analysis of variance and corrected with the Holm-Sidak multiple comparisons test [46]. Datasets significantly differing at least 95 times out of the 100 analyzed randomizations were then considered significantly different.

4.4 Results

4.4.1 Growth analysis and infection rates of EHV-1 and EHV-4 in PBMC

Three independent growth kinetic experiments were performed for EHV-1 and EHV-4. The results showed that viral titers did not increase exponentially over time as normally seen during

productive infection in cultured cells. On the contrary, titers of EHV-4 significantly decreased over time compared to EHV-1, which remained stable (Fig. 4.1a).

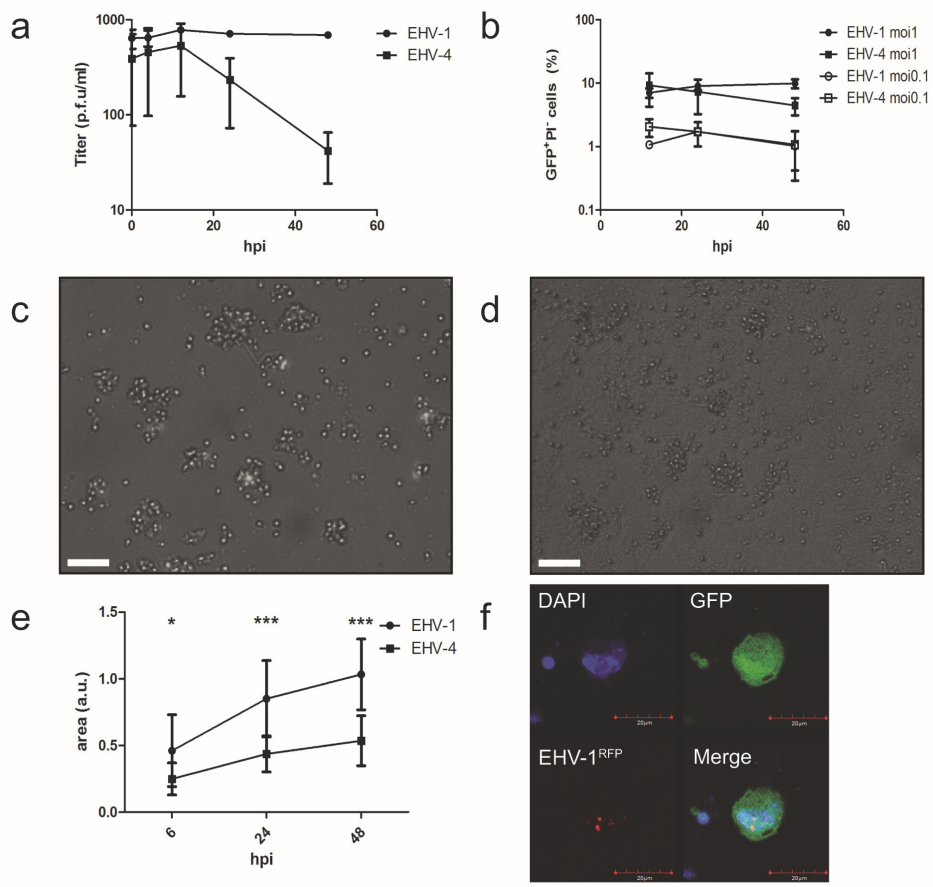

Figure 4.1. *In vitro* growth characterization of EHV-1 and EHV-4 in PBMC. (a) PBMC (10^5 cells) were infected at an MOI of 1. Infected cells and supernatants were collected and virus titers were determined at the indicated times p.i. The presented data are means ± SD of triplicate independent measurements. A significant decrease was measured for EHV-4 when compared to EHV-1 (n=3; Mann–Whitney test; P<0.01). (b) PBMC were infected at an MOI of either 0.1 or 1 of EHV-1 or EHV-4. Viral infection of PBMC was evaluated by means of flow cytometric analyses. No significant differences were measured for EHV-1 when compared to EHV-4 (n=3; Friedman test-Dunn's multiple comparison test; P>0.05). Aggregates of EHV-1- (c) or EHV-4- (d) infected PBMC at 48 h p.i. The bar represents 100 µm. (e) Infected PBMC were cultured for the indicated time intervals and showed significantly smaller aggregate sizes [aggregate size is represented in area quantified in arbitrary units (a.u.)] for PBMC infected with EHV-4 compared to EHV-1. (n=18; two-way ANOVA; *: P<0.05; ***: P<0.001). (f) PBMC were infected with EHV-1 that express RFP-labeled nucleocapsids (MOI of 5) and then analyzed by means of confocal microscopy. Blue: DAPI staining of the nucleus. Green: GFP expression indicating transcription and translation of the viral genome. Red: RFP-labeled nucleocapsids.

Table 4.1. Number of cell aggregates produced by EHV1 and EHV-4.

Hours post infection	Number of cell aggregates	
	EHV-1	EHV-4
6	103	26
24	230	86
48	272	141

The infection rates of PBMC with EHV-1 or EHV-4 did also not significantly increase over the 48 h infection period, a finding commensurate with the absence of production of infectious virus in PBMC. Comparing PBMC infection rates at 12 h p.i., we could also show that both viruses infect PBMC with similar efficiency. However, the number of infected cells increased (EHV-1) or decreased (EHV-4) over the course of 48 h when cells were infected at an MOI of 1 (Fig. 4.1b). The slight increase of EHV-1 titers could be caused by aggregation of infected PBMC, enabling cell-to-cell transfer of virus as described earlier [47]. Indeed, when the ability to aggregate was compared between EHV-1 and EHV-4, EHV-4 was not able to induce aggregation of PBMC to the same extent as EHV-1 (Table 4.1 and Fig. 4.1c-e).Finally, newly formed RFP-labeled EHV-1 nucleocapsids could be visualized within the nucleus (Fig. 4.1f). The nuclear localization of capsid proteins is indicative for *de novo* production of nucleocapsids, which is in accordance with the hypothesis of restricted replication as defined previously by van der Meulen and colleagues [18].

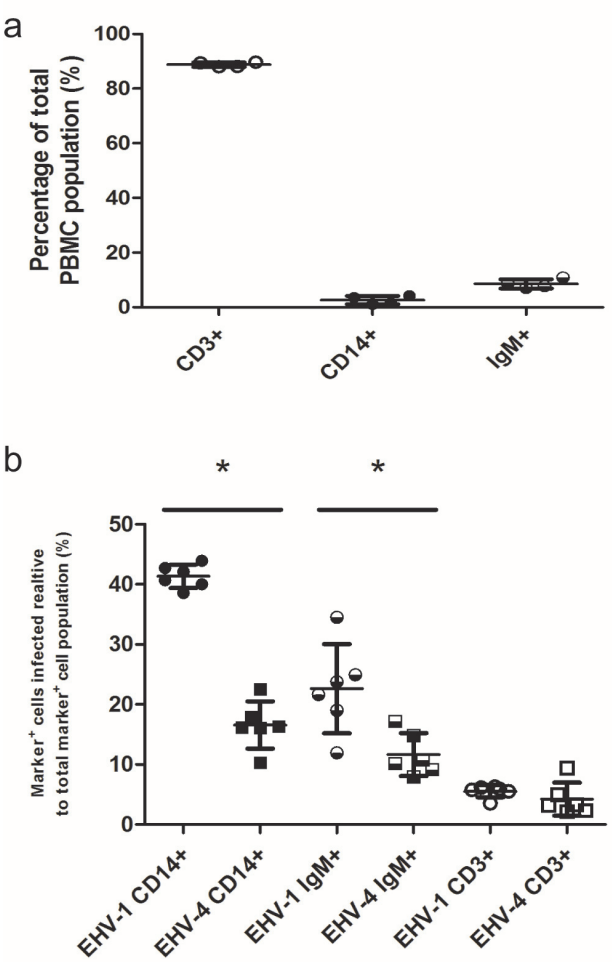

Figure 4.2. Infection rates of different PBMC subpopulations by EHV-1 and EHV-4. (a) Subpopulation composition of the PBMC; CD3+: lymphocytes; CD14+: monocytes; IgM: B-lymphocytes. (b) PBMC were infected with EHV-1 or EHV-4 (MOI=1), washed and then incubated for 24 h. After incubation, the percentage of infected cells for each subpopulation was determined relative to the total number of cells within the subpopulation by flow cytometry. Significant differences (*) were seen ($P<0.05$) between EHV-1 and EHV-4 for monocytes and B-lymphocytes where EHV-1 achieved higher infection rates (n=6; one-way ANOVA).

4.4.2 Infection rates of the different PBMC subpopulations by EHV-1 and EHV-4

The composition of the three main PBMC subpopulations (mean ± SD) was determined by flow cytometry (Fig. 4.2a). The percentages were 88.82±0.84% CD3+ T-lymphocytes, 2.60±1.47% CD14+ monocytes and 8.57±1.6% IgM+ B-lymphocytes, which are still within range of previously obtained values [18, 48]. The rate of infection of parental EHV-1 and EHV-4 was determined for each PBMC subpopulation (mean ± SD). EHV-1 was able to infect both monocytes [41.34±1.95% (EHV-1) vs. 16.57±3.94% (EHV-4)] and B-lymphocytes [22.64±7.43% (EHV-1 vs. 11.65±3.57% (EHV-4)] at significantly higher rates than EHV-4. No significant differences in infection rate between EHV-1 and EHV-4 were seen for T-lymphocytes [5.52±1.02% (EHV-1) vs. 4.22±2.73% (EVH-4)] (Fig. 4.2b).

4.4.3 gB plays an important role in direct virus transfer from infected PBMC to EC

Experiments were conducted to study virus transfer from PBMC that had been infected with parental or recombinant EHV-1 and EHV-4 in either the "contact" or "no contact" model. Parental EHV-1 was able to infect EC in the presence of neutralizing antibody only in the "contact" model (Fig. 4.3). Interestingly, EHV-4 was unable to spread (under contact conditions) from infected PBMC to EC, as evidenced by the absence of any plaque formation (Fig. 4.3), although the used EC preparations were permissive for EHV-4 infection using free infectious virus produced on ED cells (data not shown) [43].

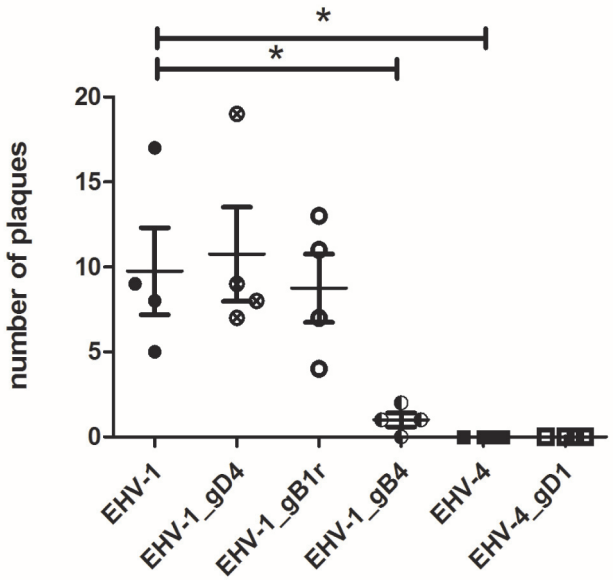

Figure 4.3. Infection of EC by PBMC-associated EHV-1 and EHV-4. PBMC were infected with recombinant or parental EHV-1 or EHV-4 (MOI=0.1). An overlay with infected PBMC was performed on EC incubated in medium containing neutralizing antibodies. After 24 h of incubation at 37°C, plaques were counted. Significant differences in plaque numbers were seen between parental EHV-1 and EHV-4 (*) and between parental/revertant EHV-1 and EHV-1_gB4 (*) (n=4; Kruskal–Wallis one-way analysis of variance; $P<0.05$).

When evaluating EHV-1_gB4 and its revertant EHV-1_gB1r, significantly decreased plaque numbers could be seen for EHV-1_gB4 compared to parental EHV-1 and the revertant EHV-1_gB1r ("contact" model; Fig. 4.3). EHV-4_gB1 could not be evaluated because adequate infection rates of PBMC could not be obtained in our experimental setup (data not shown). EHV-1_gD4 and EHV-4_gD1 did not show a significant difference when compared with their respective parental viruses (Fig.4.3). In all cases, no virus transfer from infected PBMC to EC was observed under "no contact" conditions (data not shown).

4.4.4 Tethering and rolling of infected PBMC and viral transfer to EC under flow conditions

After introducing $5x10^4$ CellVue-labeled and infected PBMC to the flow system, PBMC were seen rolling over the confluent EC monolayer as illustrated by the bright-field tiff stack video of EHV-1-infected PBMC (Supplementary Movie 1). When counting the PBMC that interacted with the EC monolayer, significant differences in the number of PBMC that tethered and rolled was seen as a function of infection. Both mock-infected [20±10.52 cells/tiff stack] and EHV-1-infected [19.46±10.35 cells/tiff stack] PBMC had similar numbers of cells rolling over the EC

monolayer. A significantly lower number of EHV-4-infected cells were rolling within the same time frame [8.3±5.3 cells/tiff stack]. Similarly, PBMC infected with EHV-1ΔUS3 [5.73±3.89 cells/tiff stack] also showed a reduced ability to tether and roll on the EC monolayer compared to parental EHV-1 [19.46±10.35 cells/tiff stack] and EHV-1_US3_4 [19.6±7.20 cells/tiff stack] (Fig. 4.4a).

The fluorescent PBMC were tracked by TrackMate throughout the consecutive images of the tiff stack. Measuring displacement between images and calculating the mean velocities for each track resulted in tracks that are color-labeled according to speed (blue: slow; to red: fast; Supplementary Movie 2). The mean velocity of rolling PBMC was measured for mock-infected PBMC, and PBMC infected with parental EHV-1, EHV-4, mutant (EHV-1ΔUS3) and recombinant (EHV-1_US3_4) viruses. The collected data were compared for differences in rolling speed as described in the Materials and Methods. We determined no significant differences in rolling speed (mean velocity ± SD) between mock-infected [mock: 37.39±13.67 μm/sec], parental [EHV-1: 43.97±16.40 μm/sec; EHV-4: 38.57±17.48μm/sec], mutant [EHV-1ΔUS3: 44.92±14.91 μm/sec], and recombinant [EHV-1_US3_4: 37.75± 13.82 μm/sec] virus-infected PBMC (Fig. 4.4b).

As already shown in the stationary setup, plaque formation was not seen for the EHV-4 infected PBMC. Only parental EHV-1 [17.33± 9.31 plaques], EHV-1_US3_4 [20±5.29 plaques] and, to a significantly lesser extent, EHV-1ΔUS3 [2±1 plaques] were able to transfer between infected PBMC and EC (Fig. 4.4c-d). Preliminary findings had not showed any differences between EHV-1_gB4 and parental EHV-1 (data not shown) and was therefore not further analyzed in this setup.

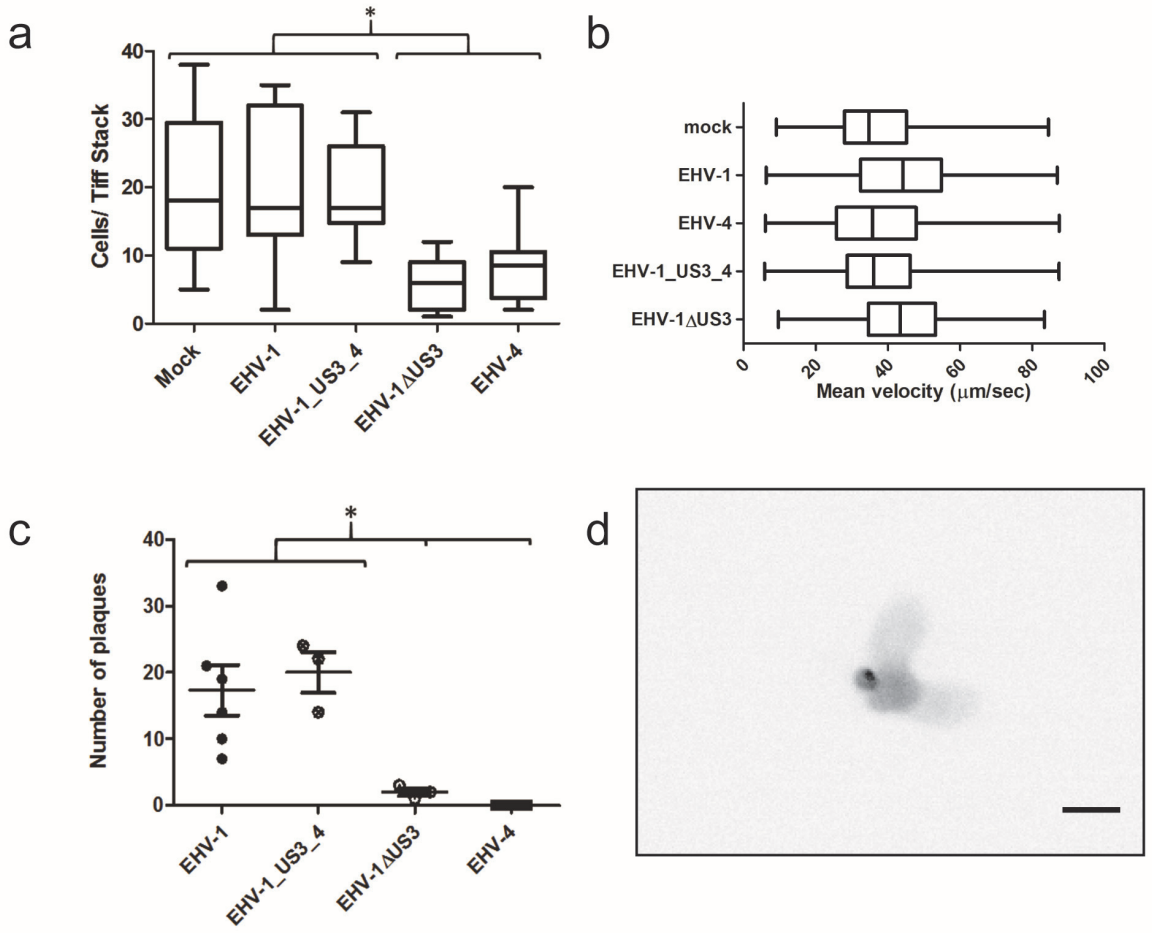

Figure 4.4. Rolling dynamics of infected PBMC and virus transfer to EC. (a) Number of infected PBMC tethering and rolling over EC. A significant reduction in rolling cells was seen for the PBMC infected with EHV-1ΔUS3 and EHV-4 (*: one-way ANOVA; $P<0.05$) compared to mock-, EHV-1- and EHV-1_US3_4-infected PBMC. (b) Mean velocities of rolling infected PBMC were compared. No significant differences were seen (Kruskal–Wallis one-way analysis of variance, Holm-Sidak multiple comparisons test; $P>0.05$). (c) twenty four hour after introducing the PBMC, plaques were counted. Significant differences in plaque numbers were seen between parental EHV-1/EHV-1_US3_4 and EHV-4 (*) and between parental EHV-1/EHV-1_US3_4 and EHV-1ΔUS3 (*: Kruskal–Wallis one-way analysis of variance; $P<0.05$). (d) Inverted black and white image obtained through epifluorescence microscopy illustrating virus transfer from infected PBMC to the underlying EC monolayer (scale bar is 20μm).

4.5 Discussion

Infection of PBMC arguably is the decisive step in the pathogenesis of EHV-1 and related herpesviruses such as PRV, because it enables the virus to evade the immune system while spreading systemically [9, 16, 17, 21-23, 49]. Interestingly, lower viral titers were seen over time for EHV-4 infected PBMC, while the titers of EHV-1-infected PBMC remained constant. Even though the differences were minor due to relatively low PBMC infection rates, this is an indication that EHV-1 and EHV-4 go through and/or induce distinctly different intracellular processes after PBMC entry. Another characteristic only seen for EHV-1 was a slightly increasing trend over time for PBMC infection rates. This could be explained by the differing

abilities of both viruses to cause aggregation of the infected PBMC, which creates intimate intercellular contact that may facilitate cell-to-cell spread of newly formed EHV-1 virions.
When the replication of EHV-1 and EHV-4 was evaluated in PBMC, the lack of increase in titers and infection rates over time indicated that there was no productive infection; however, this does not exclude that there is some productive virus replication albeit at low levels. Our results confirm the findings reported previously by Scott *et al.* and van der Meulen *et al.* [19, 50]; the latter study referred to the phenomenon as replication "in a highly restricted way". We did, however, observe differences in PBMC (total and subpopulations) infection rates between our and previous findings [18, 51]. The most important factors that could explain the discrepancies are the differences in virus strains used and the methods used for measuring infection. That low-level replication is taking place was also confirmed by the use of a recombinant virus that expresses an mRFP-labeled capsid protein, for which *de novo* production of nucleocapsids was detected in the nuclei of infected PBMC. It is still unclear why no productive infection is seen despite the expression of late gene products. Similar reports were also made for other alphaherpesviruses, such as HSV and PRV, where limited replication was seen in monocytes [52, 53]. This limited replication attributes to PBMC an important role in the pathogenesis of alphaherpesviruses [16, 18, 21]. Fully productive infection would impede normal PBMC function and possibly induce a CTL-based immune response targeting the infected PBMC, which would result in their elimination before virus could systemically spread.
We show here that monocytes were the most highly infected PBMC, which is consistent with the results of van der Meulen *et al.* [18]. We could also see differences between EHV-1 and EHV-4 in their ability to infect monocytes and B-lymphocyte subpopulations. In both cases, EHV-1 achieved significantly higher infection rates than EHV-4. This reduced capability of EHV-4 to infect monocytes and B-lymphocytes could partly explain the weak and short cell-associated viremia seen *in vivo* for EHV-4 [11] and the absence of the more severe outcomes that seem to be characteristic for EHV-1. Monocytes are the main leukocyte target of several herpesviruses, which is likely facilitated by a distinct characteristic of these cells, namely the restriction of a fully productive infection [18, 21, 49, 54, 55]. This lack of complete replication and production of virus in monocytes during the viremic phase allows the viruses to evade the immune system until they reach tissues that activate the monocytes and induce differentiation into macrophages [56].
It is known that EHV-1-infected PBMC are responsible for transporting the virus to the endothelia of the gravid uterus and the CNS where it can cause severe symptoms even resulting in death [36, 57]. We could confirm that cell-associated EHV-1 was able to infect EC through direct cell-to-cell contact, previously reported by Goehring *et al.* [42]. Strikingly, we saw that cell-associated EHV-4 was not able to infect EC in the presence of neutralizing antibody. This is of importance given the absence of vasculitis during EHV-4 infection. It is also supporting our hypothesis that EHV-1 and EHV-4 go through distinctly different intracellular processes and differentially manipulate PBMC after entry. The exchange of gB in EHV-1 with its EHV-4 counterpart had a marked effect on viral transfer between infected PBMC and EC, despite the exchange having no significant effect on viral growth *in vitro* [30]. EHV-1_gB4 showed a significantly attenuated ability to transfer to the EC monolayer indicating an important function for gB in this process of virus transfer and PBMC aggregation. These results add a new facet to the previously reported role of gB in facilitating direct cell-to-cell spread [28].
Striking differences were seen between EHV-4-, EHV-1- or mock-infected PBMC with regards to the number of PBMC able to tether and roll on EC. While EHV-1-infected PBMC were able

to tether, roll and transfer the virus to EC, many EHV-4-infected PBMC seemed unable to roll over the EC monolayer which marks another difference in the course of disease that would prevent EHV-4 from causing vasculitis. This indicates that, apart from differences in adhesion molecules on the EC surface affecting PBMC/EC interaction [15], changes in the infected PBMC also affect this process. Such changes could be influencing the expression of adhesion molecules on the surface of PBMC either through a direct effect of the virus [58] or because of apoptosis that can induce downregulation of adhesion molecules as seen in neutrophils [59]. We propose that downregulation of adhesion molecules on the cell surface could explain this reduced ability to tether and roll. This was supported by our recent findings, that EHV-4, but not EHV-1, was capable of downregulating adhesion molecules expressed on the surface of infected PBMC (Azab and Osterrieder, unpublished data). We showed also that deleting US3 decreased the ability of infected PBMC to tether and roll. This could be caused by a number of factors since the described functions of pUS3 are very diverse. pUS3-mediated actin rearrangement might be influencing adhesion molecule expression on infected PBMC [33, 35], thereby negatively influencing tethering and rolling. Furthermore, without the anti-apoptotic effects of pUS3, infected cells might also undergo apoptosis, which negatively influences cell surface adhesion molecule expression and may eliminate PBMC before EHV-1 is able to transfer to the EC. When evaluating the dynamic characteristics of infected PBMC that were still able to tether and roll, no changes were seen regarding their rolling speed. This suggests that rolling speed does not factor into the process of viral transfer. This is in accordance with previous reports, which showed that the main determinants for the leukocyte rolling velocity were the flow rate and the characteristics of the substrate on which the leukocytes roll, rather than differences between leukocytes themselves. Furthermore, it was proposed that heterogeneity in P-selectin expression on the endothelium likely plays an important role in the variation of rolling velocities [60]. It is also remarkable that EHV-1-infected PBMC show no significant differences when compared to mock-infected PBMC regarding rolling speed and number of rolling cells. Keeping immune cell function normal after infection is essential to facilitate the spread of EHV-1 throughout the body by cell-associated viremia.

The results obtained from the growth kinetics, transwell and flow chamber assays all indicate that EHV-1 and EHV-4 infection of PBMC has very different impacts on infected PBMC. It is, therefore, of great interest to screen for differences in expression of cellular proteins, for example by means of microarrays or RNA sequencing and quantitative mass spectrometry, in order to determine the underlying mechanisms for these specific phenotypes.

In summary, we have established an experimental setup that for the first time allows the evaluation of the dynamic alphaherpesvirus-infected PBMC-EC interactions. Our data support a model where circulating infected PBMC remain functioning normally with regards to their interactions with EC, thanks in part to pUS3. Once firmly attached to the EC lining of the blood vessels, virus transfer can occur between the infected PBMC and EC, which is facilitated by gB. This is then followed by productive infection in the EC lining of the blood vessel inducing a strong immune response (Fig. 4.5).

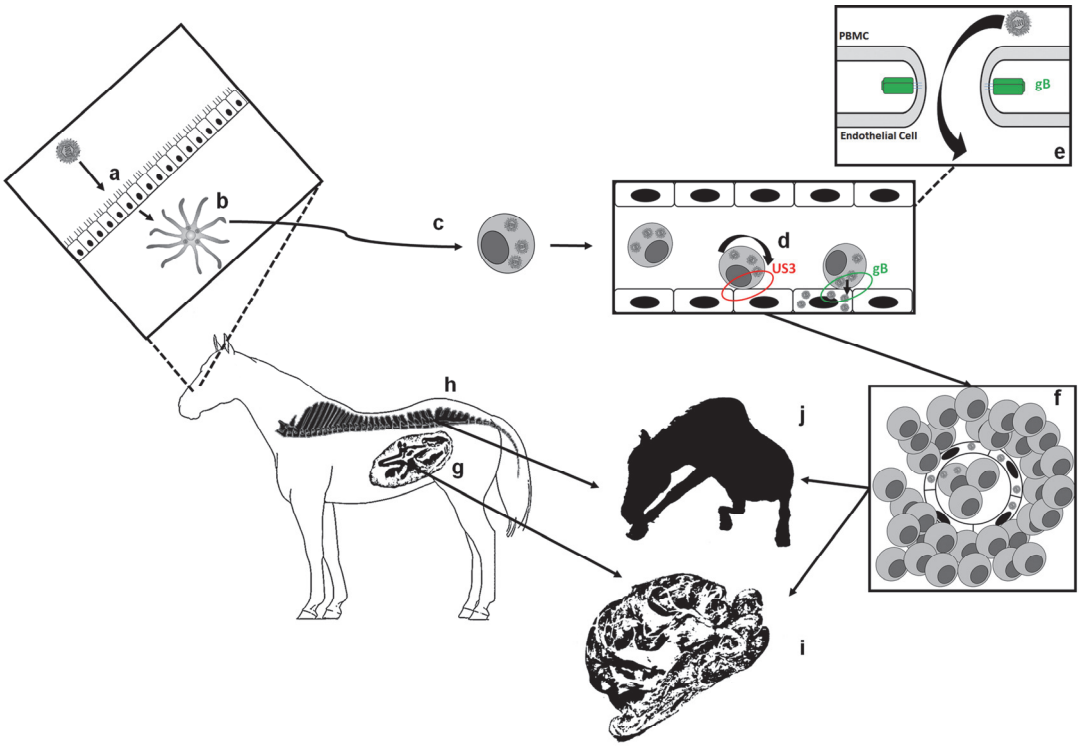

Figure 4.5. Model indicating the role of pUS3 and gB in EHV-1 pathogenesis. After initial replication in the upper respiratory tract (a), EHV-1 infects immune cells and migrates past the epithelial basement membrane (b) to the lymph nodes and blood stream. This results in high levels of EHV-1 viremia for prolonged periods of time in PBMC (c). These infected PBMC remain functioning normally with regards to their interactions with EC (d). Once firmly attached to the EC lining of the blood vessels, viral transfer can occur between the infected PBMC and the underlying EC, which is largely facilitated by gB (e). Productive EHV-1 infection induces inflammation and secondary hypoxic degeneration of the affected tissues (f). EHV-1 replication occurs mainly in blood vessels of the pregnant uterus (g) or the central nervous system (h) which can ultimately lead to abortion (i) or equine herpesvirus-1 myeloencephalopathy (EHM; j).

4.6 Competing interests

The authors declare that they have no competing interests.

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Chapter 5

5 General discussion

EHV-1 and EHV-4 are genetically highly similar, indicating that they are functionally closely related [1-3]. However, the disease outcomes after infection differ substantially. EHV-4 only causes respiratory symptoms, while EHV-1, in addition, causes more systemic symptoms such as abortion and EHM [4-10]. On the cellular level, several differences in viral-cell interaction have been described. For instance, both viruses enter cells through different pathways. More specifically, EHV-1 enters equine epithelial cells via direct fusion at the plasma membrane, while EHV-4 does so via an endocytic pathway [11]. gB is a key player in the process of cell entry. This essential fusion protein is highly conserved within the *Herpesviridae* family [12]. For gB1, it was previously reported to have an essential role in entry but also cell-cell fusion and direct cell-to-cell spread [12-14]. However, gB was never evaluated in detail for either EHV-1 and EHV-4. For this dissertation, I therefore aimed to perform a detailed characterization of the structural and functional aspects of gB1 and gB4.

5.1 The importance of gB for EHV-4 replication

Firstly, I investigated whether gB4 was essential as previously reported for gB1 [13]. After transfection with pEHV-4ΔgB, no spread from the transfected to the neighboring cells could be seen. pEHV-4ΔgB was only able to induce syncytium formation on gB-complementary cells, suggesting that EHV-4 is unable to be released from infected cells and/or enter uninfected cells. These results imply a similar role for gB as seen for HSV-1 and Kaposi's sarcoma-associated herpesvirus where gB plays a role in cell entry and egress [15, 16]. However, a role in egress is unlikely since gB is not required for this process in the case of fellow members of the *Varicellovirus* genus [17]. Taken together, the essentiality of gB remains a characteristic conserved for all studied members of the *Herpesviridae* family. Nonetheless, the underlying mechanisms by which gB4 is essential is still not known at present and needs to be further investigated.

5.2 Exchanging gB between EHV-1 and EHV-4

Previous studies on other members of the viral glycoproteins involved in cell entry, where the respective genes were exchanged between EHV-1 and EHV-4, showed striking functional differences between these highly conserved proteins of EHV-1 and EHV-4 [11, 18, 19]. The principle of exchanging genes between EHV-1 and EHV-4 proved to be a potent tool to assess functions of essential proteins.

Similar to the previous approach, I exchanged gB between EHV-1 and EHV-4. No significant changes were seen for EHV-1_gB4 when the viral growth was evaluated *in vitro*. EHV-4_gB1, on the other hand, exhibited significantly reduced growth, evidenced by the impaired viral cell-to-cell spread and reduced growth kinetics. This was in stark contrast with previous experiments where gB was exchanged between alphaherpesviruses of different natural hosts without the occurrence of an *in vitro* growth defect [20]. Perhaps the growth defect of EHV-4_gB1 was caused by structural incompatibility of gB that EHV-4 was unable to compensate for. gB4 could also have an additional role during EHV-4 replication that gB1 does not fulfill. A similar instance was previously reported where HSV-1 gB showed an additional function, which was lacking for gB of Saimiriine Herpesvirus 1 (SaHV-1). Contrary to HSV-1 gB, SaHV-1 gB was not able to interact with paired immunoglobulin-like type 2 receptor-alpha (PILRα), despite the conservation of the recognition motif within its sequence [21]. Therefore, it would be interesting

to further elucidate the structural differences between gB1 and gB4 and identify putative cellular interaction partners of gB. Another possibility is that, the authentic EHV-4 gH-gL complex could not prime gB1 as efficiently for fusion as gB4. It will, therefore, be interesting to examine whether a triple mutant, i.e. EHV-4 harboring gH1-gL1-gB1 and vice versa, will lead to any changes of the ability of the virus to effectively replicate.

5.3 The role of gB in cell tropism

Previous research has shown that gD1 can bind to several receptors and thereby determines the host range of EHV-1. This is less clear for gD4, which also uses MHC class I as an entry receptor and likely another receptor to enter Vero cells [18]. When evaluating gB for a possible role in cellular host range, no effect could be detected, suggesting that gB is not important in determining cellular host range. It seems likely that, in contrast to HSV-1 [22-24], gB of EHV-1 or EHV-4 does not need to bind to different cellular receptors to facilitate fusion. However, a role for gB, together with gC, to bind to cell surface heparan sulfate and help the attachment of virions to cells during the initial events of infection is hereby not excluded [25].

5.4 Integrin-binding motif (YGL) of gB1

gB also has a putative integrin-binding motif, tyrosine-glycine-leucine (YGL), which is conserved in PRV, MDV, EHV-1 and EHV-4, and can potentially interact with α4β7, α4β1, and α9β1 integrins [26]. YGL is also present in the VP4 spike protein of rotaviruses where it mediates cell entry [26]. Since integrin-binding motifs were shown to have significant roles during viral infection (e.g. LDI of gH1 [11]), we addressed the role of the YGL-motif in gB during EHV-1 entry.

Disrupting the integrin-binding motif YGL in gB1 did not have any effect on virus growth *in vitro*. YGL, apparently, does also not play a decisive role in determining the cell entry pathway of EHV-1. Previously, complementing results were obtained when binding between the integrin-binding motif YGL and selected known ligands α4β1 or α4β7 was mitigated using blocking antibodies. From these experiments was concluded that YGL does not need to interact with its known binding partners for infection [27]. Taken together, we concluded that gB-integrin interaction does not play an important role in cell entry or determining the cell entry pathway. It is not uncommon for integrin-binding motifs of viral proteins to lack an apparent role in viral infection. A similar lack of function was previously reported for the RGD motif of adeno-associated virus type 2 (AAV2) [28]. However, it may have a role in signaling transduction that might be needed during other steps of virus replication. Furthermore, these data do not necessarily mean that no interaction occurs between EHV-1 and the respective integrins. The integrins may serve as a receptor and/or co-receptor for viral entry, but their blockade may not have a measurable effect on virus infection.

5.5 Furin cleavage of gB

A significant reduction of plaque sizes and *in vitro* growth of EHV-4_gB1 was evident when compared to parental and revertant EHV-4. This may be attributable to the loss of functional interactions between gB and other proteins involved in virus entry. One possible cause for the loss of function/interaction may be structural differences between gB1 and gB4. Our rationale was that this could result from the different locations of the furin cleavage site within the respective gBs.

In order to evaluate this possibility, recombinant EHV-1 and EHV-4 viruses, in which the furin cleavage site (EHV-1: RRRR; EHV-4: RTRR) was either deleted or replaced with alanine amino acids, were constructed and characterized.

5.5.1 Furin cleavage of gB1

Contrary to pEHV-4Δ^{513}RTRR^{516} and pEHV-4_^{513}AAAA^{516}, no reconstitution problems occurred for pEHV-1Δ^{518}RRRR^{521} and pEHV-1_^{518}AAAA^{521}. Previous studies on furin cleavage of gB homologues of other herpesviruses resulted in a wide array of mutant phenotypes when evaluated for virus growth *in vitro* [29-34]. In accordance with previous reports on VZV, EBV and MuHV-4 [29, 33, 34], no differences in *in vitro* growth were seen for EHV-1Δ^{518}RRRR^{521} and EHV-1_^{518}AAAA^{521} when evaluated in ED cells. Contrary to the results for MuHV-4, however, no drop in EHV-1-infection rates could be detected in myeloid cells [29]. This was of particular interest for EHV-1, since the cell-associated viremic phase allows EHV-1 to spread throughout the host without being exposed to the immune system, thereby facilitating more severe secondary pathologies [35]. Taken together, our results suggest that furin cleavage of gB is of no importance for *in vitro* replication.

These results were very similar to those obtained for VZV, which is the prototype viruse of the *Varicellovirus* genus. Furthermore, partial cleavage of the mutant gB was apparent for EHV-1 as previously seen for VZV. It was proposed for VZV that this partial cleavage of gB was caused by one of two putative alternate cleavage sites. However, the functionality of these cleavage sites was never confirmed [33]. With this in mind, we identified one putative cleavage site (^{495}RSNR^{498}) upstream of the furin recognition motif (^{518}RRRR^{521}) as a promising candidate for facilitating partial gB cleavage. Interestingly, the absence of lysine or arginine in the P2 position has been reported to cause a lower cleavage efficiency [36]. This could have explained the apparent partial cleavage seen for EHV-1Δ^{518}RRRR^{521} and EHV-1_^{518}AAAA^{521}. However, after comparatively analyzing the gB expression of EHV-1_^{518}AAAA^{521}/^{495}AAAA^{498} and the parental EHV-1_^{518}AAAA^{521}, no differences could be seen. This eliminated the possibility of alternate gB cleavage by furing or any other member of the substilisin superfamily, since no other motifs fitted the requirements (Arg-X-X-Arg↓). After glycosylation was also excluded as a possible cause, mass spectrometry (MS) analyses were conducted in order to determine whether partial cleavage by another endoprotease or partial miss-folding of the protein were responsible. After evaluating this protein with MS, using different enzymatic digestions in preparation, peptides were identified ranging from the newly identified signal cleavage site to the c-terminal end of gB. These MS results argued against the possibility of alternate cleavage by an unknown endoprotease. Furthermore, an additional gB protein band, that fitted the predicted molecular weight (approximately 145 kDa) of the fully glycosylated uncleaved form of gB [37], appeared for the mutant viruses that was not present for parental EHV-1. The presence of peptides spanning the entire gB sequence in the gB protein of approximately 90 kDa, together with the presence of mature uncleaved gB of approximately 145 kDa, suggest that the changes in gB expression are caused by partial miss-folding of the fully glycosylated gB when furin cleavage is mitigated.

This is in stark contrast with the previously proposed model where gB was predicted to be cleaved at the ^{544}RLHKR^{548} motif and possibly also at the furin cleavage site [38]. This was suspected since the proposed additional motif did not fulfill the minimal requirements of the general recognition motif of the subtilisin/kexin-type serine proteinase family [39]. In addition, the exact signal peptide cleavage site was elucidated by MS of peptides derived from the approximately 75 kDa and 90 kDa gB protein of parental EHV-1 and EHV-1_^{518}AAAA^{521}, respectively. Cleavage of the signal peptide was clearly demonstrated, since no peptides corresponding to the signal peptide region were identified. The MS data indicates a signal cleavage site in position 98/99. This contradicts the previously published signal cleavage site

(^{85}AV^{86}), determined by Edman degradation [38]. The fact that this newly discovered cleavage site (^{98}TS^{99}) was only identified after elastase digestion may result from difficulties in the identification of AspN or chymotryptic peptides in the region of residues 99-117.

5.5.2 Furin cleavage of gB4

Furin-mediated cleavage of gB1 was found to be dispensable for viral replication as reported for other herpesviruses [29-34]. In contrast, reconstituting the mutant EHV-4 viruses was not successful, which we interpret as being in line with the reduced growth properties of EHV-4_gB1 [40]. These previous data, together with the current findings, more likely suggest that EHV-4 is more sensitive to changes in gB than EHV-1, or that gB4 has additional functions which are obstructed by furin cleavage mitigation. However, further studies are needed to elucidate the role of furin-mediated gB cleavage during EHV-4 replication.

5.6 gB and pUS3 functionality during the transfer of EHV-1 between PBMC and endothelial cells

After addressing the structural and functional aspects of gB on a protein level, it would also be interesting to see how these findings translate to the functions of gB during the key steps of EHV-1 pathogenesis. Infection of PBMC and subsequent transfer from these PBMC to the EC lining of the blood vessels is arguably the most important step in the pathogenesis of EHV-1 [3, 41-46]. We therefore evaluated the roles of gB and pUS3 in this process and came to some interesting conclusions.

5.6.1 No productive infection in PBMC

Infection of PBMC is a decisive step in the pathogenesis of EHV-1 and other herpesviruses, because it enables the virus to evade the immune system while spreading systemically [3, 41-46]. During the growth kinetics in PBMC, a decrease in viral titers could be seen for EHV-4, while the titers of EHV-1 remained constant. These differences were minor but significant, and indicated that EHV-1 and EHV-4 go through distinctly different intracellular processes after PBMC entry. Also the PBMC infection rate differed between EHV-1 and EHV-4, showing only for EHV-1 an increasing trend over time. This is possibly caused by the differing abilities of both viruses to cause aggregation of the infected PBMC, which enables cell-to-cell spread of newly formed EHV-1 viruses. Taken together, our results suggest that no productive infection takes place. However, this does not exclude that a certain level of replication still occurs. This was also confirmed by the use of an mRFP-expressing recombinant EHV-1 virus for which *de novo* production was detected in the nuclei of infected PBMC. However, the mechanism by which this remains restricted, despite the expression of late gene products, is still unclear. These results are in accordance with the findings reported previously by Scott *et al.* and ven der Meulen *et al.* [47, 48]. The latter referred to this phenomenon as replication in a restrictive manner. There are, however, differences in PBMC infection rates between our and previous reports [49, 50]. An important factor that explains these inconsistencies are the differences in used virus strains and the methods used for measuring infection.

Lack of productive infection was also reported for other alphaherpesviruses, such as HSV-1 and PRV, were restricted replication was seen in monocytes [51, 52]. This limited replication gives PBMC an important role in the pathogenesis of alphaherpesviruses [41, 46, 50]. If productive infection would occur in PBMC, it would impede their physiological functions and possibly induce CTL-mediated immune responses against the infected PBMC. This could result in their elimination, thereby prohibiting systemic spread. In accordance with the results of van der Meulen *et al.*, we also show that monocytes were the most highly infected PBMC [50]. Interestingly, EHV-4 was not able to infect monocytes and B-lymphocytes to the same degree as

EHV-1. This could partly explain the weaker and shorter cell-associated viremic phase seen for EHV-4 *in vivo* [53], and result in the absence of severe symptoms.

5.6.2 Dynamic aspects of cell-associated viremia of EHV-1 and EHV-4

Before viral transfer can occur from the infected PBMC to EC, initial attachment of the PBMC has to occur. Striking differences were seen between EHV-4-, EHV-1- and mock-infected PBMC with regards to the number of PBMC able to tether and roll. While EHV-1-infected PBMC were able to tether, roll and transfer the virus, many EHV-4-infected PBMC seemed unable to roll over the EC monolayer which marks another difference in the course of disease that would prohibit EHV-4 from causing vasculitis. This indicates that, apart from differences in adhesion molecules on the EC's surface affecting PBMC/EC interactions [54], also changes in the infected PBMC affect this process. Such changes could be influencing the expression of adhesion molecules on the cell surface of PBMC either through a direct effect of the virus or because of apoptosis that can induce downregulation of adhesion molecules as seen in neutrophils [55, 56]. We, therefore, proposed that downregulation of adhesion molecules on the cell surface could explain this reduced ability to tether and roll. This was supported by our recent findings, that EHV-4, but not EHV-1, induced downregulation of adhesion molecules expressed on the surface of infected PBMC (Azab and Osterrieder, unpublished data). Our findings showing that deleting US3 decreased the ability of infected PBMC to tether and roll, can be caused by a number of factors. The functions of pUS3 are very diverse. pUS3-mediated actin rearrangement might be influencing adhesion molecule expression on infected PBMC, since they are anchored in the actin-containing cytoskeleton [57-59]. This could in turn negatively influence tethering and rolling. Furthermore, without the anti-apoptotic effects of pUS3, infected cells might also undergo apoptosis, which negatively influences the cell surface adhesion molecule expression and eliminates the PBMC before EHV-1 is able to transfer to the EC. In contrast, no significant changes in rolling velocity were seen when the dynamic aspects of PBMC/EC interactions were evaluated. This is not surprising since previous findings indicated that the main determinants for the leukocyte rolling velocity are the flow rate and the substrate characteristics on which the leukocytes roll, rather than differences among the leukocytes. Furthermore, they propose that heterogeneity in P-selectin expression on the endothelium likely plays an important role in the variation of rolling velocities [60]. It should also not be overlooked how remarkable it is that EHV-1-infected PBMC show no significant differences with mock-infected PBMC regarding the number of rolling cells. Keeping immune cells functioning normal after infection is important for systemic cell-associated EHV-1 viremia.

5.6.3 gB plays an important role in direct viral transfer from infected PBMC to EC.

Infected PBMC are responsible for transporting EHV-1 to the gravid uterus and the CNS where it can cause severe symptoms and even death [61, 62]. We confirmed the results, previously published by Goehring *et al.* [63] that cell-associated EHV-1 was able to infect EC through direct cell-to-cell contact [63]. Interestingly, no EHV-4 transfer between PBMC and EC, in the presence of VNA, was reported. These results further corroborate our hypothesis that EHV-1 and EHV-4 undergo distinctly different intracellular processes after PBMC entry. This is also of great importance for explaining the absence of vasculitis during EHV-4 infection.

EHV-1_gB4 was also limited in its viral transfer between infected PBMC and EC, despite showing no defects in *in vitro* growth otherwise [40]. This significantly attenuated ability to transfer to the EC monolayer indicates an important function for gB in this process. These results add a new facet to the previously reported role of gB in facilitating direct cell-to-cell spread [13].

5.7 Final remarks and outlook

The replacement of gB1 by gB4 in EHV-1 did not lead to any significant changes in viral growth *in vitro* compared to EHV-1. However, EHV-4 seems to be unable to fully compensate the structural changes introduced by the replacement of gB4 with gB1 with respect to replication *in vitro*. Obtaining the crystal structure of gB1 and gB4 might elucidate this. However, many practical hurdles need to be overcome to achieve this. Structural aspects aside, the generation of a stable EHV-1_gB4 recombinant virus gives us the tools to address viral entry and spread of EHV-1 and EHV-4 in other cell types in the future. In addition, the evaluation of gB during EHV-1 infection in a tissue model could be attempted. It would be interesting to see how gB's role of facilitating cell-to-cell spread is reflected in a setting where three-dimensional structures and normal cell–cell contacts are maintained. We could evaluate this using equine nasal mucosal explants. This system was established by Vandekerckhove and colleagues to study equine respiratory infections including those caused by EHV-1 [64, 65]. It closely mimics the *in vivo* situation and would be ideal to avoid the problems that arise when performing infection experiments in the natural host [66].

Also, the role of furin-mediated gB cleavage during infection of this nasal explant model would be interesting to investigate since differences were seen for the phenotype of VZV during infection of skin xenografts [33]. This is worth exploring, since VZV and EHV-1, both closely related members of the genus *Varicellovirus*, also showed similar results during the *in vitro* studies. Both showed no differences for *in vitro* growth after abolishing furin-mediated gB cleavage. Contrary to what was reported for VZV, however, we concluded that furin is solely responsible for the cleavage of gB1 and that altering or deleting the motif results in partial miss-folding of gB in its mature form. The crystal structure of gB could also here help to futher study the effect of furin cleavage mitigation on the structure of gB.

In a second part of my project, we took a closer look at the PBMC/EC interactions and how equine herpesviruses transfer between them. However, we have not identified which PBMC subpopulations are predominantly responsible for this transfer. For several other herpesviruses monocytes are the main leukocyte targets, which is likely in part because of the absence of productive infection in these cells [44, 46, 50, 67, 68]. In the case of HCMV the lack of full replication in monocytes during the viremic phase allows the viruses to evade the immune system until the virus induces differentiation into macrophages through a yet unknown mechanism [69]. However, this does not explain the mode of transfer of EHV-1 from infected PBMC to EC, since this occurs before the productive stage seen for HCMV. Additional studies are needed to determine what the underlying mechanism is, used by EHV-1, to facilitate this form of viral transfer. And also which subpopulation is responsible for this viral transfer.

We also established a flow chamber setup that for the first time allows the evaluation of the dynamic virus-infected PBMC/EC interactions. We reported that PBMC, infected with either EHV-4 or EHV-1ΔUS3, showed a reduced ability to interact with the EC monolayer. We could use our flow chamber setup to determine which adhesive molecules are involved in this change of phenotype. First we would have to screen PBMC, infected with either parental EHV-1, parental EHV-4 or EHV-1ΔUS3, for downregulation of adhesive molecules previously reported to be important for PBMC rolling, such as VLA-4 and PSGL-1 [70-72]. We could then test the adhesive molecules, for which a change in cell surface presentation is found, whether their down-regulation also has functional repercussions on PBMC rolling. This could be achieved by pretreating PBMC, infected with one of our different parental or mutant viruses, with function-blocking antibodies against these adhesive molecules of interest. This type of blocking

experiments were used successfully in the past to identify different molecular players during leukocyte rolling [73, 74].
Another aspect of EHV-1 infection that can be addressed, using the flow chamber assay, is the preference of EHV-1-infected PBMC for the CNS. In a murine model, an increased adhesion of T-lymphocytes to the endothelium was seen when the EC were cultured in the presence of astrocyte-conditioned medium thereby simulating the CNS environment [75]. This, in turn, could explain the preference of EHV-1 for the CNS. It would be very interesting to see what repercussions this has for EHV-1 infection. In future experiments we could therefore evaluate how EC, under flow conditions in a CNS-like [addition of astrocyte-conditioned medium (ACM)] environment, interact with infected PBMC. We could then screen for changes in viral transfer between PBMC and EC, and how this relates to the ability to roll and the rolling speeds. However, since we would use non-equine ACM, its effect on equine primary EC should first be more generally characterized. Apart from enhanced T-lymphocyte adhesion, addition of ACM to EC cultures was characterized by the structural reorganization of the EC, resulting in the increase of tight junction formation and a decrease in monolayer permeability [assessed by means of transendothelial electrical resistance (TEER) and EC permeability for dextran and propidium] [76-78]. If similar changes in our equine EC are seen, we could continue to evaluate equine herpesvirus-infected PBMC in this setup. Interestingly, also an exposure of the EC to shear stress resulted in decreased permeability [78]. This indicates that the presence of a flow over the EC monolayer influences its morphology. It would therefore be advisable to modify the flow chamber setup so that constant culturing of the EC monolayer under flow conditions is made possible, in order to achieve a more physiologically accurate model.
The results obtained from the growth kinetics, transwell and flow chamber assays all indicate that EHV-1 and EHV-4 infection of PBMC have very different impacts on infected PBMC. It is, therefore, of great interest to screen for differences in expression of cellular proteins in the different PBMC subpopulations in order to determine the underlying mechanisms for these specific phenotypes. Several potent tools exist which could be used to achieve this goal. Quantitative methods to measure gene expression, such as Northern blots, serial analysis of gene expression (SAGE) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), are not very suited for this goal since they are either very time-consuming or only able to examine a small number of genes at a time [79-84]. Microarrays on the other hand would be very useful to examine changes in PBMC gene expression following EHV-1 and EHV-4 infection. Several equine-specific gene expression microarrays have been developed [84-86], and in at least one case also commercialized. These microarrays do have several limitations. Detection of low-prevalence sequences is difficult, and accurately quantifying sequences with a high prevalence is also challenging. In addition, a bias exists within the samples since the sample material is acquired through PCR-based amplification [87]. However, these limitations are negligible for comparative studies of known genome sequences [87, 88], which is the case here. Microarrays are therefore still a valid and affordable option to unravel the differences in PBMC gene expression after equine herpesvirus infection. When an analysis method is wanted that more accurately detects the transcriptome in correlation to the proteome of the cell, next generation RNA sequencing (RNA-Seq) would be the preferred option [88]. Several RNA-seq platforms have been developed, such as 454 pyrosequencing, Solexa/illumine genome analyzer and SOLiD [87]. The SOLiD RNA-seq platform has already been successfully used to evaluate changes in the transcriptional landscape of equine cells [89]. Despite the improved correlation of the mRNA levels measured with RNA-seq compared to the cellular protein expression, the correlation with

the proteome is still moderate. It is not possible for the mRNA levels to perfectly correlate with the protein expression since this does not account for post-transcriptional regulation [88]. The evaluation of the cellular protein levels is therefore biologically more relevant than the mRNA levels measured by microarrays and RNA-seq. MS-based quantitative analysis of equine herpesvirus-infected PBMC would therefore be to most ideal way to screen for virus-induced changes in cellular protein expression. The usefulness of MS in this respect has been established when numerous differentially expressed host proteins were identified in MDV-transformed lymphoblastoid cells using quantitative label-free MS [90]. However, these experiments are very complex and the facilities required for these experiments are not widely available yet. Taken together, several potent tools are available for analyzing the differences in functional changes of PBMC after infection with either EHV-1 or EHV-4. A number of aspects, such as budgetary constraints and accuracy, will have to be taken into account in order to determine which of these platforms is the best suited for the task at hand.

In conclusion, our data supports a model where EHV-1 infects PBMC without causing productive infection. These circulating infected PBMC remain functionally normal with regards to their interactions with EC, thanks in part to pUS3. Once firmly attached to the EC lining of the blood vessels, viral transfer can occur between the infected PBMC and EC, which is in part facilitated by gB. This is then followed by productive infection in the EC lining of the blood vessel inducing a strong immune response.

5.8 Reference

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Zusammenfassung

„Neue Erkenntnisse zur Rolle des Glykoproteins B und pUS3 während der Pathogenese des equine Herpesviruses“

Das Glykoprotein B (gB) spielt zusammen mit gD und dem gH/gL-Komplex eine wichtige Rolle während des Eintritts der Alphaherpesviren. Um eventuelle funktionale Unterschiede zwischen den Glykoproteinen gB1 von EHV-1 und gB4 von EHV-4 zu untersuchen, wurden die entsprechenden Gene zwischen EHV-1 und EHV-4 ausgetauscht. Das rekombinante, gB4 enthaltende EHV-1 Virus (EHV-1_gB4) wurde hinsichtlich seines Wachstums *in vitro*, seines Zelltropismus und des Zelleintritts untersucht, wobei keine signifikanten Unterschiede im Vergleich zum Ursprungsvirus festgestellt werden konnten. Zusätzlich wurde ein potentielles Integrin-Bindemotiv mutiert, was jedoch keinen Einfluss auf die *in vitro* Funktion von gB hatte. Im Gegensatz dazu konnte eine signifikante Reduzierung der Plaquegröße und der Virusvermehrung des gB1 enthaltendem EHV-4 Viruses (EHV-4_gB1) im Vergleich zum Ursprungsvirus und der Revertante ermittelt werden. Die Abnahme der Virusvermehrung könnte hierbei auf den Verlust der Interaktion von gB mit anderen Glykoproteinen, einschließlich gD und gH/gL, zurückzuführen sein, die am Zelleintritt beteiligt sind. Alternativ dazu könnte gB4 eine zusätzliche Funktion ausüben, die für die Vermehrung von EHV-4 von Nöten ist, jedoch nicht von gB1 übernommen werden kann. Die verringerte Vermehrungsfähigkeit von EHV-4_gB1 könnte auf einen Verlust der Interaktionsfähigkeit von gB mit anderen, am Zelleintritt beteiligten Proteinen zurückzuführen sein, wobei dies auf strukturellen Unterschieden von gB1 und gB4 beruhen könnte. Unsere Erklärung für einen strukturellen Unterschied ist, dass dieser durch eine unterschiedliche Lage der Furinschnittstelle in den entsprechenden gBs hervorgerufen werden könnte. Um den Einfluss der durch Furin vermittelten Spaltung von gB auf das Viruswachstum von EHV-1 und EHV-4 zu untersuchen, wurden die Schnittstellen mutiert. Während die Veränderung der Furin-Erkennungssequenz das Wachstum von EHV-1 nicht beeinflusste, konnte EHV-4 nicht rekonstituiert werden, was die oben beschrieben Ergebnisse bezüglich unterschiedlicher Eigenschaften von gB4 im Vergleich zu EHV-1 bestätigt. Obwohl die Vermehrung von EHV-1 nicht beeinflusst war, konnten Westernblot und massenspektrometrische Analysen zeigen, dass die Mutation der Furin-Erkennungssequenz tatsächlich die Spaltung von gB verhinderte und das Protein partiell fehlerhaft gefaltet war. Darüber hinaus konnte eine neue, bisher nicht publizierte Peptidschnittstelle zwischen den Aminosäuren ^{98}TS^{99} identifiziert werden. Daraus schließen wir, dass allein Furin für die Spaltung von gB verantwortlich ist und somit wichtig für die korrekte Faltung des Proteins.
Nachdem die strukturellen und funktionalen Aspekte von gB auf Proteinebene untersucht wurden, haben wir die Frage gestellt, wie sich die Funktion von gB auf eine der Schlüsselstellen der EHV-1 Pathogenese, nämlich der Übertagung der Viren von infizierten PBMCs (Peripheral Blood Mononuclear Cells oder mononukleäre Zellen des peripheren Blutes) auf Endothelzellen (EZ), überträgt. Infizierte PBMCs transportieren hoch effizient EHV-1, aber nicht EHV-4, zu den Endothelzellen, welche die Blutgefäße des schwangeren Uterus und des zentralen Nervensystems auskleiden, wodurch es zu Fehlgeburten bzw. Myeloenzephalopathie komme kann. Mittels eines dynamischen *in vitro* Modelles haben wir die Unterschiede von EHV-1 und EHV-4 hinsichtlich der Infektion von PBMCs und der PBMC/EZ-Interaktion untersucht. Infektionsversuche zeigten dabei, dass EHV-1 B-Lymphozyten und Monozyten besser infiziert als EHV-4. Um nun den Virustransfer von infizierten PBMCs auf Endothelzellen zu untersuchen, wurden Ko-Kultivierungsversuche durchgeführt. Ausschließlich EHV-1 wurde in

diesem System von PBMCs auf Endothelzellen übertragen, wobei hauptsächlich gB für diesen Zell-zu-Zell-Transfer verantwortlich war. Um die dynamischen Aspekte der Interaction von PBMCs und Endothelzellen zu beleuchten, wurden infizierte PBMCs unter Anwesenheit von neutralisierenden Antiköpern durch einen mit Endothelzellen bewachsenen Durchflusskanal gepumpt. Dadurch ist es möglich, den kapillaren Blutstrom zu simulieren und währenddessen das Verhalten der infizierten PBMCs durch „live cell imaging“ und automatischer Zell-Verfolgung (cell tracking) zu analysieren. Hierbei ließ sich feststellen, dass EHV-1 in der Lage war, das Anhaften und Rollen der infizierten PBMCs auf den Endothelzellen effizienter aufrechtzuerhalten als EHV-4.
Zusammengefasst lässt sich daraus folgern, dass die systemische Ausbreitung und Infektion von Endothelzellen durch EHV-1, aber nicht EHV-4, durch die Fähigkeit vermittelt wird, mononukleäre Blutzellen zu infizieren und/oder sie hinsichtlich ihres Anhaftungs- und Rollverhaltens auf Endothelzellen und der anschließenden Infektion umzuprogrammieren.

Summary

Glycoprotein B (gB) plays an important role in alphaherpesvirus cellular entry and acts in concert with gD and the gH/gL complex. To evaluate whether functional differences exist between gB1 and gB4, the corresponding genes were exchanged between the two viruses. The gB4-containing-EHV-1 (EHV-1_gB4) recombinant virus was analyzed for growth in culture, cell tropism, and cell entry revealing no significant differences when compared to parental virus. We also disrupted a potential integrin-binding motif, which did not affect the function of gB in culture. In contrast, a significant reduction of plaque sizes and growth kinetics of gB1-containing-EHV-4 (EHV-4_gB1) was evident when compared to parental EHV-4 and revertant viruses. The reduction in virus growth may be attributable to the loss of functional interaction between gB and the other envelope proteins involved in virus entry, including gD and gH/gL. Alternatively, gB4 might have an additional function, required for EHV-4 replication, which is not fulfilled by gB1. The significant attenuation of virus growth in the case of EHV-4_gB1 may be attributable to the loss of functional interaction between gB and other proteins involved in virus entry. One possible cause for the loss of function/interaction may be structural differences between gB1 and gB4. Our rationale was that this structural difference could be caused by the different locations of the furin cleavage site within the respective gBs.
To investigate the contribution of furin-mediated gB cleavage to EHV-1 and EHV-4 growth, the cleavage sites were mutated. While mitigating furin recognition motif did not affect *in vitro* growth of EHV-1, reconstitution of the mutant EHV-4 was not successful, which confirms previous results indicating different properties of gB4 when compared to gB1. Western blot and mass spectrometry analysis of mutated gB1 suggest that mutating the furin cleavage site indeed prevented gB cleavage and resulted in a partial misfolding. In addition, a novel signal peptide cleavage site was identified for gB1 between residues 98 and 99, which is different from that previously published. We conclude that furin cleavage is solely responsible for gB cleavage and involved in the protein folding. After addressing the structural and functional aspects of gB on a protein level, we looked at how this translated to the function of gB during a key step of EHV-1 pathogenesis, namely the viral transfer between infected PBMC to EC.
Infected peripheral blood mononuclear cells (PBMC) effectively transport equine herpesvirus type 1 (EHV-1), but not EHV-4, to endothelial cells (EC) lining the blood vessels of the pregnant uterus or central nervous system, a process that can result in abortion or myeloencephalopathy. We examined, using a dynamic *in vitro* model, the differences between EHV-1 and EHV-4 infection of PBMC and PBMC-EC interactions. Infection assays revealed that EHV-1 infected B-lymphocytes and monocytes more efficiently than EHV-4. In order to evaluate viral transfer between infected PBMC and EC, co-cultivation assays were performed. Only EHV-1 was transferred from PBMC to EC and viral glycoprotein B (gB) was shown to be mainly responsible for this form of cell-to-cell transfer. For addressing the more dynamic aspects of PBMC-EC interaction, infected PBMC were perfused through a flow channel containing EC in the presence of neutralizing antibodies. By simulating capillary blood flow and analyzing the behavior of infected PBMC through live fluorescence imaging and automated cell tracking, we observed that EHV-1 was able to maintain tethering and rolling of infected PBMC on EC more effectively than EHV-4. Deletion of US3 reduced the ability of infected PBMC to tether and roll compared to parental virus, which resulted in a significant reduction in virus transfer from PBMC to EC. Taken together, we conclude that systemic spread and EC infection of EHV-1, but not EHV-4, is caused by its ability to infect and/or reprogram mononuclear cells with respect to their tethering and rolling behavior on EC and consequent virus transfer.

Publications

Spiesschaert B, Osterrieder N, Azab W
Comparative Analysis of Glycoprotein B (gB) of Equine Herpesvirus Type 1 and Type 4 (EHV-1 and EHV-4) in Cellular Tropism and Cell-to-Cell Transmission.
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Spiesschaert B, McFadden G, Hermans K, Nauwynck H, Van de Walle GR
The current status and future directions of myxoma virus, a master in immune evasion.
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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 22.06.2015 **Bart Spiesschaert**