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Equine Herpesvirus Type 1: Immune Evasion and Vector Development

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Abbreviations

aa	Amino acid
ADCC	Antibody-dependent cellular cytotoxicity
AE	Alkaline endonuclease
ATCC	American type culture collection
ATP	Adenosine triphosphate
β2M	β2-microglobulin
BACs	Bacterial artificial chromosomes
BHV-1	Bovine herpesvirus type 1
BHV-5	Bovine herpesvirus type 5
bp	Base pair
BTV-8	Bluetongue virus type 8
BVDV	Bovine viral diarrhea virus
CBS	Citrate buffered saline
CF	Complement fixing
CTL	Cytotoxic T lyphocyte
DIVA	Differentiating infected from vaccinated animals
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EGFP	Enhanced green fluorescence protein
EHV-1	Equine herpesvirus type 1
EHV-3	Equine coital exanthema virus
EHV-4	Equine herpesvirus type 4
EHV-6	Asinine herpesvirus 1
EHV-7	Asinine herpesvirus 2
EHV-8	Asinine herpesvirus 3
EHV-9	Gazelle herpesvirus 1
EHM	Equine herpesvirus myeloencephalopathy
EMEM	Earle’s minimum essential medium
FcγR	Receptor for Fc domain of IgG
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic retriculum
FBS	Fetal bovine serum
gB	Glycoprotein B
HCMV	Humane cytomegalovirus
HEKT	Human embryonic kidney 293 T cells

hpi	Hour post infection
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
ICP4	Infected cell polypeptide 4
IE	Immediate early
IFA	Indirect immunofluorescence assay
$IFNAR^{-/-}$	Interferon-receptor-deficient
IgG	Immunoglobulin G
IL8	Interleukin 8
IN	Intranasal
INM	Inner nuclear membrane
IR	Internal repeat
IRES	Internal ribosome entry site
kDa	Kilodalton
KIF1A	Kinesin family member 1A
KSHV	Kaposi's sarcoma-associated herpesvirus
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
MCP	Major capsid protein
MFI	Mean fluorescence intensity
MHC-I	Major histocompatibility class I
MIP1α	Macrophage inflammatory protein 1α
MLV	Modified live virus
m.o.i.	Multiplicity of infection
mRNA	Messenger RNA
MT	Microtubule
MVA	Modified vaccinia virus
Nedd4	Neuronal precursor cell-expressed, developmentally downregulated 4
NK	Natural killer
NP	Nuclear pore
NS1	Non-structural proteins 1
OD	Optical density
ORF	Open reading frame
PAA	Phosphonoacetic acid
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

PEI	Polyethylenimine
PFU	Plaque forming units
PrV	Pseudorabies virus
PVDF	polyvinylidene fluoride
RER	Rough endoplasmic reticulum
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RK13	Rabbit kidney cells
RT	Room temperature
RT-PCR	Reverse transtription-polymerase chain reaction
SC	Subcutaneous
SDS	Sodium dodecyl sulfate
SNT	Serum neutralizing test
TAP	Transporter associated with antigen processing
TGN	Trans-Golgi network
TK	Thymidine kinase
TMD	Transmembrane domain
TR	Terminal repeat
UL	Unique long
US	Unique short
vCKBP	Viral chemokine binding protein
VEEV	Venezuelan Equine Encephalitis virus
vhs	Virion host shutoff
VLPs	Virus-like particles
VN	Virus neutralizing
VP	Virion protein
VZV	Varicella-zoster virus
WNV	West Nile virus

Zusammenfassung

Equines Herpesvirus 1 (EHV-1): Immunevasion und Vectorentwicklung

Guanggang Ma

Das equine Herpesvirus 1 (EHV-1) ist, trotz routinemäßiger Impfung, auch heutzutage ein großes Problem in der Pferdehaltung. EHV-1-Infektionen werden hauptsächlich durch die zelluläre Immunantwort kontrolliert, getragen durch $CD8^+$ zytotoxische T-Lymphozyten (cytotoxic T lymphocytes, CTL). EHV-1, wie auch weitere Vertreter der großen *Herpesviridae*-Familie, hat Strategien entwickelt, diese CTL-vermittelte Immunantwort zu umgehen, indem es die Antigenpräsentation durch den Hauptgewebeverträglichkeitskomplex Klasse I (major histocompatibility complex class I, MHC-I) stört. Im Rahmen dieser Doktorarbeit wurde ein immunmodulatorisches Protein identifiziert, welches an der Unterdrückung der MHC-I-Lokalisierung an der Zellmembran beteiligt ist. Das verantwortliche virale Protein pUL56, kodiert im offenen Leserahmen (open reading frame, ORF) EHV-1 *ORF1*, ist ein phosphoryliertes frühes Protein, mit unterschiedlichen Translationsvarianten, das vornehmlich an Membranen des Golgi-Apparates und davon gebildeten Vesikeln lokalisiert ist. Außerdem wurde gezeigt, daß die Transmembrandomäne von pUL56 essentiell für die korrekte Funktionalität und subzelluläre Lokalisierung des Proteins ist. Unabhängig davon wird die Funktion von pUL56 auch von pUL49.5 vermittelt, einem viralen Protein, das den Transporter der Antigenprozessierung (TAP) inhibiert und von EHV-1 und verwandten Viren kodiert wird. Überraschenderweise ist pUL56 allein nicht in der Lage, MHC-I herunterzuregulieren, daher benötigt es wahrscheinlich weitere, bisher unidentifizierte, virale Proteine für diese Funktion.

EHV-1 ist, wie kürzlich demonstriert, ein vielversprechender viraler Vektor für fremde Antigene. Im zweiten Teil dieser Doktorarbeit wurde der *ORF1*-Genort benutzt, um dort fremde Gene einzufügen, spezifisch die VP2- und/oder VP5-Gene des Blauzungenkrankheitvirus (blue tongue virus, BTV) Serotyp 8. BTV-8 infiziert die meisten domestizierten und freilebenden Wiederkäuer und war für einen Seuchenausbruch in Nordeuropa 2008 verantwortlich. Die konstruierten EHV-1-Rekombinanten exprimieren stabil die entsprechenden Transgene und zeigen mit dem Wildtyp identische Wachstumskurven *in vitro*. Immunisierung von Mäusen mit transgenem Virus führte zur Bildung BTV-8-spezifischer Antikörper. In einem Infektionsversuch mit einer tödlichen Dosis BTV-8 an Interferon-A-Rezeptor-defizienten ($IFNAR^{-/-}$) Mäusen überlebten 100% der mit VP2/VP5-doppelttransgenem EHV-1 geimpften Tiere, aber keines der mit VP2-einfachtransgenem EHV-1 geimpften. VP7 wurde in den transgenen Impfstoffen nicht

verwendet und ist erfolgreich als Unterscheidungsmerkmal infizierter und geimpfter Tiere (marker for differentiating infected from vaccinated animals, DIVA) benutzt worden.
Zusammengefaßt kann gesagt werden, daß das vom EHV-1 *ORF1* kodierte Protein pUL56 ein bisher unbekannter Faktor ist, mit dem das Virus der Immunantwort ausweicht. pUL56 ist an der Störung der MHC-I-Oberflächenexpression beteiligt, kann aber seine Funktion nicht außerhalb des viralen Kontextes ausüben. Ein rekombinantes EHV-1, das VP2 und VP5 vom BTV-8 im ORF1-Genort trägt, löst im Mausmodell protektive Immunität aus und kann daher eine mögliche Alternative zu bisherigen BTV-8-Impfstrategien sein.

Summary

Equine Herpesvirus Type 1: Immune Evasion and Vector Development

Guanggang Ma

Equine herpesvirus type 1 (EHV-1) remains a severe threat to horse industry despite widespread vaccination. The control of EHV-1 infection is mainly dependent on cellular immunity that is mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs). EHV-1, as its relatives in the large *Herpesviridae* family, has evolved strategies to evade CTL immunity by interfering with the major histocompatibility complex class I (MHC-I) antigen presentation pathway. In the first part of this thesis, we identified a novel immunomodulatory protein involved in the downregulation of MHC-I from the cell surface. The responsible viral protein, pUL56, which is encoded by EHV-1 open reading frame 1 (*ORF1*), is a phosphorylated early protein, which is expressed as different forms and predominantly localizes to Golgi-derived membranes. In addition, the transmembrane (TM) domain of pUL56 was shown to be indispensable for correct subcellular localization and proper function. The function of pUL56 was independent of that mediated by pUL49.5, a viral protein known to inhibit the transporter associated with antigen processing (TAP) and encoded by EHV-1 and related viruses. Surprisingly, pUL56 by itself was not capable of downregulating MHC-I and likely needs (an)other unidentified viral protein(s) to perform this action.

EHV-1 has recently been demonstrated to be a promising viral vehicle for delivery of foreign antigens. In the second part of the thesis, we utilized *ORF1* as the locus for the insertion of foreign genes, more specifically the VP2 and/or VP5 genes of bluetongue virus serotype 8 (BTV-8). BTV-8 can infect most domestic and wild ruminants species and was responsible for an epizootic in northern Europe in 2006. The EHV-1 recombinant viruses generated stably expressed the transgenes and grew with kinetics that were identical to those of parental virus *in vitro*. After immunization of mice, a BTV-8-specific neutralizing antibody response was elicited. In a challenge experiment using a lethal dose of BTV-8, 100% of interferon-receptor-deficient ($IFNAR^{-/-}$) mice vaccinated with the recombinant EHV-1 carrying both VP2 and VP5, but not VP2 alone, survived. VP7 was not included in the vectored vaccines and was successfully used as a marker for differentiating infected from vaccinated animals (DIVA).

In conclusion, EHV-1 *ORF1*-encoded pUL56 is a novel immune evasion protein involved in the interference of MHC-I surface expression, but is unable to perform its function outside of the context of viral infection. An EHV-I recombinant carrying VP2 and VP5 of BTV-8 in the

ORF1 locus is capable of eliciting protective immunity in a murine infection model and as such may be an alternative for BTV vaccination strategies.

C H A P T E R 1

Introduction

Introduction

Equine herpesvirus type 1 (EHV-1) is a highly prevalent pathogen affecting the Equidae and all breeds of horses throughout the world. The virus exerts its impact by causing a range of syndromes in horses, including mild respiratory disease, abortion in pregnant mares, death of neonatal foals and severe neurological disorders (Allen & Bryans, 1986). Despite widespread vaccinations, EHV-1 remains a severe problem for the horse industry as outbreaks continue to occur and result in death of affected animals and interruptions of horse traffic and events.

1.1 Taxonomy and genome characters

Herpesviruses represent one of the largest known virus groups. The latest taxonomy has incorporated herpesviruses into a new order, *Herpesvirales*, which is divided into three families: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae* (Davison et al., 2009). In nature, each herpesvirus is usually associated with a single host species, but it is common that one host is infected with several distinct herpesviruses. In equid populations, nine herpesviruses have been identified so far, of which EHV-1 (equine abortion virus), EHV-3 (equine coital exanthema virus), EHV-4 (equine rhinopneumonitis virus), EHV-6 (asinine herpesvirus 1), EHV-8 (asinine herpesvirus 3) and EHV-9 (gazelle herpesvirus 1) belong to the genus *Varicellovirus* of the subfamily *Alphaherpesvirinae*, while EHV-2, EHV-5 and EHV-7 (asinine herpesvirus 2) belong to the *Gammaherpesvirinae* subfamily. Several other animal herpesviruses of agricultural importance including bovine herpesvirus type 1 (BHV-1), BHV-5, suid herpesvirus 1 (Pseudorabies virus, PrV) are also allocated to the *Varicellovirus* genus and closely related with EHV-1 (Davison *et al.*, 2009).

Clinically, economically and epidemiologically, EHV-1 and EHV-4 by far are the most relevant pathogens of herpesviruses affecting equids and were considered subtypes of one and the same virus species until 1981 (Patel & Heldens, 2005). Although both viruses show a high degree of antigenic and genetic similarity, they are strikingly different in their pathogenicity. While EHV-4 infection is limited mainly to the upper respiratory tract in most of the cases, EHV-1 has a multi-organ, systemic distribution causing different diseases that range from mild rhinopneumonitis to abortion and lethal myeloencephalopathy (Patel & Heldens, 2005).

EHV-1 possesses a linear double-stranded type D DNA genome of about 150kbp with a 56.7% G+C content (Telford *et al.*, 1992). The complete genome contains at least 80 open reading frames (ORFs). Since four ORFs are duplicated, the genome is considered to contain at least 76 distinct genes with a potential to code for 77 different proteins due to splicing of ORF64 (Harty *et al.*, 1989; Telford *et al.*, 1992). The genome harbors a unique long (UL) region and a unique short (US) region, with the latter bracketed by two inverted repeat

regions, namely the internal repeat (IR) and terminal repeat (TR) (Telford *et al.*, 1992). As with the other herpesviruses, EHV-1 genomic DNA is packaged within an icosahedral capsid of approximately 125 nm in diameter, which in turn is embedded in a matrix of so-called tegument proteins and enclosed in an envelope containing a number of viral membrane (glycol)proteins (Davison, 2002).

1.2 Replication cycle

In the life cycle of herpesviruses, two general phases are found: the lytic phase, which is followed by a persistent, "latent" phase that usually lasts for the lifetime of the host. During latency, no infectious progeny and only a very limited set of viral proteins are produced. Latent virus can be activated upon stimulation and enter lytic replication (Fig. 1) resulting in production of infectious viral particles (Lehman & Boehmer, 1999). Productive infection initiates with the attachment and infectious entry of viruses into host cells, a process in which at least five viral glycoproteins are involved: glycoprotein B (gB), gC, gD and the gH-gL complex (Spear & Longnecker, 2003). Following the interaction between gD and cell surface receptors, e.g. major histocompatibility class I (MHC-I) (Kurtz *et al.*, 2010; Sasaki *et al.*, 2011), EHV-1 is able to enter cells efficiently by direct fusion at the plasma membrane or after endocytic uptake, which was shown to depend on the cell type infected (Frampton *et al.*, 2007; Hasebe *et al.*, 2009; Van de Walle *et al.*, 2008). Following penetration, viral capsids and tegument proteins are released into the cytoplasm and capsids are transported along microtubules to the the nuclear pores. After delivery of viral DNA into the cell nucleus, transcription, DNA replication, capsid assembly and DNA packaging occur. A tegument protein, VP16, transactivates the transcription of the only immediate-early protein of EHV-1, which is encoded by *ORF64*, the EHV-1 homologue to ICP4 of herpes simplex virus type 1 (HSV-1). EHV-1 ICP4 will then transactivate the expression of early genes including enzymes required for genome replication, DNA repair, deoxynucleotide metabolism and a number of other early proteins with functions not clearly understood to date. Seven of these early proteins (*UL5*, *UL8*, *UL9*, *UL29*, *UL30*, *UL42* and *UL52* gene products) are essential for viral DNA replication. Shortly after the appearance of the early proteins, genomic DNA is synthesized by a rolling-cycle mechanism. Later on, late proteins, mainly capsid proteins and some of the glycoproteins, are expressed and nucleocapsids assembled. The newly produced head-to-tail concatameric genomic DNA is then cleaved and packaged as unit-length molecules resulting in mature (C) capsids (Lehman & Boehmer, 1999). With the involvement of *UL31*, *UL34* and *US3* protein kinases, the fully assembled nucleocapsid buds out of the nucleus and is temporarily present within the perinuclear space. Primary budding results in nucleocapsids acquiring an envelope derived from the inner leaflet of the nuclear membrane (INM). The nucleocapsids lose the primary envelope when passing through the outer nuclear membrane (ONM) and the are released into the cytoplasm, where they acquire the full set of

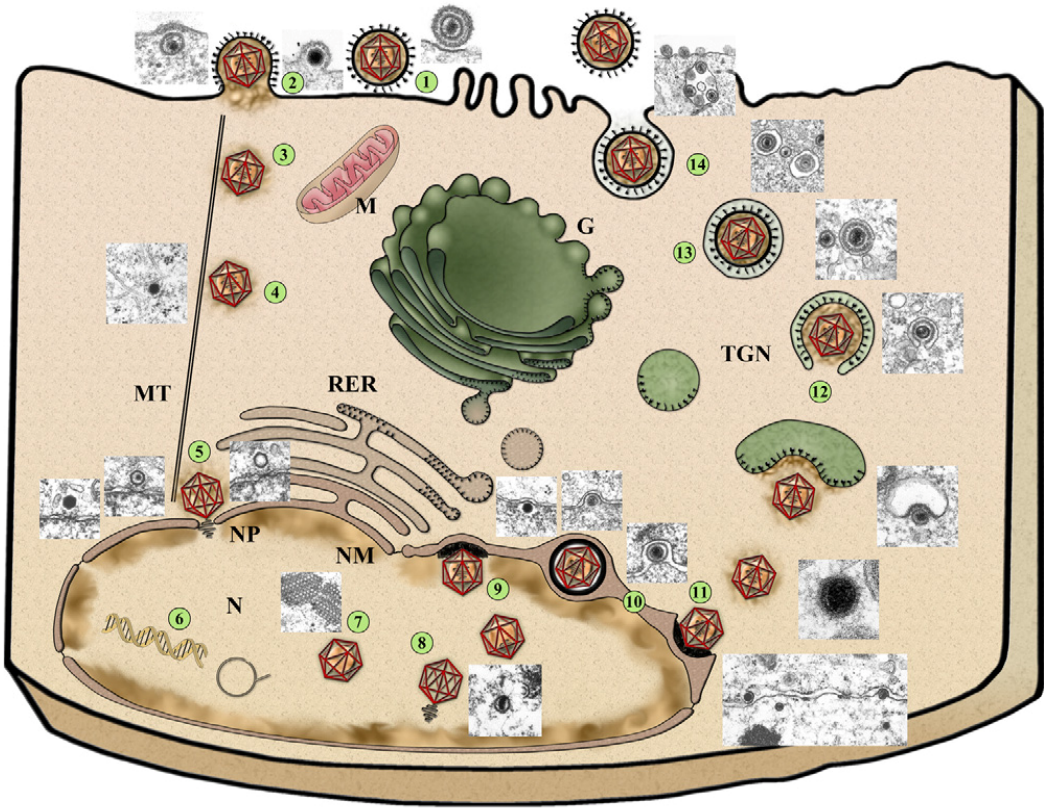

Figure 1.1 The replication cycle of the alphaherpesvirus pseudorabies virus (PrV), a close relative of EHV-1.

The replication cycle of PrV, a close relative of EHV-1 in genus *Varicellovirus*, is shown together with electron micrographs of the respective stages. (1) attachment; (2) penetration; (3) and (4) capsids transported to the nucleus (N) via microtubules (MT); (5) the viral genome released through the nuclear pore (NP); (6) transcription and genome replication; (7) and (8) genome cleavage and packaging; (9) budding at the INM; (10) and (11) fusion with the outer nuclear membrane; (12) final maturation by tegumentation and secondary envelopment via budding into the trans-Golgi network TGN; (13) transport to the cell surface; (14) release by fusion. RER, rough endoplasmic reticulum; M, mitochondrion; G, Golgi apparatus. Adopted with permission from Mettenleiter *et al*, *Virus Research* 143 (2009) 222-234.

tegument and the final (secondary) envelope via an intricated sequence of protein-protein interactions that occur both in the cytoplasm and at vesicles of the trans-Golgi and endosomal networks. The mature virions are transported to the cell surface within sorting vesicles and released by fusion of vesicles with plasma membrane (Fig. 1.1) (Mettenleiter, 2002; Mettenleiter *et al.*, 2009; Pomeranz *et al.*, 2005).

1.3 Pathogenesis and Latency

EHV-1 is highly contagious and is usually transmitted by direct contact that leads to transfer of infected saliva or nasal discharge. While rare, EHV-1 can also be transmitted by aerosol or contaminated feed or water (Allen & Bryans, 1986). After inhalation, the virus replicates in the epithelial cells of the upper respiratory tract, resulting in viral shedding and distinct herpetic lesions of the mucosal membranes. In natural and experimental infections, both EHV-1 and EHV-4 can cause respiratory disease characterized by fever, anorexia, nasal discharge and ocular discharge (Patel & Heldens, 2005). After respiratory infection, EHV-1 will invade the lamina propria and infect leukocytes and endothelial cells of blood and lymphatic vessels. Subsequently, EHV-1 spreads to local lymph nodes from which mononuclear cells – monocytes, B cells and T cells – are infected and the virus is carried to the internal organs via cell-associated viraemia. The widespread infection of the endothelial cells in the vasculature of the pregnant uterus or the central nervous systems will ultimately induce abortion or neurological disease as a result of vasculitis, thrombosis, and ischemic damage (Smith *et al.*, 1992; Wilson, 1997). It seems that the leukocyte-associated viremia and the infection of endothelial cells are key for EHV-1 pathogenicity.

In contrast to EHV-1, EHV-4 infection remains limited to the upper respiratory tract and is rarely accompanied with cell-associated viremia. The differences in the pathogenic potential between the two closely related viruses have not been fully understood. It was long thought that the difference was ascribed, at least in part, to variations in cellular tropism that is mainly determined by gD. While EHV-1 is able to infect and replicate in vascular endothelial cells, EHV-4 replication is restricted to epithelial cells of the upper respiratory tract (Patel *et al.*, 1982). Later studies showed, however, that EHV-4 is also able to replicate in endothelial cells both *in vitro* and *in vivo* (Blunden et al., 1995; Osterrieder & Van de Walle, 2010). Even in peripheral blood mononuclear cells (PBMCs) *in vitro*, EHV-4 has a similar infection efficiency as EHV-1 (Azab & Osterrieder, 2012), indicating that the difference in cellular entry does not play the decisive role in determining the tropism and hence the pathogenicity of EHV-1 and EHV-4. Interestingly, the chemokine binding protein (vCKBP), glycoprotein G (gG), expressed by EHV-1 and EHV-4 showed different chemokine binding properties, with the former being able to modulate chemokines, while the latter is not (Van de Walle *et al.*, 2009b; Van de Walle *et al.*, 2007). The inability of EHV-4 gG to modulate chemokines that are crucial for leukocyte migration was also thought to be one of the reasons why EHV-4 infection is restricted to the upper respiratory tract (Osterrieder & Van de Walle, 2010).

Over the last decade, the incidence of equine herpesvirus myeloencephalopathy (EHM) induced by EHV-1 has increased significantly in North America and Europe (Allen *et al.*, 2008; Perkins *et al.*, 2009; Pusterla *et al.*, 2009). As a result, EHM has been classified as a

potentially emerging disease by the US Department of Agriculture (USDA-APHIS, 2007). Recently, a single nucleotide exchange at position 2254 (A/G2254) in the catalytic subunit of EHV-1 DNA polymerase encoded by open reading frame 30 (*ORF30*) was found to be strongly associated with the occurrence of EHM (Goodman *et al.*, 2007; Nugent *et al.*, 2006; Van de Walle *et al.*, 2009a). Compared to the non-neuropathogenic genotype of EHV-1 (A2254), the neuropathogenic genotype (G2254) was found to replicate more efficiently in horses, resulting in higher levels and longer periods of cell-associated viraemia (Edington *et al.*, 1986; Goodman *et al.*, 2007; Van de Walle *et al.*, 2009a). It should be noted that a minor percentage of EHM cases were actually induced by EHV-1 A2254 genotype and on the other hand, G2254 substitution did not necessarily lead to EHM (Perkins *et al.*, 2009; Pronost *et al.*, 2010; Smith *et al.*, 2010; Vissani *et al.*, 2009), indicating that other viral factors might also contribute to the occurrence of EHM. Lately, the *UL24* (*ORF37*) gene product was shown to play a role in neuropathogenicity in a mouse encephalitis model (Kasem *et al.*, 2010). Besides viral genetics, host and environmental factors, e.g. the age, the physical condition and the immune status of the host and the infection route, also have a significant impact on the clinical outcome following EHV-1 infection (Nugent *et al.*, 2006).

Both EHV-1 and EHV-4 will establish life-long latency after primary infection. It is clear that the latently infected leukocytes are invisible to immune surveillance and elimination. Experimentally, latent EHV-1 and EHV-4 could be reactivated with corticosteroid treatment and mild nasal trauma (Browning *et al.*, 1988; Edington *et al.*, 1985). The main sites of latency are still controversial. Some studies demonstrated that latency was mainly in lymphoid tissues and peripheral leukocytes (Carvalho *et al.*, 2000; Chesters *et al.*, 1997; Smith *et al.*, 1998; Welch *et al.*, 1992), while some other studies claim latency is established predominantly neuronally in trigeminal ganglia (Borchers *et al.*, 1997; Borchers *et al.*, 1999; Slater *et al.*, 1994). It has been commonly recognized, however, that viruses reactivated from latency can be transmitted to other horses, causing clinical diseases and viral shedding, and therefore, play a considerable important role in the epidemiology of EHV-1 and EHV-4 (Lunn *et al.*, 2009; Patel & Heldens, 2005).

1.4 Immune response against EHV-1 infection

Understanding the immune response associated with protection against EHV-1 infection is the prerequisite to design efficient EHV-1 vaccines. Using diagnostic techniques, it has been shown that both virus neutralizing (VN) and complement fixing (CF) antibodies are elicited starting approximately 2 weeks after field or experimental infection with EHV-1 (Doll & Bryans, 1962; Thomson *et al.*, 1976). Primarily, the EHV-1 glycoproteins, gB, gC, gD, gH and the EHV-1 unique gp2 were found to be immunodominant in natural infections of horses (Crabb *et al.*, 1991). The efficacy of the circulating antibody in the protection of EHV-1

infection, however, is not satisfactory. While some studies found that there was an inverse correlation between VN antibody titer and nasal shedding, some other studies did not record this correlation (Patel & Heldens, 2005). Although the data are controversial, it is usually agreed that antibody mediated immunity plays a role in neutralizing cytolytically replicating EHV-1, reducing virus shedding in the nasal mucus, and as a result, reducing clinical symptoms, especially in respiratory disease. Apart from that, humoral immunity is not considered the most effective mechanism in protection against abortion and neurological disease. Despite the presence of high level of antibodies after repeated vaccinations, abortion and neurological disease still occur.

Cellular immunity mediated by CD8+ cytotoxic T lymphocytes (CTLs) is an essential defense mechanism against many virus infections. The frequency of precursor CTLs specific for EHV-1 antigens has been found to be correlated with protection against disease (Kydd et al., 2003). Compared to the short-lived antibody response, CTL activity is of long duration after EHV-1 infection (Kydd *et al.*, 2003). Having known the importance of CTL activity in immune defense, preliminary attempts have been done to identify CTL epitopes encoded by EHV-1 proteins using outbred ponies expressing predominantly the equine leucocyte antigen A3 haplotype (Soboll *et al.*, 2003). The only viral protein that was found to be able to constantly induce CTL activity in this study was the immediate early protein (IE, ICP4) encoded by *ORF64* (Soboll *et al.*, 2003). Several glycoproteins, including gC, gD, gI and gL, were also found to contain CTL epitopes, but the findings were obtained from individual ponies only and need to be confirmed (Allen *et al.*, 1995). One can expect that if EHV-1-infected lymphocytes are efficiently recognized by CTLs, virus elimination could be achieved. Unfortunately, it was revealed that the lymphocytes isolated from animals exposed to virulent strains of EHV-1 showed immunosuppression and failed to respond optimally to incubation with either inactivated or live viruses (Charan *et al.*, 1997; Hannant *et al.*, 1999). Together with the fact that latently infected leukocytes and neuronal cells can be inactivated a long time after the primary infection, it is strongly suggested that some viral factors have rendered the virus the ability to evade the immune system. Elucidation of the immune evasion strategies used by EHV-1 will definitely benefit the optimization of EHV-1 vaccines.

1.5 Immune evasion of EHV-1 and other herpesviruses

EHV-1, like other herpesviruses, establishes lifelong infection after primary inoculation. It is generally accepted that the long-term persistence in the presence of an active host immune system must have been facilitated by the immunomodulatory strategies that have been developed by the viruses during their long history of co-evolution with the hosts. In the last decade, some of the mechanisms that herpesviruses utilize to counteract the host's innate and acquired immune response have been elucidated.

1.5.1 Evasion from humoral immunity

As one of the most important immune defense mechanisms, antibody-dependent humoral immunity plays a crucial role in the neutralization of cell-free viruses as well as the clearance of virus-infected cells. To clear virus-infected cells, virus-specific antibody needs to recognize viral antigens that are expressed on the cell surface, so that complement-mediated cell lysis and cell-mediated cytotoxicity can occur. It is clear that strong, albeit short-lived, antibody responses can be elicited after vaccination or EHV-1 infection in horses, but the recognition of EHV-1-infected cells by antibody seemed to be problematic. Both *in vitro* and *in vivo* studies have shown that the majority of EHV-1-infected PBMCs do not express viral envelope proteins on the cell surface. As a result, these cells are insensitive to antibody-dependent elimination (van der Meulen *et al.*, 2006a; van der Meulen *et al.*, 2003). Interestingly, it was found that viral envelope proteins were even undetectable intracellularly and only IE and some early proteins were present, indicating that the replication of EHV-1 in circulating PBMCs is restricted to early phase or that PBMCs expressing viral envelope proteins might be selectively removed from the circulation (van der Meulen *et al.*, 2006a). Since EHV-1-infected PBMCs are still able to transmit infectious viruses to susceptible cells by direct contact, it is likely that this contact, e.g. between PBMCs and endothelial cells, might provide signals to trigger the late phase replication of EHV-1 in PBMCs (van der Meulen *et al.*, 2006a).

While the majority of EHV-1-infected PBMCs do not express viral envelope proteins, a minority of PBMCs still does. *In vitro* studies showedthat most of these PBMCs were still resistant to the lysis mediated by antibody and complement (van der Meulen *et al.*, 2003). One of the possibilities is that certain EHV-1 envelope proteins interfere with the action of complement. It has been reported that EHV-1 gC is an envelope protein that can interfere with the activation of the complement cascade by binding to complement component C3 (Huemer *et al.*, 1995). Similar findings were also reported for gC of numbers of other herpesviruses, including EHV-4 (Azab *et al.*, 2010; Huemer *et al.*, 1995), PrV (Huemer *et al.*, 1992), BHV-1 (Huemer *et al.*, 1993) and HSV-1 (Fries *et al.*, 1986). HSV-1 gC has been studied in detail and was demonstrated to bind complement C3 and its activation products, C3b, iC3b, C3c resulting in an acceleration of the decay of the alternative C pathway C3 convertase (Fries *et al.*, 1986; Kostavasili *et al.*, 1997). HSV-1 gC also interferes with C5 and properdin (P) binding to gC (Fries *et al.*, 1986; Kostavasili *et al.*, 1997). *In vivo* studies confirmed that HSV-1 gC protect the virus from complement-mediated neutralization (Lubinski *et al.*, 1999; Lubinski *et al.*, 2002; Lubinski *et al.*, 1998).

To ensure efficient evasion from antibody-dependent immunity, herpesviruses are equipped with another envelope protein, gE, a receptor for the Fc domain of immunoglobulin G (IgG)

(FcγR), and thus interfere with C1q binding and antibody-dependent attack (Dubin *et al.*, 1991). HSV-1 gE forms a heterodimeric complex with gI that binds to the IgG Fc domain with higher affinity than gE alone (Johnson & Feenstra, 1987). *In vitro*, HSV-1 FcγR has been demonstrated to protect the virus from antibody-dependent complement-mediated neutralization, antibody-dependent cellular cytotoxicity (ADCC) and Fc-mediated attachment of granulocytes to virus-infected cells (Dubin *et al.*, 1991; Nagashunmugam *et al.*, 1998; Van Vliet *et al.*, 1992). By using an HSV- 1 gE mutant that functions normally in cell-to-cell spread but lost Fc binding ability, it was shown that gE promotes immune evasion from IgG Fc-mediated activity *in vivo* (Lubinski *et al.*, 2011). The gE/gI complex of PrV, but not BHV-1, has also been shown to bind IgG Fc domain and aid to protect PrV-infected cells from complement-mediated lysis (Favoreel *et al.*, 1997; Van de Walle *et al.*, 2003; Whitbeck *et al.*, 1996). Whether the gE/gI complex of EHV-1 also interferes with Fc-mediated activities is yet to be elucidated.

1.5.2 Evasion from CTL immunity

CTL-based immunity is dependent on the efficient recognition of viral peptides presented by MHC-I on the cell surface. After virus infection, viral proteins, primarily the defective forms of newly synthesized proteins, will be degraded into small peptides by the multi-subunit proteasome complex in a ubiquitin-dependent manner (Hershko & Ciechanover, 1998; Voges *et al.*, 1999). The small peptides generated by the proteasome are then transported into the lumen of endoplasmatic retriculum (ER) by the transporter associated with antigen processing (TAP) 1 and 2. In the ER, the peptides are loaded to the MHC-I α chain associated with β2-microglobulin (β2M) and form stable tri-molecular complex (Androlewicz *et al.*, 1993; Ortmann *et al.*, 1994). Once the peptides are successfully loaded, the MHC-I complex is released and transported along the secretory pathway via the Golgi apparatus to the cell surface where a CTL response will be triggered. MHC-I molecules that fail to bind peptides are re-routed to the cytosol where they become degraded by the proteasome (York & Rock, 1996).

While CTL-based immunity is an efficient defense mechanism, herpesviruses have developed multiple strategies to target almost every step of the MHC-I antigen processing and presentation pathway (Griffin *et al.*, 2010). The interference of antigen presentation starts immediately after virus entry into host cells. The virion-associated *UL41* gene product of alphaherpesviruses, known as virion host shutoff (vhs) protein, and the alkaline endonuclease (AE) of gammaherpesviruses can induce global degradation of host mRNA, resulting in the shortage of antigen presentation pathway components and reduced levels of cell surface MHC-I expression (Everly *et al.*, 2002; Rowe *et al.*, 2007; Zuo *et al.*, 2008). Some herpesvirus proteins can hamper the generation of virus-derived peptides, e.g. the M27

product of murine cytomegalovirus (MCMV) and EBNA1 of Epstein-Barr virus (EBV), by blocking the formation of immunoproteasome components or their own proteasomal degradation (Hoyt *et al.*, 2006; Khan *et al.*, 2004). The transport of peptides by TAP is a common target for herpesvirus proteins involved in the interference of MHC-I presentation pathway. TAP-inhibitors have been found in all the three subfamilies of the *Herpesviridae*. Although TAP inhibition seems to be a conserved activity among herpesviruses, it is achieved by different viral proteins with diverse mechanisms (Griffin *et al.*, 2010). For HSV-1 and HSV-2, the *US12* gene product (known as ICP47), which is unique to the *Simplexviruses*, serves as a high-affinity peptide-binding competitor by associating with the peptide-binding site of TAP (Früh *et al.*, 1995; Hill *et al.*, 1995). For members of the *Varicellovirus* genus including BHV-1, PrV, EHV-1 and EHV-4, the products of the *UL49.5* orthologues (pUL49.5) were recently identified as a novel class of TAP inhibitors (Koppers-Lalic *et al.*, 2005; Koppers-Lalic *et al.*, 2008). While BHV-1 pUL49.5 targets TAP for proteasomal degradation, EHV-1 and EHV-4 pUL49.5 interfere with the binding of ATP to TAP (Koppers-Lalic *et al.*, 2008). Interestingly, although all herpesviruses seem to encode a *UL49.5* homologue, the capacity to inhibit TAP was found to be restricted only to a subgroup of varicelloviruses (Verweij *et al.*, 2011). Other TAP inhibitors include *US6* product of humane cytomegalovirus (HCMV) and EBV BNLF2α, which inhibit ATP-binding to TAP (Hewitt *et al.*, 2001; Hislop *et al.*, 2007). MHC-I molecules are also targeted by HCMV *US2* and *US11* products, which direct MHC-I molecules from ER to the cytosol for degradation (Wiertz *et al.*, 1996a; Wiertz *et al.*, 1996b). The HCMV *US3* product targets tapasin, a companion protein that links TAP to immature MHC-I and governs peptide loading (Jones *et al.*, 1996). The *ORF66* protein of VZV, a homolog of HSV-1 *US3* kinase, can retain MHC-I complexes in the Golgi (Eisfeld *et al.*, 2007). Even after MHC-I complexes find their ways to reach the cell surface, they can be internalized by viral E3 ubiquitin ligases, kK3 and kK5, of Kaposi's sarcoma-associated herpesvirus (KSHV) (Coscoy *et al.*, 2001). Thus, herpesviruses effectively reduce cell surface MHC-I expression and evade from CTL-mediated cell lysis by multiple mechanisms.

EHV-1 was also shown to downregulate MHC-I cell surface expression in equine embryonic lung cells (NBL6) (Rappocciolo *et al.*, 2003) and to inhibit TAP-mediated peptide transport in equine kidney primary fibroblasts (Ambagala *et al.*, 2004) after infection *in vitro*. The downregulation of surface MHC-I was also observed in EHV-1-infected PBMCs *in vitro*, but not *in vivo*. The controversial results might be due to the restricted replication of EHV-1 in PBMCs *in vivo* relative to *in intro*, and to the fact that only the absolute percentage of MHC-I positive PBMCs, instead of the amount of MHC-I per infected PMBC, was addressed in these studies (van der Meulen *et al.*, 2006a; van der Meulen *et al.*, 2006b). Rappocciolo et al. reported that an early viral protein(s) was responsible for the observed surface MHC-I downregulation, probably by enhancing endocytosis of MHC-I molecules (Rappocciolo *et al.*,

2003). pUL49.5 is the only EHV-1 protein identified to modulate the MHC-I-restricted antigen-presenting pathway by blocking ATP-binding to TAP. When pUL49.5 was over-expressed in a stably transfected cell line, TAP activity was inhibited and cell surface MHC-I was indeed downregulated (Koppers-Lalic *et al.*, 2008). During infection, EHV-1 pUL49.5 was also able to inhibit TAP, but it was not clear whether this inhibition resulted in downregulation of surface MHC-I. It is unlikely, albeit possible, that pUL49.5 enhances MHC-I internalization from the cell surface. As described above, the vhs protein (*UL41* gene product) and *US3* protein kinase can interfere with MHC-I antigen presentation in certain alphaherpesviruses. But there is no evidence for EHV-1 showing that pUL41 is involved in MHC-I downregulation (Rappocciolo *et al.*, 2003). The role of EHV-1 pUS3 in MHC-I downregulation is also unclear. Still, it could be assumed that yet unknown EHV-1 early proteins exist that can induce MHC-I downregulation. Interestingly, the downregulation of cell surface MHC-I by EHV-1 seems to be locus- or allele-specific (Rappocciolo *et al.*, 2003), which is important because the virus-infected cells could avoid the action of natural killer (NK) cells.

1.5.3 Interference with cytokine and chemokine responses

Cytokines and chemokines are hormone-like messengers that regulate the development and expression of the broad array of immune responses and therefore play a critical role in innate and adaptive immunity (Banyer *et al.*, 2000). By mimicking host cytokines, chemokines and their receptors, viruses will be able to evade detection and destruction by the host immune system (Alcami, 2003). *Alpha*-, *beta*- and *gammaherpesviruses* are known to encode chemokine binding proteins (vCKBP) or their receptors (Bryant *et al.*, 2003; Lalani *et al.*, 2000; Murphy, 2001). For members of alphaherpesviruses, including EHV-1 but not EHV-4, the envelope protein gG, which is present both at cell membrane and in solution, is able to bind a broad range of chemokines with high affinity and block their activity (Bryant *et al.*, 2003). *In vitro* studies showed that EHV-1 gG inhibited equine interleukin 8 (IL8) and KC (the murine orthologue of IL8)-induced migration of equine neutrophils. Using a murine infection model, it was shown that neutrophil migration in the lungs was significantly inhibited by EHV-1 gG (Van de Walle *et al.*, 2007). Furthermore, EHV-1 gG was shown to interfere with the proinflammatory chemokine macrophage inflammatory protein 1α (MIP1α)-induced migration of murine macrophages *in vitro* and *in vivo* (Van de Walle et al., 2008).

In summary, EHV-1, along with other herpesviruses, has developed multiple strategies to evade antibody-, CTL- and NK cell-mediated immunity. As a result, EHV-1-infected cells cannot be completely cleared and re-infection can occur shortly after primary infection. The importance of CTL-mediated immunity has been highlighted by the discovery of an

impressing number of herpesvirus proteins that are involved in the interference of MHC-I antigen presentation pathway. But for EHV-1 specifically and alphaherpesviruses in general, the immune evasion proteins targeting MHC-I antigen presentation pathway have not been completely identified, which also encouraged us to identify such activities of EHV-1 proteins.

1.6 EHV-1 vaccination

Since the virus was first isolated in the early 1930s, vaccines have been developed, including inactivated vaccines, traditional modified live virus (MLV) vaccines, recombinant (gene-deleted) live vaccines, live vectored vaccines, DNA vaccines and so on (MINKE *et al.*, 2004).

1.6.1 Current vaccines

Both inactivated and MLV vaccines are commercially available currently. Inactivated EHV-1 vaccines were initially prepared from virus grown in equine fetal tissues or hamsters in the late 1950s. These vaccines were abandoned because of their adverse side effects and poor immunogenicity (Doll & Bryans, 1963; Doll *et al.*, 1959). Subsequently, an MLV vaccine was developed using hamster adapted live virus, but it retained residual virulence and caused abortion if given at later times of gestation (Doll, 1961). At the same time, Mayr et al. developed another MLV vaccine derived from strain RacH (Mayr *et al.*, 1968), which is still in commercial use today. The RacH strain was isolated from an aborted fetus in Europe and attenuated by 256 passages on primary swine kidney cells (Mayr *et al.*, 1968). During continuous passage, the virus underwent genomic alterations, of which the deletion of both copies of gene 67 (encoding IR6 protein) was found as the major determinant of its attenuation (Osterrieder *et al.*, 1996a; Osterrieder *et al.*, 1996b). RacH-based MLV vaccines are licensed as Rhinomune (initially by Pfizer Animal Health, now by Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) and represents the only EHV-1 MLV vaccine in North America at this time. This vaccine has a good safety record and can protect horses against clinical disease, but its efficacy in preventing viremia, abortion and neurological disease is unclear (Kydd *et al.*, 2006). Another well-characterized candidate MLV vaccine strain is Kentucky A (KyA). Similar to RacH, KyA was also attenuated by continuous passages in murine L-M cells and underwent considerable genomic changes including the deletion of gene 1 and 2 and a major deletion in the US region of its genome (Rosas *et al.*, 2006). Recently, a temperature sensitive MLV vaccine based on a German abortion isolate was developed. Upon vaccination with this vaccine, virus shedding, cell-associated viremia and mild clinical signs were observed and the protection efficacy against challenge was not consistent in different studies (Patel *et al.*, 2003a; Patel *et al.*, 2004; Patel *et al.*, 2003b). Concerning the safety of MLV vaccines, the currently available EHV-1 vaccines are made almost exclusively from inactivated preparations, the majority of which are combination

vaccines. Only three products (Pneumabort K and Duvaxyn-1,4 by Fort Dodge and Prodigy by Intervet) offer claims to protect against EHV-1-induced abortion. Vaccines that are labeled for prevention of EHV-1-induced neurological diseases are still not available so far (Rosas *et al.*, 2006). As stated above, protective immunity against EHV-1 infection requires a CTL-based more than a neutralizing antibody response. Inactivated vaccines, while being able to induce high levels of neutralizing antibody, are weak inducers of cell-mediated immunity. Therefore, inactivated vaccines provide only partial clinical and virological protection against respiratory infection by EHV-1 or EHV-4, but do not fully prevent cell-associated viremia and abortion.

1.6.2 Novel vaccines

The introduction of virus mutagenesis has made possible the design of novel and potentially more effective EHV-1 vaccine candidates, including gene deletion mutants, live-vectored vaccines and DNA vaccines. By deleting virulence-associated genes, several EHV-1 mutants were constructed and tested for vaccine efficacy. An EHV-1 mutant with the deletion of gE/gI was found to be safe for horses, but only provided partial protection against EHV-1 challenge (Matsumura *et al.*, 1998). The mutant lacking the thymidine kinase (TK) gene was unable to prevent viremia after EHV-1 challenge (Slater *et al.*, 1993; Tewari *et al.*, 1993). EHV-1 mutants devoid of gB or gM were apathogenic for mice and provided protection against challenge infection, but the safety and protection efficacy are unknown in horses (Neubauer *et al.*, 1997). A recombinant canarypox virus expressing EHV-1 gB, gC, and gD genes significantly reduced virus shedding after challenge, but failed to prevent cell-associated viremia (Minke *et al.*, 2006). Although the IE protein is a CTL target for an equine MHC-I allele expressed by the A3 haplotype (Soboll *et al.*, 2003), the expression of IE alone by using vaccinia virus-based vector was unable to protect against challenge infection (Paillot *et al.*, 2006). The efficacy of DNA vaccines has also been evaluated. A combination of plasmids expressing gB, gC and gD or plasmids expressing the IE protein and the early UL5 gene product were used to immunize ponies and were shown to induce only limited immune responses and protection against EHV-1 challenge (Soboll *et al.*, 2006). All in all, while a lot of novel vaccines have been developed, their protective efficacy is still not satisfactory. Ideal vaccines should be able to induce both high levels of neutralizing antibody and a strong CTL immune response. From this point of view, MLV vaccines, if they are safe, should be the prime targets. Since EHV-1 is known to modulate immune response, the deletion of those genes involved in immune evasion, e.g. gG, gp2 and UL49.5, in addition to the deletion of virulence-associated genes, should increase immunogenicity and might result in robust and long-lived immunity.

1.7 EHV-1 as a live vector

Large DNA viruses as well as some RNA viruses have been developed as delivery vectors for immunization and gene therapy, among which are poxviruses, adenoviruses, retroviruses and several human herpesviruses (Borst & Messerle, 2000; Goins *et al.*, 2004; Gunzburg & Salmons, 1995; Moss, 1996; Paoletti, 1996). RNA viruses as vectors are limited by their restricted capacity to package nucleic acids. Poxviruses and adenoviruses are well-studied and proven to be stable and effective vaccine vectors for the delivery of foreign antigens and induction of robust immune responses. However, poxviruses might induce complications ranging from mild reactions to fatal encephalitis in immunocompromised individuals (Rosas *et al.*, 2006). Efficient transduction of human cells using adenovirus vectors require high titers of viruses, which can be contaminated by helper viruses and cause toxic reactions in patients (Rosas *et al.*, 2006). Another common problem using poxviruses, adenoviruses and human herpesviruses like HSV-1 and HCMV as vectors is the pre-existing anti-vector immunity due to natural infection or vaccination (Rosas *et al.*, 2006).
Recently, the potential of EHV-1 as a delivery vector for immunization has been evaluated (Trapp *et al.*, 2005). EHV-1, like other herpesviruses, has a high packaging capacity due to numbers of non-essential genes that can be replaced by foreign genes. Surprisingly, it was discovered that EHV-1 had a broad host range in cultured cells and was able to enter and express transgenes in both dividing and non-dividing cells of different origins including equine, rodent, rabbit, mouse, baby hamster, avian, bovine, porcine, canine, feline and human cells. Importantly, EHV-1 was not neutralized by human sera containing high titers of antibodies against human herpesviruses, an indicator of an absence of anti-vector immunity (Trapp *et al.*, 2005). Last, but not least, several strains of EHV-1, including the vaccine strain RacH, have been constructed as infectious bacterial artificial chromosomes (BACs) by replacing gp2-encoded *ORF71* with a mini-F sequence (Goodman *et al.*, 2007; Rudolph *et al.*, 2002; Rudolph & Osterrieder, 2002). Together with the lately invented two-step Red-mediated recombination technique (also called *en passant* recombination), the EHV-1 genome can be easily maintained and manipulated in *E. coli* (Tischer *et al.*, 2006).

Using EHV-1 RacH as a live vector, the gene encoding H3 haemagglutinin of equine influenza virus (EIV) and genes encoding immunogenic proteins of several *Flaviviruses*, e.g. the prM and E proteins of West Nile virus (WNV), the four structural proteins (C, E^{rns}, E1 and E2) of bovine viral diarrhea virus (BVDV) and the structural proteins E3-E2-6K-E1 of Venezuelan Equine Encephalitis virus (VEEV) were inserted into the virus genome cloned as a BAC. These recombinant viruses stably expressed the transgenes *in vitro* without apparent growth defects and induced protective immune responses against the corresponding pathogens in mice or the target animals *in vivo* (Rosas et al., 2008a; Rosas et al., 2007a; Rosas et al., 2008b; Rosas et al., 2007b). EHV-1 RacH is safe to be used as a MLV vaccine in

horses, and accordingly, the safety of RacH-based recombinant vaccines in animals other than horses also seems to be acceptable (Rosas *et al.*, 2007a; Rosas *et al.*, 2008b). From these findings, it was concluded that EHV-1 can be used as a novel virus vector. It should be noted that in these studies, the foreign genes were inserted to replace the *egfp* gene in the mini-F sequence. As a result, the mini-F sequence, which contains a chloramphenicol restistence gene, was not removed from the final recombinant viruses and the gp2-encoding ORF71 was not repaired. Future application of EHV-1 as a vector should consider other loci in the genome for insertion of foreign genes and generate markerless recombinant vaccines.

1.8 Outline of the thesis

This thesis consists two major parts. Firstly, we focus on the immune evasion properties of EHV-1 with respect to MHC-I-dependent CTL-mediated immunity. We were successful to identify a protein, namely pUL56 encoded by *ORF1*, as a novel immune evasion protein that plays a dominant role in downregulating cell surface expression of MHC-I. The protein was further characterized and shown to be a phosphorylated early protein, contain a second in-frame start codon, and was localized primarily to Golgi vesicles. pUL56 functions independently of other known immune evasion proteins like pUL49.5 and pUS3. Interestingly, pUL56 by itself cannot downregulate MHC-I, but instead, it needs a yet unknown viral parter(s) to fulfill its functions. Secondly, we utilized the non-essential *ORF1* locus as the insertion site for two foreign genes, VP2 and/or VP5 of bluetongue virus type 8 (BTV-8), and generated an EHV-1 live vector. The EHV-1 recombinant was able to stably express the transgenes *in vitro* and induce BTV-8-specific antibody responses *in vivo*. After lethal challenge with BTV-8, the recombinant simultaneously expressing VP2 and VP5 was shown to provide complete protection of immunized mice. In general, the following main findings are presented in this thesis:

1 Discovery of a novel immune evasion protein in the *Alphaherpesvirinae*;
2 Identification of the protein, pUL56 as a phosphorylated early protein;
3 The inability of pUL56 by itself in downregulating cell surface MHC-I;
4 Identification of the subcellular localization of pUL56;
5 The importance of pUL56 transmembrane domain;
6 Use of the *ORF1* locus as the novel insertion site for generating EHV-1-vectored vaccines;
7 EHV-1 can be used as an alternative live vaccine vector for protection against *Orbivirus* infection.

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CHAPTER 2

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Identification and characterization of equine herpesvirus type 1 (EHV-1) pUL56 and its role in virus-induced downregulation of MHC class I

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2.1 Summary

Major histocompatibility complex class I (MHC-I) molecules play an important role in host immunity to infection by presenting antigenic peptides to cytotoxic T lymphocytes (CTL), which recognize and destroy virus-infected cells. Members of *Herpesviridae* have developed multiple mechanisms to avoid CTL recognition by virtue of downregulation of MHC-I on the cell surface. We report here on an immunomodulatory protein involved in this process, pUL56, which is encoded by *ORF1* of equine herpesvirus type 1 (EHV-1), an alphaherpesvirus. We show that EHV-1 pUL56 is a phosphorylated early protein, which is expressed as different forms and predominantly localizes to Golgi membranes. In addition, the transmembrane (TM) domain of the type II membrane protein was shown to be indispensable for correct subcellular localization and a proper function. pUL56 by itself is not functional with respect to interference with MHC-I and likely needs (an)other unidentified viral protein(s) to perform this action. Surprisingly, pUL49.5, an inhibitor of the transporter associated with antigen processing (TAP) and encoded by EHV-1 and related viruses, appeared not to be required for pUL56-induced early MHC-I downmodulation in infected cells. In conclusion, our data identify a new immunomodulatory protein, pUL56, involved in MHC-I downregulation, which is unable to perform its function outside of the context of viral infection.

2.2 Introduction

Efficient recognition and destruction of infected cells by antigen-specific cytotoxic $CD8^+$ T lymphocytes (CTL) is an important host defense mechanism in the control of viral and certain bacterial infections. The CTL-based defense relies on the recognition of pathogen-derived peptides presented on the surface of the infected cells by major histocompatibility complex class I (MHC-I). The MHC-I antigen presentation pathway originates in the cytosol, where viral proteins are processed into small peptides in the proteasome and translocated into the lumen of the endoplasmatic retriculum (ER) by the transporter associated with antigen processing (TAP) 1 and 2. In the ER, a tri-molecular complex is formed between the newly

synthesized MHC-I heavy chains, the small viral peptides and $ß_2$-microglobulin ($ß_2$M) (Androlewicz *et al.*, 1993; Ortmann *et al.*, 1994). Once the peptides are successfully loaded, the MHC-I complex is released and transported to the cell surface where a CTL response will be triggered. MHC-I molecules that fail to bind peptides are re-routed to the cytosol where they become degraded by the proteasome (York & Rock, 1996).

Viruses, especially members of *Herpesviridae,* have evolved mechanisms that target multiple steps in the MHC-I antigen presentation pathway to evade from immune recognition. A number of viral proteins involved in this process have been identified and studied extensively in the *Beta- and Gammaherpesvirinae* (Griffin *et al.*, 2010). In the case of *Alphaherpesvirinae*, the product of the herpes simplex virus (HSV) *US12* gene (ICP47) and the products of the *UL49.5* orthologues of several members of the varicelloviruses were shown to interfere with the TAP-mediated peptide transport via different mechanisms (Koppers-Lalic *et al.*, 2005; Koppers-Lalic *et al.*, 2008; Verweij *et al.*, 2011). In the case of bovine herpesvirus type 1 (BHV-1), pUL49.5 alone is sufficient to downregulate MHC-I expression during virus infection . In contrast, the *UL49.5* product of human varicella-zoster virus (VZV), while capable of interacting with TAP, is unable to block peptide transport or modulate MHC-I cell surface expression (Koppers-Lalic *et al.*, 2005; Koppers-Lalic *et al.*, 2008). Instead, the VZV *ORF66* protein kinase contributes to MHC-I downregulation by delaying MHC-I maturation during its transport from the endoplasmic reticulum (ER) through the Golgi network to the plasma membrane (Eisfeld *et al.*, 2007). Finally, in the case of a third virus from this group, namely pseudorabies virus (PRV), it has been reported that MHC-I downregulation is not or only partly dependent on *UL49.5* and *US3* gene products, indicating that other, unidentified viral proteins can also be involved in this viral immune evasion process (Eisfeld *et al.*, 2007).

We here focused on another member of the *Varicellovirus* genus, namely equine herpesvirus type 1 (EHV-1). Transmitted by aerosol, EHV-1 is a highly contagious pathogen of horses causing upper respiratory disease, late stage abortion in mares and neurological disorders (Deruelle *et al.*, 2009). Despite regular vaccinations, EHV-1 remains a constant threat to horses worldwide, mainly because the immune responses induced after both infection and vaccination are not fully protective (Allen & Bryans, 1986; Burki *et al.*, 1990). EHV-1 infection is controlled mainly by the action of CTLs that attack virus-infected cells. The frequency of precursor CTLs specific for EHV-1 antigens is correlated with protection against disease (Mumford *et al.*, 1987). However, EHV-1 has also developed mechanisms to avoid CTL recognition by virtue of a massive downregulation of cell surface MHC-I molecules (Ambagala *et al.*, 2004; Kydd *et al.*, 2003). The UL49.5 product is the only EHV-1 protein identified to modulate the MHC-I-restricted antigen-presenting pathway in E.derm

UL49.5^{EHV-1} cells. The cells stably express EHV-1 pUL49.5, which was shown to block ATP-binding to TAP and inhibit transport of proteasome-generated peptides into the ER. This blockade resulted in a downregulation of MHC-I on the cell surface of transfected cells (Rappocciolo *et al.*, 2003). Recently, we observed that infection with EHV-1 strain RacL11 resulted only in mild downregulation of MHC-I although it encodes a fully functional pUL49.5 protein (Koppers-Lalic *et al.*, 2008). These results were in stark contrast to another EHV-1 strain, namely Ab4, which was reported to induce a massive downregulation of MHC-I in infected equine dermal cells (NBL6) (Rudolph *et al.*, 2002b). This strain-dependent pattern led us to hypothesize that one or more unknown viral proteins participate in downregulation of MHC-I mediated by EHV-1.

In the present study, we demonstrated that the *ORF1* gene encodes a novel immune evasion protein that plays a major role in MHC-I downregulation. This *ORF1*-encoded protein, pUL56, is a phosphorylated type II transmembrane protein expressed with early kinetics and localized predominantly in Golgi membranes. The transmembrane (TM) domain of EHV-1 pUL56 was shown to be indispensable for correct subcellular localization of pUL56 and function.

2.3 Materials and methods

2.3.1 Cells and viruses

Rabbit kidney (RK13) cells, human HeLa cells and human embryonic kidney 293 (HEKT) cells were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom AG), 100 U/ml penicillin and 0.1 mg/ml streptomycin (1% Pen/Strep, Sigma). The equine skin fibroblast cell line NBL6 (ATCC) was maintained in EMEM supplemented with 20% FCS, 29 mg/ml L-glutamine, 1 % nonessential amino acids (Biochrom AG) and 1% Pen/Strep. EHV-1 strains Ab4 and RacL11 were constructed previously as bacterial artificial chromosomes (BAC) by insertion of mini-F sequences containing and an *egfp* marker gene in lieu of gene 71, which encodes glycoprotein gp2 (Goodman *et al.*, 2007; Rappocciolo *et al.*, 2003). BAC-reconstituted parental viruses Ab4G, RacL11G and all mutant viruses were generated and propagated in RK13 cells.

2.3.2 Antibodies

Monoclonal mouse anti-MHC-I antibody (mAb) H58A (isotype IgG2a) and anti-CD44 mAb BAT31A (isotype IgG1) were purchased from VMRD and used at a 1/100 dilution. Rabbit anti-EHV-1 UL49.5 polyclonal (p)Abs, mouse anti-EHV-1 gM mAbs F6, mouse anti-EHV-1 gp2 and biotinylated equine polyclonal anti-EHV-1 antibodies have been previously

described (Rudolph *et al.*, 2002a; van der Meulen *et al.*, 2003; von Einem *et al.*, 2004). Rabbit anti-EHV-1 pUL56 pAbs (1/500) were designed and produced by GenScript Corporation (NJ, USA). The β-actin (13E5) rabbit mAbs (1/2500) were from Cell Signaling Technologies. Alexa Fluor 647 conjugated goat anti-mouse IgG (1/500), Alexa Fluor 488 conjugated goat anti-rabbit IgG (1/500), Texas Red conjugated goat anti-rabbit (1/100) and streptavidin-PE (1/200) were from Invitrogen and HRP-labeled goat anti-rabbit and goat anti-mouse IgG (1/10000) from SouthernBiotech. Goat anti-mouse Cy5 was obtained from Zymed.

2.3.3 Viral mutagenesis

Two-step Red-mediated (*en passant*) recombination was used to create mutant and revertant viruses (Rudolph *et al.*, 2002b). Detailed lists of the primers used for generating the mutant viruses are given in Table 2.1. The genotypes of all virus mutants and revertants were confirmed by restriction enzyme analyses (RFLP) and nucleotide sequencing. In addition, the expression of gp2 was restored in the Ab4 parental virus (vAb4), Ab4GΔ1 (vAb4Δ1), Ab4GΔ1_TM (vAb4Δ1_TM) mutants and the parental RacL11 virus (vRacL11) by co-transfection of RK13 cells with BAC DNA and plasmid DNA containing full-length gene 71, followed by plaque purification (Tischer *et al.*, 2006). Restoration of gp2 was confirmed by western blot analysis of virus-infected cells using the anti-EHV1 gp2 mAb 8B6, exactly as previously described (Rudolph & Osterrieder, 2002; Van de Walle *et al.*, 2009).

2.3.4 Construction of the pNTAP-UL56 plasmid and transfection

To express pUL56, vector pCeMM-NTAP (GS) containing the *egfp* gene under control of the HCMV IE promoter was used (von Einem *et al.*, 2004). Briefly, the *ORF1* gene was PCR-amplified from Ab4 DNA using primers NTte (5'-aaaaaagtcgacaatgagacccgagggagtttcgcggggcc-3') and NTf (5'-aaaaaagtcgacttatttctccttcttgccgtttgtta ac-3') (underlined sequence indicates the *Sal*I restriction site), and cloned into the *Sal*I-compatible *Xho*I restriction site of pCeMM-NTAP (GS). The resulting pNTAP-UL56 construct clones were confirmed by RFLP, using *Hind*III and *Nco*I, and sequencing, using NTAP-ORF_F (5'-ccggtgagctggagcagcta-3') and NTAP-ORF1_F (5'-cttcggccagtaacgttagg-3') (data not shown). HEKT or HeLa cells were transfected using the Fugene HD reagent, according to the protocol recommended by the supplier (Promega).

2.3.5 Flow cytometry

NBL6 cells were mock-infected or infected with the various viruses at a multiplicity of infection (m.o.i.) of 5. At different time points (0, 2, 4, 6, 8, 12, 16h) post infection (hpi), (mock)-infected cells were trypsinized, resuspended in phosphate-buffered saline (PBS)

containing 2.5% fetal bovine serum (FBS) and 0.02% sodium azide (FCM buffer). Cells were incubated for 40 min on ice with anti-MHC-I or anti-CD44 mAbs in FCM at a 1/100 dilution. After three times of washing, the cells were incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (1/500) and analyzed immediately using a FACSCalibur flow cytometer (Becton Dickinson). For each sample, at least 10,000 cells were evaluated and all data were corrected for unspecific binding using isotype controls. Only EGFP-positive cells were analyzed in the case of virus-infected cells. The expression levels of surface MHC-I were presented as mean fluorescence intensities (MFI).

For the combined transfection-infection experiments, HeLa or HEKT cells were first transfected with the pNTAP-UL56 or empty NTAP vector using Fugene HD and infected with vAb4 or vAb4Δ1 at an m.o.i of 10. Twenty-four hpi, cells were collected, washed and stained for MHC-I expression as described above. To identify the infected cells, a biotinylated anti-EHV1 antibody was used, followed by streptavidin-PE. Only double-positive cells, with EGFP expression as a marker for successful transfection and PE as a marker for infection, were analyzed for cell surface MHC-I expression, which was presented as MFI.

Table 2.1 Primers used for generating the different deletion mutant viruses

Purpose	Description[a]	Sequence (5'-3')[b]
Deletion of *ORF1*	ORF1_F	CGAGGGAGTTTCGCGGGGCCGCGCCTCCTCTGTCTCCATCAAGGAGAAATAAAACGACTG**TAGGGATAACAGGGTAATCG**
	ORF1_R	ATTAACCTTTGCGGTACTACAGTCGTTTTATTTCTCCTTGATGGAGACAGAGGAGGCGC**GCCAGTGTTACAACCAATTAACC**
Deletion of *ORF2*	ORF2_F	GTACCGCAAAGGTTAATCGCATTTATTTTTACATGCACTCATTGTGGTAGGGTTTGAGAC**TAGGGATAACAGGGTAATCG**
	ORF2_R	CGTGGAGGAGGCGCATACACGTCTCAAACCCTACCACAATGAGTGCATGTAAAAATAAAT**GCCAGTGTTACAACCAATTAACC**
Deletion of *ORF1*&*ORF2*	ORF1&2_F	CGAGGGAGTTTCGCGGGGCCGCGCCTCCTCTGTCTCCATCGGTAGGGTTTGAGACGTGTA**TAGGGATAACAGGGTAATCG**
	ORF1&2_R	TCCAGCGTGGAGGAGGCGCATACACGTCTCAAACCCTACCGATGGAGACAGAGGAGGCGC**GCCAGTGTTACAACCAATTAACC**
Deletion of the start codon of *UL49.5*	UL49.5_F	TGCTCGCGCTCTCCATCTCTCACCACACTCAACCACCACGCTGTCCACGAGATTCGTGAC**TAGGGATAACAGGGTAATCGATTT**
	UL49.5_R	GGCAGGCGAGAATGGCCAGCGTCACGAATCTCGTGGACAG CGTGGTGGTTGAGTGTGGTG**CCAGTGTTACAACCAATTAACC**
Deletion of the first ATG of *ORF1*	ORF1^{1stATG} F	TCCACCTGCACCTTTTCCATCTCCTCTCCAACTCGCCGCCAGACCCGAGGGAGTTTCGCG**TAGGGATAACAGGGTAATCGATT**
	ORF1^{1stATG} R	AGACAGAGGAGGCGCGGCCCCGCGAAACTCCCTCGGGTCTGGCGGCGAGTTGGAGAGGAG**CCAGTGTTACAACCAATTAACC**
Deletion of the second ATG of *ORF1*	ORF1^{2ndATG} F	GGGAGTTTCGCGGGGCCGCGCCTCCTCTGTCTCCATCTCCTGCCCACCGCCGCCCAATGG**TAGGGATAACAGGGTAATCGATT**
	ORF1^{2ndATG} R	CCAGCGATGCGCGGCGCGCCCCATTGGGCGGCGGTGGGCAGGAGATGGAGACAGAGGAGG**CCAGTGTTACAACCAATTAACC**
Deletion of the major part (841bp) of *US3*	US3_F	AACCGACTGGTTTTCCACTACGAGCGACGCGTCAGAATCGTAATTTCAACGCTCAAGATA**GGATGACGACGATAAGTAGGGATAAC**
	US3_R	TCGAGGAAACTCCTCCGGATTTATCTTGAGCGTTGAAATTACGATTCTGACGCGTCGCTCG**GGGTAA TGCCAGTGTTACAACCA**
Deletion of the predicted catalytic domain of *UL13* (aa 221-380)	UL13_F	TACCTTTACAGTGAACCCAGAGATGCACTACAGGCGCGTGTCTATGGCCATGCGGGCGGA**GGATGACGACGATAAGTAGGGATAAC**
	UL13_R	CGCCGGTATCAATTTCAAACTCCGCCCGCATGGCCATAGACACGCGCCTGTAGTGCATCT**GGGTAATGCCAGTGTTACAACCA**
Deletion of the transmembrane region (aa 170-192) of *ORF1*	ORF1TM_F	CTCCGATCCCCACCAGACCTCTCGAAGGAGACAGAACCCGAGTTCGTTAACAAACGGCAA**TAGGGATAACAGGGTAATCG**
	ORF1TM_R	AGTCGTTTTATTTCTCCTTCTTGCCGTTTGTTAACGAACTCGGGTTCTGTCTCCTTCGAGG**CCAGTGTTACAACCAATTA**

[a] directionality of the primer: F: forward, R: reverse;

[b] bold sequence indicates the template binding region of the primers for PCR amplification with pEPkanS; aa: amino acid, bp: base pairs

2.3.6 Western blot analyses

NBL6 cells were infected with parental or mutant viruses at an m.o.i of 5, in the presence or absence of the viral DNA synthesis inhibitor phosphonoacetic acid (PAA, 300 μg/ml, Alfa Aesar). The virus was allowed to attach for 1h at 4°C, followed by a penetration step for 1h at 37°C. The cells were then treated with citrate buffered saline (CBS, pH 3.0) for 3 min to remove remaining virus on the cell surface and washed with PBS. At different time points after infection, cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1%SDS) with protease inhibitor cocktail (Roche) and benzonase (Novagen). The proteins were separated by SDS-PAGE (10%-15%) and transferred to polyvinylidene fluoride (PVDF) membranes. To exclude non-specific binding, membranes were blocked with PBS containing 0.05% Tween 20 (PBST) supplemented with 5% non-fat dry milk overnight at 4°C. After blocking, membranes were incubated with anti-pUL56 (1/500), anti-pUL49.5 (1/500), anti-gM (1/500) or anti-actin (1/2500) antibodies for 1h at RT. After 3 washing steps with PBST, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/10000) or goat anti-mouse IgG (1/10000) for 1h at RT. Membranes were washed again three times with PBST and the reactive bands were visualized using ECL Plus Western blotting detection system (Amersham Pharmacia). To examine pUL56 phosphorylation, cell lysates were treated with lambda protein phosphatase (λ-PPase, New England Biolabs) for 30 min at 30°C before electrophoresis.

2.3.7 Immunofluorescence

To control for proper pUL56 expression in transfected HEKT cells, cells were incubated with anti-ORF1 pAbs (1/200) in blocking buffer for 1h at RT and then, after 3 washing steps, incubated with goat anti-rabbit Texas Red (1/100) for 1 h at RT. Cells were analyzed with a confocal laser scanning microscope (Leica TCS SP2 laser scanning spectral confocal system, Leica Microsystems GmbH, Wetzlar) and the Leica confocal software.

To evaluate intracellular expression of pUL56 in infected cells, NBL6 cells were grown on coverslips coated with 0.5mg/ml Collagen A (Biochrom AG) in ddH2O (pH 3.5) and infected with parental vAb4, vAb4Δ1 or vAb4Δ1_TM mutant viruses at an m.o.i. of 0.1 for 20 h. To stain the Golgi apparatus, cells were incubated with 5μM BODIPY®TR C_5-ceramide complexed to BSA (Invitrogen) in HBSS/Hepes (Hank's Balanced Salt solution with 10mM Hepes, pH 7.4) for 30 min at 4°C, washed once with medium and incubated in fresh medium for 1h. To stain the ER, cells were incubated with 1mM ER-Tracker™ Red (BODIPY®TR glibenclamide, Invitrogen) in HBSS/Hepes for 30 min at 37°C and incubated in fresh medium for 1h. After 1 h of incubation, cells were fixed with 3.5% PFA for 5 min at RT, followed by

permeabilization with 0.1% Tween 20 for 15 min. After blocking with 10% normal goat serum for 1 h, cells were incubated with anti-pUL56 pAbs (1/200) for 1 h at RT. After 3 washing steps, cells were incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG (1/5000) for 1 h at RT. Finally, after 3 washing steps, Vectashield® mounting medium with DAPI (Vector Laboratories) was added to cells and the coverslips were inspected under a Zeiss Axio Imager M1 microscope (Zeiss, Germany).

2.4 Results

2.4.1 The ability of EHV-1 to downregulate MHC-I is virus strain-dependent

It was reported earlier that the EHV-1 strain Ab4 is able to induce robust downregulation of MHC-I molecules on the surface of infected NBL6 cells (Bürckstümmer *et al.*, 2006), a phenomenon confirmed here (Fig. 2.1A). Expression of the cell surface protein CD44, used as a control, was unaffected during infection (Fig. 2.1A). In contrast to Ab4, the EHV-1 strain RacL11 resulted in only moderate downregulation of MHC-I surface expression (Fig. 2.1A&B). This inability could not be attributed to differences in expression of the *UL49.5*

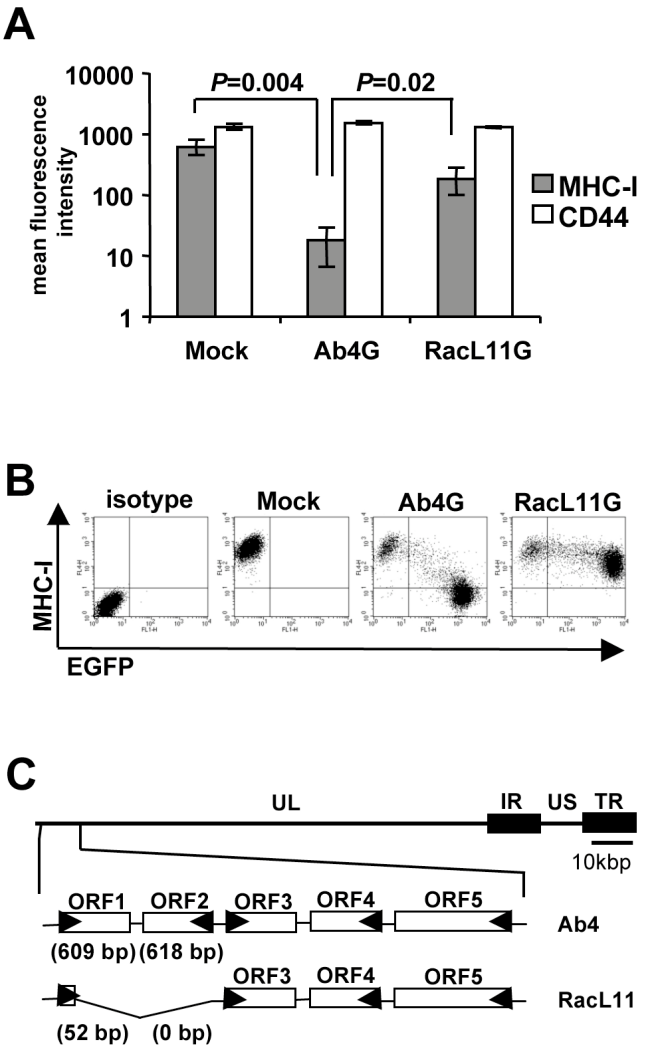

Figure 2.1 The ability of EHV-1 to downregulate MHC-I is virus strain-dependent. (A) NBL6 cells were infected with EHV-1 Ab4G or RacL11G. Cell surface MHC-I expression was analyzed at 16hpi using flow cytometry. Primary anti-MHC-I mAb H58A and anti-CD44 mAb were used, followed by goat anti-mouse Alexa fluor 647. **(B)** Dot plots from a representative experiment depicting MHC-I surface expression in mock- and virus-infected NBL6 cells. Quadrant gates were established using isotype controls and mock-infected cells respectively. **(C)** Genomic organization of EHV-1 strains Ab4 and RacL11. A detailed organization of the leftmost open reading frames (ORFs) of the unique long (UL) region of the two virus strains is shown. US: unique short; IR: internal repeat: TR: terminal sequence analyses repeat.

gene product, since RacL11 encodes a functional pUL49.5 (Rappocciolo *et al.*, 2003) and failed to reveal differences between the *UL49.5* genes of Ab4 and RacL11 (data not shown). Hence, these results indicated that (an)other, yet unidentified, protein(s) account for the difference in MHC-I downregulation capabilities of EHV-1 strains. Compared to Ab4, RacL11 specifies a shortened unique-long segment (U_L) of its genome and the deletion was mapped to the extreme left genomic terminus (Koppers-Lalic *et al.*, 2008). When comparing the sequence of this region of RacL11 to that of Ab4 (GenBank accession # NC_001491.2), we discovered a deletion of 1283bp, resulting in the absence of the majority of *ORF1* and the complete *ORF2* (Fig. 2.1C). EHV-1 *ORF1* is predicted to encode a protein of 202 amino acids (aa) in length, which shares homology with pUL56 orthologues of related alphaherpesviruses (Hubert *et al.*, 1996; Tai *et al.*, 2010; Telford *et al.*, 1998). EHV-1 *ORF2* is predicted to encode a 205 aa protein, has a positional counterpart in VZV, and likely arose from the same ancestor as *ORF1* (Baumeister *et al.*, 1995). We hypothesized that the two ORFs are plausible candidates to be involved in MHC-I downregulation.

2.4.2 pUL56-mediated MHC-I downregulation is dependent on virus infection but independent of pUL49.5

To determine whether the proteins encoded by *ORF1* and/or *ORF2* play a role in MHC-I downregulation, deletion mutants were generated based on the Ab4 strain and cell surface expression levels of MHC-I evaluated. To this end, the majority of ORF1, the complete ORF2 or a combination of both were deleted, resulting in the recombinant viruses Ab4GΔ1, Ab4GΔ2 and Ab4GΔ1_2, respectively (Fig. 2.2A). Infecting NBL6 cells with these mutant viruses showed that MHC-I levels on the surface of cells infected with Ab4GΔ1_2 or Ab4GΔ1 were restored (Fig. 2.2B). In contrast, MHC-I levels remained low after infection with Ab4GΔ2 (Fig. 2.2B), indicating that the *ORF1* but not the *ORF2* gene product is involved in MHC-I downregulation. Parental Ab4G and the revertant viruses Ab4GΔ1R and Ab4GΔ1_2R were included as positive controls, and infection of NBL6 cells resulted in a significant decrease of MHC-I expression (Fig. 2.2B).

In order to analyze whether pUL56 encoded by *ORF1* is able to downregulate MHC class I by itself, we constructed a pUL56-expressing plasmid pNTAP-UL56. Upon transfection of HEKT cells with the plasmid, transfection efficiencies of 55.4 ± 3.7% were achieved and co-expression of pUL56 and EGFP was confirmed by fluorescence microscopy (Fig. 2.2C). Transfection with the empty vector pNTAP was included as a control, and EGFP positive cells did not show any pUL56 expression (Fig. 2.2C). Next, the MHC-I levels in HeLa or HEKT cells were analyzed after transfection with pNTAP-UL56 or the empty pNTAP vector. Surprisingly, no MHC-I downregulation was observed in pUL56-transfected cells (Fig. 2.2D), despite the fact that infection of the cells with Ab4G, with infection efficiencies of 69.9 ±

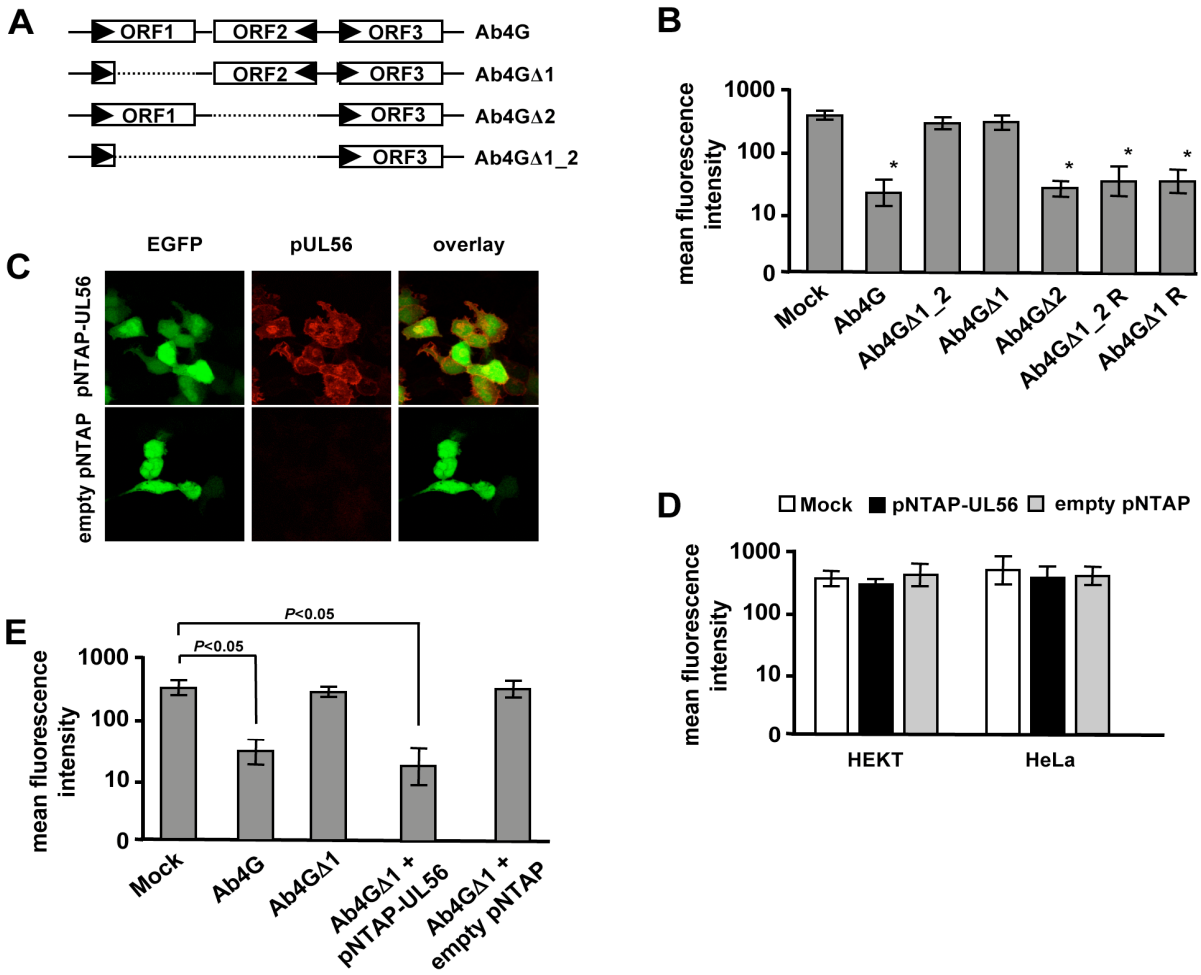

Figure 2.2 pUL56-mediated MHC-I downregulation is dependent on virus infection. (A) Shown is a schematic representation of the structure of *ORF1* and *ORF2* in wild type Ab4 as well as in the deletion mutant viruses. **(B)** NBL6 cells were infected with EHV-1 Ab4G, Ab4GΔ1_2, Ab4GΔ1, Ab4GΔ2 or the revertant viruses Ab4GΔ1_2 R and Ab4GΔ1 R. Cell surface MHC-I expression was analyzed at 16hpi using flow cytometry. Primary anti-MHC-I mAb H58A was used, followed by goat anti-mouse Alexa fluor 647. **P*<0.05. **(C)** HEKT cells were transfected with pNTAP-UL56 or the empty pNTAP for 48h. Immunofluorescence stainings of pUL56 expression in transfected cells (EGFP-positive) were performed using the rabbit anti-EHV-1 pUL56 pAbs, followed by goat anti-rabbit-Texas Red. **(D)** HEKT and HeLa cells were transfected with pNTAP-UL56 or the empty pNTAP. Cell surface MHC-I expression was analyzed at 48hpi using flow cytometry. Primary anti-MHC-I mAb H58A was used, followed by goat anti-mouse Cy5. **(E)** HeLa cells were transfected with pNTAP-UL56 or the empty pNTAP and 24h later, infected with Ab4GΔ1. Cell surface MHC-I expression was analyzed 24h later using flow cytometry. Primary anti-MHC-I mAb H58A was used, followed by goat anti-mouse Cy5. To identify infected cells, biotinylated anti-EHV-1 Ab were used, followed by streptavidin-PE. Mock- and Ab4G-infected cells were included as controls.

5.5%, resulted in massive downregulation of MHC-I (data not shown). This led us to speculate that pUL56 can only function properly in the presence of virus infection. To verify this assumption, HeLa and HEKT cells were transfected with pNTAP-UL56 followed by infection with vAb4GΔ1. The results showed that the combination of transfection and infection led to a significant downregulation of MHC-I in HeLa (*P*=0.001, Fig. 2.2E) as well as HEKT cells (*P*=0.01, data not shown). In contrast, infection of the same cells with vAb4GΔ1, with infection efficiencies of 80.6 ± 1.5 %, alone or after transfection of empty pNTAP vector only slightly downregulated MHC-I in both cell lines (Fig. 2.2E). These data suggest that pUL56 needs one or more viral partners or cellular partners induced by virus infection to efficiently downregulate cell surface MHC-I.

It was shown previously that EHV-1 pUL49.5 can modulate MHC-I cell surface expression in pUL49.5-expressing equine E.derm cell lines (Telford *et al.*, 1998). We therefore examined whether the observed pUL56-induced MHC-I downregulation was perhaps dependent on expression of pUL49.5. Recombinant Ab4 viruses were generated where the *UL49.5* gene was inactivated by deletion of the start codon, alone or in combination with deletion of *ORF1*, and the resulting viruses were named Ab4GΔ49.5 and Ab4GΔ1_49.5, respectively. Western blot analyses confirmed that expression of pUL49.5 and/or pUL56 was abrogated in the corresponding mutant viruses (Fig. 2.3A). Analyses of surface MHC-I expression of NBL6 cells at 16 hpi revealed that the absence of pUL49.5 had no effect on the virus' ability to

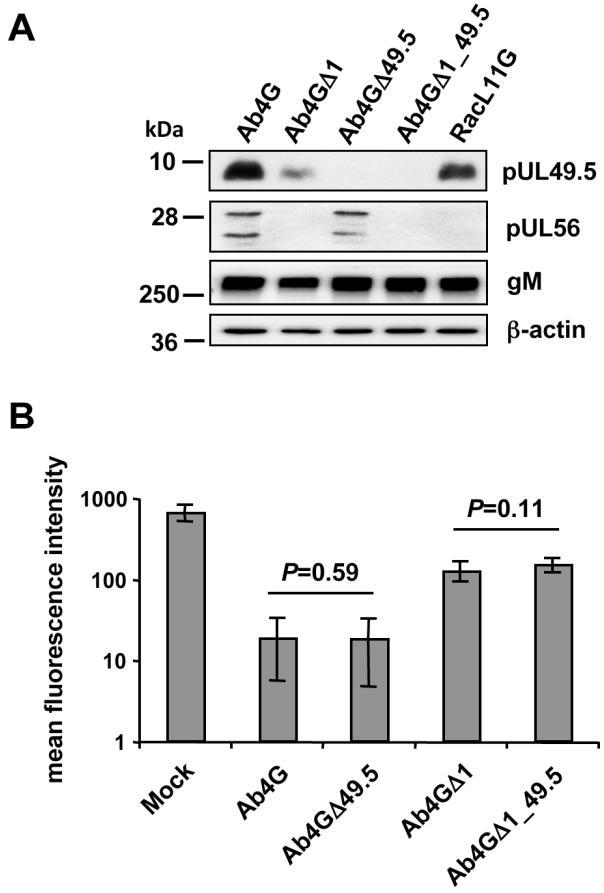

Figure 2.3 pUL56-mediated downregulation of MHC-I is pUL49.5-independent. (A) Cells were mock-infected or infected with parental Ab4G or the mutant viruses Ab4GΔ1, Ab4GΔ49.5 and Ab4GΔ1_49.5, and cell lysates were prepared at 16hpi. Western blotting was performed to detect pUL49.5 using anti-EHV-1 UL49.5 polyclonal (p)Abs, pUL56 using anti-EHV-1 UL56 pAbs or gM using the mAb F6. The mAb 13E5 was used to detect β-actin, which was included as a loading control. **(B)** NBL6 cells were mock-infected or infected with Ab4G, Ab4GΔ1, Ab4GΔ49.5 or Ab4GΔ1_49.5. At 16hpi, cell surface expression of MHC-I was analyzed using flow cytometry.

downregulate MHC-I (Fig. 2.3B). Based on these results, we concluded that pUL56-mediated downregulation of MHC-I during infection is independent of pUL49.5.

2.4.3 pUL56 is a phosphorylated early protein

It was previously reported that downregulation of MHC-I by EHV-1 was mediated by an early gene product (Koppers-Lalic *et al.*, 2008). To examine the kinetics of MHC-I downregulation as well as the expression profile of pUL56, we performed time course studies. NBL6 cells were infected with Ab4G at an m.o.i of 5, and at different time points after infection, surface MHC-I and pUL56 expression were determined. The results showed that MHC-I downregulation started as early as 2 hpi, was complete by 8 hpi and maintained throughout the replication cycle (Fig. 2.4A). Expression of the surface marker CD44 was not altered at all time points tested. In parallel, we examined the kinetic correlation between MHC-I downregulation and pUL56 expression. At 2 hpi, one single pUL56-specific band of approximately 22kDa in size was detected, a finding compatible with the expected molecular mass of pUL56 (Fig. 2.4B). From 4 hpi onwards, a second band appeared with an apparent molecular mass of about 28kDa and both moieties reached maximum levels at 8 hpi (Fig. 2.4B). Besides these two prominent bands, two fainter bands, with apparent molecular masses

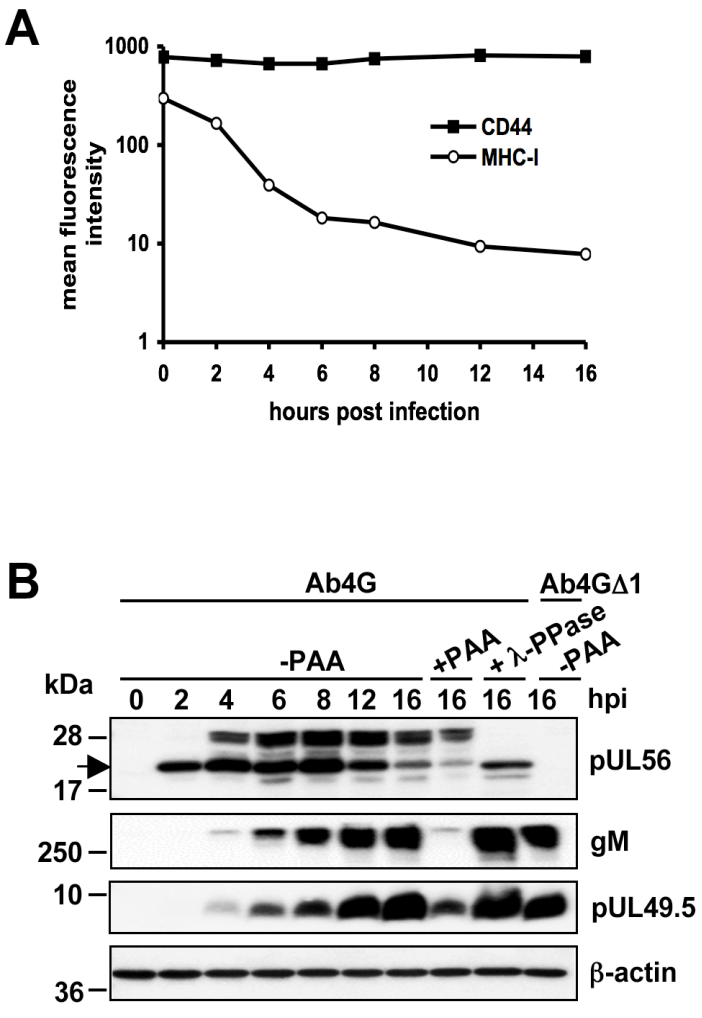

Figure 2.4 pUL56 is a phosphorylated early protein, expressed as different isoforms. (A) Untreated or PAA-treated NBL6 cells were infected with Ab4G parental virus and at the indicated time points, MHC-I expression on the cell surface was analyzed with anti-MHC-I mAb H58A. CD44 expression was included as a control. **(B)** NBL6 cells, treated or not treated with PAA, were infected with Ab4G or Ab4GΔ1 and at the indicated time points, cell lysates were prepared. Western blot analyses were performed to detect pUL49.5 using anti-EHV-1 UL49.5 polyclonal (p)Abs, pUL56 using anti-EHV-1 pUL56 pAbs or gM using the mAb F6. The mAb 13E5 was used to detect β-actin, which was included as a loading control.

of 20kDa and 25kDa, were also detected (Fig. 2.4B). Based on the fact that no specific signal was detected in Ab4GΔ1-infected cells, we concluded that all bands are products of the *ORF1* gene and that pUL56 is expressed as different forms. In the presence of phosphonoacetic acid (PAA), an inhibitor of viral DNA synthesis,expression of pUL56 was only slightly reduced at 16 hpi, indicating that pUL56 expression is independent of DNA synthesis and encoded by a gene with (immediate) early kinetics. As expected, expression of the late viral protein gM, which was used as a control, was significantly inhibited by PAA (Fig. 2.4B). When infected cell lysates were treated with the phosphatase λ-PPase, all higher molecular mass pUL56 moieties disappeared, whereas the 22kDa as well as the smaller proteins remained unaffected, suggesting that the observed 25kDa and 28kDa proteins represent phosphorylated forms of pUL56 (Fig. 2.4B). In contrast, pUL49.5 only became expressed from 4 hpi onwards (Fig. 2.4B), which is clearly later than the onset of MHC-I downregulation (Fig. 2.4A). Moreover, PAA treatment partially inhibited pUL49.5 expression, indicating that EHV-1 pUL49.5 is expressed with early-late kinetics. These results were an additional indication that pUL56, but not pUL49.5, is involved in the early downregulation of MHC-I during EHV-1 infection.

2.4.4 pUL56 contains an additional, functional in-frame start codon

It was reported that the *UL56* gene product of HSV-2 is detectable as several species during infection due to phosphorylation and utilization of an additional in-frame start codon upstream of the canonical AUG (Rappocciolo *et al.*, 2003). Similar to HSV-2, we showed that EHV-1 pUL56 is also phosphorylated and expressed as several protein species that makes use of an additional in-frame AUG start codon in the *ORF1* gene. This additional in-frame AUG is located 17 codons downstream of the predicted start codon (Fig. 2.5A) and initiation at this second AUG would theoretically result in a 20 kDa protein as observed during the course of infection (Fig. 2.4B). In order to determine whether this pUL56 form is produced by translation initiated from the second AUG, the first or second in-frame start codons was deleted in the Ab4 virus. pUL56 production, as well as cell surface MHC-I expression were analyzed after infection of NBL6 cells. The pUL56 expression profile (Fig. 2.5B, lanes 5 & 6) was indistinguishable from that of parental virus (Fig. 2.5B, lanes 1 & 2) when the second in-frame start codon was deleted, indicating that all its forms are a result of translation initiation from the first AUG. Still, one single unphosphorylated protein, with a molecular mass between the 20kDa and 22kDa species, was detected upon deletion of the first AUG (Fig. 2.5B, lanes 3 & 4), indicating that the second in-frame AUG can also be used as an alternative initiation site. The expression levels of this unphosphorylated truncated protein, however, were considerably lower when compared to the pUL56 species that result from initiation at the first AUG (Fig. 2.5B). Regardless, this pUL56 moiety was still able to induce significant downregulation of MHC-I (P=0.009), albeit less effective than the downregulation induced by

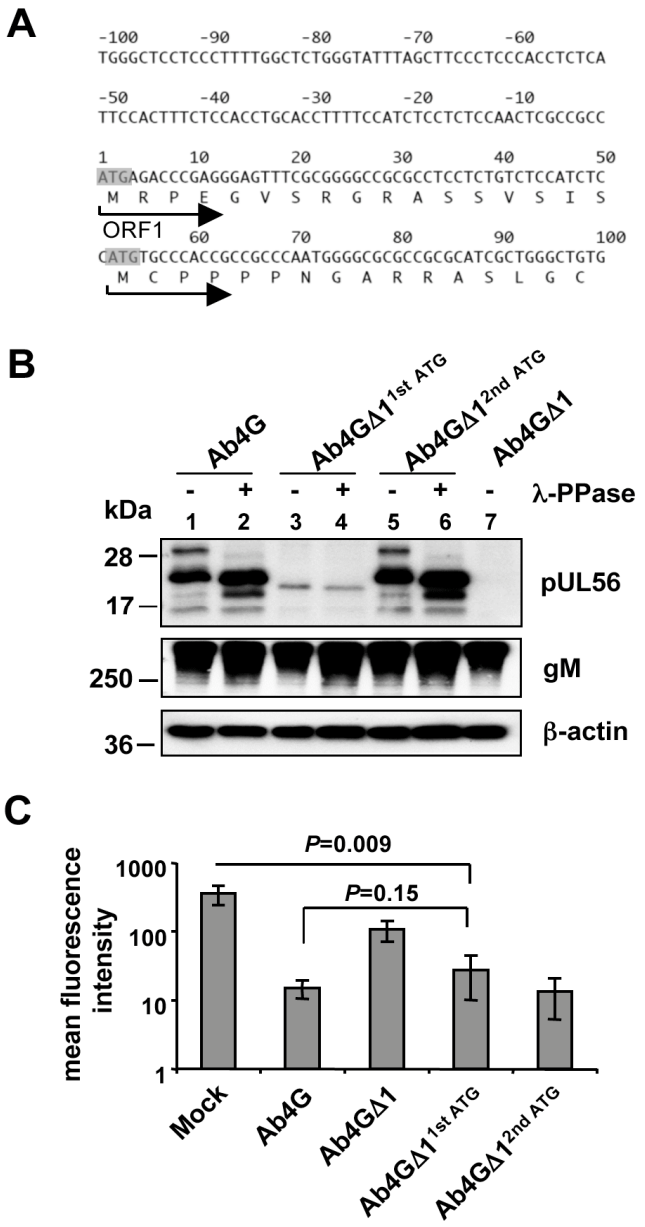

Figure 2.5 pUL56 contains an additional, functional in-frame start codon. (A) The upstream and N-terminal DNA sequence of *ORF1* is shown. The start codon of the complete *ORF1* and the second in-frame start codon are indicated in shadow. The proposed encoded amino acid sequences are shown as single-letter codes. The arrows indicate the initiation direction from the first or second start codon. **(B)** NBL6 cells were infected with Ab4G parental virus, Ab4GΔ1^{1stATG}, Ab4GΔ1^{2ndATG} or Ab4GΔ1. At 16 hpi, cell lysates were treated or not treated with λ-PPase. Western blot analyses were performed to detect pUL56 using anti-EHV-1 UL56 pAbs or gM using the mAb F6. The mAb 13E5 was used to detect β-actin, which was included as a loading control. **(C)** NBL6 cells were either mock-infected or infected with Ab4G, Ab4GΔ1^{1stATG}, Ab4GΔ1^{2ndATG} or Ab4GΔ1 and at 16hpi, cell surface expression of MHC-I was analyzed using flow cytometry.

parental Ab4G virus (Fig. 2.5C). Based on the results, we concluded that pUL56 can be translated from the second in-frame AUG, which results in a truncated but still functional protein, and that phosphorylation as well as the N-terminal 17 aa are not required for pUL56 to downregulate MHC-I. Whether production of truncated pUL56 also occurs *in vivo* remains to be determined.

2.4.5 pUL56 is not phosphorylated by viral US3 or UL13 kinases

Alphaherpesviruses have two viral serine/threonine kinases, which are encoded by the *US3* and *UL13* genes, respectively (de Wind *et al.*, 1992; Koshizuka *et al.*, 2002). Given that the *US3*-encoded kinases of PRV and VZV have been reported to contribute to MHC-I

downregulation in certain cell types (Deruelle *et al.*, 2009; Purves *et al.*, 1987) and EHV-1 pUL56 is a phosphoprotein, we asked if one of these two viral kinases phosphorylated pUL56 and if their expression may affect cell surface MHC-I levels. We therefore generated two kinase mutants, Ab4GΔS3 and Ab4GΔ13, by replacing the majority of the *US3* gene (841bp) or sequences encoding the predicted catalytic domain of UL13 (aa 221-380) with the kanamycin-resistance gene (*aphAI*) (Fig. 2.6A). Infection of NBL6 cells with the generated mutants demonstrated that pUL56 was still phosphorylated (Fig. 2.6B) and that MHC-I downregulation was not affected by the deletion of *US3* or *UL13* (Fig. 2.6C). Taken together, the data indicated that pUL56 is not phosphorylated by either of the two viral kinases and that they do not have detectable contributions to the EHV-1-induced MHC-I downregulation in NBL6 cells.

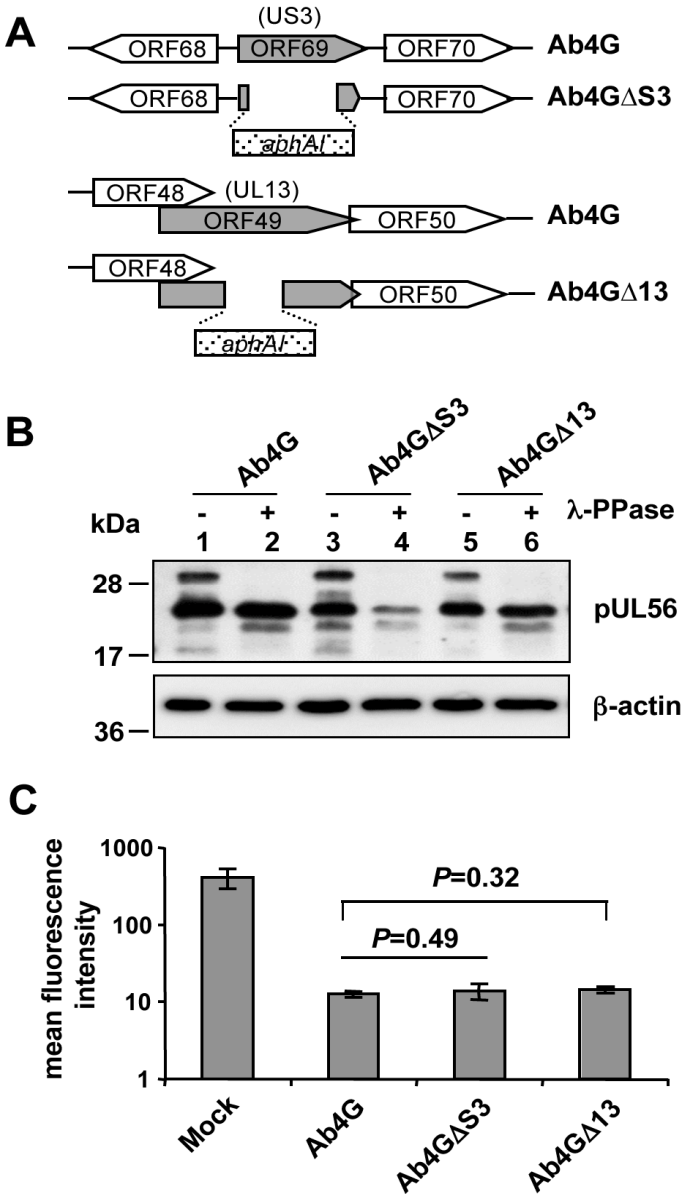

Figure 2.6 pUL56 is not phosphorylated by the viral protein kinases pUS3 or pUL13. (A) Shown is a schematic representation of the structure of *US3* and *UL13* in wild type as well as in the deletion mutant viruses. The kanamycin-resistence gene is indicated by *aphAI* (dotted box). (B) NBL6 cells were infected with Ab4G, Ab4GΔS3 or Ab4GΔ13. At 16 hpi, cell lysates were treated or not treated with λ-PPase. Western blotting was performed to detect pUL56 using anti-EHV-1 UL56 pAbs. The mAb 13E5 was used to detect β-actin, which was included as a loading control. **(C)** NBL6 cells were either mock-infected or infected with Ab4G, Ab4GΔS3 or Ab4GΔ13 and at 16hpi, cell surface expression of MHC-I was analyzed using flow cytometry.

2.4.6 pUL56 is predominantly localized in the Golgi complex and its transmembrane domain is essential for subcellular localization and function

The HSV-2 pUL56 homologue has been previously identified as a type II transmembrane protein (Eisfeld *et al.*, 2007). Using the TMHMM server v. 2.0. program (http://www.cbs.dtu.dk/services/TMHMM), EHV-1 pUL56 was also predicted to represent a type II transmembrane protein (Fig. 2.7A). We therefore investigated the intracellular localization of the protein and the importance of the TM domain. To this end, an *ORF1* mutant, Ab4GΔ1_TM, was created that lacked the aa sequence 170-192, which was mapped to the TM domain (Fig. 2.7A). Moreover, parental and mutant viruses were reconstituted such that the *gp2* gene was repaired were infected with vAb4 and fluorescent labels were used to visualize the Golgi complex or the ER. We found that pUL56 accumulated in the Golgi complex but not in the ER in cells infected with wild-type virus (Fig. 2.7B&C, a, b, c). In contrast, pUL56 exhibited a diffuse appearance in the cytoplasm when the TM domain was deleted (Fig. 2.7B&C, d, e, f), indicating that the TM domain is required for correct subcellular localization. vAb4Δ1-infected cells were included as a control and showed no pUL56 expression (Fig. 2.7B&C, g). In addition, flow cytometric analyses showed that MHC-I expression levels in cells infected with the TM mutant virus were identical to those infected with Ab4GΔ1 (P=0.8), the deletion mutant unable to express pUL56 (Fig. 2.7D). Western blot analyses showed that only unphosphorylated forms of pUL56 were expressed by the Ab4GΔ1_TM mutant (Fig. 2.7E, lane 3, 4). Taken together, our results show that pUL56 ispredominantly localized in the Golgi complex and that the TM domain is essential for correct subcellular localization and protein function.

2.5 Discussion

Downregulation of cell surface MHC-I is an effective survival strategy for herpesviruses to escape from the host's immune response and helps establish lifelong persistence. For alphaherpesviruses, although they constitute the largest subfamily of the *Herpesviridae*, the viral proteins involved in MHC-I downregulation are less well understood. We here report that alphaherpesviral pUL56 is a novel immune evasion protein that allows efficient downregulation of cell surface MHC-I expression.

The *UL56* gene family encodes tail-anchored membrane proteins and is present in most members of the *Alphaherpesvirinae* subfamily, except for BHV-1 and BHV-5 (Koshizuka *et al.*, 2002), indicating that the encoded proteins may share similar functions . EHV-1 pUL56, like its characterized counterparts, is dispensable for virus growth *in vitro*, but was recently shown to attenuate Ab4 pathogenicity *in vivo* (Ushijima *et al.*, 2008). These findings are in agreement with those in HSV-1 and HSV-2, where pUL56 is also dispensable for virus

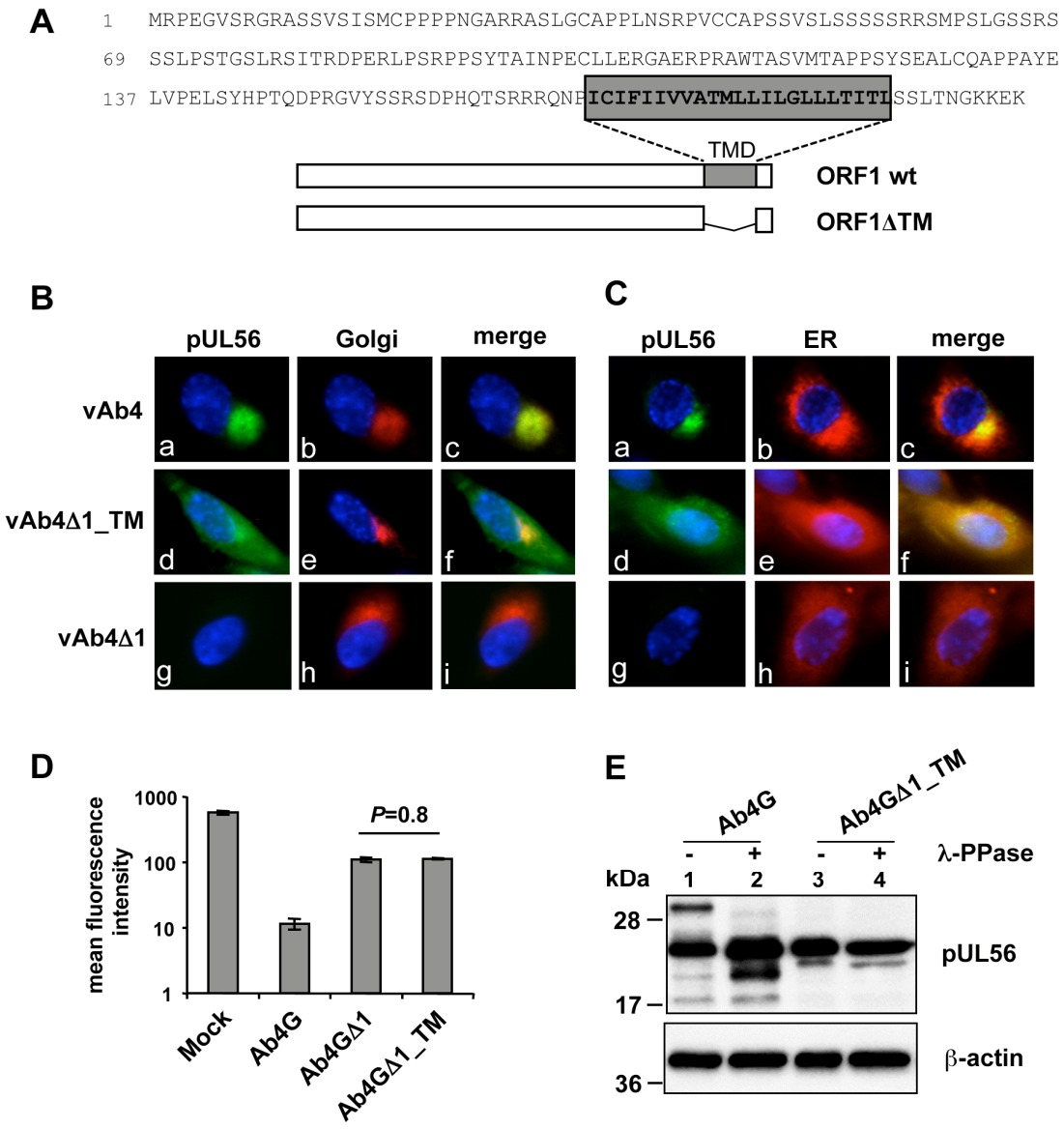

Figure 2.7 pUL56 is predominantly localized in the Golgi complex and its TM domain is essential for subcellular localization and function. (A) The amino acid sequence of the *ORF1* gene product pUL56 is shown as single-letter codes. The predicted transmembrane domain (TMD) is indicated in the grey box. The structure of the TM deletion mutant is shown below. **(B)** NBL6 cells were infected with vAb4 parental or the *ORF1* mutants vAb4Δ1 or vAb4Δ1_TM for 16h. Immunofluorescence stainings of the Golgi compartment with BODIPY®TR C_5-ceramide complexed to BSA or the ER with BODIPY®TR glibenclamide were performed. **(C)** NBL6 cells were either mock-infected or infected with Ab4G, Ab4GΔ1 or Ab4GΔ1_TM and at 16hpi, cell surface expression of MHC-I was analyzed using flow cytometry. **(D)** NBL6 cells were infected with Ab4G or Ab4GΔ1_TM and at 16hpi, cell lysates were treated or not treated with λ-PPase. Western blot analyses were performed to detect pUL56 using anti-EHV-1 pUL56 pAbs. The mAb 13E5 was used to detect β-actin, which was included as a loading control.

growth *in vitro*, but contributes to pathogenicity *in vivo* (Soboll Hussey *et al.*, 2011). It is unclear whether pUL56 orthologues in HSV-1 and HSV-2 are also responsible for the downregulation of cell surface MHC-I, but may explain the prominent role of pUL56 in HSV-1 and HSV-2 pathogenicity.

It was reported previously that an early EHV-1 gene product, alone or in combination with other proteins, causes MHC-I downregulation (Kehm *et al.*, 1996). We here demonstrate that pUL56 is a phosphorylated early protein that plays a dominant role in the process. Surprisingly, we failed to observe MHC-I downregulation in transfection experiments with a vector expressing pUL56, indicating that pUL56 needs (an) additional viral early protein or virus-induced cellular partner(s) to completely fulfill its function. HSV-1 and HSV-2 pUL56 are known to be components of the viral envelope (Koshizuka *et al.*, 2002; Rappocciolo *et al.*, 2003) and we also demonstrated the presence of pUL56 in purified EHV-1 virions (data not shown). However, based on the fact that UV-inactivated EHV-1 is unable to downregulate MHC-I (Kehm *et al.*, 1998), we exclude the possibility that virion-associated pUL56 or other structural proteins are capable of downregulating MHC-I during virus entry, although MHC-I was shown to be one of the entry receptors for EHV-1 entry (Rappocciolo *et al.*, 2003). Our results seem to suggest that only newly synthesized pUL56 functionally interacts with its partner(s). The pUL56 orthologue of HSV-2 has been reported to interact with pUL11, a viral tegument protein with dynamic membrane-trafficking properties (Koshizuka *et al.*, 2006; Sasaki *et al.*, 2011), KIF1A, a neuron-specific cellular kinesin (Loomis *et al.*, 2001) and Nedd4, a member of the E3 ubiquitin ligase family (Koshizuka *et al.*, 2005; Ushijima *et al.*, 2009; Ushijima *et al.*, 2010). In future studies, we will address potential interactors of EHV-1 pUL56, including viral and cellular proteins, and the consequences of such interactions.

We discovered that MHC-I downregulation in EHV-1-infected cells occurs independently of pUL49.5. The pUL49.5 orthologues of several members of the genus *Varicellovirus*, including EHV-1, have been identified as inhibitors of TAP activity. Using pUL45.9-expressing cell lines, it was shown that pUL49.5 robustly inhibits peptide transport into the ER lumen and precludes migration of peptide-loaded MHC-I complexes to the cell surface (Ushijima *et al.*, 2008). In a virus background, EHV-1 pUL49.5 was also shown to inhibit TAP, but the inhibitory effect was much milder and the consequences of pUL49.5-TAP interference on surface MHC-I expression remained elusive (Koppers-Lalic *et al.*, 2008). It should be noted that a significant population of surface MHC-I in NBL6 cells remain stable for more than 24 hours, even when the transport of newly synthesized MHC-I to the cell surface is blocked (Koppers-Lalic *et al.*, 2008). This finding may explain that the dramatic downregulation of MHC-I at early times of infection is likely not caused by interference of pUL49.5 with TAP. Consistent with this speculation, we here provide evidence that pUL49.5

does not have a readily detectable effect on MHC-I levels on the cell surface during virus infection, at least at early times of infection. A possible explanation for the discrepancy in the results could be that the long-term effect of the pUL49.5-TAP interference are masked by the massive and early effects of pUL56. It is tempting to speculate that removal of MHC-I from the surface of EHV-1-infected cells is the consequence of a coordinated and functional cooperation of pUL56 and pUL49.5: In the early stages of infection, before most viral peptides are loaded onto and presented by MHC-I, pUL56 will remove the majority of the molecules from the cell surface. In a second step, pUL56 would cooperate with pUL49.5 in preventing MHC-I with bound viral peptides to present their viral cargo to CTLs.

It has been reported previously that cell surface MHC-I molecules are not completely removed by EHV-1 as shown with different antibodies used to detect expression and the downregulation of MHC-I may also be locus or allele-specific (Rappocciolo *et al.*, 2003). Although, at first glance, incomplete downregulation may seem disadvantageous for the virus in its attempt to escape from CTLs, it will prevent the action of natural killer (NK) cells. Cell surface expression of MHC-I molecules provides inhibitory signals to NK cells and modulation of MHC-I presentation could sensitize infected cells for NK-mediated cytotoxicity (Rappocciolo *et al.*, 2003). Avoiding NK cell responses by selectively downregulating MHC-I alleles, which do not provide inhibitory signals, or by expressing non-functional MHC-I decoys is known for beta- and gamma-herpesviruses (Marcenaro *et al.*, 2011) and similar functions may be at play during alphaherpesvirus infections as well.

Phosphorylation by protein kinases is a common post-translational modification that regulates protein functions. Combining the facts that EHV-1 pUL56 is a phosphoprotein and is functional only in the virus context, we hypothesized that phosphorylation of pUL56 by a viral protein kinase might be required to fulfill its function. However, we found that even unphosphorylated and truncated pUL56 was still able to efficiently downregulate MHC-I, suggesting that phosphorylation is actually not essential for pUL56. In contrast to what have been reported for PRV and VZV (Deruelle *et al.*, 2009; Griffin *et al.*, 2010), neither of the viral kinases, pUS3 and pUL13, had any detectable effect on the downregulation of surface MHC-I in NBL6 cells. One possibility is that EHV-1 pUS3 might also play a role in delaying MHC-I maturation as was described for the VZV pUS3 orthologue, the gene 66 product (Eisfeld *et al.*, 2007), an effect that would likely be undetecable in the early stages of infection. Alternatively, the function of EHV-1 pUS3 may be cell-type dependent. Taken together, our data clearly show that pUL56 plays a dominant role in removing surface MHC-I in fashion that is clearly independent of pUS3, pUL13 and pUL49.5.

We found that pUL56 is predominantly localized in the Golgi compartment and that subcellular localization is dependent on the presence of its TM domain. This intracellular localization is similar to what has been reported for HSV-2 pUL56. Indirect immunofluorescence showed that HSV-2 pUL56 was mainly located to Golgi and other cytoplasmic vesicles in infected cells (Eisfeld *et al.*, 2007). In contrast, pUL56 was not detected in the ER, which was somewhat anticipated because pUL56 does not contain a double-arginine ER retention motif at its N-terminus (Koshizuka *et al.*, 2002). Furthermore, a significant amount of HSV-2 pUL56 was also shown to be associated with lipid rafts (Schutze *et al.*, 1994), the organizing centers for signal transduction and membrane trafficking (Koshizuka *et al.*, 2002). These findings indicate that alphaherpesviral pUL56 orthologues may play its role by sorting and trafficking of MHC-I. Rappocciolo et at (Brown & London, 1998) suggested that EHV-1 infection may induce enhanced endocytosis of surface MHC-I based on their observation that EHV-1 resulted in significantly greater loss of surface MHC-I when compared to treatment of uninfected cells with inhibitors blocking either protein synthesis or transport of newly synthesized protein to cell surface. Whether this interesting phenomenon is caused by pUL56 is yet to be elucidated.

In conclusion, we have identified a novel immune evasion protein encoded by the *UL56* gene family of alphaherpesviruses. We demonstrate that pUL56 is a phosphorylated early protein and plays a dominant role in the downregulation of cell surface MHC-I in the early stages of infection. While independent of pUL49.5, pUS3 and pUL13, pUL56 by itself is unable to downregulate MHC-I. Our current work is focused on elucidating the underlying mechanism of pUL56-mediated MHC-I downregulation.

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CHAPTER 3

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An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model

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3.1 Summary

Bluetongue virus (BTV) can infect most species of domestic and wild ruminants causing substantial morbidity and mortality and, consequently, high economic losses. In 2006, an epizootic of BTV serotype 8 (BTV-8) started in northern Europe that caused significant disease in cattle and sheep before comprehensive vaccination was introduced two years later. Here, we evaluate the potential of equine herpesvirus type 1 (EHV-1), an alphaherpesvirus, as a novel vectored DIVA (differentiating infected from vaccinated animals) vaccine expressing VP2 of BTV-8 alone or in combination with VP5. The EHV-1 recombinant viruses stably expressed the transgenes and grew with kinetics that were identical to those of parental virus *in vitro*. After immunization of mice, a BTV-8-specific neutralizing antibody response was elicited. In a challenge experiment using a lethal dose of BTV-8, 100% of interferon-receptor-deficient ($IFNAR^{-/-}$) mice vaccinated with the recombinant EHV-1 carrying both VP2 and VP5, but not VP2 alone, survived. VP7 was not included in the vectored vaccines and was successfully used as a DIVA marker. In summary, we show that EHV-1 expressing BTV-8 VP2 and VP5 is capable of eliciting a protective immune response that is distinguishable from that after infection and as such may be an alternative for BTV vaccination strategies in which DIVA compatibility is of importance.

3.2 Introduction

Bluetongue virus (BTV), the prototype of the genus *Orbivirus* within the family *Reoviridae*, is the causative agent of bluetongue disease in many species of domestic ruminants, especially sheep. The virus is highly infectious but not contagious; it is transmitted to ruminants by biting midges belonging to the genus *Culicoides* (Mellor *et al.*, 2000). BTV has a genome composed of 10 segments of double-stranded RNA that encode for 7 structural proteins (VP1-VP7) and 5 non-structural proteins (NS1, NS2, NS3/3a, NS4) (Belhouchet *et al.*, 2011). Of these, VP2 and VP5 are the major structural proteins forming the outer capsid of the virus particle. VP2 is the main determinant of BTV serotype and is responsible for

receptor binding, hemagglutination, and induction of serotype-specific neutralizing antibodies, while VP5 influences virus neutralization through its conformational interaction with VP2 (DeMaula *et al.*, 2000; Schwartz-Cornil *et al.*, 2008). VP7 and VP3 are the major core proteins and play important roles with respect to the structural integrity of virions (Schwartz-Cornil *et al.*, 2008). At present, 26 distinct serotypes of BTV (BTV-1 to -26) are recognized, between which there is only little cross-protection, a fact that complicates vaccination strategies (Hofmann *et al.*, 2008; Maan *et al.*, 2011; Schwartz-Cornil *et al.*, 2008).

BTV was thought to circulate only in tropical and sub-tropical regions; however, an unusual epizootic of BTV serotype 8 (BTV-8) started in central and northern Europe in 2006, affecting both sheep and cattle. During the following years, BTV-8 has spread rapidly throughout Central Europe and caused massive economic losses (Saegerman *et al.*, 2008; Zientara *et al.*, 2010). For safety reasons, only inactivated whole virus vaccines against several serotypes of BTV, including serotype 8, are now commercially available in Europe. The vaccines are highly efficacious in reducing clinical disease and BTV circulation (Zientara *et al.*, 2010). Preparations of structurally intact BTV virions induce a broad immune response to virtually all BTV structural proteins and, in some cases, even non-structural proteins dependent on the production system (Alpar *et al.*, 2009). Widespread use of such vaccines, therefore, confounds serological diagnosis and surveillance. The inability to differentiate between infected and vaccinated animals is of concern particularly in cattle, which are usually asymptomatic after BTV infection but can still spread the virus (Roy *et al.*, 2009). One possible approach to a vaccination strategy that allows the differentiation of infected from vaccinated animals (DIVA) is the use of recombinant vaccines expressing only a subset of BTV proteins. Proteins not included in the vaccine can then be used as negative markers. Promising results were reported using diverse poxviruses as live vectors for delivery of BTV antigens, mainly VP2, VP5 and VP7 (Boone *et al.*, 2007; Lobato *et al.*, 1997; Perrin *et al.*, 2007; Wade-Evans *et al.*, 1996).

This study presents a different approach, using equine herpesvirus type 1 (EHV-1) as the delivery vector. EHV-1, a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* (Davison *et al.*, 2009), is endemic in many horse populations and causes mild to severe clinical disease that includes rhinopneumonitis, abortion and neurological disorders (Allen & Bryans, 1986). EHV-1 has a double-stranded DNA genome 150 kbp in length, with numerous nonessential genes that allow insertion of foreign sequences. The potential of EHV-1 as a universal immunization vector is highlighted by its high efficiency in delivering foreign genes in a wide variety of cells and the lack of anti-vector immunity in non-equine hosts (Rosas *et al.*, 2006; Trapp *et al.*, 2005). EHV-1 strain RacH, attenuated by continuous passaging on primary swine kidney cells, is currently used as a modified live

vaccine (MLV) against EHV-1 infection in the US and Europe and has an excellent safety record (Rosas *et al.*, 2007b). This vaccine strain has been established as an infectious bacterial artificial chromosome (BAC), which makes manipulation of the virus genome easily accessible (Rudolph & Osterrieder, 2002). Based on the RacH strain, live-vectored vaccines have been developed against various viruses, which were shown to induce both humoral and cellular immune responses and provide protection in vaccinated animals, including mice, dogs and cattle (Rosas *et al.*, 2008a; Rosas *et al.*, 2007a; Rosas *et al.*, 2008b; Rosas *et al.*, 2007b; Said *et al.*, 2011).

Lately, interferon a/β receptor-deficient ($IFNAR^{-/-}$) mice have been established as a novel small animal model of BTV infection (Calvo-Pinilla *et al.*, 2009a). $IFNAR^{-/-}$ mice are impaired in their innate immune responses (Muller *et al.*, 1994) and were shown to be highly susceptible to BTV infection, but they can be completely protected by vaccination, making them an ideal tool for BTV vaccine research (Calvo-Pinilla *et al.*, 2009a).

Here, we describe the construction and evaluation of two EHV-1 RacH-based recombinant vaccines against BTV-8 expressing the immunodominant outer capsid protein VP2 alone or in combination with VP5. We show that both recombinant EHV-1 mutants stably express the transgenes and induce a BTV-8-specific neutralizing antibody response. In the $IFNAR^{-/-}$ mouse model, VP2 alone was unable to protect mice against BTV-8 challenge; however, substantial protection was observed when VP2 and VP5 were used in combination. VP7 was not included in the recombinant vaccines and was used as a DIVA marker.

3.3 Materials and Methods

3.3.1 Ethics statement

This study was carried out in strict accordance with German legislation on animal protection (Tierschutzgesetz). The experimental procedures were approved by the respective Ethics Committees in the federal states of Berlin (Permit No. LAGESO I C 112 – 0424/08) and Mecklenburg-Vorpommern (Permit No. LALLF M-V TSD/7221.3-1.1-058/10). The animal care facilities and programs of Freie Universität Berlin and the Friedrich-Loeffler-Institut meet all legal requirements. The experiments were carried out as approved by the Ethics Committees and all efforts were made to minimize suffering.

3.3.2 Cells and viruses

Rabbit kidney (RK13) cells (Rosas *et al.*, 2007b; Trapp *et al.*, 2005) and Vero cells (RIE0015, Collection of Cell Lines in Veterinary Medicine, FLI; derived from CCL 81, ATCC, Manassas, VA, USA) were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U penicillin ml^{-1} and 0.1 mg

streptomycin ml^{-1}. EHV-1 parental viruses HΔgp2 (EHV-1 strain RacH in which gp2-encoding gene71 was replaced with a mini-F sequence) (Rudolph & Osterrieder, 2002), rRacH1 (HΔgp2 in which gene 71 was restored), rH_VP2 (recombinant RacH expressing VP2 of BTV-8) and rH_VP2_5 (recombinant RacH expressing both VP2 and VP5 of BTV-8) were propagated and titrated in RK13 cells. The BTV-8 strain used in this study was a 2008 German isolate passaged two times on Vero cells (Eschbaumer *et al*, 2009).

3.3.3 Plasmids and BAC mutagenesis

The complete VP2 and VP5 genes of BTV-8 were commercially synthesized after codon optimization (Genscript) and cloned as pUC57-BTV2 and pUC57-BTV5. The VP2 gene was then PCR-amplified using Finnzymes' Phusion high-fidelity DNA polymerase (New England BioLabs) with two oligonucleotides VP2-F/VP2-R (Table 3.1) and cloned into the *Bam*HI and *Xba*I sites of pEP-CMV-in to generate pEP-VP2. pEP-CMV-in was previously constructed from pcDNA3 (Invitrogen) by inserting the kanamycin resistance gene *aph*AI and a 18bp *I-Sce*I restriction site flanked with two 50-bp duplicated sequences into the cytomegalovirus (CMV) promoter (Tischer *et al.*, 2006). Two fragments (161bp and 226bp in length) flanked with *I-Ceu*I restriction sites were amplified from either side of the ORF1/2 deletion region in the RacH genome and cloned into pUC19 to generate pUC19-ORF1/2. The VP2-expressing cassette was released from pEP-VP2 by digestion with *Spe*I and *Sph*I, and cloned into the *Spe*I/*Sph*I sites of pUC-ORF1/2 to generate transfer plasmid pUC19-ORF1/2-VP2. By digesting with *I-Ceu*I, the VP2-expressing cassette with two flanking fragments was released from pUC19-ORF1/2-VP2 and used for the later recombination. To construct a VP5 transfer plasmid, an IRES sequence was amplified and cloned into the *Eco*RI and *Xba*I sites of pUC57-BTV5, resulting in plasmid pUC57-IRES-VP5. The kanamycin resistance gene *aphAI* and an *I-Sce*I restriction site with two flanks of 40-bp each were inserted using the *Xba*I site of pUC57-IRES-VP5 to generate transfer plasmid pUC57-IRES-kan-VP5.

The EHV-1 BAC pRacH1, derived from vaccine strain RacH, was constructed to harbor an inversion of the mini-F sequence relative to the parental clone pRacH described elsewhere (Rudolph & Osterrieder, 2002) to increase genomic stability (Rosas *et al.*, 2007b). Here, pRacH1 was further modified by replacing the HCMV promoter upstream of *egfp* in the mini-F cassette with human elongation factor promoter 1α (EF-1α) to avoid potential recombination with the HCMV promoter present in the transfer plasmids. BAC mutagenesis was conducted using a two-step Red-mediated (*en passant*) strategy (Tischer *et al.*, 2006). Briefly, the EF-1α promoter with the *I-Sce*I-*aphAI* cassette was amplified from pEP-EF1-in using oligonucleotides EF1-ep1/EF1-ep2 (Table 3.1), which carry sequences homologous to those flanking the HCMV promoter within mini-F sequences present in pRacH1. The PCR product was gel-purified and electroporated into *E. coli* GS1783 (Tischer *et al.*, 2010) cells

harboring pRacH1. After the first recombination, the EF-1α promoter and *I-Sce*I-*aphAI* cassette were inserted into pRacH1, and kanamycin-resistant intermediates were obtained. For the second recombination, 1% arabinose was used to induce expression of the homing endonuclease *I-Sce*I, resulting in the cleavage of the *I-Sce*I restriction site upstream of the *aphAI* gene and, ultimately, the excision of the kanamycin cassette. The modified BAC was termed pH1_EF1. With the same strategy, the VP2 expression cassette was inserted in lieu of ORF1/2 of pH1_EF1 through recombination between BAC DNA and the fragment released by digestion with *I-Ceu*I from pUC19-ORF1/2-VP2, resulting in pH1_EF1_VP2. To insert the VP5 gene into pH1_EF1_VP2, two oligonucleotides VP5-ep1/VP5-ep2 (Table 3.1) were used to amplify IRES-VP5 and the *I-Sce*I-*aphAI* cassette from pUC57-IRES-kan-VP5. With another round of *en passant* recombination, the amplicon was inserted into pH1_EF1_VP2 utilizing 40-bp homology flanks present in the primers. The resulting pH1_EF1_VP2_5 BAC contained the VP2 and VP5 genes of BTV-8 separated by the IRES sequence and controlled by the common upstream HCMV IE promoter.

Table 3.1 Oligonucleotides used in this study

Primers	Sequences (5' to 3')
VP2-F	CGCGGATCC ATGGAGGAGCTGGCTATCCCC
VP2-R	GCTCTAGA TTACACGTTCAGAAGCTTCGTAAGC
ORF1 Fw	CAGTGAATTCGACGTAACTATAACGGTCCTAAGGTAGCGAATTTTTCCATTGGGCCCCTCCC
ORF1 Rv	CGCCTGCAGCTACTAGT TGGAGATGGAGACAGAGGAGG
ORF3 Fw	GATC GCATGC CCCGGGGCTAAAAAGCTGCGT
ORF3 Rv	GATCAAGCTGACGTAACTATAACGGTCCTAAGGTAGCGAAGGAGCAGCAGGCCCCCATCGA
EF1-ep1	TTTTGCGCACGGTTATGTGGACAAAATACCTGGTTACCCAGGCCGTGCCGGCACGTTAACCGGGCTCGTGAGGC TCCGGTGCCCGTCA
EF1-ep2	TGGTGGCGACCGGTAGCGCTAGCGGATCTGACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCTCACGACA CCTGAAATGGAAGA
IRES Fw	CAGT GAATTC GCCCCTCTCCCTCCCCCCCCCCT
IRES Rv	ATGC TCTAGA ATTATCATCGTGTTTTTCAAAGGAA
VP5-in Fw	ATGCTCTAGATCCAATATGGGCAAAATCATTAAGAGCCTGTCCCGTTGGATGACGACGATAAGTAGGGATAAC
VP5-in Rv	ATGC TCTAGA GGG TAA TGC CAG TGT TAC AAC CA
VP5-ep1	CATGTCTTTGGTAACGATGAGATGCTTACGAAGCTTCTGAACGTGTAAGCCCCTCTCCCTCCCCCCCCCCT
VP5-ep2	GCGAGCTCTAGCATTTAGGTGACACTATAGAATAGGGCCCTCTAGATCAGGCATTGCGAAGAAACAATGGG
pRacH-xx-F	CCCTCTACGGTTTTCTTCGAGGCCG
pRacH-xx-R	CCTAGGCGATGTGTGCAGCCGAGGC

The strategy for the BAC mutagenesis is illustrated in Figure 3.1. The correct construction of BAC mutants was assessed by restriction fragment length polymorphism (RFLP) analysis and

sequencing. Virus reconstitution was performed by transfecting 1 µg of BAC DNA into RK13 cells using polyethylenimine (PEI) (Polysciences). gp2 expression of the reconstituted viruses was restored by co-transfection of 1 µg BAC DNA and 10 µg plasmid p71 in RK13 cells. The recombinant EHV-1 viruses expressing VP2 alone or VP2 and VP5 were named rH_VP2 and rH_VP2_5, respectively.

3.3.4 Indirect immunofluorescence assays (IFA) and western blotting

For IFA, confluent RK13 cells in a 6-well plate were infected with rH_VP2, rH_VP2_5 or parental virus rRacH1 at a multiplicity of infection (m.o.i.) of 0.0001. One hour post infection (p.i), viruses were removed and infected cells were overlaid with 1.5% methylcellulose (Sigma) in EMEM-2% FBS. After 48 h of incubation at 37°C, cells were washed with 1x phosphate-buffered saline (1xPBS, 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4) and fixed in 3.5% paraformaldehyde in PBS for 30 min at room temperature (RT), followed by a 5 min incubation in PBS containing 30 mM glycine and another 5 min for permeabilization using PBS with 0.1% Triton X-100. After washing with PBS, cells were blocked with PBS-3% bovine serum albumin (BSA) for 30 min at RT and then incubated with monoclonal antibody (mAb) 13C10 against BTV-8 VP2 (a gift from Dr. Malte Dauber, Friedrich-Loeffler-Institut, Insel Riems, Germany) or 3B12 against EHV-1 gp2 (Meyer & Hubert, 1988) for 1 h at RT. After extensive washing (3 times for 10 min), the secondary antibody (Alexa Fluor568-conjugated goat anti-mouse IgG, Invitrogen) was added at a dilution of 1:2,000 in PBS-3% BSA and incubated for 1 h at RT. After washing, the fluorescence signal was inspected under an inverted fluorescence microscope and recorded with a digital camera (Axiovert 25 and Axiocam, Zeiss).

For western blot analyses, RK13 cells were infected with the recombinant or parental EHV-1 viruses and Vero cells with BTV-8 wild type virus. Twenty-four hours p.i., cells were collected and resuspended in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1%SDS) with a protease inhibitor cocktail (Roche) and benzonase (Novagen). Proteins of cell lysates were separated using sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking the membrane with 5% non-fat dry milk in PBS, the membrane was incubated with mAb 13C10 against BTV-8 VP2, a sheep anti-BTV-8 hyperimmune serum or mAb 3G4 against EHV-1 major capsid protein (MCP) in blocking buffer for 1 h. The secondary antibody was rabbit anti-mouse IgG-HRP (1:1,000) (SouthernBiotech) or goat anti-sheep IgG conjugated with HRP (1:1,000) (Santa Cruz Biotechnology). Reactive bands were visualized by enhanced chemoluminescence (Amersham ECL plus, GE healthcare).

3.3.5 *In vitro* growth properties

To compare the *in vitro* growth properties of the recombinant viruses with EHV-1 parental virus, plaque areas and single-step growth kinetics were determined. Plaque areas were measured after infection of RK13 cells at an m.o.i. of 0.0001 and overlaid with 1.5% methylcellulose in EMEM–2% FBS. Three days p.i., IFA using mAb A8 against EHV-1 gM (Rudolph & Osterrieder, 2002) was performed. For each virus, 50 plaques were photographed and mean plaque sizes were analyzed by using ImageJ software (http://rsb.info.nih.gov/ij/). For determining single-step growth kinetics, RK13 cells seeded in 24-well plates were infected at an m.o.i. of 5. The viruses were allowed to attach for 1 h at 4°C, followed by a penetration step of 1.5 h at 37 °C. After washing twice with PBS, infected cells were treated with ice-cold citrate buffered saline (pH 3.0) for 3 min to remove residual virus. At different time points (0, 4, 8, 12, 24, 36, 48 h p.i.), supernatants and infected cells were collected separately. Extracellular and cell-associated virus titers were determined by plaque assays. Single-step growth curves were computed from three independent experiments.

3.3.6 Immunization and challenge infection of mice

In the first animal experiment, we determined whether the recombinant viruses induced a BTV-8-specific humoral immune response. Three-week-old female Balb/c mice (Harlan) were allocated randomly to five groups of 6 mice each. Each mouse was primed and booster-immunized with 1 x 10^5 plaque forming units (PFU) of virus in a 3-week interval. Group I was inoculated intranasally (IN) with rH_VP2. For Group II, the same virus was used, but the application was subcutaneous (SC). Group III and IV were vaccinated with rH_VP2_5 IN or SC, respectively. The mice in the control group were immunized with parental rRacH1. Blood was collected from mice 1 day before immunization (day-1) and on days 14, 21, 28, and 35. Serum was prepared by centrifugation at 5000 rpm for 2min. The antibody titers were examined in a standard SNT (Savini *et al.*, 2007).

In the second animal experiment, the protective efficacy of the vectored vaccines against BTV-8 challenge was evaluated using 36 male $IFNAR^{-/-}$ mice (on a C57BL/6 genetic background), which were provided by Dr. Markus Keller, Friedrich-Loeffler-Institut, Insel Riems, Germany. Two groups of eight mice were inoculated with rH_VP2 or rH_VP2_5. Four mice received parental rRacH1. Eight mice served as positive vaccination controls and were injected with 50 µl of a commercially available inactivated BTV-8 vaccine (BTVPUR AlSap™ 8, Merial, Lyon, France; licensed for use in domestic ruminants in the European Union). A control group of four mice was mock-vaccinated with 100 µl of PBS. Another group of four mice was kept in the same room as the other mice, but was not vaccinated (environmental control). The immunizations were given SC twice three weeks apart. Three

weeks after the second application, all mice except the environmental control group were challenged by subcutaneous injection of 5 x 10^3 $TCID_{50}$ of BTV-8 in cell culture medium. The intraperitoneal LD_{50} of this strain for $IFNAR^{-/-}$ mice is about 10^{-1} $TCID_{50}$ (M. Eschbaumer, unpublished observations). The challenge dose was confirmed by titration on Vero cells.

3.3.7 Tissue sample preparation

Mice were weighed every morning, checked for signs of disease at least twice a day, and dead mice were removed from the cages immediately. Mice that met pre-defined exit criteria (apathy, ruffled fur and ocular discharge or over 20% weight loss) were anesthetized with isoflurane and killed by cervical dislocation. The whole spleen was removed from dead or euthanized animals, suspended in 1 ml of serum-free MEM containing antibiotics and antimycotics, weighed and stored at -70 °C until analysis.

3.3.8 RNA extraction and quantitative RT-PCR

For RNA extraction and virus titration, the samples were thawed and homogenized with steel balls in a TissueLyser (Qiagen, Hilden, Germany). Homogenized spleen samples were centrifuged, and the supernatants removed. One hundred microliters of supernatant were added to 300 µl of MagNA Pure LC lysis/binding buffer (Roche Diagnostics) and total nucleic acid was extracted using a MagNA Pure LC according to the manufacturer's instructions. BTV-8 RNA was detected by semi-quantitative RT-PCR (RT-qPCR). Of 100 µl of total nucleic acid eluate, 5 µl were used for RT-qPCR. Amplification was performed with the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems/Ambion) using a "pan-BTV" genome segment 5 PCR with primers and probes exactly as described (Toussaint *et al*, 2007). In vitro transcribed BTV-8 segment 5 RNA standard (B. Hoffmann, unpublished; protocol available upon request) was used for absolute quantification. BTV-8 virus titers in spleen samples of mice were determined by end-point titration of supernatants on Vero cells. Differences in virus titers between groups were statistically evaluated with a Kruskal-Wallis rank sum test.

3.3.9 Detection of VP7 antibody using ELISA

At the end of the experiment (two weeks after challenge infection), blood samples were taken from all surviving mice. Blood was collected in plain tubes. After coagulation, samples were centrifuged and sera were harvested and stored at -70 °C until analysis. Antibody levels to BTV-8 core capsid protein VP7 were determined with the ID Screen BT Competition ELISA (ID Vet). The optical density values (ODs) of samples are evaluated by comparing them to

the kit negative control. All samples with an OD of up to 50% of the negative control are positive. Samples with higher ODs are considered doubtful (50% to 60%) or negative (over 60%).

3.4 Results

3.4.1 Construction of recombinant viruses

EHV-1 ORF1 and ORF2 have been shown to be dispensable for virus growth, are absent in the vaccine strain RacH (Rosas *et al.*, 2008b; Rosas *et al.*, 2007b; Soboll Hussey *et al.*, 2011; Van de Walle *et al.*, 2010) and were chosen as the target region for transgene insertion. To avoid potential recombination of the CMV promoter upstream of *egfp* in mini-F sequences with the CMV promoter controlling transgene expression, parental pRacH1 was modified such that the CMV promoter upstream of *egfp* was replaced with an EF-1α promoter using two-step Red mutagenesis. Based on the modified pH1_EF1 BAC, a BTV-8 VP2 expression

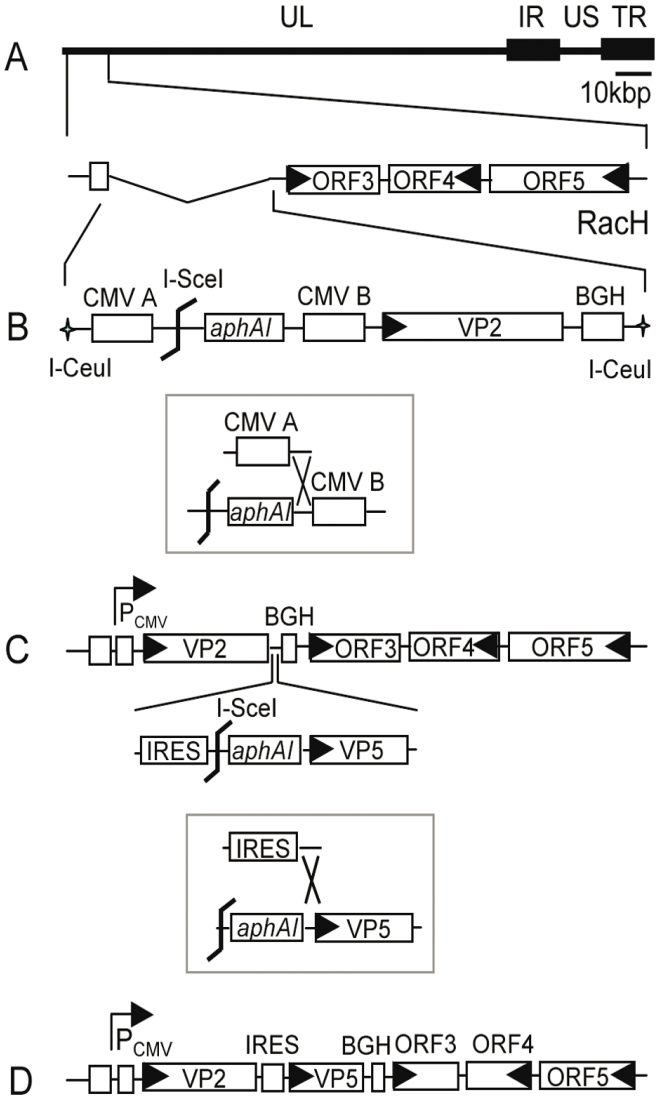

Figure 3.1 Schematic illustration of the construction strategies of the recombinant viruses. (A) The organization of the left terminus of the EHV-1 RacH genome showing that ORF1 and ORF2 are absent. UL: unique long; US: unique short; IR: internal repeat; TR: terminal repeat. (B) A fragment released from transfer plasmid pEP-VP2 by *I-Ceu*I digestion was used to recombine with RacH genome, resulting in an intermediate kanamycin (*aphAI* cassette)-resistant BAC clone. After *I-Sce*I digestion, kanamycin was removed in the following step of *en passant* mutagenesis (in box) to generate VP2-expressing virus. (C) With another round of *en passant* mutagenesis, VP5 gene with an IRES sequence upstream were inserted in between VP2 and BGH polyA, and a final construct expressing both VP2 and VP5 (D) was generated.

cassette was inserted in the ORF1/2 deletion region, resulting in pH1_EF1_VP2. In a next step, the BTV-8 VP5 gene with an upstream internal ribosome entry site (IRES) was inserted downstream of VP2, resulting in pH1_EF1_VP2_5 (Fig. 3.1). The intervening IRES sequence serves as a ribosome-binding site for the internal initiation of translation in a cap-independent fashion (Wong *et al.*, 2002). VP2 and VP5 were separated by the IRES sequence such that the two genes could be co-expressed as a single transcriptional unit under the control of the common upstream HCMV IE promoter. The correct genotype of all mutant BACs was confirmed by RFLP analysis using *Bam*HI or *Hin*dIII digestion. Upon transfection of BAC DNA into RK13 cells, the recombinant viruses, rRacH1_EF1, rH_EF1_VP2 and rH_EF1_VP2_5 were reconstituted. The expression of gp2 was repaired in the viruses by co-transfection of RK13 cells with viral DNA and plasmid DNA containing full-length gene 71, and the final mutants, rRacH1, rH_VP2 and rH_VP2_5 were generated. The VP2 and VP5 genes were sequenced in the recombinant viruses, which ensured that no mutations occurred during the recombination processes (data not shown).

3.4.2 Transgene expression and *in vitro* growth properties of the recombinant viruses

To determine whether the recombinant viruses expressed VP2 and VP5, IFA and western blot analyses were performed. Using VP2 mAb 13C10, a specific signal could be detected in cells infected with either rH_VP2 or rH_VP2_5, but not in cells infected with the parental rRacH1 virus. As a control, EHV-1 gp2 expression could be detected in cells infected by either of these viruses (Fig. 3.2A). Because a specific mAb against VP5 was not available, the expression of VP5 could not be tested using IFA. In western blot analyses using sheep anti-BTV-8 hyperimmune sera, a specific band with a size of around 60 kDa could be detected in lysates of rH_VP2_VP5-infected RK13 cells and BTV-8-infected Vero cells but not in thosefrom rH_VP2- or rRacH1-infected cells (Fig. 3.2B). We concluded from the specificity of detection and the size of the reactive band that VP5 was expressed from rH_VP2_VP5 but not from the other two viruses. Consistent with the IFA results, VP2, with a predicted mass of 106kDa, could be detected in RK13 cells infected with rH_VP2 or rH_VP2_VP5, but not in those infected with rRacH1 (Fig. 3.2B). Both VP2 and VP5 recombinant proteins were shown to co-migrate with wild-type virus proteins from Vero cells infected with BTV-8 (Fig. 3.2B). Expression of VP2 and VP5 remained stable during continuous virus passage in RK13 cells as tested by both IFA and western blotting after 10 passages.

Knowing that the transgenes were stably expressed, the *in vitro* growth properties of the recombinant viruses were compared with those of parental virus rRacH1. The ability of the viruses to spread from cell to cell was determined by comparison of relative plaque areas. With the insertion of the VP2 expression cassette or VP2 and VP5 in combination, the recombinant viruses displayed reduced plaque areas that were about 20% smaller than those

formed by rRacH1 when measured on day 3 p.i. (P<0.05) (Fig. 3.2C). To further examine the replication properties of the recombinant viruses, single-step growth kinetics were determined. We could demonstrate that both extracellular and intracellular titers of rH_VP2 and

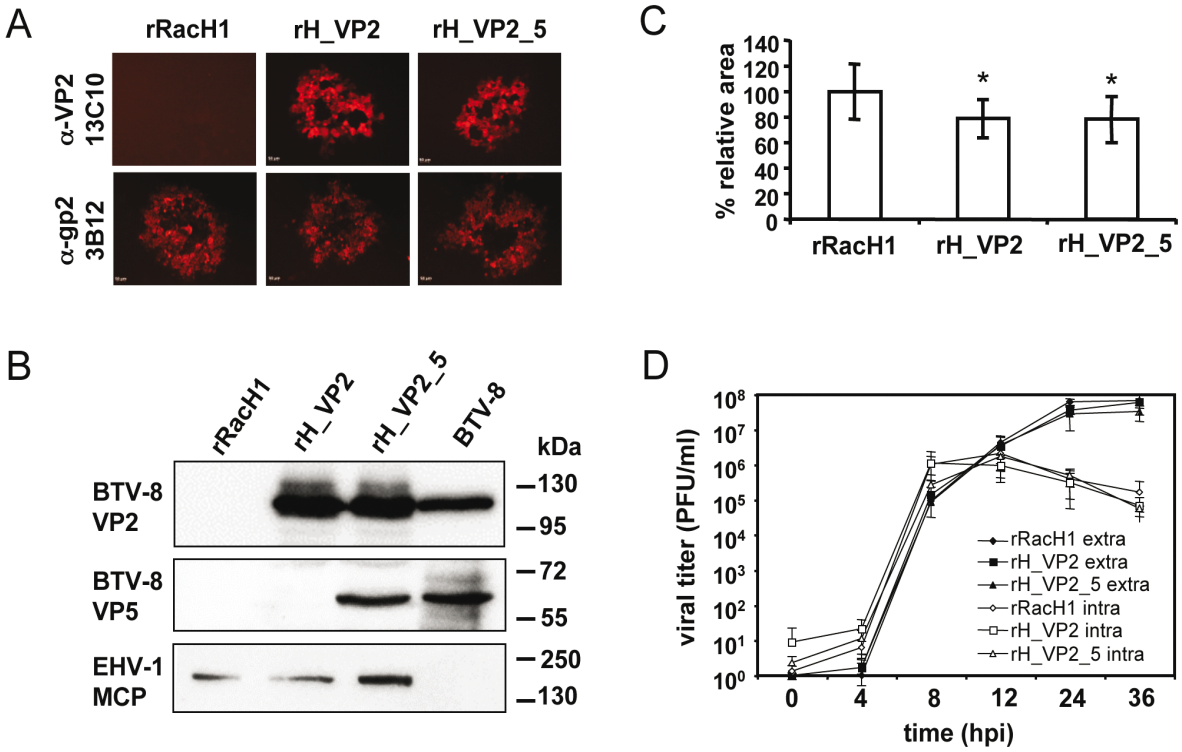

Figure 3.2 Expression of the transgenes and *in vitro* growth properties.

(A) RK13 cells were infected with parental rRacH1, rH_VP2 or rH_VP2_5 at an m.o.i of 0.0001. Two days post infection, cells were fixed and incubated with anti-VP2 mAb 13C10 or anti-EHV-1 gp2 mAb 3B12, followed by Alexa Fluor 568-conjugated goat anti-mouse IgG. Fluorescence signal was inspected under the inverted fluorescence microscope. Bar indicates 50μm. (B) Cell lysates infected by rRacH1, rH_VP2, rH_VP2_5 or BTV-8 were separated by 10% SDS-PAGE and analysed by Western blot. Expression of VP2 and VP5 was detected using primary antibody 13C10 and sheep anti-BTV-8 hyperimmune sera, respectively. EHV-1 MCP was used as a control and detected with mAb 3G4. (C) RK13 cells were infected by the individual virus at an m.o.i of 0.0001 and overlaid. Three days post infection, plaques were photographed and the areas were measured. For each virus, at least 50 plaques were measured. The relative plaque area was compared to that of rRacH, which was set as 100%. * P<0.001. (D) The single-step growth kinetics of these viruses was analysed. RK13 cells were infected by the viruses at an m.o.i of 5. Extracellular and intracellular virus titres were determined at the indicated time points.

rH_VP2_5 were comparable with those of parental rRacH1 during a 36 h period (Fig. 3.2D). The results revealed that rH_VP2 and rH_VP2_5 were only slightly impaired in terms of cell-to-cell spread, but that infectious virus production was not affected by the insertion and expression of the BTV-8 antigens.

3.4.3 Vaccination with rH_VP2 and rH_VP2_5 induces a neutralizing antibody response against BTV-8

To determine if the recombinant viruses can induce neutralizing antibody responses against BTV-8, pilot vaccination of Balb/c mice was conducted. The mice were immunized twice by the IN or SC route with rH_VP2, rH_VP2_5 or rRacH1, and the neutralizing antibody titers were measured using a standard serum neutralization test (SNT). Neutralizing antibodies were detected in mice inoculated with either rH_VP2 or rH_VP2_5, but not in mice that had been inoculated with rRacH1 (Fig. 3.3). While neutralizing antibodies were detected as early as day 14 in mice immunized by the SC route with rH_VP2 or in mice vaccinated with rH_VP2_5 by either route, neutralizing activity was detectable only from day 35 (2 weeks after booster immunization) in mice receiving rH_VP2 IN. For rH_VP2_5, the antibody titer after immunization by the SC route was also higher, albeit not significantly, than that after IN immunization, with a maximum difference on day 35 (P=0.12, T-test). When the two recombinant viruses were compared, the neutralizing antibody response induced by rH_VP2_5, although slightly weaker at day 14 and day 28 than that induced by rH_VP2 given by the SC route, reached significantly higher levels on day 35 (P=0.04, T-test) (Fig. 3.3). On the basis of these results, we concluded that both rH_VP2 and rH_VP2_5 were able to induce a specific anti-BTV-8 antibody response *in vivo* and that rH_VP2_5 was more effective than rH_VP2 with respect to inducing a neutralizing antibody response.

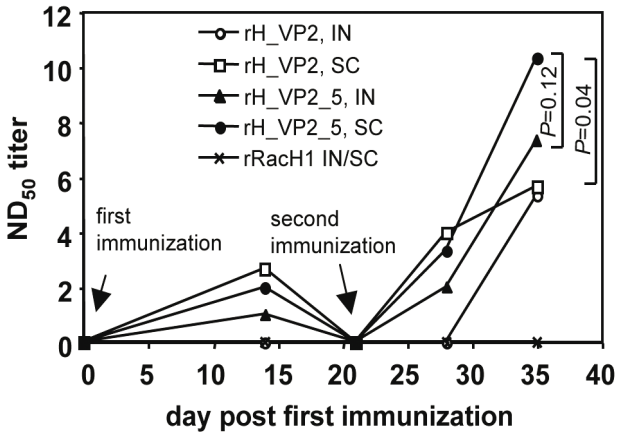

Figure 3.3 Neutralising antibody response was induced by the recombinant viruses. Three-week old female Balb/c mice were prime/booster immunised with rRacH1, rH_VP2 or rH_VP2_5. At the indicated days (0, 14, 21, 28, 35), mice were bled and the antibody was examined using standard serum neutralisation test. IN: intranasal; SC: subcutaneous.

3.4.4 rH_VP2_5, but not rH_VP2, protects mice against BTV-8 challenge

To evaluate the protective efficacy of the recombinant viruses against BTV-8 challenge, we utilized IFNAR$^{-/-}$ mice as the infection model. After each injection, the applied dose of all vaccine inocula was confirmed by plaque assays on RK13 cells. In the first immunization, mice received 1.24 x 10^6 plaque forming units (PFU) of rRacH1, 0.96 x 10^6 PFU of rH_VP2 and 0.80 x 10^6 PFU of rH_VP2_5. The booster doses were 1.25 x 10^6 PFU, 1.05 x 10^6 PFU, and 1.67 x 10^6 PFU, respectively. Even though IFNAR$^{-/-}$ mice are highly susceptible to a

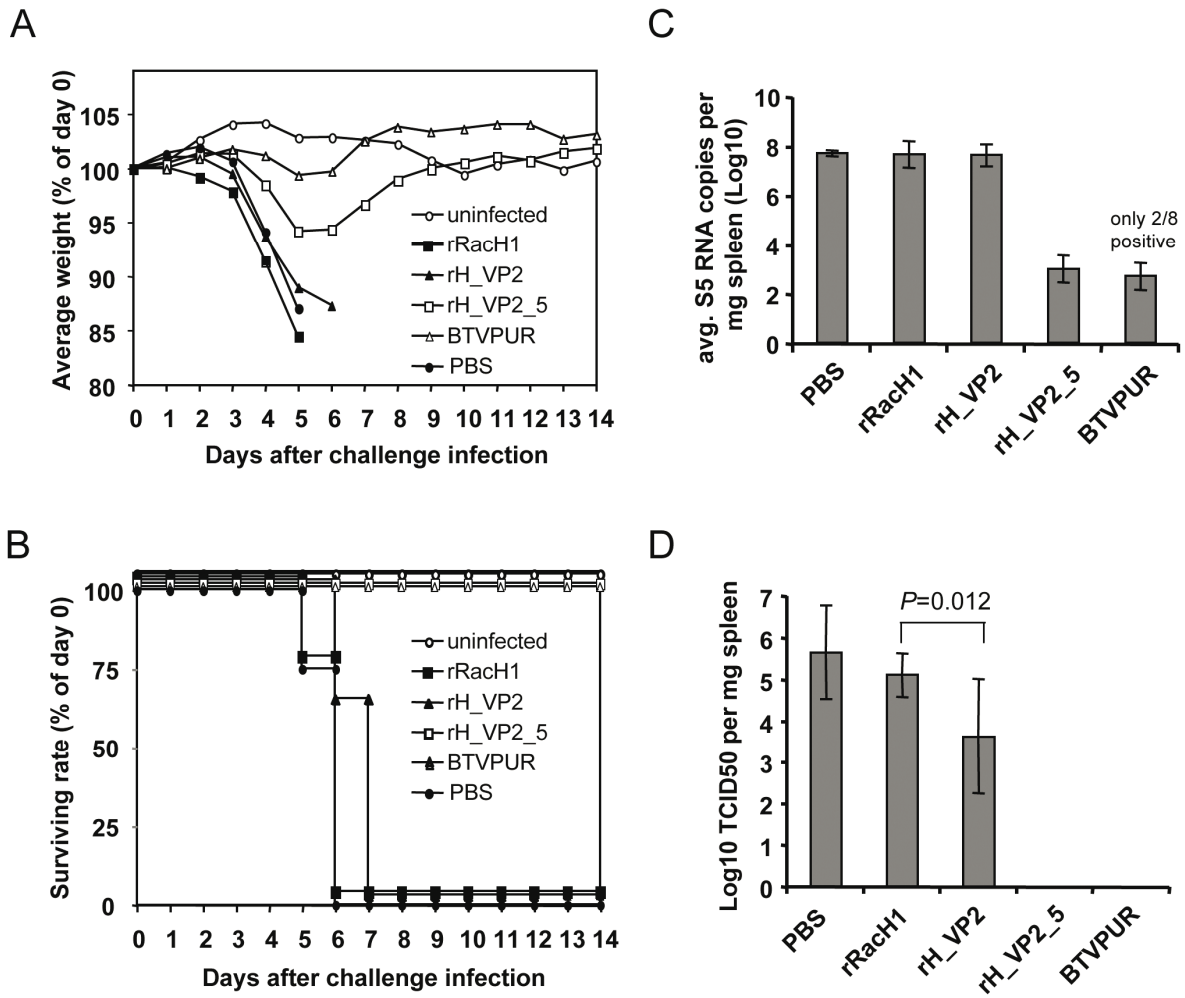

Figure 3.4 Protective efficacy of the recombinant vaccines against BTV-8 challenge in IFNAR$^{-/-}$ mice. (A) Average group weights after challenge. (B) Survival rate of IFNAR$^{-/-}$ mice after BTV-8 challenge infection. (C) BTV-8 RNA in spleen samples taken 14 days after challenge was quantified using real-time RT-PCR targeting segment 5. The average segment 5 RNA copies per mg of spleen are shown. (D) BTV-8 virus titers in spleen samples were determined by end-point titration of supernatants on Vero cells and shown as average $TCID_{50}$ per mg of spleen. Differences in virus titres between the groups were statistically evaluated with a Kruskal-Wallis rank sum test.

number of virus infections (Muller *et al.*, 1994), all vaccinations were well tolerated and no adverse effects were observed in any of the mice. After challenge, all mock-vaccinated mice as well as mice that had received rRacH1 rapidly lost weight and died or had to be euthanized by day 6. The course of disease was slightly delayed in mice vaccinated with rH_VP2, but all mice in this group also died by day 7 after challenge infection (Fig. 3.4A&B). In contrast, mice in the rH_VP2_5 group only transiently displayed mild disease (stilted gait, ruffled coat) and weight loss (about 5%) by day 5, but fully recovered by day 9 (Fig. 3.4A&B). All mice vaccinated with the commercially available inactivated vaccine and the environmental controls stayed healthy and survived until the end of the experiment (Fig. 3.4A&B).

All spleens of mice that had succumbed to BTV-8 infection contained large amounts of viral RNA (over 1 x 10^7 segment 5 copies per mg of spleen tissue). There was no significant difference in viral RNA levels between mice that succumbed to the infection in different groups (P>0.05). On average, spleens of mice vaccinated with rH_VP2_5 contained almost 50,000-fold less BTV-8 RNA than the spleens of mice vaccinated with rRacH1 (Fig. 3.4C). Only two of eight mice vaccinated with the inactivated vaccine were positive for BTV RNA.

In contrast, up to $10^{6.8}$ $TCID_{50}$ of BTV-8 per mg of tissue were found in spleens of perished mice. On average, spleens of mice in the PBS group contained $10^{5.6\pm1.1}$ $TCID_{50}$ per mg of tissue, compared to $10^{5.1\pm0.6}$ in the rRacH1 group, and $10^{3.6\pm1.4}$ in the rH_VP2 group. The difference between the rH_VP2 group and the other two groups was statistically significant (P<0.05) (Fig. 3.4D). No infectious virus could be re-isolated from the spleens of mice that

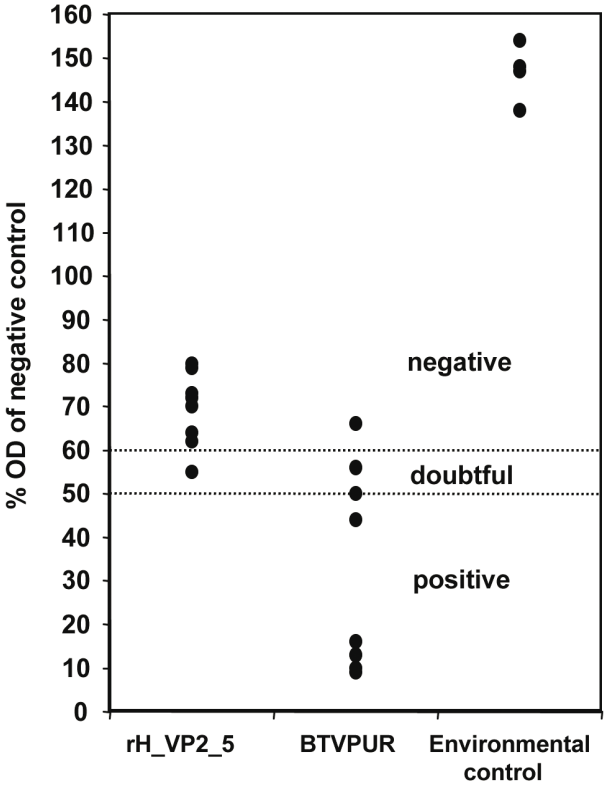

Figure 3.5 Detection of VP7 antibody from survived mice using ELISA.

At the end of the experiment, antibody levels to BTV-8 VP7 were determined from blood samples of the surviving mice (rH_VP2_5, BTVPUR AlSap™ 8, and the environmental groups) with the ID Screen BT Competition ELISA. The OD values of samples were evaluated by comparing them to the kit negative control. All samples with an OD of up to 50% of the negative control are antibody-positive. Samples with higher ODs are considered doubtful (50% to 60%) or negative (over 60%).

had been vaccinated with rH_VP2_5 or BTVPUR.

Serum samples from surviving mice were tested in a VP7-specific ELISA, but blood samples of mice succumbing to infection were not available. All environmental controls stayed completely negative in the ELISA. Mice that had been vaccinated with rH_VP2_5 showed a clear, albeit weak, reaction in the test upon challenge infection. Seven of eight, however, remained on the negative side of the cut-off, compared to only one in the BTVPUR group (Fig. 3.5).

Taken together, our data suggest that the EHV-1 recombinant virus expressing VP2 and VP5 in combination but not VP2 alone can protect against BTV-8 challenge and that the recombinant vectored vaccine can be used as a DIVA when combined with a commercial VP7-specific ELISA.

3.5 Discussion

Different vaccine strategies against BTV have been previously developed, including inactivated whole virus preparations, MLV vaccines, virus-like particles (VLPs) and vectored vaccines, of which only inactivated and MLV vaccines have been commercialized (Zientara *et al.*, 2010). Both inactivated and MLV vaccines are highly efficacious in inducing long-term protection in ruminants (Schwartz-Cornil *et al.*, 2008; Wäckerlin *et al.*, 2010). For inactivated vaccines, however, the cost of production is high and repeated immunization is needed; for MLV vaccines, the risk of causing adverse reactions in vaccinated ruminants and the potential reassortment with circulating wild-type viruses are of concern. The main argument against the use of either standard inactivated or MLV vaccines is, however, that they are not DIVA vaccines (Schwartz-Cornil *et al.*, 2008).

In contrast to the traditional inactivated or MLV vaccines, a DIVA strategy can be achieved using VLPs or live-vectored vaccines. VLPs are self-assembling structural viral proteins without BTV nucleic acids, which are safe as a reversion to virulence or genomic reassortment is impossible. Multivalent BTV vaccines are also possible when VLPs are administered as a mixture (Roy, 2004). High production costs and stability issues with the assembled particles, however, have so far prevented commercialization of this approach.

On the other hand, vectored vaccines based on well-established poxvirus delivery systems have been developed. Since VP2 is an immunodominant protein harboring the most important neutralizing epitopes, it is usually the first candidate to be included in the vectored vaccines, alone or in combination with other proteins. A recombinant canarypox virus vectored vaccine co-expressing VP2 and VP5 of BTV-17 was shown to induce a sterilizing

immune response in sheep (Boone *et al.*, 2007). With a replication-competent capripox virus encoding for VP2, VP7, NS1 and NS3 of BTV-2 individually, only partial protection was observed (Perrin *et al.*, 2007). Based on these findings, we chose VP2, alone or in combination with its partner in the virus capsid, VP5, as transgenes expressed by recombinant EHV-1.

The potential of EHV-1 as a universal vector for immunization has been previously evaluated. It was shown to be very efficient in non-equine animals, mainly due to its capacity to deliver foreign genes in cells of various species and the lack of pre-existing anti-EHV-1 immunity (Trapp *et al.*, 2005). EHV-1 has been used to deliver bovine viral diarrhea virus (BVDV) structural proteins in cattle and was shown to induce neutralizing antibodies that were correlated with reduced viremia and virus shedding (Rosas *et al.*, 2007a). While immunization of cattle with the recombinant EHV-1 induced neutralizing antibodies against EHV-1 as well, no cross-reactivity with bovine herpesvirus type 1 (BHV-1), a major pathogen of cattle, was observed (Rosas *et al.*, 2007a). This is of critical importance if a DIVA compatibility for BHV-1 is also needed. On the other hand, the lack of pre-existing anti-EHV-1 immunity in non-equine ruminants will avoid interference with the vector itself. This might be an issue with other vector systems such as capripox virus (Perrin *et al.*, 2007) and bovine herpesvirus type 4 (BHV-4) (Franceschi *et al.*, 2011) that naturally occur in ruminants.

The limitations of BTV vaccine trials in natural hosts, such as the high cost for large animals, the need for large animal facilities of biosafety level 3 and the paucity of knowledge about their immune systems, has been overcome recently by the establishment of a small animal model, IFNAR$^{-/-}$ mice (Calvo-Pinilla *et al.*, 2009a). Due to a deficiency in the β subunit of the IFN-α/β receptor, the mice are highly susceptible to many virus infections but can be protected by immunization (Muller *et al.*, 1994), making them a versatile tool to evaluate the immune response and protection conferred by vaccination. Recently, IFNAR$^{-/-}$ mice have been used to test BTV vectored vaccines based on recombinant modified vaccinia virus (MVA) and BHV-4 (Franceschi *et al.*, 2011; Calvo-Pinilla *et al.*, 2009b).

We found that recombinant RacH co-expressing VP2 and VP5 of BTV-8 protected IFNAR$^{-/-}$ mice against a lethal challenge infection. The mice displayed only transient and mild signs of discomfort, but fully recovered by day 9 after infection. No infectious virus was found in the mice, and viral RNA loads were dramatically reduced. With VP2 alone, the course of disease was slightly delayed, but all mice eventually succumbed to BTV-8 challenge. A similar result was reported when VP2 alone was expressed by a BHV-4 vector, even though the challenge dose in that experiment was much lower than the one used here (Franceschi *et al.*, 2011). These findings suggest that VP2 alone, while able to induce a neutralizing antibody response,

can hardly provide complete protection against BTV infection. The degree of protection, however, is much greater when VP2 is used together with the minor outer capsid protein VP5 (Lobato *et al.*, 1997; Roy *et al.*, 1990). This synergy may be derived from their close interaction in the virus particle, suggesting a strong conformational/folding dependence of VP2 on VP5. The presence of neutralizing epitopes not only in VP2 but also in VP5 has been postulated (DeMaula *et al.*, 2000), but we did not test for antibodies to VP5 alone here.

The discriminatory potential of ELISAs based on non-structural proteins has already been demonstrated (Anderson *et al.*, 1993; Barros *et al.*, 2009), and those assays would be theoretically suited for a DIVA concept with existing inactivated vaccines. The recent BTV-8 vaccination campaigns, however, have shown that the practical value of a non-structural protein antibody ELISA is very limited, particularly in animals that have been vaccinated and revaccinated repeatedly. The carryover of non-structural proteins from culture system used to produce the vaccines often results in antibodies to viral non-structural proteins in vaccinated animals. While some of the inactivated vaccines in the market are highly purified, not all manufacturers include this cost-intensive step in their production process (Alpar *et al.*, 2009). In consequence, attempts at establishing a commercial NS1 ELISA were not successful. Vaccination with inactivated vaccines from different companies led to an increased number of unspecific results in the test, which, consequently, was not released by the manufacturer.

If adequate protection can be achieved without using VP7, the well-established VP7 antibody ELISAs that are routinely used for BTV diagnosis can be used for DIVA. We demonstrate that the recombinant EHV-1 carrying both VP2 and VP5 protects mice against lethal BTV-8 challenge. While the highly sensitive real-time RT-PCR assay detected low levels of viral RNA in mice immunized with rH_VP2_5, no infectious virus was present. Compared to mice vaccinated with a standard inactivated vaccine, the rH_VP2_5 mice displayed only minimal levels of VP7 antibody even after challenge.

Together with the availability of infectious clones of EHV-1 vaccine strain RacH, which allows rapid generation of transgene-expressing vaccines, an approach based on vectored vaccines is a valuable alternative for protection against orbivirus infections, especially when a DIVA regimen is required.

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CHAPTER 4

Summary and Discussion

Summary and Discussion

4.1 Evasion of EHV-1 from CTL-mediated immunity

The inability of vaccination in preventing EHV-1 infection has long been a concern for equine veterinarians and virologists. One of the main reasons for the continued struggle is the immune evasion strategies developed by this virus, especially with respect to evasion from MHC-I-dependent CTL-mediated immunity, which still is correlated with protection against disease (Kydd *et al.*, 2003). A number of proteins produced by members of the herpesivrus family have been shown to interfere with MHC-I antigen presentation pathway. However, different viruses do so by strikingly different mechanisms (Griffin *et al.*, 2010). For the *Alphaherpevirinae*, four proteins, which are the gene products of *UL41* (vhs protein), *UL49.5*, *US3* and *US12* (ICP47) (Eisfeld *et al.*, 2007; Everly *et al.*, 2002; Früh *et al.*, 1995; Koppers-Lalic *et al.*, 2005), have been previously found to account for the CTL evasion. While ICP47 is unique for the *Simplexviruses*, homologues of the other three genes are present in EHV-1. The vhs protein of HSV-1 and BHV-1 exerts its influence by non-specifical degradation of host mRNA, including that encoding MHC-I and other components of the antigen presentation pathway (Everly *et al.*, 2002; Koppers-Lalic *et al.*, 2001). However, the vhs protein of EHV-1 does not seem to induce a detectable decrease of MHC-I cell surface expression (Rappocciolo *et al.*, 2003). The identification of *UL49.5* gene products as a novel class of TAP inhibitors in the *Varicelloviruses* resulted in progress in our understanding of CTL evasion strategies by this group of viruses. The EHV-1 *UL49.5* product inhibits TAP activity by inhibiting ATP binding, but an EHV-1 mutant lacking *UL49.5* is still able to downregulate surface MHC-I (Koppers-Lalic *et al.*, 2008). The *US3* kinase of PrV and VZV have been shown to induce surface MHC-I downregulation in a cell type-dependent manner (Deruelle *et al.*, 2009; Eisfeld *et al.*, 2007). In PK15 cells, PrV-induced MHC-I downregulation is only partially dependent on pUL49.5, but independent of pUS3, suggesting that a yet unidentified early protein(s) are involved (Deruelle *et al.*, 2009). It has also been shown that one or more early EHV-1 protein(s) induces the downregulation of cell surface MHC-I (Rappocciolo *et al.*, 2003). In this thesis, we clearly identified an early protein, pUL56 encoded by *ORF1*, as the major determinant of cell surface MHC-I downregulation, while both pUL49.5 and pUS3 did not have detectable effects on the cell surface MHC-I expression at least in the early time of the infection (chapter 2).

4.2 Identification of pUL56 as a novel immune evasion protein

The discovery of pUL56 as an immune evasion protein originated from the observation that EHV-1 strain RacL11, in stark contrast to the Ab4 strain, only induced mild downregulation of cell surface MHC-I. Sequence alignment showed that both viruses harbor the *UL41*, *UL49.5* and *US3* genes without sequence variations, indicating that other genes that were

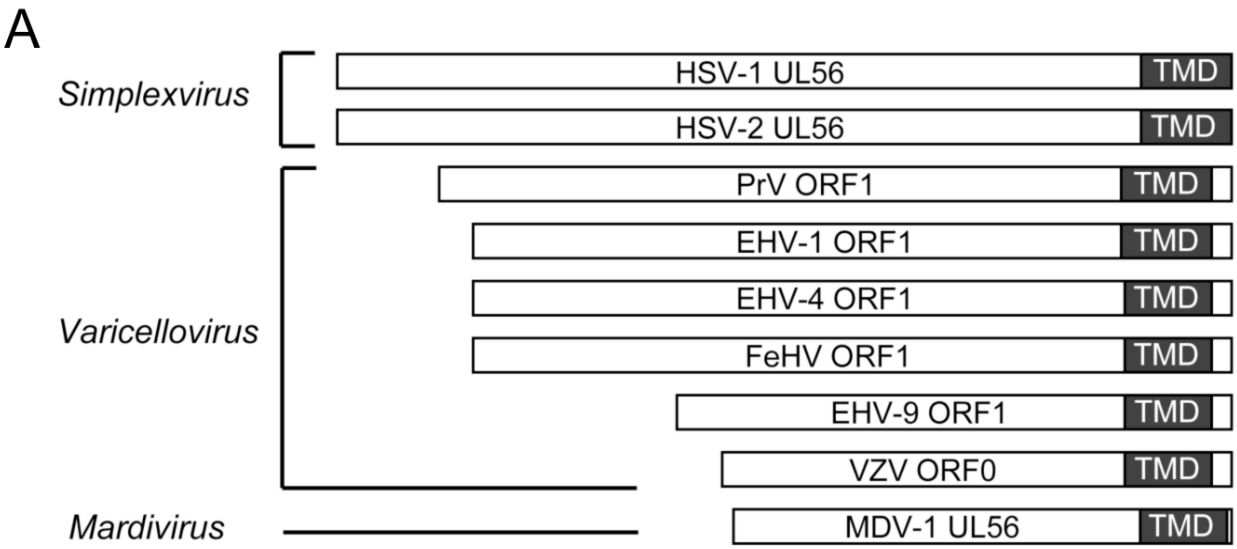
mutated or absent in RacL11 that are responsible for its impaired ability of downregulating MHC-I. For RacL11 and some of its derivatives including RacH, the left UL terminus is shortened compared to that of the Ab4 strain (Hubert *et al.*, 1996). Following this clue, we found that *ORF1* and *ORF2* were missing in this region in RacL11 strain. Coincidentally, the same deletion was also demonstrated in another strain, the MLV candidate KyA (Rosas *et al.*, 2006), which was also impaired in downregulation of MHC-I (data not shown). Both ORF1 and ORF2 gene products are predicted type II transmembrane proteins with a C-terminal hydrophobic domain, indicating they might share similar functions. However, we were able to show that only the *ORF1* product (pUL56) was associated with MHC-I downregulation. We identified that EHV-1 pUL56 was a phosphorylated early membrane protein, which is consistent with the earlier observation that MHC-I downregulation was an early event in virus infection and, hence, induced by an early protein(s). In contrast, EHV-1 pUL49.5 is expressed later than pUL56 and was classified as an early-late protein. Deletion mutants lacking either UL49.5 or US3 are still able to induce dramatic surface MHC-I downregulation, in fact in a manner that is indistinguishable from wild type virus, which strongly suggests that pUL56, but not pUL49.5 and pUS3, plays the dominant role in downregulating surface MHC-I.

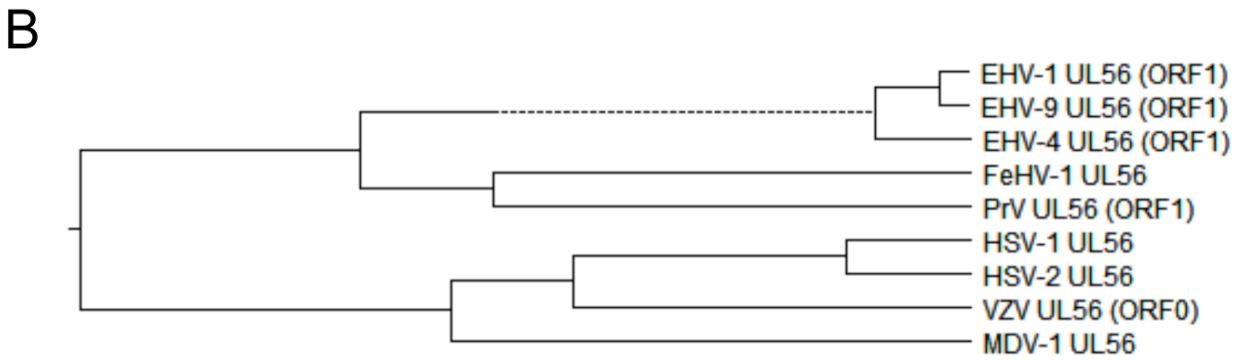
4.3 The functions of pUL56 homologues in other alphaherpesviruses

The EHV-1 *ORF1* gene product is the homologue of pUL56 in HSV-1 and is present in all alphaherpesviruses except for BHV-1 and BHV-5 (Ushijima *et al.*, 2010), indicating that its function might be conserved. Very little information is available concerning the protein's function. The *UL56* homologue in PrV is known to be dispensable for virus growth *in vitro* (Baumeister *et al.*, 1995; Tai *et al.*, 2010). For VZV, the positional and sequence homologue of *UL56* is *ORF0* (or *ORF S/L*) (Koshizuka *et al.*, 2010). Interestingly, the vaccine strain vOka contains a mutated version of *ORF0* compared to its parental virus pOKa, which might contribute to its attenuation (Koshizuka *et al.*, 2010). VZV *ORF0* is required for efficient growth both *in vitro* and *in vivo*. Detailed analysis showed that *ORF0* contained a specific cleavage/packaging element at the extreme 5' terminus that is important for the processing of viral DNA, and the growth defect of *ORF0* mutant was only partially due to the loss of *ORF0* function (Kaufer *et al.*, 2010; Zhang *et al.*, 2007). This element does not seem to exist in EHV-1 *ORF1*, because EHV-1 mutants lacking *ORF1* did not show growth defects compared to wild-type virus. For HSV-1 and HSV-2, *UL56* is dispensable for virus growth and were previously shown to be important for pathogenicity. HSV-1 strains lacking *UL56* are substantially less neuroinvasive (Berkowitz *et al.*, 1994; Kehm *et al.*, 1996). More specifically, the C-terminal hydrophobic region of UL56 is important for HSV-1 neurovirulence (Kehm *et al.*, 1996). Accordingly, we discovered that the TM domain (TMD) of EHV-1 pUL56 is essential for its function in downregulating MHC-I. Furthermore, HSV-2 pUL56 was shown to interact with KIF1A (Koshizuka *et al.*, 2005), a neuron-specific member of the kinesin-3

family involved in the axonal transport of synaptic vesicle precursors (Okada *et al.*, 1995), leading to the hypothesis that pUL56 protein affects vesicular trafficking in infected cells. Later on, an interaction between HSV-2 pUL56 and the viral pUL11 protein was demonstrated by an *in vitro* pull-down assay, however, the function of this interaction remains unclear (Koshizuka *et al.*, 2006). More recently, HSV-2 pUL56 protein was found to interact with members of Nedd4 family of E3 ubiquitin ligases, including Nedd4 and itch, through its three PY motifs and resulted in the ubiquitination and ultimate degradation of these E3 ligases, while UL56 itself was not ubiquitinated (Ushijima *et al.*, 2008; Ushijima *et al.*, 2010). Ubiquitin-mediated protein modifications regulate a variety of cellular processes, including protein turnover and trafficking, endocytosis and transcription factor activation (Weissman, 2001). These findings suggest that HSV-2 pUL56 regulates the Nedd4 family of E3 ligases, but the biological consequence of this regulation is yet to be elucidated. The identification of EHV-1 pUL56 as an immune evasion protein will guide us to investigate the functions of this group of proteins in a new direction.

While pUL56 homologues are present in most of the alphaherpesviruses, the similarity of their amino acid sequences is rather limited. The highest similarity (67.3%) is found between the two closely related viruses, EHV-1 and EHV-4. Although the amino acid identity between EHV-1 and EHV-9 pUL56 is as high as 97.2%, EHV-9 pUL56 is actually a N-terminally truncated version relative to EHV-1 pUL56 (Fig.4.1A). The similarity of pUL56 proteins of EHV-1 and other alphaherpesviruses ranges from 15.9% to 20.8% only. In the varicelloviruses, the VZV ORF0 product represents the shortest member of the UL56 family. Structurally, all the pUL56 members possess C-terminal TMD (Fig.4.1A), which are essential for their cellular localization and functions. As expected, pUL56 members of varicelloviruses, except for VZV, share the closest phylogenetic relationship (Fig.4.1B), suggesting that their functions are most likely conserved. Sequence alignment revealed several highly conserved regions across the whole pUL56 family, especially the two PY motifs, one ‘PPSY’ from amino acid position 121 to 124 and the other ‘PPAY’ from 132 to 135 in EHV-1 (shown in dotted windows in Fig. 4.1C). Another PY motif (PPSY) is found between positions 91 to 94, which is only conserved in EHV-1, -4, -9, feline herpesvirus type 1 (FHV-1) and PrV (shown in dotted window in Fig. 4.1C). It should be noted that (1) the first two PY motifs in HSV-1 and HSV-2 (shown in solid windows) (Ushijima *et al.*, 2008) have different positions from those of EHV-1 and (2) VZV ORF0 gene product possesses only one PY motif (Fig. 4.1C). The preservation of PY motifs suggests that the interaction of pUL56 with Nedd4 family E3 ligases might be a conserved feature of this protein family.


A
Simplexvirus
HSV-1 UL56
TMD
HSV-2 UL56
TMD
Varicellovirus
PrV ORF1
TMD
EHV-1 ORF1
TMD
EHV-4 ORF1
TMD
FeHV ORF1
TMD
EHV-9 ORF1
TMD
VZV ORF0
TMD
Mardivirus
MDV-1 UL56
TMD


B
EHV-1 UL56 (ORF1)
EHV-9 UL56 (ORF1)
EHV-4 UL56 (ORF1)
FeHV-1 UL56
PrV UL56 (ORF1)
HSV-1 UL56
HSV-2 UL56
VZV UL56 (ORF0)
MDV-1 UL56

C

Figure 4.1 Comparison of EHV-1 UL56 and its homologues in other alphaherpesviruses.
(A) Schematic illustration of the structures of UL56 family in a selection of alphaherpesviruses. TMD, transmembrane domain. (B) Phylogenetic relationship of UL56 family members. The tree was generated based on UL56 amino acid sequences using Megalign of the sequence analysis software DNAStar. (C) Sequence alignment of UL56 proteins. The matching amino acids are marked in shadows. The PY motifs of HSV UL56 are shown in solid windows, and the PY motifs in other viruses are shown in dotted windows.

4.4 The putative mechanism(s) of pUL56-mediated MHC-I downregulation

The mechanism(s) of pUL56-mediated downregulation of surface MHC-I is still unclear so far. We report here that EHV-1 pUL56 is unable to downregulate MHC-I by itself, but instead, it needs the virus background to fulfill its functions, clearly indicating that EHV-1 pUL56 requires a viral partner(s) or a cellular protein(s) that are modified by other viral proteins. The downregulation of surface MHC-I by EHV-1 occurs at early times after infection. Therefore, the interaction partner(s) of pUL56 should also be an early protein(s) with comparable expression kinetics as pUL56. The only viral protein that was shown to interact with pUL56 is the product of *UL11* in HSV-2 (Koshizuka *et al.*, 2006). For EHV-1, pUL11 is known as an early-late tegument protein and is localized predominantly in *trans*-Golgi network (Schimmer & Neubauer, 2003), where pUL56 is also localized. Whether EHV-1 pUL56 interacts with pUL11 is unknown, whereas our preliminary data showed that pUL11 is not the viral partner pUL56 needed to downregulate MHC-I, because an EHV-1 mutant lacking pUL11 expression is still able to induce dramatic MHC-I downregulation (unpublished data). As stated above, pUL56 function is also independent of pUL49.5 and pUS3. The interaction between pUL56 and the unknown viral partner(s) must be functional, but not necessarily physical. In order to find the functional viral partner(s) of pUL56, screening of the early viral proteins across EHV-1 genome is warranted.

Rappocciolo et al. observed that MHC-I downregulation by EHV-1 infection was greater than that in non-infected cells treated with inhibitors inhibiting protein transport to Golgi, and that surface MHC-I had a half-life of more than 24 hours in non-infected cells (Rappocciolo *et al.*, 2003). In combination with our finding that EHV-1 induces MHC-I downregulation at early times after infection, we speculate that EHV-1-induces downregulation of surface MHC-I, an effect mainly induced by pUL56, by enhancing endocytosis. Up to now, such an effect was only described for gammaherpesviruses. For KSHV, two E3 ubiquitin ligases, kK3 and kK5, catalyze K63-linked polyubiquitination of the cytoplasmic tail of MHC-I heavy chain and induce endocytosis in a clathrin-dependent fashion (Coscoy & Ganem, 2000; Duncan *et al.*, 2006). For EBV, endocytosis of MHC-I is induced by BILF1, a viral glycoprotein with the

properties of a constitutively signaling G-protein-coupled receptor (GPCR) that is predominantly localized at the plasma membrane (Paulsen *et al.*, 2005; Zuo *et al.*, 2009; Zuo *et al.*, 2011). The finding that HSV-2 pUL56 interacts with Nedd4 family E3 ligases led us to consider the relationship between this interaction and pUL56-mediated MHC-I downregulation. The first questions to be answered are whether HSV-2 pUL56 also induces MHC-I downregulation like EHV-1 pUL56 or whether EHV-1 pUL56 also interacts with equine Nedd4 family. The whole picture of the mechanisms would be elucidated if the interaction of EHV-1 pUL56 with equine Nedd4 E3 ligases could be confirmed and/or the unknown viral partner(s) could be discovered. Both these avenues are currently followed in our research.

4.5 Deletion of immune evasion genes is anticipated to increase vaccine efficacy

The discovery of immune evasion genes within viral genomes will definitely benefit the improvement of MLV vaccines. The control of herpesvirus infections is mainly dependent on CTL-mediated immunity, which is counteracted by a number of immune evasion proteins. Evidence has accumulated that deletion of these immune evasion genes will make the viruses less virulent and induce stronger immune responses. When mice were infected with a KSHV mutant lacking kK3, the number of latently infected spleen cells was reduced and the frequency of virus-specific $CD8^+$ CTLs was increased (Stevenson *et al.*, 2002). A BHV-1 mutant with the deletion of *UL50*, *UL49.5* and *UL49* was significantly attenuated and protected vaccinated calves against secretion of challenge virus better than any other live BHV-1 vaccines, indicating that a stronger anti-BHV-1 T cell response was elicited (Liang *et al.*, 1997). The immune responses following infection of ponies with an EHV-1 *ORF1/2* deletion mutant was also evaluated (Soboll Hussey *et al.*, 2011). Both EHV-1 wild type and the mutant led to cytokine responses and suppression of proliferative T-cell responses in ponies on day 7 post infection; however, the mutant EHV-1 caused significantly shorter primary pyrexia and reduced nasal shedding, and a less pronounced decrease in PBMC IL-8 and increased T-bet responses relative to the wild-type virus (Soboll Hussey *et al.*, 2011). The remaining suppression of T-cell proliferation may be caused by other immune evasion proteins such as pUL49.5. A better understanding of the correlation between T-cell responses and immune evasion genes could be achieved by using double deletion mutant lacking both ORF1 and UL49.5.

4.6 Development of EHV-1-vectored vaccines: the second generation

Since EHV-1 has been developed as a universal vector to deliver foreign genes, the efficacy of the vector in inducing immune responses should be concerned. The EHV-1 vaccine strain RacH, together with its parental virus RacL11, have a natural deletion of *ORF1* in their

genomes. The deletion of *ORF1* might also contribute to the attenuation of RacH strain. The first generation of RacH-vectored vaccines was developed by the insertion of foreign genes in replace of *egfp* gene in the mini-F backbone. The resulting recombinant viruses retain mini-F sequences, which might cause problems due to the presence of resistance genes. In this thesis (chapter 3), we developed the second generation of live-vectored vaccine by inserting the foreign genes, e.g. VP2 and/or VP5 genes of BTV-8 under the control of HCMV promoter and BGH polyA sequence, into the *ORF1* deletion region. The whole mini-F gene was eventually removed by repairing gp2-encoding *ORF71*, and as such, 'clean' recombinant viruses were generated. Lately, another MLV vaccine was developed based on a recent EHV-1 strain, NY03, which was isolated during an abortion storm in New York in 2003 (Van de Walle *et al.*, 2010). EHV-1 NY03 was modified to imitate the deletions in RacH, including the deletion of *ORF1*, both copies of *IR6* and the cytoplamic tail of gp2. The NY03 mutant was able to induce robust EHV-1 specific immune responses and significantly reduce clinical signs, nasal shedding and viremia levels in challenged horses. NY03-based MLV was further used as a novel live vaccine vector to express the H3 protein of equine influenza virus and was shown to induce a robust serological immune responses (Van de Walle *et al.*, 2010). Theoretically, EHV-1 can be used to insert multiple foreign genes at different loci. Besides *ORF1*, other genes relating to immune evasion could also be targeted for insertion of foreign genes, for example, *UL49.5* and *UL41* (vhs). In general, EHV-1 as a live vector has been improved with the discovery of immune evasion genes and will be more promising to use in the future.

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Selbeständigkeitserklärung

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

Ort, den 08.11.2012 Unterschrift Guanggang Ma