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**Equine CD172a⁺ monocytic cells,
the ‘Trojan Horse’ for equine herpesvirus type 1
(EHV-1) dissemination in the horse**

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Thesis submitted in fulfillment of the requirements for the degree of
Doctor in Veterinary Sciences (PhD), February 2016

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TABLE OF CONTENTS

Chapter 1	11
Introduction	11
1. Equine herpesvirus type 1	13
1.1. <i>Introduction</i>	13
1.2. <i>History</i>	13
1.3. <i>Taxonomy</i>	13
1.4. <i>Virion structure</i>	15
1.5. <i>Cell infection</i>	16
1.6. <i>Replication cycle</i>	18
2. Pathogenesis of EHV-1 infection	22
2.1. <i>Introduction</i>	22
2.2. <i>Primary replication in the upper respiratory tract (URT)</i>	22
2.3. <i>Replication in the draining lymph nodes and cell-associated viremia</i>	23
2.4. <i>Secondary replication in the pregnant uterus, CNS and/or eye</i>	26
2.5. <i>Latency and reactivation</i>	28
2.6. <i>Factors determining the development of abortion vs EHM</i>	30
3. Clinical signs	31
3.1. <i>Respiratory disease</i>	31
3.2. <i>Abortion</i>	31
3.3. <i>Neurological disease</i>	32
3.4. <i>Ocular disease</i>	33
4. Immunity to EHV-1 infection	34
4.1. <i>Introduction</i>	34
4.2. <i>Innate immunity</i>	34
4.3. <i>Adaptive immunity</i>	35
5. Diagnosis	36
6. Treatment	37
7. Control and prevention	38
7.1. <i>Management</i>	38
7.2. <i>Vaccination</i>	39
8. Immune evasive strategies of EHV-1	43
8.1. <i>Introduction</i>	43
8.2. <i>Evasion from humoral immunity</i>	43

8.3. <i>Evasion from CTL immunity</i>	45
8.4. <i>Evasion from NK cell activity</i>	46
8.5. <i>Interference with cytokine and chemokine responses</i>	46
8.6. <i>Cell-to-cell spread</i>	47
8.7. <i>Conclusions</i>	48
9. References	49
Chapter 2	61
Aims of the thesis	61
Chapter 3	65
Replication of EHV-1 in CD172a⁺ monocytic cells	65
Chapter 4	113
Entry of EHV-1 into CD172a⁺ monocytic cells	113
Chapter 5	147
EHV-1 enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells	147
Chapter 6	185
General discussion	185
Chapter 7	209
Summary-Samenvatting	209
Curriculum Vitae	217
Acknowledgments	221

LIST OF ABBREVIATIONS

Akt	protein kinase B
ANOVA	analysis of variance
BBB	blood-brain barrier
BCA	bicinchoninic acid
BM	basement membrane
BoHV	bovine herpesvirus
C3b	complement factor 3b
CD	cluster of differentiation
CF	complement fixation
CNS	central nervous system
CO ₂	carbon dioxide
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
DC	dendritic cells
Dio	dioctadecyloxacarbocyanine perchlorate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
dpi	day post-inoculation
E	early
EBV	Epstein-Barr virus
EC	endothelial cells
EEL	equine embryonic lung cells
EHM	equine herpesvirus myeloencephalopathy

EHV	equine herpesvirus
ELISA	enzyme-linked immunosorbent assay
EMPF	equine multinodular pulmonary fibrosis
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
Fil	filipin III
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
gB, gC	glycoprotein B, glycoprotein C
h	hour
HA	hemagglutinin
HAART	highly active anti-retroviral therapy
HAT	histone acetyltransferase
(H)CMV	(human) cytomegalovirus
HDAC	histone deacetylase
HIV	human immunodeficiency virus
hpa	hour post-adhesion
hpi	hour post-inoculation
HS	heparan sulfate
HSV	herpes simplex virus
hTERT	human telomerase reverse transcriptase
HVEM	herpesvirus entry mediator
IE	immediate-early
IF	immunofluorescence

IFN	interferon
Ig	immunoglobulin
IL-2	interleukin 2
KSHV	Kaposi's sarcoma-associated herpesvirus
L	late
LAT	latency associated transcript
LM	leukocyte medium
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
m β CD	methyl- β -cyclodextrin
MHC	major histocompatibility complex
MLV	modified live vaccine
MOI	multiplicity of infection
MVV	Maedi-Visna virus
N	neurovirulent
NA	neuraminidase
NaBut	sodium butyrate
NK	cell natural killer cell
NN	non-neurovirulent
ORF	open reading frame
PAA	phosphonoacetate
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction

PFA	paraformaldehyde
PI(3)K	phosphatidylinositol 3-kinase
PRV	pseudorabies virus
RC	replicative compartment
RK-13	rabbit kidney epithelial cells
RNAi	RNA interference
ROCK	Rho-associated coiled-coil kinase
RPMI	Roswell Park Memorial Institute
RSD	arginine-serine-aspartic acid
SD	standard deviation
SiRNA	small inhibitory RNA
SN	serum neutralization
SNP	single nucleotide polymorphism
SV40LT	simian virus 40 large T-antigen
TAP	transporter associated with antigen processing
TCID ₅₀	tissue culture infectious dose with a 50% endpoint
TK	thymidine kinase
TLR	toll-like receptor
TNF- α	tumor necrosis factor-alpha
TSA	trichostatin A
URT	upper respiratory tract
vCKBP	viral chemokine binding protein
VN	virus neutralizing
VP16	virion protein 16

Chapter 1.

Introduction

1. Equine herpesvirus type 1

1.1. Introduction

Equine herpesvirus type 1 (EHV-1) is a ubiquitous pathogen in horses. The virus is responsible for respiratory disorders, abortion, neonatal foal death and neurological disorders (Allen & Bryans, 1986). As current vaccines do not provide full protection against severe symptoms, EHV-1 is still a major threat for the horse industry, causing serious economic losses every year.

1.2. History

In 1933, Dimock and Edwards documented epidemic virus abortion in mares in Kentucky (Dimock & Edwards, 1933). The virus was first designated as ‘equine abortion virus’ and later renamed equine herpesvirus type 1. EHV-1 was isolated for the first time in 1966 from cases of abortion and paralysis (Saxegaard, 1966). Until 1981, EHV-1 and EHV-4 were considered as two subtypes of the same virus, namely EHV-1 (Patel & Heldens, 2005). Only in 1988, the distinction between both viruses was recognized by the International Committee for Taxonomy of Viruses (Roizman *et al.*, 1992).

1.3. Taxonomy

EHV-1 belongs to the herpesviruses, which represents 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). *Herpesviridae* incorporates viruses of mammals, birds and reptiles and the members of this family share four significant biological properties: (i) they encode a variety of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins, (ii) synthesis of viral DNA and encapsidation occur in the nucleus, (iii) productive infection results in cell

destruction and (iv) they are able to remain latent in their hosts (Roizman & Baines, 1991).

The family of *Herpesviridae* is divided into three distinct subfamilies: alpha-, beta- and gamma-herpesviruses based on their differences at the level of gene content, host range, duration of the reproductive cycle, spread in cell culture, destruction of infected cells and capacity to establish latency. The *Alphaherpesvirinae* have a broad host range and a short replication cycle with a rapid destruction of infected cells. They spread rapidly in cell culture and establish latency primarily in sensory ganglionic neurons. The *Betaherpesvirinae* are characterized by a narrow host range, a slow replication cycle and a frequent enlargement of infected cells (cytomegaly). These viruses can establish latency in different tissues including secretory glands, kidneys, and lymphoreticular cells. The host-range of *Gammaherpesvirinae* is restricted and viruses remain latent in lymphoid tissues (Roizman, 1996).

In equid populations, nine herpesviruses have been identified so far, of which EHV-1, EHV-3, EHV-4, 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 (Davison *et al.*, 2009; Patel & Heldens, 2005). EHV-1 shares its classification with other animal herpesviruses of agricultural importance including bovine herpesvirus type 1 (BoHV-1), bovine herpesvirus type 5 (BoHV-5) and suid herpesvirus 1 (Pseudorabies virus, PrV) (Davison *et al.*, 2009).

Of all equid herpesviruses, EHV-1 and EHV-4 are clinically, economically and epidemiologically the most relevant pathogens. Although both viruses show a high degree of antigenic and genetic similarity, they are strikingly different in their pathogenicity. While EHV-1 infection results in respiratory disease, abortion, fatal neonatal illness and/or neurological disorders, EHV-4 infection is limited mainly to the upper respiratory tract (Allen & Bryans, 1986; Ostlund, 1993). In contrast, EHV-2, -3 and -5 are considered of less economic and veterinary importance. EHV-2 is widespread throughout the equine population and has been implicated in immunosuppression in foals, upper respiratory tract disease, conjunctivitis, general malaise and poor performance but its precise role as a pathogen is still unclear (Gleeson & Studdert, 1977). EHV-3 causes an acute and self-limiting venereal

infection of external genitalia but the prognosis for clinical recovery is generally good, although some mares and more rarely stallions can show recurrent coital exanthema (Blanchard *et al.*, 1992). EHV-5 infection has an unclear pathogenicity and distribution among the horse population. It has been reported that EHV-5 infections associate with equine multinodular pulmonary fibrosis (EMPF) but the role of EHV-5 in EMPF is still unclear (Williams *et al.*, 2007).

1.4. Virion structure

Like most herpesviruses, EHV-1 particles have a size of 200-250 nm and consist of four main structural components: genome, capsid, tegument and envelope. The general structure of a herpesvirus particle is given in Figure 1.

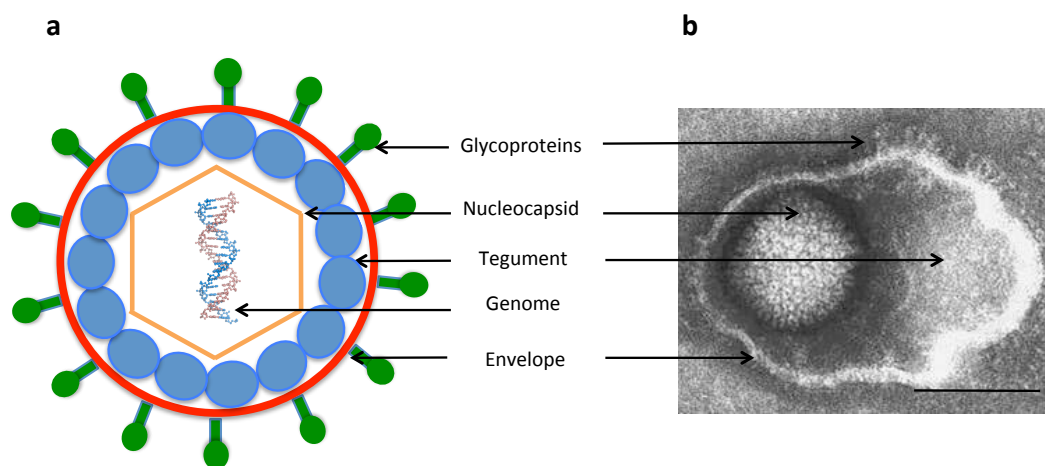


Figure 1. Structure of an EHV-1 virion. (a) Schematic representation. (b) Transmission electron microscopic image of a herpesvirus particle. The bar represents 100 nm. Image courtesy of University of Michigan Health System.

Genome

The viral genome consists of a linear double stranded DNA of 150 kbp. The complete genome contains at least 76 open reading frames (ORFs). The genome consists of a long unique region (U_L) flanked by a short inverted repeat (TR_L/IR_L) linked to a short unique region (U_S) flanked by an inverted repeat (TR_S/IR_S) (Roizman *et al.*, 1992; Telford *et al.*, 1992).

Capsid

The genome is enclosed in an icosahedral capsid consisting of 162 capsomers (12 pentons, 150 hexons) (Perdue *et al.*, 1974). Genome and capsid together form the nucleocapsid.

Tegument

The nucleocapsid is surrounded by the tegument, a proteinaceous matrix that lines the space between the nucleocapsid and the envelope. The tegument is encoded by at least 15 viral genes.

Envelope

Nucleocapsid and tegument are enclosed by an envelope that forms the outer layer of the virus. The envelope consists of an irregularly shaped bilayer of phospholipids that is derived from the *trans*-Golgi network of the host cell and in which different glycoproteins are embedded. For EHV-1, 12 glycoproteins have been characterized of which 11 have been found to be homologous to other alphaherpesvirus glycoproteins (Herpes simplex virus (HSV), PRV or BoHV). Therefore, these 11 glycoproteins have been named according to the nomenclature established for HSV (Roizman & Furlong, 1974). EHV-1 encodes an additional glycoprotein, gp2. An overview of the different EHV-1 glycoproteins with their main functions is provided in Table 1.

1.5. Cell infection

EHV-1 has a broad host spectrum *in vitro* as the virus can replicate in cultured cells of various origins, such as equine, human, swine, bovine, canine, feline and rabbit. *In vivo*, EHV-1 occasionally infects domestic cattle, captive cervids, zebras and donkeys (Abdelgawad *et al.*, 2014; Chowdhury *et al.*, 1988; Crandell *et al.*, 1988; Pusterla *et al.*, 2012; Rebhun *et al.*, 1988). EHV-1 primarily infects epithelial cells, mononuclear cells in lymphoid tissue and peripheral blood (PBMC) and endothelial cells of inner organs (Osterrieder & Van de Walle, 2010). EHV-1 can infect cells via extracellular EHV-1 virions or by direct cell-to-cell spread of EHV-1 from infected cells to uninfected cells. In contrast, the natural host range of EHV-4 is restricted to horses and replicates poorly in non-equine cells (Azab & Osterrieder, 2012).

Table 1. Overview of the EHV-1 glycoproteins and their functions. Red box indicated gp2, a viral glycoprotein that is unique to EHV-1/-4.

Glycoproteins	Function	References
gB	-Involved in attachment -Essential for virus penetration/ fusion process -Cell-to-cell spread -Antigenic properties	Cai <i>et al.</i> , 1988 Wellington <i>et al.</i> , 1996b Neubauer <i>et al.</i> , 1997 Ahmed <i>et al.</i> , 1993
gC	-Involved in initial non-essential binding of virus and in release of virions -Binds with C3 of the complement cascade -Haemagglutination activity	Osterreider <i>et al.</i> , 1999 Huemer <i>et al.</i> , 1995 Andoh <i>et al.</i> , 2015
gD	-Essential for stable binding of virus to the cell and subsequent fusion -Determine cell tropism by binding to cellular receptors -Cell-to-cell spread	Csellner <i>et al.</i> , 2000 Azab <i>et al.</i> , 2012 Wellington <i>et al.</i> , 1996a
gE	-Involved in cell-to-cell spread and virus egress -Virulent protein -Immunomodulatory properties: gE/gI complex acts as a Fc receptor?	Matsumura <i>et al.</i> , 1998 Tsujiurai <i>et al.</i> , 2009
gG	-Viral chemokine binding protein -Interference with leukocyte migration in tissues	Bryant <i>et al.</i> , 2003; Van de Walle <i>et al.</i> , 2007, 2008b Thormann <i>et al.</i> , 2012; Osterreider & Van de Walle, 2010
gH	-Fusion regulatory factor -Cellular host range and integrin binding -Cell-to-cell spread	Atanasiu <i>et al.</i> , 2010; Chowdary <i>et al.</i> , 2010 Azab <i>et al.</i> , 2013 Azab <i>et al.</i> , 2012
gI	-Forms with gE a functional entity	Matsumura <i>et al.</i> , 1998
gK	-Essential for viral egress and cell-to-cell spread -Facilitate virus penetration and syncytium formation	Neubauer & Osterreider, 2004
gL	-Forms with gH a functional entity -Important for correct folding and trafficking and function of gH	Eisenberg <i>et al.</i> , 2012 Fan <i>et al.</i> , 2009
gM	-Virus penetration and cell-to-cell spread	Osterreider <i>et al.</i> , 1996
gN	-Forms with gM a functional entity -Immunomodulatory protein (TAP-inhibiting molecule)	Verweij <i>et al.</i> , 2011
gp2	-Unique glycoprotein for EHV-1 -Virus attachment and egress -Immunomodulatory protein <i>in vivo</i>	Sun <i>et al.</i> , 1996 Rudolph & Osterreider, 2002 Smith <i>et al.</i> , 2005

1.6. Replication cycle

A general overview of the alphaherpesvirus productive replication cycle, valid for EHV-1, is depicted in Figure 2.

Viral attachment

In order to infect a cell, EHV-1 must bind to the cell surface. The initial attachment of EHV-1 is mediated by interactions of positively charged residues on EHV-1 gB and gC with heparan sulfate proteoglycans present on the cell surface, which are negatively charged (Spear & Longnecker, 2003). This interaction is relatively unspecific, reversible and serves to concentrate the virus at the cell surface. After initial attachment, the interaction with gD and a putative receptor on the cell is required to stabilize the binding and promote virus entry (Csellner *et al.*, 2000; Frampton *et al.*, 2005). The interaction with an entry receptor is highly specific and irreversible and triggers the fusion between the viral envelope and the cellular membrane. Currently, the receptor of EHV-1 has still not been clearly identified. While some studies have shown that EHV-1 uses the same viral glycoproteins utilized by other alphaherpesviruses, such as HSV-1 and PRV, for binding and entry into permissive cells, other studies have demonstrated that EHV-1 uses a unique entry receptor that is distinct from HSV-1 and PRV receptors.

To date, three classes of entry receptors for HSV and PRV gD have been identified, which include herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor family; nectin-1 and nectin-2, two members of the immunoglobulin superfamily; and a modified form of heparan sulfate called 3-*O*-sulfated heparan sulfate (Spear *et al.*, 2000). Frampton *et al.*, 2005, demonstrated that EHV-1 could still replicate in cells lacking entry receptors HVEM, nectin-1 and nectin-2, ruling out that these surface molecules are EHV-1 receptors. Other authors have identified the Major Histocompatibility Complex Class I (MHC-I) as a functional entry receptor for EHV-1 gD in equine dermal and equine brain microvascular endothelial cells (Kurtz *et al.*, 2010; Sasaki *et al.*, 2011). However, MHC-I molecules are expressed on the cell surface of all nucleated cells (David-Watine *et al.*, 1990), which makes it impossible to explain the clear, delineated tissue tropism of EHV-1 in horses. As an explanation for this inconsistency, it was suggested that not all equine MHC-I alleles serve as functional EHV-1 entry receptors and that host factors other than MHC class

I may be involved in the observed EHV-1 tropism (Sasaki *et al.*, 2011). In the meantime, a study from Azab *et al.*, 2012 showed that EHV-1 uses different receptor(s) than MHC I to infect PBMC, further demonstrating that additional research is needed to clearly establish the receptor(s) of EHV-1 on the different equine target cells.

Viral entry

Once attached, EHV-1 penetrates the cells either by direct fusion of the virus envelope with the plasma membrane at neutral pH or by non-classical endocytosis/phagocytosis pathways. Receptor engagement results in a conformational change in gD and leads to the activation of the heterodimeric gH-gL complex, which in turn primes gB for fusion (Spear & Longnecker, 2003). EHV-1 can enter cells, such as rabbit kidney epithelial cells (RK-13) and equine dermal cells (ED) via direct fusion with the plasma membrane (Frampton *et al.*, 2007). In addition, EHV-1 can enter via the endocytic/phagocytic pathway into Chinese hamster ovary cells (CHO-K1) and PBMC and α_v integrins are involved in this type of viral entry (Van de Walle *et al.*, 2008a). Moreover, EHV-1 can enter equine brain microvascular endothelial cells (but not ED cells) via caveolar endocytosis, suggesting that EHV-1 entry pathways are cell-type dependent (Hasebe *et al.*, 2009). Glycoprotein gH and cellular $\alpha_4\beta_1$ integrins are important determinants in the choice of alphaherpesvirus entry pathways (Azab *et al.*, 2013).

Irrespective of the entry route, naked EHV-1 capsids are released into the cytosol after the virus fuses with the plasma membrane/endosomal membrane. Successful infection through either mechanism requires the activation of Rho-associated coiled-coil kinase 1 (ROCK1) (Frampton *et al.*, 2007).

Viral transport to the nucleus

Consequent to fusion between the two lipid membranes, the capsid is released into the cytoplasm of the cell and transported along microtubules to the nucleus. Viral DNA is released in the nucleus via the nuclear pore complex (Padeloup *et al.*, 2009; Sodeik *et al.*, 1997).

Gene expression and replication

Upon delivery of the viral genome in the nucleus, the viral DNA circularizes and transcription is initiated. As for all alphaherpesviruses, EHV-1 transcription is tightly regulated in a cascade-manner where the immediate-early (IE), early (E) and late (L) genes are sequentially transcribed (Gray *et al.*, 1987a; 1987b; Caughman *et al.*, 1985; Purewal *et al.*, 1994). The IE protein (IEP) upregulated by a tegument ETIF protein (ETIFP) (Elliot and O'Hare, 1995) *trans*-activates early and some late genes (Kim *et al.*, 1997; Smith *et al.*, 1992; 1994), and also downregulates its own promoter (Kim *et al.*, 1995). Early genes are expressed before DNA replication and encode enzymes that are required for genome replication, DNA repair and nucleotide metabolism and a number of other proteins with yet unknown functions. Viral DNA replication occurs in the nucleus. Late genes are expressed after DNA replication has initiated and typically encode viral structural proteins, such as capsid proteins and envelope glycoproteins.

Virion assembly and egress

EHV-1 nucleocapsids are assembled in the nucleus prior to viral DNA encapsidation. The newly produced head-to-tail concatameric genomic DNA is then cleaved and packaged as unit-length molecules resulting in mature capsids (Lehman & Boehmer, 1999). Nucleocapsids leave the nucleus by budding through the inner nuclear membrane into the perinuclear space, thus acquiring a primary envelope. This primary envelope is then lost by fusion with the outer nuclear membrane, resulting in the release of naked nucleocapsids into the cytoplasm (Granzow *et al.*, 2001). The naked nucleocapsids acquire their final tegument in the cytoplasm. A second envelopment process takes place in the trans-Golgi network, which contains all viral envelope proteins. The mature virions are transported to the cell surface within secretory vesicles and released via exocytosis in the extracellular space (Mettenleiter, 2002; Mettenleiter *et al.*, 2009). The infectious virus can also directly infect other cells via cell-to-cell spread. This strategy allows the virus to escape from antibodies, complement and phagocytes. EHV-1 glycoproteins gB, gD, gE/gI, gK and gM are mainly involved in this process.

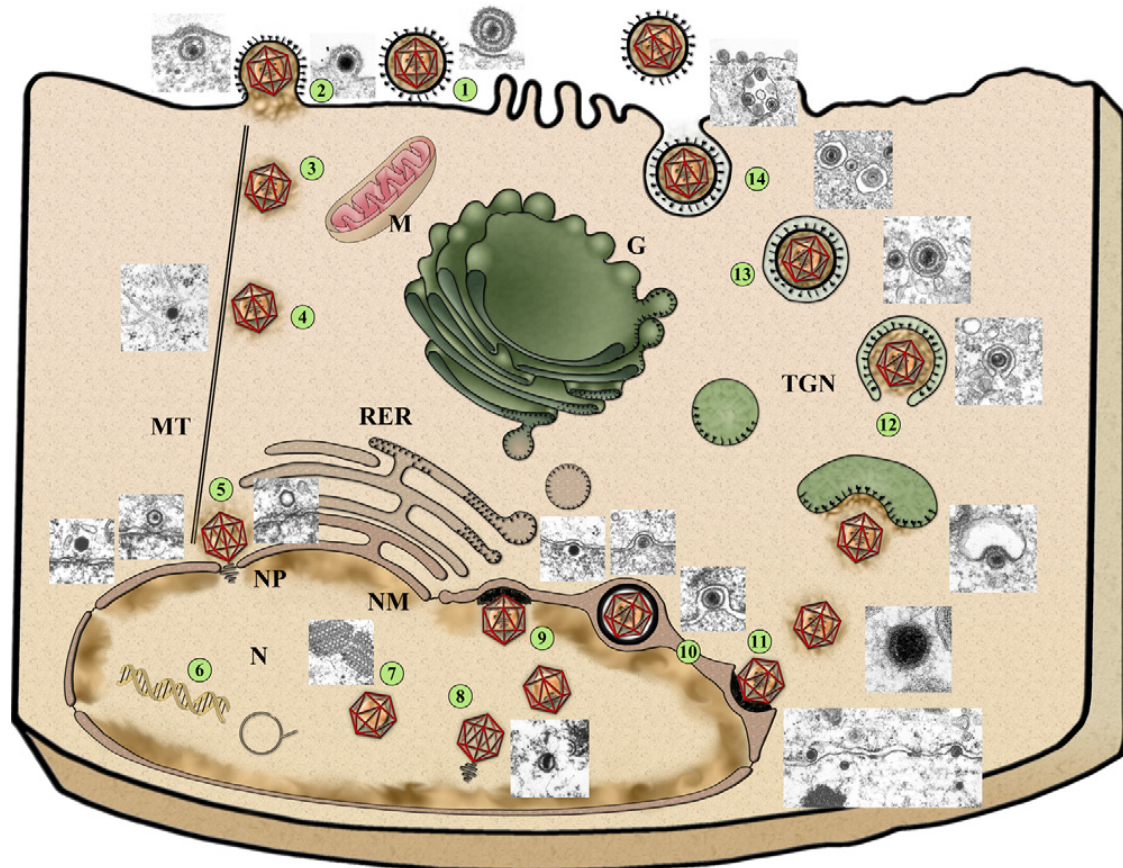


Figure 2. The replication cycle of the alphaherpesvirus pseudorabies virus (PrV), a close relative of EHV-1, associated with electron micrographs. (1) and (2) Attachment and penetration of PRV into the cytoplasm of the host cell. (3) and (4) Nucleocapsids are transported to the nucleus (N) via interaction with microtubules (MT). (5) The viral genome is released in the nucleus through the nuclear pore (NP). (6) Transcription and viral DNA replication occur in the nucleus. (7) and (8) The genome is cleaved and packaged into newly formed capsids, which leave the nucleus by budding into the inner nuclear membrane (9). (10) and (11) Nucleocapsids lose the primary envelope when passing through the outer nuclear membrane and naked nucleocapsids are released into the cytoplasm. (12) The nucleocapsids acquire their secondary envelope via budding into the trans-Golgi network (TGN). (13) The mature virions are transported to the cell surface within sorting vesicles and released via exocytosis in the extracellular space (14). Rough endoplasmic reticulum (RER); mitochondrion (M); golgi apparatus (G); nuclear membrane (NM). Adapted from Mettenleiter et al., 2009.

2. Pathogenesis of EHV-1 infection

The pathogenesis of EHV-1 is illustrated in Figure 3.

2.1. Introduction

EHV-1 is a highly contagious pathogen and is usually transmitted via direct contact with infectious secretions (saliva, nasal discharge) or via inhalation of infectious aerosols (Patel *et al.*, 1982). Fetal or placental tissues that contain large virus loads may also serve as a possible source of infection (Reed & Toribio, 2004).

2.2. Primary replication in the upper respiratory tract (URT)

Upon entry into the host, EHV-1 first replicates in a plaquewise manner in the epithelial cells lining the URT, including nasal septum, turbinates, nasopharynx, soft palate and trachea (Fig 3.1) (Gryspeerd *et al.*, 2010; van Maanen, 2002). *In vivo*, EHV-1 induced plaques were observed in the epithelium of the nasal mucosa starting from 2 till 7 days post-inoculation (dpi) (Gryspeerd *et al.*, 2010). In *ex vivo* experiments, single infected epithelial cells were visible at 12 hpi and EHV-1 induced plaques were observed starting from 24 hpi in the epithelium of equine nasal and nasopharyngeal explants (Vandekerckhove *et al.*, 2010). Primary EHV-1 infection of several tissues of the URT results in the destruction and erosion of the epithelium and in nasal shedding starting from 1 to 10-14 dpi (Edington *et al.*, 1986; Gibson *et al.*; Gryspeerd *et al.*, 2010).

Following infection of the respiratory epithelium, EHV-1 crosses the basement membrane (BM) by the use of single infected leukocytes (Edington *et al.*, 1986; Kydd *et al.*, 1994). These cells were mainly identified as CD172a⁺, cells originating from the myeloid lineage (monocytes, macrophages and dendritic cells) (Gryspeerd *et al.*, 2010; Vandekerckhove *et al.*, 2010). This invasion mechanism is distinct from other *alphaherpesviruses*, such as PRV and BoHV-1, which spread in a plaquewise manner across the BM. PRV and BoHV-1 can cause severe respiratory symptoms while EHV-1 causes a self-limited respiratory infection. It was proposed that the different

mechanisms of invasion between PRV/ BoHV-1 and EHV-1 might correlate with their different clinical outcomes (Glorieux *et al.*, 2009; Steukers *et al.*, 2012).

Similar to EHV-1 infection, EHV-4 can infect and replicate in the epithelium of the URT following direct contact with infectious secretions or via inhalation of infectious aerosols. However, EHV-4 infection remains mostly restricted to the URT and does not extend beyond the local lymph nodes (Patel *et al.*, 1982).

2.3. Replication in the draining lymph nodes and cell-associated viremia

Upon crossing the BM, EHV-1 can penetrate connective tissues and reach the bloodstream and the draining lymph nodes. Within 24-48 hours after infection, infectious virus as well as viral antigens have been detected in submandibular, retropharyngeal and bronchial lymph nodes. EHV-1 infection is amplified in the draining lymph nodes with discharge of infected leukocytes, via the efferent lymph, into the blood circulation (Figure 3.2) (Kydd *et al.*, 1994). As a result, EHV-1 initiates a cell-associated viremia in PBMC that allows it to disseminate within the host. Viremia can be detected starting from 1 dpi and persists for at least 14 days (Gryspeerd *et al.*, 2010). The cell-associated viremia is a prerequisite for the dissemination of EHV-1 to target organs such as the pregnant uterus and/or central nervous system (CNS).

During cell-associated viremia, all PBMCs subpopulations have been shown to be susceptible to EHV-1 infection both *in vivo* and *in vitro*. However, the degree of susceptibility to EHV-1 infection between PBMC subpopulations varies between studies. An early *in vivo* study using PBMC collected from EHV-1 infected horses identified the T-lymphocyte population as the primary PBMC subpopulation infected with EHV-1 during viremia (Scott *et al.*, 1983). A more recent *in vivo* study using neurovirulent and non-neurovirulent EHV-1 strains identified CD172a⁺ monocytic cells and to a lesser extent T- and B-lymphocytes as the main carrier cells of both EHV-1 strains in the blood (Gryspeerd *et al.*, 2010). A possible explanation for this discrepancy could be the choice of EHV-1 strain used and/or the method to isolate PBMC. Scott and colleagues used an American strain isolated from an aborted fetus and separated the leukocyte population by glass-bead column. Moreover, no direct characterization of the leukocyte subpopulations was performed. In contrast,

Gryspeerd and colleagues used two different Belgian strains and isolated PBMC by density centrifugation on Ficoll-Paque. Specific markers for monocytic, T- and B-cells were used to characterize PBMC subpopulations. *In vitro* studies showed that all PBMC subpopulations are susceptible to EHV-1 infection but that monocytic cells are the main target cells infected in resting PBMCs (van der Meulen *et al.*, 2000). Dendritic cells (DC) are also susceptible to EHV-1 infection *in vitro* (Siedek *et al.*, 1997; Siedek *et al.*, 1999). After mitogen stimulation, T-lymphocytes showed an increased susceptibility to EHV-1 infection (van der Meulen *et al.*, 2000). It was suggested that blastic transformation of T-lymphocytes, induced *in vitro* by mitogens, may provide a signal for the virus to start its replication, and thus lead to increased susceptibility of T-lymphocytes to EHV-1 infection. This is consistent with an early *in vivo* study from McCulloch *et al.* (1993) demonstrating an increase in the percentage of blastic cells in the blood after experimental infection with EHV-1. This might explain the earlier results of Scott's *in vivo* study that pointed to T-lymphocytes as the predominant PBMC subpopulations infected with EHV-1. Another *in vitro* study demonstrated a difference in leukocyte tropism between neuro- and non-neurovirulent EHV-1 strains (Goodman *et al.*, 2007). This is in striking contrast with the *in vivo* study of Gryspeerd *et al.* (2010). This might be due to differences in the frequency of infected PBMC between *in vitro* (10-30%) and *in vivo* situations (1-10 positive cells/10⁷ PBMC) (Goodman *et al.*, 2007; van der Meulen *et al.*, 2006). The majority of PBMC do not show viral envelope proteins on their surface during viremia (Gryspeerd *et al.*, 2012; van der Meulen *et al.*, 2003). This suggests that EHV-1 misuses these cells to spread through the body without being detected by the immune system.

In contrast to EHV-1, cell-associated viremia is extremely rare during EHV-4 infections. Thus, EHV-4 is only very rarely associated with abortion and neurological disorders (Matsumura *et al.*, 1992; Osterrieder & Van de Walle, 2010; Patel & Heldens, 2005; Verheyen *et al.*, 1998).

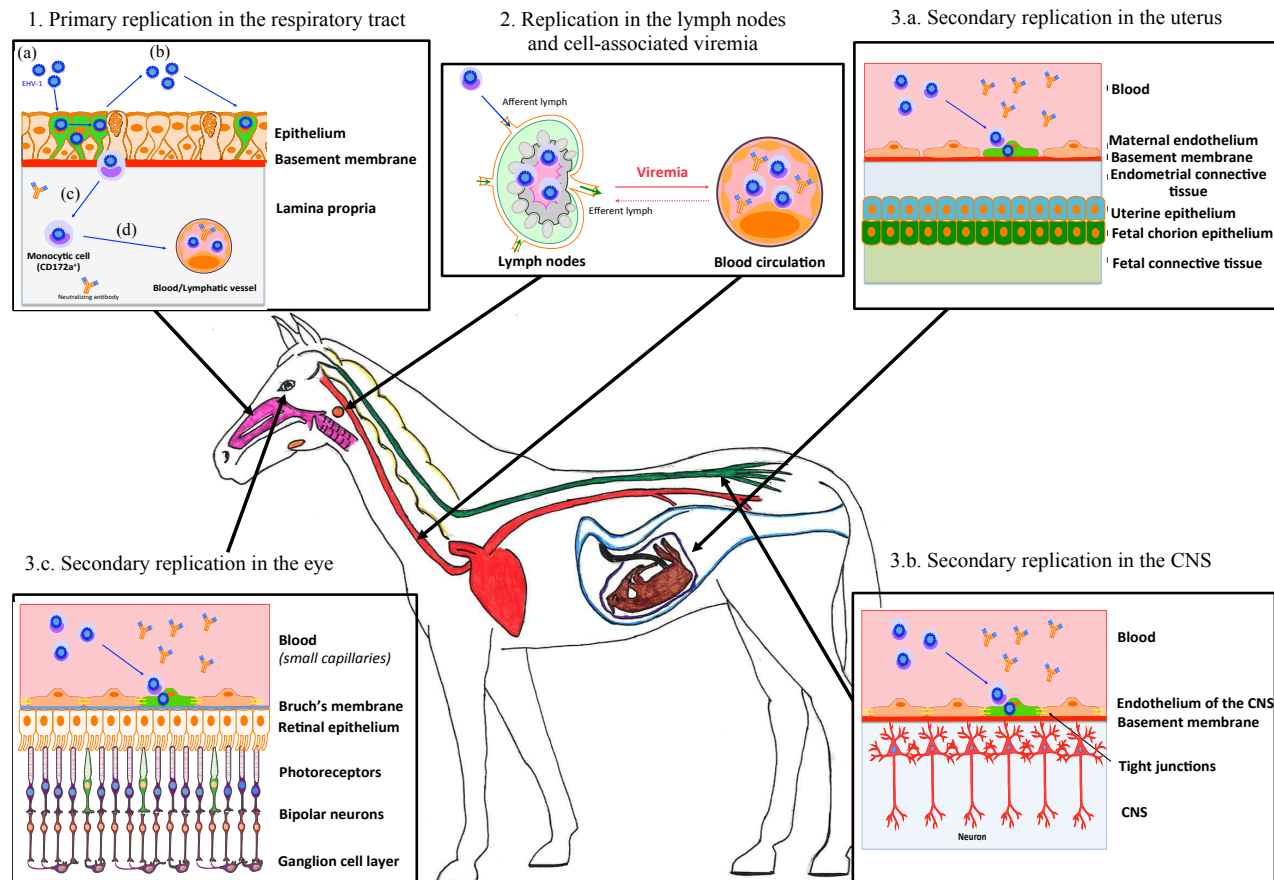


Figure 3: Schematic overview of the pathogenesis of EHV-1. (1) EHV-1 first replicates in the epithelial cells of the URT; (a) EHV-1 infection; (b) viral spread and shedding; (c) EHV-1 crosses the BM by the use of single immune cells; (d) viral dissemination (2) Upon crossing the BM, EHV-1 penetrates the connective tissues and reaches the bloodstream and draining lymph nodes. (3) Via a cell-associated viremia in PBMC, EHV-1 is transported to target organs such as the pregnant uterus (3.a), CNS (3.b) and/or eye (3.c) where it initiates a secondary replication in the endothelial cells lining the blood vessels of these organs. Pink = respiratory tract; red = blood circulation; orange = lymph nodes; yellow = vertebrae; green = spinal cord; blue = uterus.

2.4. Secondary replication in the pregnant uterus, CNS and/or eye

Once in the blood circulation, infected leukocytes can adhere and subsequently transfer EHV-1 to the endothelial cells (EC) lining the blood vessels of target organs such as the pregnant uterus or CNS. The infection of EC located in the vasculature of the late-gravid uterus or CNS is mediated by cell-to-cell contacts between infected PBMC and EC and was found to occur even in the presence of virus neutralizing antibodies (Goehring *et al.*, 2011; Smith *et al.*, 2001).

The migration of leukocytes from the bloodstream into perivascular tissue, so called leukocyte extravasation, is a dynamic process, which has been widely studied in humans. According to the current paradigm, this interaction involves the sequential engagement of leukocyte and endothelial adhesion molecules. First, selectins and their carbohydrate counter ligands mediate leukocyte tethering and rolling. Then, leukocyte integrins and their ligands, including immunoglobulin-like intercellular adhesion molecules, mediate firm leukocyte adhesion. Chemokines play a role in firm adhesion by activating integrins on the leukocyte cell surface (Chavakis *et al.*, 2009). In the final stage of leukocyte extravasation, leukocytes migrate through the endothelium into the tissues. A schematic overview of the distinct steps of the leukocyte recruitment to EC is provided in Figure 4.

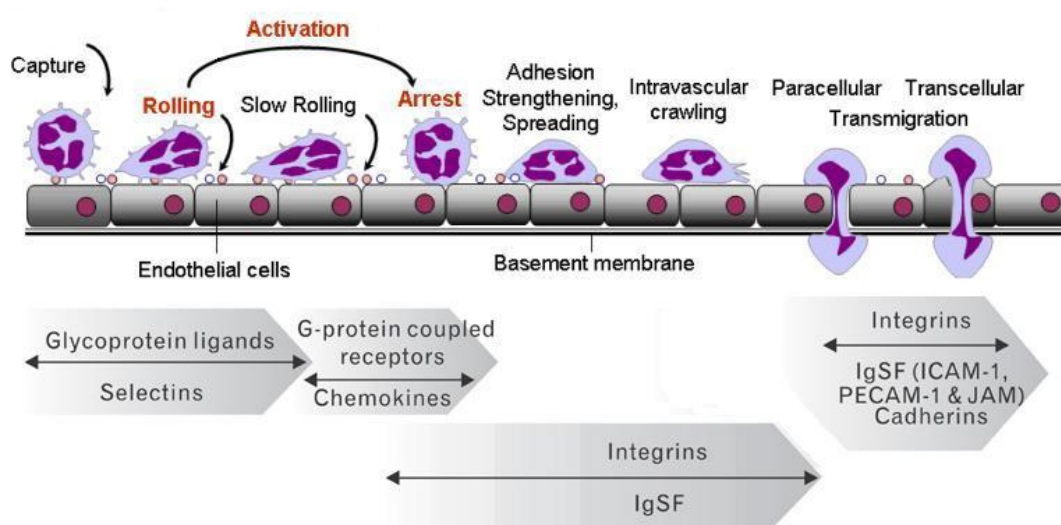


Figure 4: Schematic overview of the multistep process of leukocyte recruitment to the endothelium. Adapted from Rivera-Nieves *et al.*, 2008.

It is likely that the recruitment of leukocytes to the endothelium of target organs plays a crucial role in the pathogenesis of EHV-1. It has been suggested that activation of adhesion molecules present on both leukocytes and EC surfaces is a prerequisite for the transfer of EHV-1 from leukocytes to the endothelium (Smith *et al.*, 2001). However, the exact nature of the adhesion molecules present on the surface of both equine leukocytes and EC has not been examined yet; a task hampered by the lack of antibodies against specific adhesion molecules that cross-react with equine cells. Moreover, it is still unknown whether there are differences in the adhesion molecules expressed on EC of target organs compared to other organs or there are variations in their expression levels, which could explain why EHV-1 targets specific organs in the horse.

Secondary replication in the EC of the pregnant uterus can cause vasculitis and multifocal thrombosis that particularly affects small arteriolar branches in the glandular layer of the endometrium at the base of the microcotyledons (Fig 3.3a) (Edington *et al.*, 1991; Smith *et al.*, 1992; Smith *et al.*, 1993). This leads to avascular necrosis and edema of the endometrium. A widespread EC infection may cause detachment of the fetal membranes, thus leading to the abortion of a virus-negative fetus. Less extensive uterine vascular pathology may allow EHV-1 to invade the fetus through the uteroplacental barrier and lead to the abortion of a virus-infected fetus. In EHV-1 positive fetuses, infected EC can be detected in blood vessels of the umbilical cord and allantochorion (Smith *et al.*, 1997; Smith *et al.*, 1993). The viral aborted fetus can show multiple lesions, including multifocal hepatic necrosis, subcutaneous edema, pleural fluid accumulation, pulmonary edema and splenic enlargement (Corner *et al.*, 1963; Machida *et al.*, 1997). At late stage of gestation, transplacental EHV-1 infection could result in the delivery of a live infected foal that may die within few days.

Secondary replication in the EC lining the blood vessels of the CNS can cause vasculitis with or without local haemorrhage and thrombo-ischemic necrosis, in the brain and spinal cord (Fig 3.3b) (Edington *et al.*, 1986; Wilson, 1997). The lack of nutrients and oxygen may cause degeneration of the nervous tissues and lead to the development of equine herpes myeloencephalopathy (EHM).

Secondary replication in the vasculature of the eye can cause multifocal chorioretinal lesions in infected horses (Fig 3.3c) (Matthews, 2004; Slater *et al.*, 1992). This type

of lesion is typically caused by endothelial damage with subsequent ischemic injury to the chorioretina that may result from direct infection of the vascular endothelium following viremia.

2.5. Latency and reactivation

Latency by alphaherpesviruses is an important epidemiological strategy that ensures survival and spread of the virus within the natural host population (Whitley & Gnann, 1993). Upon lytic infection in the URT, EHV-1 enters a latent state in lymphoid tissues draining the respiratory tract, CD8⁺ lymphocytes and neurons of the trigeminal ganglia (Baxi *et al.*, 1995; Edington *et al.*, 1994; Gibson *et al.*, 1992; Slater *et al.*, 1994b; Smith *et al.*, 1998a; Welch *et al.*, 1992). The CD8⁺ lymphocytes are defined as a predominant site of latency and it is estimated that 1/50 000 PBMC are latently infected (Chesters *et al.*, 1997). The CD8⁺ lymphocytes undergo a transition from productive to latently infected cells, which usually takes between 2 to 3 weeks (Slater *et al.*, 1994a). During latency, the transcription of EHV-1 genome is restricted. Like for HSV-1, only latency-associated transcripts (LAT) accumulate in the latently infected cells and no infectious virus is produced (Roizman *et al.*, 1992). EHV-1 can be reactivated from latency upon corticosteroid treatment and natural stimuli such as stress, weaning and castration (Burrows & Goodridge, 1984; Edington *et al.*, 1985; Slater *et al.*, 1994b). Upon reactivation months or years after primary infection, EHV-1 is delivered back to the URT and can start a lytic infection depending on the local immune status of the epithelium. This can result in virus shedding with the potential to infect other horses. A cell-associated viremia is also initiated prior to or following infection of the respiratory epithelium and may result in dissemination of EHV-1 to the pregnant uterus or the CNS to cause abortions and/or neurological disorders. EHV-1 induced abortions usually occur in single mares within a group, suggesting that abortion has resulted from viral reactivation rather than from newly acquired respiratory infection (Doll & Bryans, 1963). The majority of horses (>60%) are latently infected by EHV-1 (Lunn *et al.*, 2009). Latently infected cells are masked from immune surveillance and constitute a permanent reservoir of the virus that is difficult to eliminate by conventional antiviral therapies and vaccine strategies.

Interestingly, studies have linked chromatin remodeling and histone acetylation/deacetylation events to the regulation of promoter activity, the control of viral replication and maintenance of viral latency during herpesvirus infections (Knipe & Cliffe, 2008; Sinclair *et al.*, 2006; Lu *et al.*, 2003; Danaher *et al.*, 2005). Acetylation of histones typically results in open chromatin, which allows cellular and viral transcription, while deacetylation generally causes the chromatin to close and therefore repress transcription (Figure 5). The acetylation of histones is carried out by a group of enzymes known as histone acetyl transferases (HAT) and histone deacetylases (HDAC) catalyse the reverse reaction (Berger *et al.*, 2007).

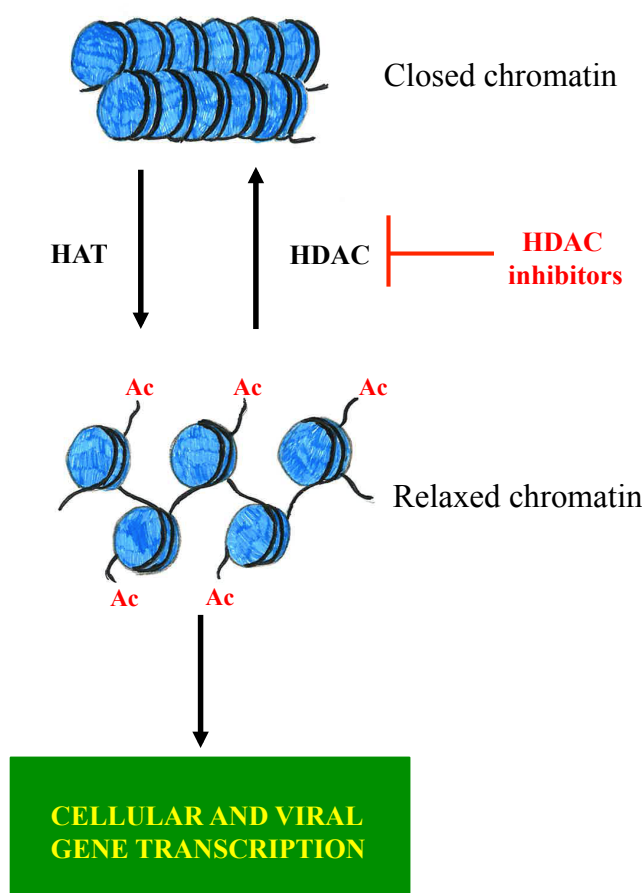


Figure 5: Chromatin remodeling by histone acetylation/ deacetylation events. HAT= histone acetyltransferase; HDAC=histone deacetylase; Ac= acetyl group.

It has been demonstrated that, during HSV-1 latency, lytic gene expression is repressed and the viral genome (with the exception of the LAT gene) associates with heterochromatin (Deshmane *et al.*, 1989). In contrast, during lytic infection, HSV-1 DNA is associated with hyperacetylated histones at promoter regions and the chromatin adopts a more disordered state (relaxed chromatin), which directly

correlates with the activation of viral transcription (Kent *et al.*, 2004). HDAC regulate HSV-1 latency directly by inducing histone deacetylation and reducing histone acetylation at HSV-1 promoter sites (Kubat *et al.*, 2004). HDAC inhibitors induce global histone acetylation resulting in HSV-1 transcription and reactivation from latency (Danaher *et al.*, 2005). So far, the control of EHV-1 replication and reactivation from latency by HDACs is largely undefined.

2.6. Factors determining the development of abortion vs EHM

The cell-associated viremia is a prerequisite for the development of severe EHV1 symptoms. The magnitude and duration of the viremia are important factors determining whether a horse will develop abortion or neurological disorders (Allen & Breathnach, 2006). However, severe symptoms do not necessarily follow viremia. During EHV-1 outbreaks, the incidence of abortions is higher (approx. 50%) than the incidence of EHM (10%) (Goehring *et al.*, 2006). A combination of viral, host and environmental factors has been proposed to contribute to the development of these severe symptoms.

First of all, it was demonstrated that a single nucleotide polymorphism (SNP) in the catalytic subunit of the viral DNA polymerase, causing a substitution of asparagine (N) by aspartic acid (D) at amino acid position 752, is significantly associated with the neurovirulent potential of naturally occurring strains (Nugent *et al.*, 2006). It was shown that more than 95% of non-neuropathogenic outbreaks were caused by virus strains encoding DNA pol N752, and more than 86% of neuropathogenic outbreaks are caused by strains encoding D752. This finding was confirmed by targeted mutation of the D₇₅₂ to the N₇₅₂ genotype in a neurovirulent isolate which resulted in attenuation of virulence, reduced levels of viremia and reduced capacity to cause neurological disorders (Goodman *et al.*, 2007). However, both viruses presented comparable peak titers of virus shedding, suggesting that both viruses can spread in a similar way into the horse population. Besides, a 23-year retrospective study of the prevalence of neurologic EHV-1 demonstrated that 24% of the EHM outbreaks are caused by N₇₅₂ EHV-1 strains and 2% of the EHV-1-induced abortions are caused by D₇₅₂ genotype strains (Perkins *et al.*, 2009).

Secondly, the development of abortion may depend on the hormonal activity and immune status of mares in late term pregnancy. Indeed, it was demonstrated that the

expression of adhesion molecules is induced by cytokines (IL-2) and hormones (17-oestradiol and equine chorionic gonadotropin) present in the local environment of late stage of pregnancy (Smith *et al.*, 2002). Moreover, the production of high levels of cortisone, progesterone and oestrogens at late stage of pregnancy may alter the immune system of the mare (Smith *et al.*, 1996). So far, the host and environmental factors determining the occurrence of EHM are still unclear. Goehring *et al.* (2006) showed that the age, breed and gender of the horse and seasonal influences (late autumn, winter and spring) might affect the risk of EHM outbreaks.

3. Clinical signs

An overview of the different symptoms following EHV-1 infection is shown in Figure 6.

3.1. Respiratory disease

Respiratory EHV-1 infection is usually mild or subclinical in adult horses. However, young horses can develop more severe clinical signs consisting of fever, nasal discharge, swelling of the submandibular and retropharyngeal lymph nodes and sometimes anorexia, depression and conjunctivitis with ocular discharge (Allen & Bryans, 1986; Coggins, 1979; Patel *et al.*, 1982). The nasal discharge can become mucopurulent, due to secondary bacterial infections, often associated with isolation of β -haemolytic streptococci. This may contribute to the development of rhinopneumonitis (Thomson *et al.*, 1979; Pusterla *et al.*, 2009; Dunowska *et al.*, 2014). The incubation period for respiratory symptoms varies between 1 to 10 dpi. Disease may occur in individual horses, or in a group of animals. EHV-4 also causes respiratory tract disease, which is clinically indistinguishable from EHV-1 (Ostlund, 1993).

3.2. Abortion

EHV-1 induced abortion typically occurs in the last trimester of pregnancy, although one case of abortion has been reported in the fourth month of gestation (Allen &

Bryans, 1986; Prickett, 1969). EHV-1 infected mares can abort without showing any clinical respiratory signs (Allen, 2002). Following viremia, the incubation period can vary between 9 days and 4 months after infection. Depending on the spread of EHV-1 infection to the endometrial blood vessels, infected mares can abort a virus-positive or virus-negative fetus. During abortion, the placenta is usually expelled together with the fetus that is often still enveloped in its amniotic membrane. The fetus usually dies from asphyxia due to the separation of the placenta from the endometrium. Fetuses that are still alive usually succumb quickly by respiratory insufficiency due to virus-induced pulmonary lesions. If infection occurs during late gestation, the fetus may be born alive, a condition known as neonatal foal disease. These foals develop jaundice and respiratory distress and mostly die within a few days (Murray *et al.*, 1998; Perkins *et al.*, 1999). Following abortion, the virus is rapidly cleared from the genital tract and the reproductive potential of the mare is usually not affected. Abortion outbreaks can occur over a period of several weeks. Due to reactivation of latent virus, mares may also abort several months or years after a primary infection (Allen *et al.*, 1999).

3.3. Neurological disease

EHV-1-induced neurological disease is not restricted by age, sex and pregnancy and can also occur in foals and stallions (Greenwood & Simson, 1980). Clinical signs can vary from mild ataxia, swaying, stumbling and falling to complete paralysis (Stierstorfer *et al.*, 2002; van Maanen *et al.*, 2001). The hind limbs are generally the most affected even though some cases of quadriplegia have been observed. Other clinical signs include bladder infection, fecal and/or urinary incontinence, head tilting, tail paralysis, oedema of testis and blindness (Borchers *et al.*, 2006; Jackson *et al.*, 1977; van Maanen *et al.*, 2001). The incubation period of EHV-1 induced neurological disorders can vary between 6 to 8 days (Jackson & Kendrick, 1971; Mumford *et al.*, 1994). The endotheliotropism of EHV-1 is central to the neurological disease (Edington *et al.*, 1986; Kohn & Fenner, 1987; Patel *et al.*, 1982; Whitwell & Blunden, 1992). EHM is the result of an inflammatory cascade (expanded levels of immune complexes and cytokines derived from cytotoxic T lymphocytes (CTL)) that is associated with EHV-1 infection of the endothelial cells of the CNS (Jackson & Kendrick, 1971; Wilson, 1997). The prognosis for non-recumbent horses is favorable.

However, horses that remain recumbent for more than 24 hours usually develop fatal complications and are euthanized.

3.4. Ocular disease

EHV-1 can also induce chorioretinopathy, causing permanent focal or multifocal lesions of the chorioretina in a substantial proportion of infected horses. Most EHV-1-induced ocular infections are subclinical but sometimes, diffuse lesions may lead to extensive retinal destruction and blindness. The frequency of EHV-1 ocular lesions varies between 50 to 90% in experimentally infected horses (Hussey *et al.*, 2013). It was proposed that EHV-1 infection of the ocular vasculature may offer an alternative model to study EHM because the vasculature of the equine ocular fundus is likely to be physiologically and anatomically similar to that of the CNS, with tight junctions analogous to the “blood brain barrier” (Matthews, 2004).

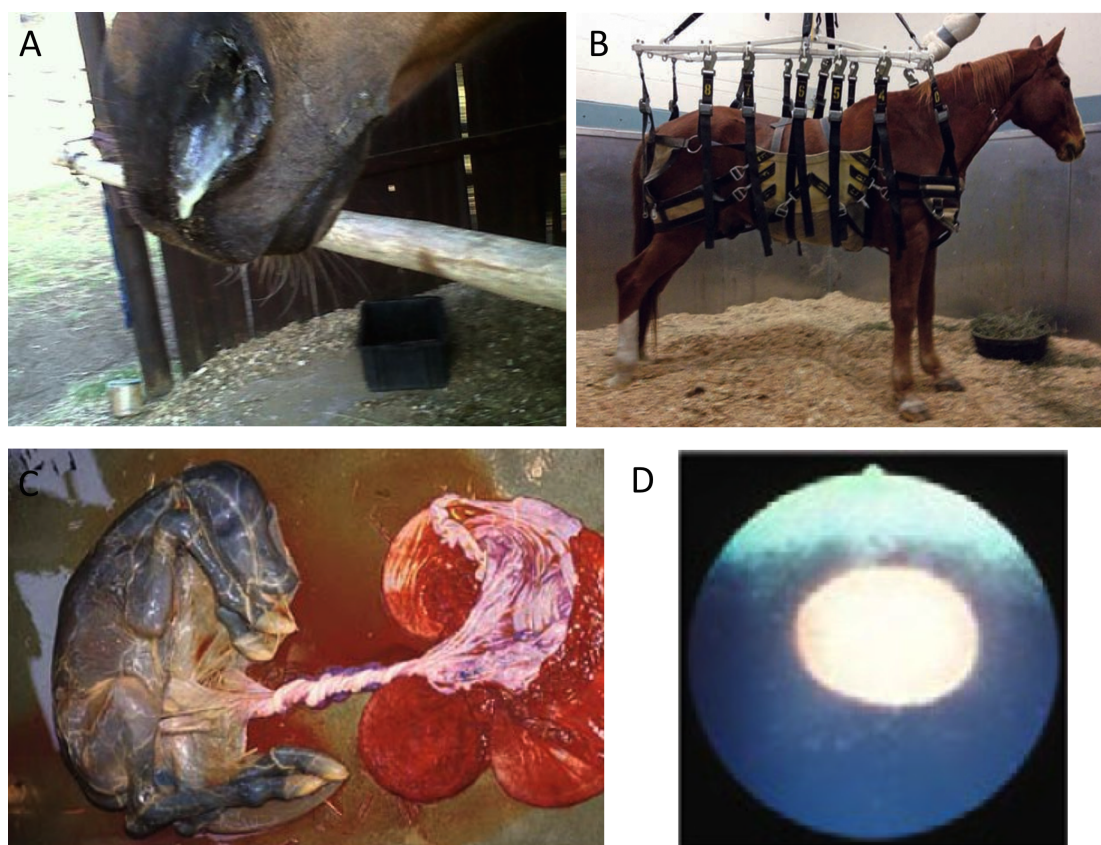


Figure 6. Clinical signs associated with EHV-1. (A) Respiratory disease (<http://www.ckequinehospital.com/>); (B) Neurological disease (<http://www.vetsonline.com/>); (C) Abortion (<http://www.ca.uky.edu/>); (D) Chorioretinopathy (Hussey *et al.*, 2013).

4. Immunity to EHV-1 infection

4.1. Introduction

Herpesviruses are experts in establishing lifelong infections of immunocompetent hosts. Understanding the immune response associated with protection against EHV-1 infection is essential to design efficient EHV-1 vaccines. Immunity to EHV-1 can be established after a natural infection or after vaccination. However, protective immunity against re-infection by EHV-1 is short-lived (4 to 8 months) (Doll *et al.*, 1955).

4.2. Innate immunity

Innate immune response mechanisms provide a first line of host defense upon viral infection and have been shown to be critically important for protection and induction of adaptive immune responses. So far, equine innate immunity to EHV-1 infection has been poorly characterized.

The respiratory mucosa is the first line of defense against EHV-1 infection. Airway epithelial cells regulate both innate and adaptive immunity through production of functional molecules and via physical interactions with cells of the immune system. They express pattern recognition receptors including toll-like receptors (TLR) that bind to pathogens and trigger the secretion of cytokines and chemokines that subsequently lead to the activation and recruitment of innate immune cells (macrophages, neutrophils, DC, natural killer cells (NK)) to the site of infection (Kato & Schleimer, 2007). EHV-1 infection of an *in vitro* equine respiratory epithelial cell system showed an increased expression of TLR3 and 9 as well as inflammatory cytokines (IL-1, TNF- α , IFN- α and IL-6) and chemokines (IL-8 and MCP-1) (Soboll Hussey *et al.*, 2014). The most effective mechanisms of the innate response against viral infections are mediated by IFN and by the activation of NK cells. Equine type 1 interferon (IFN α/β) has been detected in nasal secretions and serum during the first and second week after experimental infection of ponies with EHV-1 (Chong & Duffus, 1992; Edington *et al.*, 1989). The production of type I IFN was directly associated with the self-limited EHV-1 infection in the respiratory

epithelium and the duration of nasal shedding (Gryspeerd *et al.*, 2010). NK cells helped by DC are central components of the innate reaction to several herpesvirus infections such as HSV-1 or HCMV but it was suggested that these cells do not have an impact on EHV-1 infection (Chong & Duffus, 1992).

4.3. Adaptive immunity

The mucosal immune system consists of specialized local inductive sites such as the MALT (mucosal associated lymphoid tissue) where adaptive immune responses are likely to be initiated. Virus-specific IgA dominate the mucosal antibody response. Virus-specific IgG can also be detected in respiratory secretions but their levels rapidly decline. This is caused by inflammation-dependent exudation of virus specific serum IgG antibody into the airway lumen, which ceases abruptly upon restoration of the respiratory epithelium (Breathnach *et al.*, 2001). Serum antibody (virus neutralization, complement fixation, IgM and IgG) responses to EHV-1 infection are usually short-lived. Both virus neutralizing (VN) (mostly against gD, gB and gC) and complement fixing (CF) antibodies are elicited starting from 2 weeks after field or experimental infection with EHV-1 (Doll & Bryans, 1962; Thomson *et al.*, 1976). CF antibodies are short-lived and do not last more than 3 months while VN antibodies can last longer than a year. The role of circulating antibodies in protection is still controversial. Some studies did not find a correlation between the level of VN antibody and the level of virus shedding in nasal secretions while some others reported that the presence of VN antibodies prior to infection reduces the amount and duration of virus shedding (Gleeson & Coggins, 1980; Hannant *et al.*, 1993; Heldens *et al.*, 2001).

Still, the humoral response is generally not sufficient to efficiently protect against EHV-1 symptoms and cellular immunity mediated by CD8⁺ cytotoxic T lymphocytes (CTLs) is considered to be an essential defense mechanism against EHV-1 infection. CTLs have been shown to be important in controlling both primary and latent EHV-1 infection (Allen *et al.*, 1995; Smith *et al.*, 1998b). The frequency of precursor CTLs specific for EHV-1 antigens was found to directly correlate with protection against disease (Kydd *et al.*, 2003; O'Neill *et al.*, 1999). EHV-1 specific CTLs remain detectable for more than a year after infection (Allen *et al.*, 1995). CTL can recognize antigen that are presented by polymorphic MHC I receptors expressed on all

nucleated cells. It was demonstrated that the IE gene elicits CTL responses by encoding a protein that contains CTL epitopes and the CTL activity was found to be restricted to the ELA-A haplotype (Soboll *et al.*, 2003). Although CTL activity was found to be efficient in controlling cell-associated viremia and in reducing severe symptoms, EHV-1 can still cause viremia in the presence of virus-specific antibodies and CTL-precursors in the host (Kydd *et al.*, 2006a; O'Neill *et al.*, 1999). This suggests that EHV-1 uses specific immune evasive strategies to persist in its host (See Chapter 1.8).

5. Diagnosis

Since EHV-1 is a highly contagious pathogen, rapid diagnostic tests for the detection of EHV-1 infections are critical to guide management strategies, especially during outbreaks of abortions and EHM. In virological laboratories, diagnosis of EHV-1 infections is commonly based on virus culture and isolation followed by immunoidentification of the isolated virus. Virus can be isolated from nasal and nasopharyngeal swabs collected during the early stages of the respiratory disease, from unclotted blood samples or from fetal tissues (liver, lung, thymus and spleen) collected from aborted fetuses. EHV-1 can be isolated on a variety of cell lines (such as rabbit kidney epithelial cells (RK-13), ED cells, equine embryonic lung cells (EEL)) and a positive EHV-1 isolation is characterized by the appearance of cytopathic effects (CPE) in inoculated cultures within 7 days. As EHV-1 and EHV-4 infections can both cause CPE in equine cell cultures, positive identification of virus isolates is usually performed by immunofluorescence with type-specific monoclonal antibodies. Several rapid diagnostic techniques based on enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) or immunohistochemical staining with peroxidase are also available for detection of EHV-1 infection with their own advantages and limitations (Drummer *et al.*, 1995; Varrasso *et al.*, 2001; Whitwell *et al.*, 1992). They can supplement traditional diagnostic methods but are often restricted to specialized reference laboratories.

Serological diagnosis of recent infection by EHV-1 is based on a 4-fold or greater increase in antibody titers in paired sera taken during acute illness and 2-3 weeks later during the convalescent stage of the disease (Kydd *et al.*, 2006b). The 'acute phase' sera from mares after abortion or from horses with neurological disorders may already contain maximal titers of EHV-1 antibody with no increase in titers detectable in sera collected at convalescence. However, serological testing of paired serum samples from clinically unaffected horses in the herd may provide useful information for the retrospective diagnosis of EHV-1 within the herd. Serological tests include serum-neutralization test (SN), ELISA or complement-fixation (CF) tests but these tests do not distinguish between antibodies to EHV-1 and EHV-4. Thus, a type-specific ELISA has been developed which is based on differences in the C-terminal portion of the glycoprotein G of both viruses (Crabb *et al.*, 1995).

6. Treatment

Currently, there is no efficacious antiviral treatment available for EHV-1 infections and treatment is mainly limited to supportive therapy. Studies assessing the efficacy of standard anti-herpesvirus drugs, including acyclovir or its derivative valacyclovir, which has improved oral bioavailability, did not show any reduction in clinical signs, viral shedding and viremia of EHV1-infected ponies, even after continuous administration at high doses (Garré *et al.*, 2009). However, it has been reported that (1'S,2'R)-9-[[10,20-bis(hydroxymethyl) cycloprop-10-yl]methyl]guanine (A-5021), a guanosine analogue that possesses anti-herpetic activity against most human herpesviruses could inhibit EHV-1 replication both *in vitro* and *ex vivo* (Glorieux *et al.*, 2012). No EHV-1 induced plaques were observed in the epithelium of infected nasal explants and no single infected mononuclear cells were detected below the BM. Thus, it was proposed that therapy with A5021 might reduce respiratory disease and protect against viremia. The efficacy of A5021 against EHV-1 infection still needs to be evaluated in the horse.

An alternative therapeutic approach is the development of RNA interference (RNAi) mediated by small inhibitory RNA (siRNA). siRNA's have recently been intensively examined for their ability to prevent and/or treat viral infections. siRNA's bind to

complementary target mRNA and, upon interaction with the cellular RNA interference mechanisms, specifically target these sequences for degradation, resulting in inhibition of protein expression (Dykxhoorn & Lieberman, 2006).

Interestingly, synthetic siRNAs against envelope gB and the origin-binding protein helicase were designed, and it was reported that RNA interference reduced plaque formation in cell cultures, clinical signs (weight loss) and viral replication within lung tissue and in a murine model of EHV-1 infection (Fulton *et al.*, 2009). Thus, siRNA treatment of in contact or exposed horses could be applied during an outbreak of EHV-1 to reduce virus shedding within the herd and could be a promising complementation to vaccination. It is believed that treatment costs for siRNA would be similar to most protein-based therapies like antibodies.

Although it has been reported that metaphylactic intranasal administration of siRNA targeted against critical EHV-1 genes did not reduce clinical disease, nasal shedding of virus and viremia in horses experimentally infected with EHV-1, euthanasia necessitated in case of neurological disease was significantly reduced after application of EHV-1 specific siRNA (Brosnahan *et al.*, 2009). Still, some caution should be taken in the interpretations of these results and the experiments should be reproduced in which host factors are more intensively monitored and controlled in order to provide additional support for the effectiveness of RNA interference as a suitable therapy to combat EHV-1 infections.

The medical treatment of horses with EHM mainly focuses on decreasing the inflammation associated with the induced vasculitis. The use of corticosteroids and anti-inflammatory drugs for a short duration has been recommended in EHM treatment, although no data are available that demonstrate their potential efficacy.

7. Control and prevention

7.1. Management

Control measures for managing herpesviruses in horses aim at: (1) preventing or reducing the likelihood of an outbreak of abortion or neurological disease; (2) limiting the spread of EHV-1 within the horse population.

Recommended herd management practices for prevention of abortion or neurological disorders in pregnant mares have been published as a 'code of practice' (Allen, 2002) (codes.hblb.org.uk; <http://www.aaep.org/>), which is updated annually and described by the acronym SISS. The actions recommended are divided into 4 categories:

- (a) Segregation of the pregnant mare population from all other horses from the premises.
- (b) Isolation of new mares arriving on the farm for a period of no less than 3 weeks (quarantine).
- (c) Subdivision of the pregnant mares into small foaling groups by stage of gestation.
- (d) Stress reduction by avoiding physical stress such as prolonged transportation of mares in late stage of pregnancy, weaning and social disruption.

Recommended measures to limit the spread of EHV-1 following an outbreak of EHV-1 abortion or neurological disease are described by the acronym DISH and consist of:

- (a) Disinfection of the contaminated area of the aborted foetus and cleaning of the aborting mare.
- (b) Isolation of the aborting mare from other horses, particularly mares in late stage of pregnancy.
- (c) Submission of clinical samples for laboratory diagnosis.
- (d) Implementation of hygienic procedures to restrict the spread of infection.

7.2. Vaccination

Vaccination is one of the most cost-effective approaches to prevent EHV-1 related disease, in combination with management measures. Successful vaccination should aim to: (1) reduce viral replication in the URT, thus minimizing viral shedding and respiratory symptoms, (2) reduce/eliminate cell-associated viremia and limit the reactivation of the virus, thus preventing the development of secondary severe symptoms (abortion and/or neurological disease) (van der Meulen *et al.*, 2007). Vaccination against EHV-1 infection requires both humoral and cellular immune responses.

Over the last 50 years, extensive research has aimed at the development of efficient vaccines including inactivated vaccines, traditional modified live virus (MLV) vaccines, recombinant live vaccines, live-vectored vaccines and DNA vaccines (Minke *et al.*, 2004). So far, none of the commercially available vaccines provide full protection against EHV-1 infection. While current vaccines can reduce viral shedding and the severity of respiratory symptoms upon challenge infection of vaccinated horses, they do not guarantee full protection against viremia and severe EHV-1 symptoms (Burrows & Goodridge, 1984; Goodman *et al.*, 2006; Heldens *et al.*, 2001). Here, we will briefly review the potential and limitations of the current commercially available vaccines and experimental vaccines in the prevention of EHV-1 infection.

Current vaccines

Inactivated EHV-1 vaccines have been the main type of vaccine commercially available in Europe and in the United States. Inactivated vaccines either contain inactivated whole virus or viral envelope glycoproteins, in combination with an adjuvant. Subunit and inactivated vaccines are characterized by their absence of pathogenicity and virus replication. Inactivated vaccines can stimulate high titres of serum neutralizing antibody, which can reduce the amount and duration of virus shedding and can also prime the mucosal compartment (Breathnach *et al.*, 2001). However, these vaccines fail to stimulate CTL responses, which have been associated with protection against EHV-1 infection (Kydd *et al.*, 2003).

In the 80-90s, studies examined the efficacy of Pneumabort-K vaccine, a whole virus inactivated and oil-adjuvanted vaccine containing EHV-1, administered to pregnant mares, yearlings and two-year old ponies (Burrows & Goodridge, 1984) (Burki *et al.*, 1990). None of these studies demonstrated a significant reduction in clinical signs, viremia and EHV-1-induced abortions between vaccinated and control mares. However, a study from Goehring *et al.* (2010) demonstrated a significant reduction in the number of days of viremia upon EHV-1 challenge of vaccinated ponies.

Several vaccination/challenge studies have also been performed in pregnant mares and naïve foals with Duvaxyn 1,4, a whole inactivated virus and carbomer-adjuvanted vaccine containing EHV-1 and EHV-4 (Foote *et al.*, 2002; Heldens *et al.*, 2001). Duvaxyn 1,4 was shown to induce both VN and CF antibody responses in foals and pregnant mares after 1 or 2 vaccinations. After experimental infection,

clinical signs and the duration of virus shedding were reduced in vaccinated pregnant mares and foals while the duration of cell-associated viremia was only reduced in vaccinated foals. In addition, the incidence of EHV-1 induced abortions was reduced in vaccinated mares. However, responses to inactivated EHV-1 vaccines are variable in the field. EHV-1 has been shown to circulate in vaccinated populations of mares and their unweaned foals in several countries (Foote *et al.*, 2006a; Foote *et al.*, 2004). Recently, Bresgen *et al.* (2012) demonstrated a reduction in the number of abortions in vaccinated mares. In Belgium, an EHV-1 inactivated vaccine is currently marketed as Equip 1,4 (Zoetis).

Besides inactivated vaccines, live attenuated vaccines are also used to prevent EHV-1-induced disease. These vaccines offer several advantages, namely the induction of a cell-mediated immune response (CTL response), relatively long duration of immunity and the induction of local mucosal immunity. However, the risk of reversion to virulent EHV1 strains remains a concern for this strategy of vaccination. Prevaccinol is a commercial live-attenuated vaccine in Europe (Merck MSD-Intervet, Munich, Germany) developed by attenuation of the RacH strain upon serial passages on swine embryonic kidney cells. This live virus was passaged further in rabbit kidney cells and then equine dermal cells and licensed as Rhinomune in the United States (Boehringer Vetmedica, Inc., St Joseph, MO). These live attenuated vaccines have a good safety record and can protect horses against clinical disease, but their efficacy in preventing viremia, abortion and neurological disease are unclear (Kydd *et al.*, 2006b). Protection against neurological disease under experimental conditions and abortions under field conditions has been reported by using these type of vaccines (Bresgen *et al.*, 2012; Goodman *et al.*, 2006).

Experimental vaccines

Novel vaccines, which are not commercially available yet, have been designed and tested to prevent EHV-1 infection. Similar to RacH, a vaccine based on the live-attenuated Kentucky A (KyA) strain was obtained by serial passage on murine fibroblast cells. This vaccine could only reduce the duration of viremia in vaccinated horses upon challenge (Matsumura *et al.*, 1996). A temperature sensitive live-attenuated EHV-1 vaccine was also developed based on a German abortion isolate. Upon vaccination, virus shedding, cell-associated viremia and mild clinical signs were observed but the protection efficacy against subsequent challenge was not

consistent in different studies (Patel *et al.*, 2003a; Patel *et al.*, 2004; Patel *et al.*, 2003b). Vaccination with an EHV-1 modified live vaccine virus based on a recent non-neurovirulent strain, NY03, showed promising results in terms of reduction of clinical signs, nasal shedding and viremia levels in horses (Van de Walle *et al.*, 2010). By deleting virulence-associated genes, several EHV-1 mutants were constructed and tested for vaccine efficacy. A mutant lacking the thymidine kinase (TK) gene was unable to prevent viremia after EHV-1 challenge (Slater *et al.*, 1993; Tewari *et al.*, 1993). A live attenuated vaccine based on a gE⁻/gI⁻ mutant was unable to reduce the amount and duration of viremia (Matsumura *et al.*, 1998). Later, a vaccination trial with a gE⁻ strain showed a reduction of respiratory symptoms, load of viral shedding and viremia in ponies upon challenge (Tsujimura *et al.*, 2009). Novel inactivated vaccines based on the expression of EHV-1 specific glycoproteins presented by immune stimulating complexes (ISCOM) have also been designed. Mares and foals vaccinated with a subunit vaccine containing both gB and gD mixed with the ISCOM reduced virus shedding but not viremia after experimental infection (Foote *et al.*, 2006b). A recombinant canarypox-based vaccine expressing EHV-1 gB, gC, and gD genes markedly reduced virus shedding after challenge but failed to protect against cell-associated viremia (Minke *et al.*, 2006). A recombinant poxvirus vector encoding the IE protein, a known CTL target protein, could stimulate cell-mediated immune responses but failed to protect against challenge infection (Paillot *et al.*, 2006). DNA vaccination with plasmids coding for gB, gC, gD and IE were shown to induce a limited immune response and protection against EHV-1 challenge of vaccinated ponies (Soboll *et al.*, 2006).

Overall, none of the current and experimental vaccines can completely eliminate virus shedding and/or cell-associated viremia, and consequently, fully protect against EHV-1 induced abortion and neurological disease. Up till now, inactivated vaccines are the only vaccines that claim protection against EHV-1 induced abortions if administered during pregnancy at fifth, seventh and ninth months of gestation (Heldens *et al.*, 2001). Foals should be vaccinated over 3-5 months of age, with a second immunization 4 to 6 weeks later and a third dose at 10-12 months. As immunity following vaccination is short-lived, it is recommended to re-vaccinate the animal at 6-month intervals. No vaccines that claim protection against EHV-1-induced neurological diseases are available on the market. This is partially due to the lack of reliable experimental models that can induce neurological disorders in horses.

Moreover, some studies have reported vaccination as a potential risk factor for the development of EHM, supporting the theory of an immune-mediated pathogenesis for EHM (Henninger *et al.*, 2007). Despite the controversy, it is important to be clear that general vaccination at herd level plays an important role in reducing viral shedding, limiting spread of EHV-1 infection and enhancing herd immunity (Pusterla & Hussey, 2014). The induction of protective immunity against EHV-1 infection still remains a challenge and is likely caused by immunomodulatory properties of the virus. Thus, future vaccine strategies may benefit from a better understanding of EHV-1 viral immune evasive strategies and should focus on stimulating both CTL responses and mucosal/plasma VN antibodies.

8. Immune evasive strategies of EHV-1

8.1. Introduction

One strategy mastered by all herpesviruses, is the capacity to establish life-long latency in immunocompetent hosts. Herpesvirus persistence is facilitated by dedicated viral immune evasion mechanisms that evolved during their long history of co-evolution with the host. This sub-chapter will give a brief overview on the recent knowledge of the specific immune evasive strategies used by EHV-1.

8.2. Evasion from humoral immunity

Following infection, viral glycoproteins are usually expressed at the plasma membrane of the infected cells. Virus-specific antibodies can recognize viral antigens on the cell surface and thus, the antibody-dependent immune system (complement-mediated cell lysis and cell-mediated cytotoxicity) can clear the infected cells. However, EHV-1 is able to interfere with antibody-dependent lysis by hampering proper viral protein expression on the cell surface.

Both *in vitro* and *in vivo* studies have shown that the majority of EHV-1-infected PBMCs do not express viral glycoproteins on their cell surface (van der Meulen *et al.*, 2006; van der Meulen *et al.*, 2003). Only IE and some E proteins were expressed

in the cell nuclei. Later, a study from Gryspeerdt et al. (2012) showed that the expression of late gC and gD protein was seriously hampered in EHV-1 infected leukocytes both *in vitro* and *in vivo*. These studies indicate that there is an early block(s) in the replication cycle of EHV-1 in target cells that may protect infected leukocytes from efficient recognition by the immune system and hence allow these carrier cells to reach target organs. This has been already described for other herpesviruses such as pseudorabies virus (PRV) and human cytomegalovirus (HCMV) (Nauwynck & Pensaert, 1994; Rice *et al.*, 1984). Contact between PBMC and endothelial cells has been suggested to provide signals to trigger the late phase replication of EHV-1 in PBMC (van der Meulen *et al.*, 2006).

However, the minority of PBMC that expressed viral glycoproteins at their cell surface was still resistant to the antibodies and complement, indicating that EHV-1 uses another strategy to evade complement pathway activation (van der Meulen *et al.*, 2003). Indeed, EHV-1 gC has been reported to bind C3, the pivotal component of the alternative complement cascade, with highest affinity to C3 of the natural host (Huemer *et al.*, 1995). This alternative pathway can be activated in the absence of antibodies and is crucial during early infection when humoral immunity is not achieved. Binding of gC on EHV-1 virions with C3 results in inhibition of downstream events of the alternative complement pathway, thus protecting EHV-1 from complement-mediated cell lysis. Similar findings were also described for gC of HSV-1, EHV-4, BoHV-1 and PrV (Azab *et al.*, 2010; Huemer *et al.*, 1992; Huemer *et al.*, 1993).

The expression of a viral Fc receptor, consisting of the viral gE/gI complex, has been widely studied for HSV-1 and PRV, as part of an immune evasion from antibody-dependent immunity. Binding of gE/gI to the constant portion of IgGs (Fc) tethers the antibody in a position where it cannot trigger downstream immune functions (steric hindrance) and also participate in antibody bipolar bridging (ABB) such that the Fabs bind a viral antigen and the Fc binds gE-gI. This gE/gI complex of HSV-1, PRV, but not BoHV-1, has been shown to protect infected cells from complement-mediated cell lysis (Favoreel *et al.*, 1997; Nagashunmugam *et al.*, 1998; Whitbeck *et al.*, 1996; Whitley & Gnann, 1993). So far, it is not clear whether the gE/gI complex of EHV-1 interferes with Fc-mediated activities.

8.3. Evasion from CTL immunity

Another aspect of immune evasion activity of herpesviruses is their ability to interfere with CTL-mediated cell lysis. This is clearly illustrated by the fact that EHV-1 can cause a viremia in the presence of virus-specific antibodies and CTL precursors (Kydd *et al.*, 2006b; O'Neill *et al.*, 1999). CTLs form an essential part of the immune defense against many virus infections. CTL-based immunity is dependent on the efficient recognition of viral peptides presented by MHC-I on the infected cell surface (Townsend & Bodmer, 1989). Upon infection, viral proteins are degraded in the cytoplasm by the proteasome and transported into the ER via the transporter associated with Ag processing (TAP). In the ER, the peptides are loaded to the MHC-I α chain associated with β 2-microglobulin (β 2M) and form stable tri-molecular complex that are expressed on the cell surface (Androlewicz *et al.*, 1993; Ortmann *et al.*, 1994). This complex can be recognized by CTL, which will induce lysis of the infected cells (Williams *et al.*, 2002).

Like other alphaherpesviruses, EHV-1 has been reported to down-regulate MHC-I cell surface expression in equine cells (Ambagala *et al.*, 2004; Rappocciolo *et al.*, 2003). EHV-1 has been shown to down-regulate MHC class I via two classes of genes, IE and E, which enhance endocytosis of MHC-I molecules (Rappocciolo *et al.*, 2013). In addition, Ambagala *et al.* (2004) demonstrated that an early EHV-1 protein was responsible for the down-regulation of MHC-I molecules by interfering with the peptide transport activity of TAP. This down-regulation directly resulted in a reduced availability of peptides in the ER and thus, in a reduced maturation and cell surface presentation of tri-molecular complexes (peptide/MHC-I/ β 2M). The EHV-1 pUL49.5, pUL56 and pUL43 proteins have been shown to modulate cell surface MHC-I expression (Koppers-Lalic *et al.*, 2005; Ma *et al.*, 2012, Huang *et al.*, 2015). EHV-1 pUL49.5 is a small type I transmembrane protein that interacts with gM and inhibits the formation of peptide-loaded MHC-I molecules by preventing ATP binding to TAP. pUL56, a type II transmembrane protein, cooperates with pUL43 to enhance the internalization of MHC-I through dynamin-dependent endocytosis (Huang *et al.*, 2014, 2015). EHV-1 infection also resulted in down-regulation of MHC-I molecules in infected PBMC *in vitro*. However, after an *in vivo* infection, no difference in the level of MHC-I expression was observed between infected and control ponies (van der Meulen *et al.*, 2003). The absolute percentage of MHC-I

positive PBMCs was determined rather than the amount of MHC-I per infected cell, which could explain this discrepancy.

After an *in vivo* infection, EHV-1 was also shown to inhibit T cell responses (Hannant *et al.*, 1991). The immunosuppressive activity was associated with the presence of soluble TGF β 1 in the serum of infected ponies (Charan *et al.*, 1997). Another study showed that the immunosuppression of T cell function was associated with circulating PBMC and/or their products rather than circulating soluble factors such as antigen or immune complexes (Hannant *et al.*, 1999).

8.4. Evasion from NK cell activity

Herpesviruses downregulate surface MHC class I molecules in order to avoid recognition by CTL. However, the loss of MHC class I molecules renders the cells susceptible to NK cells. The activation of NK cells is regulated through the integration of signals delivered by inhibitory and/or activating receptors, where particular MHC-I molecules represent important ligands for inhibitory NK cell receptors (Biassoni *et al.*, 2001). EHV-1 may be able to evade detection by and activation of NK cells by lowering the concentration of some MHC-I molecules on infected cells and by affecting the expression of MHC-I molecules in an allele-specific manner, as has been suggested for PRV (Rappocciolo *et al.*, 2003; Sparks-Thissen & Enquist, 1999).

8.5. Interference with cytokine and chemokine responses

Viral infection stimulates the production of cytokines and chemokines that play a crucial role in the regulation of innate and adaptive immune response (Guidotti & Chisari, 2000). It is not surprising that herpesviruses, including EHV-1 divert the cytokine/chemokine responses for their own benefit (Alcami, 2003). EHV-1 gG has been identified as a chemokine binding protein (vCKBP), which binds to a broad range of chemokines, such as CXCL1 and IL-8, with high affinity and subsequently blocks their activity (Bryant *et al.*, 2003). EHV-1 gG deleted mutant was shown to induce a more pronounced inflammatory response in horses compared to the wild-type virus (von Einem *et al.*, 2007). In addition, a study from Van de Walle *et al.*,

(2008b) demonstrated that EHV-1 gG was able to inhibit migration of equine neutrophils in response to equine recombinant IL-8.

Interestingly, no chemokine binding activity has been attributed to the gG protein of EHV-4 despite its 58% identity with the gG protein of EHV-1. It has been suggested that the immunomodulatory property of EHV-1 gG may contribute to its ability to cause systemic infection in contrast to EHV-4 (Bryant *et al.*, 2003; Van de Walle *et al.*, 2007).

8.6. Cell-to-cell spread

Enveloped viruses can spread via two distinct routes, either through the cell-free aqueous environment or by direct cell-cell contact. While transmission by cell-free virus can allow the spread across long distances within the infected host, viruses often rely on cell-to-cell spread to bypass several barriers (antibody-mediated immune responses, anti-viral restriction factors) present in the cell-free path for efficient spreading.

The intraneuronal spread of HSV is an essential component in disease pathogenesis. For instance, HSV-1 can spread in both directions along neurons, and their capsids can undergo anterograde and retrograde transport along microtubules (Smith *et al.*, 2001). Bidirectional transport along neurons allows herpesvirus to reach ganglia to establish latency. Upon reactivation, viruses can travel back to the periphery at the primary site of infection. Glycoproteins gH and gL, along with gB and gD, are required for cell-to-cell spread of HSV-1 (Even *et al.*, 2006). The complex gE/gI is required for efficient spread of HSV-1 in epithelial cells and neuronal tissues (Dingwell *et al.*, 1994; Dingwell *et al.*, 1995). In addition, gK has also been shown to play an essential role in cell-to-cell spread in corneal and trigeminal ganglia cells (David *et al.*, 2008).

Like other herpesviruses, EHV-1 can spread from cell-to-cell and this mode of transmission plays a crucial role in the pathogenesis of EHV-1 infections. Cell-to-cell contacts between PBMC and EC of the pregnant uterus and CNS are essential for the transfer of EHV-1 in the presence of neutralizing antibodies (Goehring *et al.*, 2011; Smith *et al.*, 2001). The glycoproteins gB, gD, gE/gI, gK and gM are mainly involved in this process (Table 1).

8.7. Conclusions

EHV-1 has evolved multiple immune evasion strategies to persist in its host. This helps to explain the short-lived immunity after primary infection and the inefficacy of current vaccines to prevent EHV-1 infection. Thus, a better understanding of the interplay between EHV-1 and the host immune system is crucial for a rational design of vaccination strategies.

9. References

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Chapter 2.

Aims of the thesis

After primary replication in the epithelial cells of the upper respiratory tract, EHV-1 disseminates through the body via a cell-associated viremia in PBMC to target organs such as the pregnant uterus or central nervous system. Secondary replication in the endothelial cells lining the blood vessels of those organs can cause vasculitis and ischemic thrombosis and may lead to severe clinical signs such as abortion and/or neurological disorders. Up till now, commercially available (inactivated and live-attenuated) vaccines do not provide full protection against EHV-1 associated disease. These vaccines fail to completely prevent virus shedding and cell-associated viremia, and consequently fail to protect against severe EHV-1 signs. Indeed, EHV-1 can cause a viremia despite the presence of virus-neutralizing antibodies and CTL precursors. Like other herpesviruses, EHV-1 has developed multiple strategies to evade antibody- and cell-mediated immunity in order to survive and spread in its host. It is clear now that a detailed understanding of the complete pathogenesis of EHV-1 and its immune evasive strategies are needed to design efficient and adequate vaccines as well as antiviral therapeutics.

In vivo and *ex vivo* studies have identified CD172a⁺ monocytic cells as one of the main carrier cells of EHV-1 in the URT and blood. In addition, it was shown that the expression of particular late proteins in EHV-1-infected PBMC was hampered at early stages of infection. Based on these results, the general aim of this thesis was to investigate in detail *in vitro* how EHV-1 hijacks specific CD172a⁺ monocytic cells and modulates its replication in order to disseminate within the host.

Therefore the specific aims of this thesis are presented below.

- The first aim was to examine EHV-1 replication kinetics in equine CD172a⁺ monocytic cells from the nasal mucosa and blood, and compare these with replication kinetics in rabbit kidney epithelial cells (RK-13), a cell line known to be fully susceptible to EHV-1 infection (Chapter 3).
- Because in Chapter 3 a restriction of EHV-1 replication in CD172a⁺ monocytic cells was observed, the second aim of this thesis was to investigate whether this restriction occurred at the level of binding and entry (Chapter 4).

- Third, to determine the mechanism(s) underlying the transmission of EHV-1 from CD172a⁺ monocytic cells to endothelial cells (EC). We studied the ability of EHV-1 inoculated CD172a⁺ cells to adhere and subsequently transmit EHV-1 infection to equine venous EC in the presence of virus neutralizing antibodies (Chapter 5).

Chapter 3.

Replication of EHV-1 in CD172a⁺ monocytic cells

*A. Replication of non-neurovirulent EHV-1 is delayed in
CD172a⁺ monocytic cells and controlled by histone
deacetylases*

Adapted from

Laval, K., Favoreel, H. W. and Nauwynck, H. J.

Journal of General Virology (2015) 96 (118-130).

Abstract

Equine herpesvirus type 1 (EHV-1) replicates in the epithelial cells of the upper respiratory tract and disseminates through the body via a cell-associated viremia in monocytic cells, despite the presence of neutralizing antibodies. However, the mechanism by which EHV-1 hijacks immune cells and uses them as ‘Trojan horses’ in order to disseminate inside its host is still unclear. Here, we hypothesize that EHV-1 delays its replication in monocytic cells in order to avoid recognition by the immune system. We compared replication kinetics *in vitro* of EHV-1 in RK-13, a cell line fully susceptible to EHV-1 infection, and primary horse cells from the myeloid lineage (CD172a⁺). We found that EHV-1 replication was restricted to 4% of CD172a⁺ compared to 100% in RK-13. In susceptible CD172a⁺, the expression of immediate-early (IEP) and early (EICP22) proteins was delayed in the cell nuclei by 2-3 hpi compared to RK-13, and the formation of replicative compartments by 15 hpi. The viral production in CD172a⁺ was significantly lower (from 10^{1.7} to 3.1 TCID₅₀/10⁵ inoculated cells) than in RK-13 (from 10⁵ to 5.7 TCID₅₀/10⁵ inoculated cells). Less than 0.02% of inoculated CD172a⁺ produced and transmitted infectious virus to neighbour cells. Pre-treatment of CD172a⁺ with inhibitors of HDAC activity increased and accelerated viral protein expression at very early time of infection and induced productive infection in CD172a⁺. Our results demonstrated that the restriction and delay of EHV-1 replication in CD172a⁺ is part of an immune evasive strategy and involves silencing of EHV-1 gene expression associated with histone deacetylases.

Introduction

Viruses are defined as obligatory intracellular parasites. They replicate inside the host cell using the host metabolic machinery. Following a primary infection, viruses can elicit a strong immune response. The host's immune system employs a variety of strategies to eliminate the virus (Vossen *et al.*, 2002). However, during co-evolution with their host, viruses have developed an impressive array of immune evasion mechanisms to escape their elimination by the host's immune system (Alcami & Koszinowski, 2000; Favoreel *et al.*, 2003). One strategy mastered by all herpesviruses, is the capacity to establish life-long latency in immunocompetent hosts. During periods of reactivation, the virus may spread to contact individuals. During latency, the virus remains hidden from the immune system and can persist in its host (Efstathiou & Preston, 2005).

Equine herpesvirus type 1 (EHV-1), a member of the *Alphaherpesvirinae*, is a major pathogen of horses worldwide. The virus is responsible for respiratory disorders, abortion, neonatal foal death and neurological disorders (Allen & Bryans, 1986; Patel & Heldens, 2005; Dunowska, 2014). After a primary replication in the epithelial cells of the upper respiratory tract, the virus can spread through the basement membrane to the connective tissues by the use of individual infected migrating monocytic cells (Vandekerckhove *et al.*, 2010; Gryspeerdt *et al.*, 2010). The virus disseminates via a cell-associated viremia in peripheral blood mononuclear cells (PBMC) to target organs such as the pregnant uterus or central nervous system. There, the virus initiates a secondary replication that may lead in some cases to severe symptoms such as abortion and/or neurological disorders (Edington *et al.*, 1991; Smith *et al.*, 1996; Stierstorfer *et al.*, 2002). Current vaccines do not provide full protection as EHV-1 can cause a viremia in the presence of virus-specific antibodies and CTL precursors (Kydd *et al.*, 2006; O'Neill *et al.*, 1999). This shows that recognition of EHV-1-infected carrier cells by the immune system is seriously hampered. Indeed, like other herpesviruses, EHV-1 appears to have developed sophisticated immune evasive strategies to counteract the immune response and to facilitate spread in an immune horse (van der Meulen *et al.*, 2006).

Previous *in vivo* and *ex vivo* studies of the pathogenesis of EHV-1 in the equine upper respiratory tract identified CD172a⁺ monocytic cells as one of the main carrier

cells of EHV-1. It was found that the expression of some late proteins in EHV-1-infected carrier monocytic cells is hampered at early stages of infection (Gryspeerd *et al.*, 2012; van der Meulen *et al.*, 2000). These results suggest that infected leukocytes in the upper respiratory tract with restricted late viral protein expression could be the source of the cell-associated viremia. An early block in the replication cycle of EHV-1 may protect infected leukocytes from efficient recognition by the immune system and hence allow these carrier cells to reach target organs. Interestingly, retroviruses, such as human immunodeficiency virus (HIV), Maedi-Visna virus (MVV) and other herpesviruses such as pseudorabies virus (PRV), human cytomegalovirus (HCMV), are known to influence early response systems by blocking several steps of their replication cycle in their target cells in order to evade immune responses and promote viral pathogenesis (Bergamaschi & Pancino, 2010; Thormar, 2005; Nauwynck & Pensaert, 1994; Rice *et al.*, 1984). However, until now, the mechanism of how EHV-1 uses CD172a⁺ cells as “Trojan horses” to evade the immune system and disseminate within the host has not been described.

In order to understand the immune evasive strategies used by EHV-1, *in vitro* EHV-1 replication kinetics in equine nasal mucosal and blood CD172a⁺ monocytic cells, two major target cells, were examined and compared with replication kinetics in rabbit kidney epithelial cells (RK-13), a cell line known to be fully susceptible to EHV-1 infection.

Material and methods

Virus

The Belgian EHV-1 non-neurovirulent strain 97P70 first isolated in 1997 from the lungs of an aborted fetus was used in this study (van der Meulen *et al.*, 2000). Virus stocks used for inoculation were at the 6th passage; 5 passages in equine embryonic lung cells (EEL) and 1 subsequent passage in RK-13.

Cells

A. Isolation of equine blood CD172a⁺ cells

Healthy horses, between 8 to 10 years old were used as blood donors. Horses were seropositive for EHV-1. The collection of blood was approved by the ethical committee of Ghent University (EC2013/17). Blood was collected by jugular venipuncture on heparin (15U ml⁻¹) (Leo) and diluted in an equal volume of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Gibco). PBMC were isolated by density centrifugation on Ficoll-Paque (d=1.077g ml⁻¹) (GE Healthcare, Life Sciences) at 800xg for 30 min at 18°C. The interphase cells, containing the PBMC, were collected and washed three times with DPBS. Cells were resuspended in leukocyte medium based on Roswell Park Memorial Institute (RPMI, Gibco) supplemented with 5% fetal calf serum (FCS) (Grainer), 1% penicillin, 1% streptomycin, 0.5% gentamycin (Gibco). Afterwards, cells were seeded on 24-well plates (Nunc A/S) at a concentration of 10⁶ cells per ml and cultivated at 37°C with 5 % CO₂. After 12 h, non-adhering lymphocytes were removed by washing cells three times with RPMI. The adherent cells consisted of > 90% of monocytic cells, as assessed by flow cytometry after indirect immunofluorescence staining with a mouse monoclonal (mAb) anti-CD172a (VMRD, clone DH59B, 1:100, IgG1) directed against cells from myeloid lineage, followed by goat anti-mouse IgG FITC (Molecular probes, 1:100).

B. Isolation of equine nasal mucosal CD172a⁺ cells

Tissues of the deep intranasal part of the septum were collected from horses, between 4 to 7 years old at the slaughterhouse and transported to the lab as described previously (Vandekerckhove *et al.*, 2011). The isolation of equine nasal mucosal CD172a⁺ cells was adapted from the protocol described by Bannazadeh Baghi *et al.* (2014). Mucosal explants were cut into small pieces of 0.5 cm² and incubated in medium based on DPBS, 5% FCS, 1% penicillin, 1% streptomycin, 0.5% gentamycin and supplemented with 1 mM EDTA (VWR), at 150 rpm for 30 min at 37°C on a shaker. Tissues were washed twice in RPMI to inactivate EDTA and incubated in digestion medium based on DPBS, 5% FCS, 1% penicillin, 1% streptomycin, 0.5% gentamycin, supplemented with 1 µg ml⁻¹ DNase type I (Stem cell Technologies) and 1 mg ml⁻¹ collagenase type IV (Life Technologies) at 150 rpm for 2 h at 37°C on a shaker. The cell suspension was passed through a 100 µm cell strainer to remove undigested tissues and centrifuged at 400×g, 10 min at 4°C. Cells were resuspended in DPBS and layered onto Ficoll-Paque (d=1.077g ml⁻¹) as described above.

The CD172a⁺ cell fraction was sorted from the total mucosal leukocyte population using magnetic-activated cell sorting (MACS) approach. Briefly, the isolated mucosal leukocyte population was first incubated with a mAb anti-CD172a for 20 min at 4°C, followed by a goat anti-mouse IgG antibody-coated magnetic beads MicroBead Kit (Miltenyi Biotec, Ltd). After 20 min incubation at 4°C, cells were washed with 1 ml of PBS buffer and centrifuged at 300×g, 10 min at 4°C. The cell pellet was resuspended in 1 ml ice-cold elution buffer (DPBS supplemented with 1 mM EDTA and 2% FCS) and the cell suspension was brought onto a LS column. The unlabeled CD172a⁻ cells pass through the column, whereas the CD172a⁺ cells remain in the column due to the magnetic field. After three washings with 1 ml of PBS buffer, the column was removed from the magnetic field and the CD172a⁺ cell fraction was eluted. The purity of the CD172a⁺ cell fraction was >95%, as assessed by flow cytometry, as described previously.

C. Rabbit kidney epithelial (RK-13) cells

RK-13 cells were used as a control for the blood and nasal CD172a⁺ cells in this study and were maintained in Modified Eagle's medium (MEM) supplemented with antibiotics and 5% FCS.

D. Cell viability

Cell viability was determined by flow cytometry, using $1\mu\text{g ml}^{-1}$ propidium iodide (Sigma-Aldrich), prior to virus inoculation and was $> 90\%$ in all cell populations.

EHV-1 inoculation

Cell populations were inoculated *in vitro* with EHV-1 strain 97P70 at a MOI of 5 in $200\mu\text{l}$ leukocyte medium for 1 h at 37°C with 5% CO_2 . Cells were gently washed twice with RPMI to remove the inoculum and further incubated with fresh medium. Mock infections were carried out in parallel. At 1, 3, 5, 7, 9, 12 and 24 hours post-inoculation (hpi), cells were collected for quantification of EHV-1 infected cells, viral production and production of infectious EHV-1 by immunofluorescence staining and virus titration, respectively.

Where indicated, $400\mu\text{M}$ of phosphonoacetate (PAA), an inhibitor of viral DNA polymerase, was added at the time of inoculation and maintained in medium throughout the course of infection. Histone deacetylase inhibition was achieved by pre-treating cells with 100 nM trichostatin A (TSA) or 0.5 mM of sodium butyrate (NaBut) for 2 h at 37°C prior to EHV-1 inoculation. HDAC inhibitors were maintained throughout the course of infection. All inhibitors were purchased from Sigma-Aldrich. The concentration of inhibitors used in this study did not decrease the cell viability ($> 95\%$) determined by flow cytometry, as described above.

Virus titration

To quantify EHV-1 replication, both intracellular and extracellular virus titers were determined at different hpi. The supernatant containing the extracellular virus was collected, centrifuged at $400\times g$ for 10 min at 4°C and stored at -70°C until titration. Cell extracts were harvested by centrifugation at $400\times g$ for 10 min at 4°C , followed by three cycles of freezing and thawing to lyse the cells. For the inactivation curve, EHV-1 was incubated for different times at 37°C . Virus titers were conducted on RK-13 cells, incubated at 37°C for 7 days. The virus titer was calculated as 50% tissue culture infective dose (TCID_{50}) according to the Reed and Muench formula (1938).

Cocultivation assay

A cocultivation assay was used to detect and quantify EHV-1 producing CD172a⁺ cells by co-cultivation of these EHV-1-inoculated CD172a⁺ cells with a permissive equine cell culture (RK-13), where a semi-solid overlay technique was applied. Briefly, EHV-1-inoculated and mock-inoculated cells were harvested at 12 hpi. 10⁵ cells per ml were tenfold diluted in leukocyte medium and 0.5 ml of each dilution was added on RK-13 monolayers in a 6-well plate and overlaid with a 0.94% carboxymethylcellulose medium (Sigma-Aldrich) prepared in RPMI-2X and centrifuged at 800xg for 30 min at 18°C, as described previously by Van der Meulen *et al.* (2000). Cells were further incubated for 5 days at 37°C, 5% CO₂. The cell monolayers were stained with 5% crystal violet and the number of plaques was counted. The percentage of infected cells producing infectious EHV-1 was calculated based on the number of plaques counted and the number of cells seeded per ml according to the volume plated. This experiment was performed three times.

Indirect immunofluorescence staining of EHV-1 proteins

To determine which kinetic classes of proteins were expressed in EHV-1-infected cells, a double immunofluorescence staining was performed on cells fixed in 100% methanol at -20°C for 20 min. Cells were incubated for 1 h at 37°C with a rabbit polyclonal Ab anti-IEP (1:1000) (Smith *et al.*, 1994; Jang *et al.*, 2001) to detect immediate early protein (IEP) expression and either a mouse monoclonal anti-EICP22 (K2 to IR4) (1:500), anti-gB (4B6) (1:100) or anti-gC (1B6) (1:100) antibodies against early (EICP22), late gB and gC proteins expression, respectively. The IEP and EICP22 antibodies were kindly provided by Dr. O'Callaghan (USA). The 4B6 and 1B6 were provided by Dr. N. Osterrieder (Germany) and Dr. H. Huemer (Austria), respectively. Subsequently, samples were incubated for 50 min at 37°C with goat anti-rabbit IgG FITC (1:100) or goat anti-mouse IgG Texas-Red[®] (1:100) antibodies (Molecular probes). All antibodies were diluted in DPBS. The nuclei were counterstained with Hoechst 33342 (10 µg ml⁻¹; Molecular Probes) for 10 min at 37°C. As negative control, mock-inoculated cells were stained following the

aforementioned protocols. In addition, appropriate isotype-matched controls were included. The percentage of viral antigen positive cells was calculated based on three hundred cells counted in distinct fields. Samples were analyzed by confocal microscopy (Leica TCS SP2 Laser scanning spectral confocal system, Leica microsystems GmbH, Germany). All individual images were representative of the cell population infected.

Statistical analysis

Data were analyzed with GraphPad Prism 5 software (GraphPad software Inc., San Diego, CA, USA). Analyzed data for statistical significance were subjected to a two-way analysis of variance (ANOVA). All results shown represent means and standard deviation (SD) of three independent experiments. Results with p -value ≤ 0.05 were considered statistically significant.

Results

1. EHV-1 replication is delayed in CD172a⁺ cells compared to RK-13 cells

In a first experiment, equine nasal mucosal and blood CD172a⁺ cells were examined for their ability to support EHV-1 replication compared to RK-13 cells. To this end, the expression kinetics of the three major classes proteins: immediate-early (IEP), one early (EICP22) and two late (gB and gC) proteins were evaluated by indirect immunofluorescence staining.

In EHV-1-inoculated RK-13 cells, IEP was detected in the nuclei of 3% of the cells as early as 3 hpi, the EICP22 protein in the nuclei of 5% of the cells as early as 7 hpi and the late gB and gC glycoproteins in the cytoplasm of 2% of the cells starting from 7 and 9 hpi, respectively (Fig. 1a). The number of RK-13 cells positive for all viral proteins increased over time. At 12 hpi, 64% of the cells were found IEP-positive, 37% EICP22-positive, 32 and 10% gB- and gC-positive. At 24 hpi, 100% of the cells were positive for all viral proteins.

In EHV-1 inoculated equine nasal mucosal and blood CD172a⁺ cells, IEP was first detected at 5 hpi. The number of IEP-positive cells was significantly higher in blood CD172a⁺ cells than in nasal mucosal CD172a⁺ cells at 5 and 7 hpi (p -value < 0.01) (Fig. 1b and 1c). From 5 hpi to 7 hpi, the percentage of IEP-positive cells increased from 0.3 to 0.9% and 2.8 to 3.6% in nasal mucosal and blood CD172a⁺ cells, respectively. A maximum of 3.6 and 3.8% of IEP-positive cells was reached at 7 and 12 hpi in blood and nasal mucosal CD172a⁺ cells. The EICP22 protein was first detected at 7 hpi in 0.2% of blood CD172a⁺ cells and at 9 hpi in 0.6% of equine nasal mucosal CD172a⁺ cells. Similarly to IEP expression, the percentage of EHV-1-inoculated CD172a⁺ cells expressing EICP22 did not increase after 12 hpi. At 24 hpi, the EICP22 protein was expressed in 2 and 1.3% of blood and nasal mucosal CD172a⁺ cells, respectively. The late gB protein was only detected in 0.3 % of blood CD172a⁺ cells at 12 hpi and in 0.6 and 1.3% of blood and nasal mucosal CD172a⁺ cells at 24 hpi. The late gC protein was only found expressed in 0.3% of nasal mucosal CD172a⁺ cells at 24 hpi. The use of a higher MOI (50) did not alter the number of IEP-positive cells (Fig. 2). Besides, EHV-1 replication was highly restricted in CD172a⁺ cells, independently of the abortigenic strains used (Fig. 3).

Overall, these results demonstrate that the replication of EHV-1 in equine nasal mucosal and blood CD172a⁺ cells is delayed based on the kinetics of viral protein appearance in these cells.

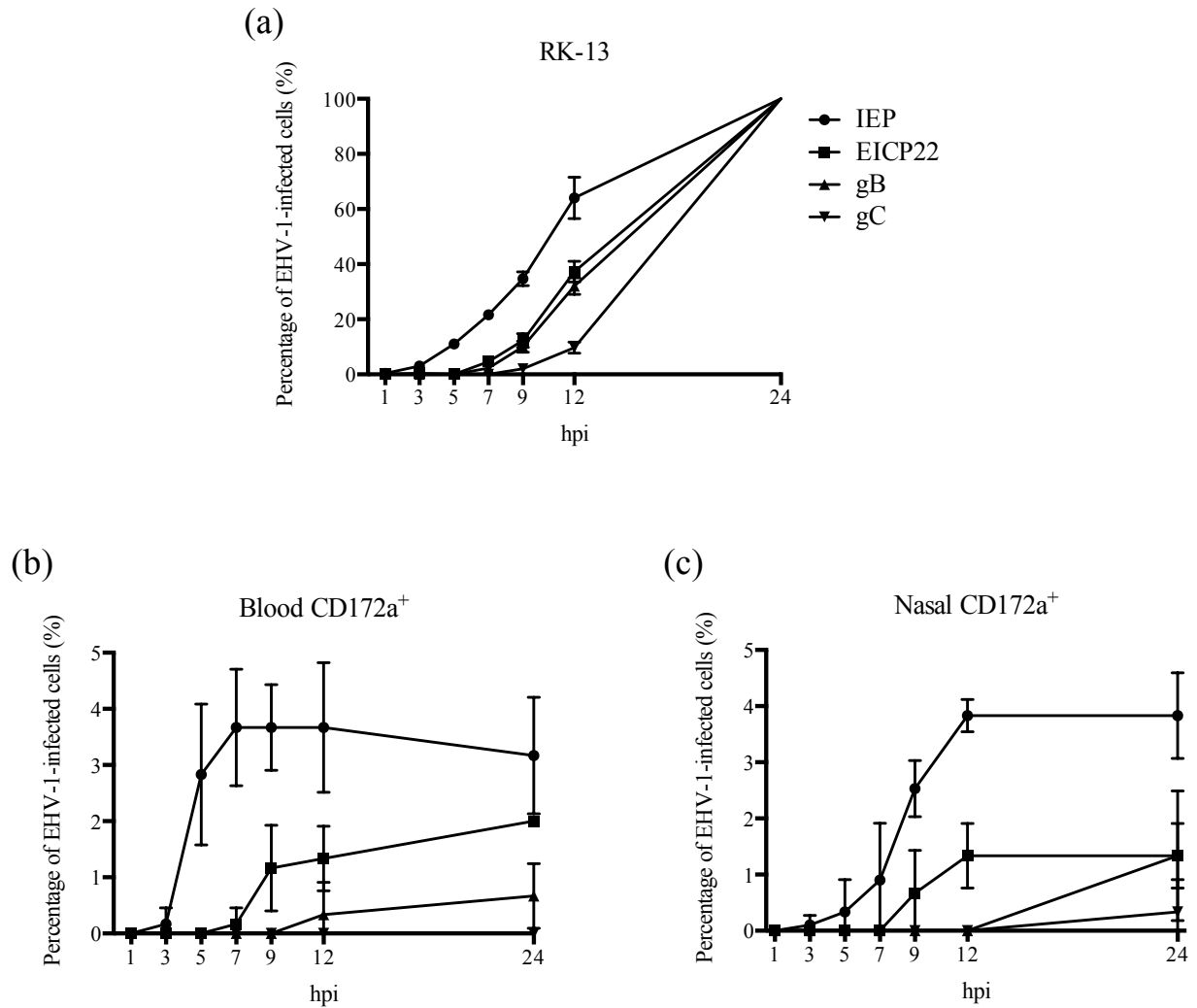


Figure 1: Expression kinetics of EHV-1 proteins in (a) RK-13 cells, (b) nasal mucosal and (c) blood CD172a⁺ cells. Immunofluorescence stainings of immediate-early (IEP), early (EICP22) and late (gB and gC) proteins were performed in triplicate and data are represented as means \pm SD.

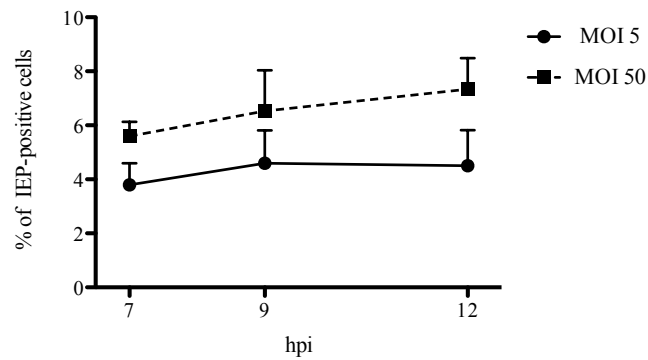


Figure 2: Expression kinetics of IEP in blood CD172a⁺ cells inoculated with EHV-1 at a MOI of 5 or 50. Experiment was performed in triplicate and data are represented as means \pm SD. No statistical differences were observed between the two groups by a two-way ANOVA test.

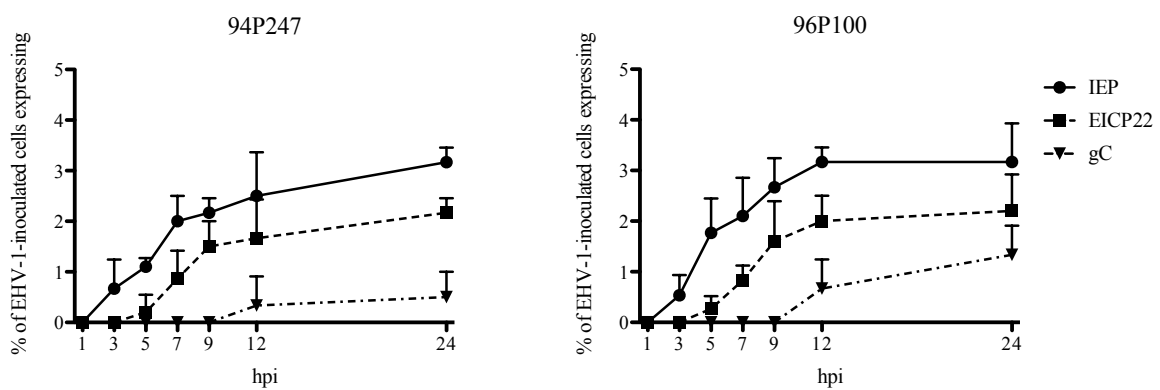


Figure 3: Expression kinetics of EHV-1 proteins in blood CD172a⁺ cells inoculated with EHV-1 abortigenic 94P247 and 96P100 strains. Immunofluorescence staining of IEP, EICP22 and gC proteins were performed in triplicate and data are represented as means \pm SD.

2. Spatio-temporal distribution of EHV-1 viral proteins in CD172a⁺ compared to RK-13 cells

To determine whether the delay of EHV-1 replication in CD172a⁺ cells was correlated to a change in the distribution of EHV-1 viral proteins within the cell, we analyzed the spatio-temporal distribution dynamics of IEP, EICP22, gB and gC in EHV-1-inoculated CD172a⁺ and RK-13 cells by immunofluorescence staining. The colocalization of IEP and EICP22 was also examined by means of immunofluorescence double staining.

In EHV-1-inoculated RK-13 cells, a weak and diffuse IEP signal was first detected at 1 hpi with a few foci of intranuclear staining, with intensity increasing by 3 and 5 hpi (Fig. 4). At 7 hpi, IEP colocalized in one or two small globular structures while IEP was also found specklingly distributed in the rest of the cell nuclei (see arrows). The localization of IEP in globular structures coincided with the appearance of EICP22 in the nuclei of cells. Both IEP and EICP22 colocalized in nuclear globular structures resembling to pre-replicative sites at 7 hpi. However, the IEP showed a change in nuclear distribution as infection progressed. The IEP signal progressively dispersed in the cytoplasm and the size of the nuclear globular compartments increased and varied from 0-2 to 2-5 μm between 7 and 9 hpi. At 12 hpi, IEP signal was found associated in massive globular compartments (5-8 μm), resembling to replicative compartments, which occupy most of the nucleus space, while IEP was found dispersed in the cytoplasm of infected RK-13 cells. EICP22 did not colocalize with IEP in the nucleus of cells but rather formed a shell around IEP-associated compartments. The IEP expression progressed from individual IEP foci to large compartments each consisting of several IEP foci. To confirm whether the formation of large IEP-associated compartments in the nuclei of EHV-1-inoculated RK-13 cells was dependent on viral DNA replication; we compared the nuclear distribution of IEP in EHV-1-inoculated cells in the presence or absence of phosphonoacetate (PAA), a specific inhibitor of the viral DNA polymerase. As expected, in PAA-treated cells, only small-punctuated IEP-associated nuclear structures were observed at later stage of infection while no large IEP-associated compartments were detected (data not shown). These results confirmed that IEP associates within distinct structures, called pre-replicative sites (7 hpi) that mature into replicative compartments (9 and 12 hpi) as infection progressed and which constitute sites for viral DNA synthesis. Finally, we examined the

expression pattern of late gB and gC in EHV-1-inoculated RK-13 cells. They were found exclusively expressed in the cytoplasm, in restricted areas and their expression intensified as infection progressed (data not shown). At 24 hpi, EHV-1-infected RK-13 cells were completely destroyed as confirmed by the presence of CPE, which did not allow any further examination of viral protein expression pattern.

In both EHV-1-inoculated nasal mucosal and blood CD172a⁺ cells, a weak IEP signal was first detected in some parts of the nucleus which intensity increased from 5 to 7 hpi (Fig. 5 and 6). At 9 hpi, IEP signal was diffuse throughout the nucleus and cytoplasm of blood CD172a⁺ cells while in nasal mucosal CD172a⁺ cells, IEP signal was still found exclusively restricted in some nuclear areas. However, in both cases, this correlated with the appearance of EICP22, which colocalized with IEP in the nucleus of both CD172a⁺ cell types. At 12 hpi, a strong IEP staining was observed in the nucleus and at a lesser extent in the cytoplasm of both cell types while no IEP-associated replicative compartments were detected. The EICP22 was found similarly expressed as at 9 hpi. Interestingly, the expression of IEP within replicative compartments and surrounded by EICP22 were only detected at 24 hpi in the nucleus of both CD172a⁺ cell types (see arrows). Finally, the cytoplasmic localization of gB and gC late proteins remained unchanged in nasal mucosal and blood CD172a⁺ cells compared to RK-13 cells (data not shown). These results showed that the delay of EHV-1 replication in CD172a⁺ cells compared to RK-13 cells is correlated to a change in the spatio-temporal distribution dynamics of EHV-1 viral proteins within the cell.

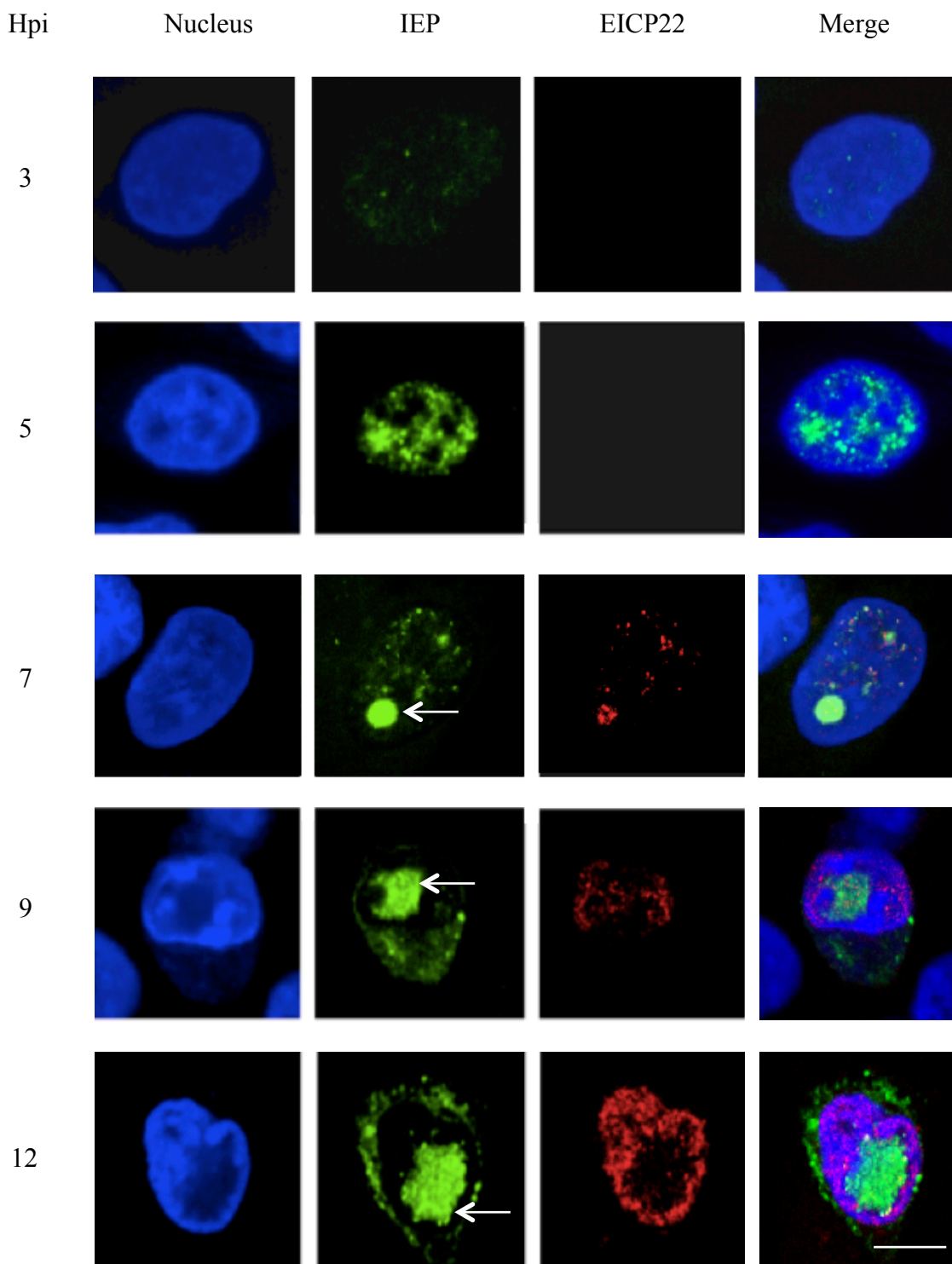


Figure 4: Double immunofluorescence of EHV-1 IEP (green) and EICP22 (red) proteins in RK-13 cells. Nuclei are counterstained with Hoechst (blue). Each image represents a single section through a cell. Arrows indicate the formation of IEP-associated replicative compartments. Scale bar = 10 μ m.

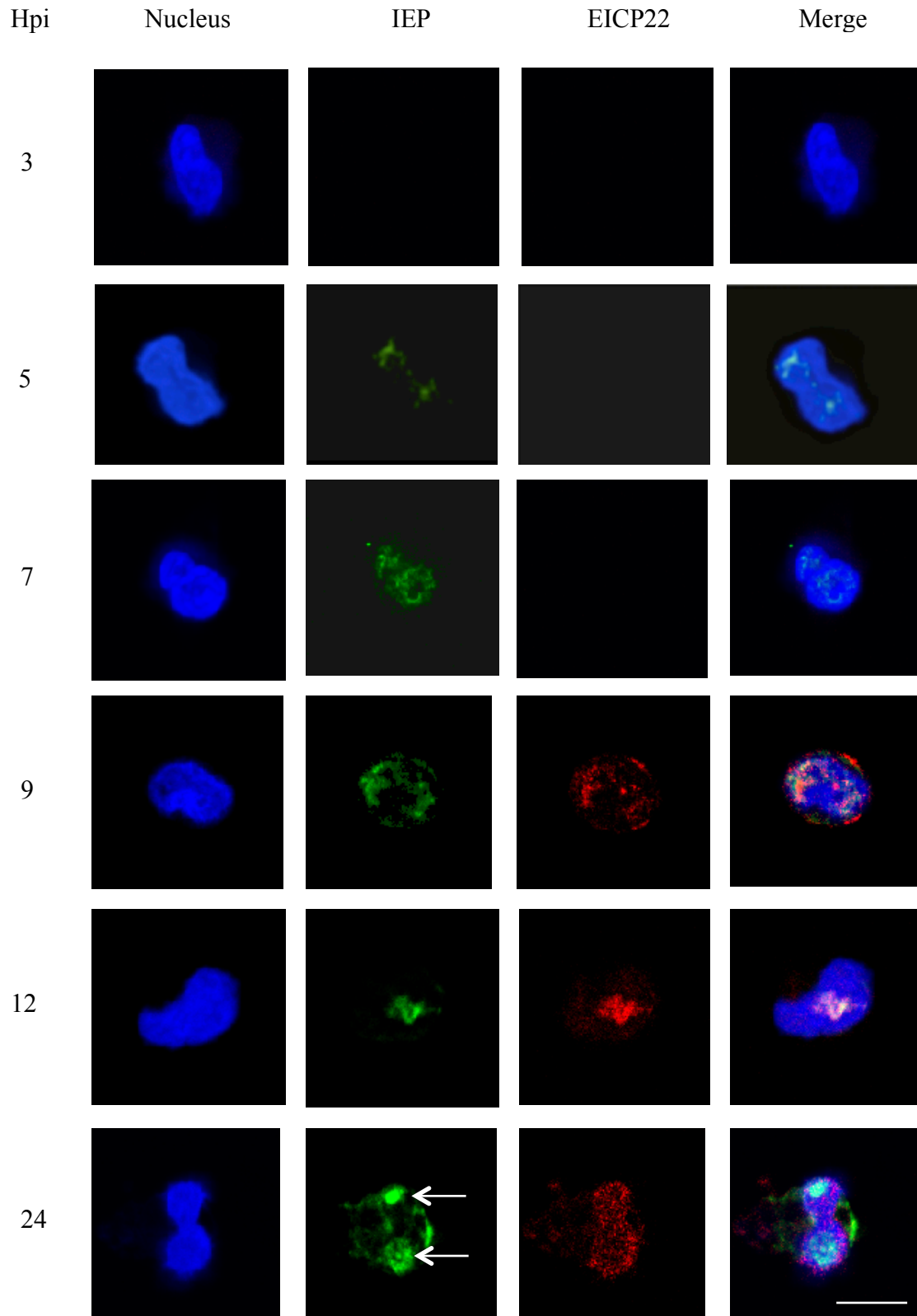


Figure 5: Double immunofluorescence of EHV-1 IEP (green) and EICP22 (red) proteins in nasal mucosal CD172a⁺ cells. Nuclei are counterstained with Hoechst (blue). Each image represents a single section through a cell. Arrows indicate the formation of IEP-associated replicative compartments. Scale bar = 10 μ m.

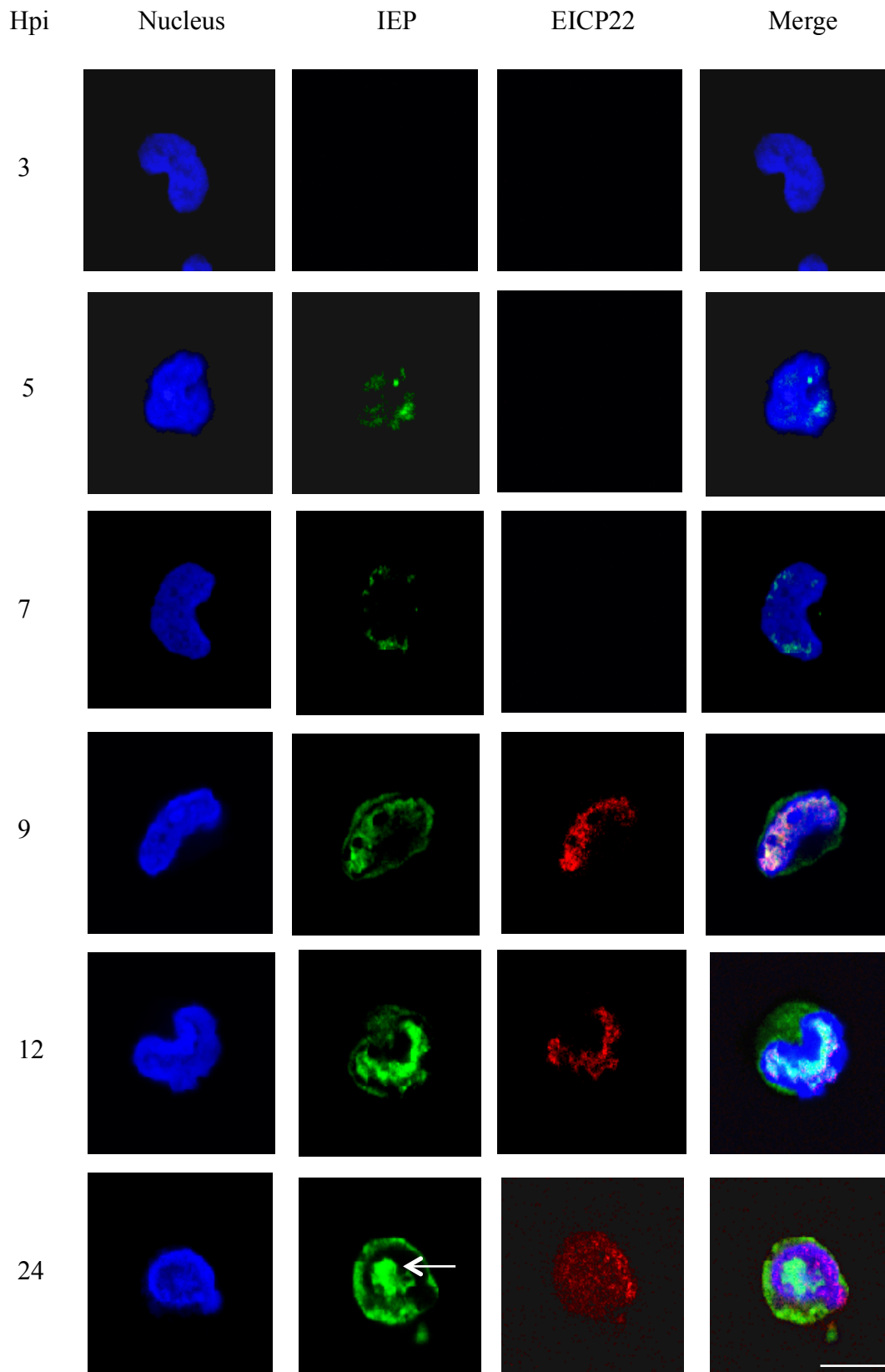


Figure 6: Double immunofluorescence of EHV-1 IEP (green) and EICP22 (red) proteins in blood $CD172a^+$ cells. Nuclei are counterstained with Hoechst (blue). Each image represents a single section through a cell. Arrows indicate the formation of IEP-associated replicative compartments. Scale bar = 10 μ m.

3. Kinetics of EHV-1 production in CD172a⁺ cells compared to RK-13 cells

To determine the kinetics of viral production in blood and nasal mucosal CD172a⁺ cells compared to infected RK-13 cells, virus titrations of supernatants (extracellular fraction) and cells (intracellular fraction) were performed.

No significant increase in intracellular and extracellular virus titers was observed in all cell types before 9 and 12 hpi, respectively. At 12 hpi, virus titration of the intracellular fraction showed a 10-fold lower virus titer in blood and nasal mucosal CD172a⁺ compared to RK-13 cells (p -value < 0.01) but no significant difference in extracellular virus titer was observed between infected CD172a⁺ and RK-13 cells (Fig. 7). At 24 hpi, virus titration of the extracellular fraction revealed a 100-fold and 1000-fold lower virus titer in blood ($10^{3.1}$ TCID₅₀/10⁵ inoculated cells) (p -value < 0.01) and nasal CD172a⁺ cells ($10^{2.4}$ TCID₅₀/10⁵ inoculated cells) (p -value < 0.001) compared to RK-13 ($10^{5.7}$ TCID₅₀/10⁵ inoculated cells). In addition, virus titration of the intracellular fraction showed a 1000-fold and 10000-fold lower titer in blood (10^2 TCID₅₀/10⁵ inoculated cells) and nasal CD172a⁺ cells ($10^{1.7}$ TCID₅₀/10⁵ inoculated cells) than in RK-13 (10^5 TCID₅₀/10⁵ inoculated cells). No increase in extracellular and intracellular virus titers was observed in nasal mucosal CD172a⁺ cells over time post-inoculation. The virus remained infectious throughout the experiment as confirmed by the inactivation curve.

Next, we evaluated and compared the percentage of cells that produced and transmitted infectious EHV-1 to neighbor cells, between infected CD172a⁺ and RK-13 cells. Co-cultivation of inoculated CD172a⁺ with RK-13 cell monolayers revealed that less than 0.01% and 0.02% of nasal mucosal and blood CD172a⁺ cells respectively, were productive. The number of EHV-1-producing CD172a⁺ cells was significantly less than the number of EHV-1-producing RK-13 cells (31%) (p -value < 0.0001).

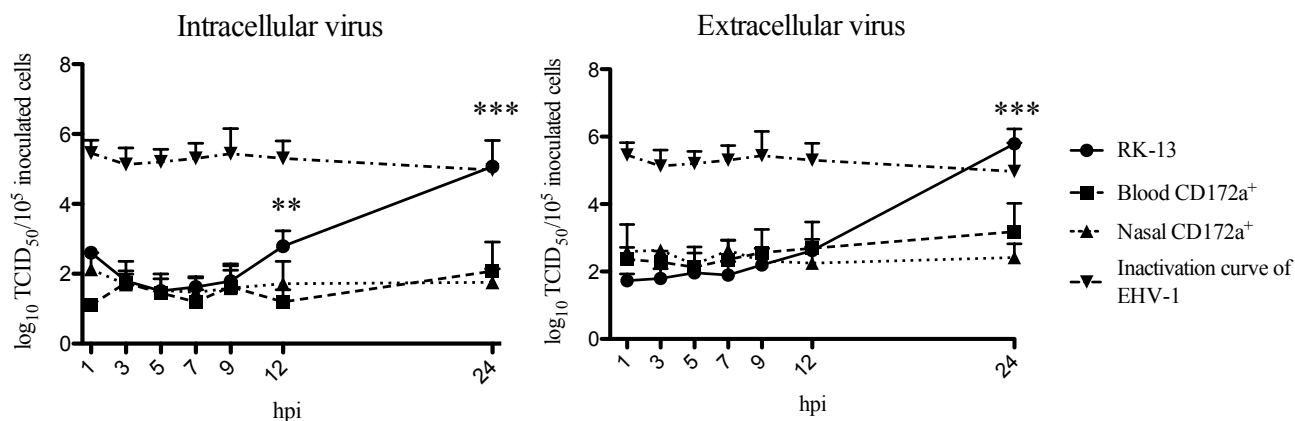


Figure 7: Kinetics of EHV-1 virus production in nasal mucosal and blood CD172a⁺ cells compared to RK-13 cells. Error bars show \pm SD and a two-way ANOVA test was performed to evaluate significant differences between RK-13 and both CD172a⁺ cell types. ** denotes a p -value < 0.01 between RK-13 and blood CD172a⁺ cells and *** a p -value < 0.001 between RK-13 and both CD172a⁺ cell types.

4. HDAC inhibitors relieve the delay of EHV-1 proteins expression in CD172a⁺ cells at very early time of infection

Next, we determined whether histone deacetylases (HDACs) were involved in the temporary block of EHV-1 protein expression in CD172a⁺ cells at early time of infection. We tested whether treatment of EHV-1-inoculated CD172a⁺ cells with the deacetylase inhibitors trichostatin (TSA) or sodium butyrate (NaBut) increased IEP, EICP22 and gB protein expression in these cells at early time of infection. These experiments were performed using blood CD172a⁺ cells only as the kinetics of EHV-1 replication was found similar in both nasal and blood CD172a⁺ cells.

We found that the number of IEP-, EICP22- and gB-positive blood CD172a⁺ cells was significantly higher in NaBut- and TSA-treated CD172a⁺ cells compared to untreated cells from 7-9 to 24 hpi (p -value < 0.05) (Fig. 8). In NaBut-treated cells, IEP was detected in the nuclei of 0.6% of the cells as early as 1 hpi, EICP22 protein in the nuclei of 1.6% of the cells as early as 5 hpi and gB in the cytoplasm of 2.3% of the cells starting from 7 hpi. At 12 hpi, 12% of the cells were found IEP-positive, 9% EICP22-positive and 7.3% gB-positive. At 24 hpi, 14% of the cells were positive for all viral proteins. Similar results were observed in TSA-treated cells. The titration of supernatant of NaBut- and TSA- treated cells showed a significant rise in virus titer compared to non-treated cells at 12 and 24 hpi, respectively (p -value < 0.05) (Fig. 9).

This result confirmed productive EHV-1 infection in these cells. TSA and NaBut cell treatment did not affect the kinetics of EHV-1 protein expression in EHV-1-inoculated RK-13 cells (data not shown).

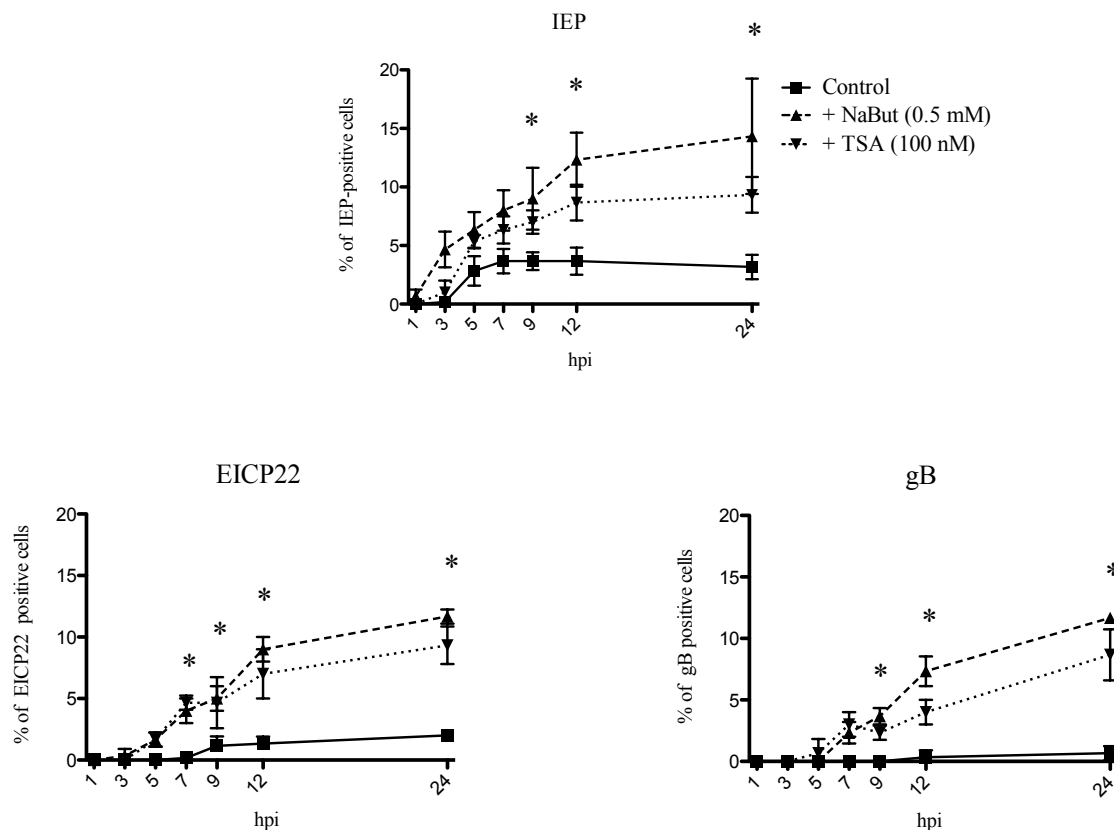


Figure 8: Replication kinetics of EHV-1 in blood CD172a⁺ cells following treatment with HDAC inhibitors, trichostatin A (TSA) and sodium butyrate (Nabut). Experiments were performed in triplicate. Error bars show \pm SD and a two-way ANOVA test was performed to evaluate significant differences between HDAC inhibitor-treated and untreated cells, * denotes a p -value < 0.05 .

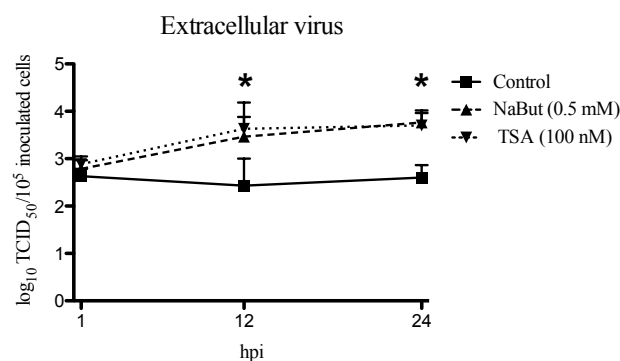


Figure 9: EHV-1 extracellular virus titer in NaBut- and TSA-treated blood CD172a⁺ cells compared to untreated cells at 1, 12 and 24 hpi. Experiment was performed in triplicate. Error bars show \pm SD and a two-way ANOVA test was performed to evaluate significant differences between HDAC inhibitor-treated and untreated cells, * denotes a P -value < 0.05 .

EHV-1 proteins were expressed earlier in HDAC inhibitor-treated CD172a⁺ cells than in untreated cells and comparable to those found in non-treated and EHV-1-infected RK-13 cells. At 1 hpi, IEP signal was bright and homogenously distributed in the nucleus of NaBut-treated cells within few foci of intranuclear staining. At 7 hpi, EICP22 was found expressed in the cell nuclei around newly formed IEP-associated compartments (Fig. 10). At 12 hpi, cells harbored a massive nuclear IEP-associated replicative compartment and gB was found expressed in the cytoplasm and at the plasma membrane of the cell (Fig 11a). From 12 to 24 hpi, intercellular contacts were observed between CD172a⁺ cells, which resulted in the formation of ‘ring-shaped’ cell clusters, consisting of at least two to 20 cells. A single cell containing a nuclear IEP-associated replicative compartment initiated intercellular contacts with neighbor cells via membrane extensions that were found IEP- and gB- positive (Fig. 11b). Recruited neighbor cells progressively formed clusters that were characterized by a cytoplasmic IEP expression as well as a cell surface and polarized cytoplasmic gB expression while no IEP-associated replicative compartment was detected in these neighbor cell nuclei (Fig. 11c).

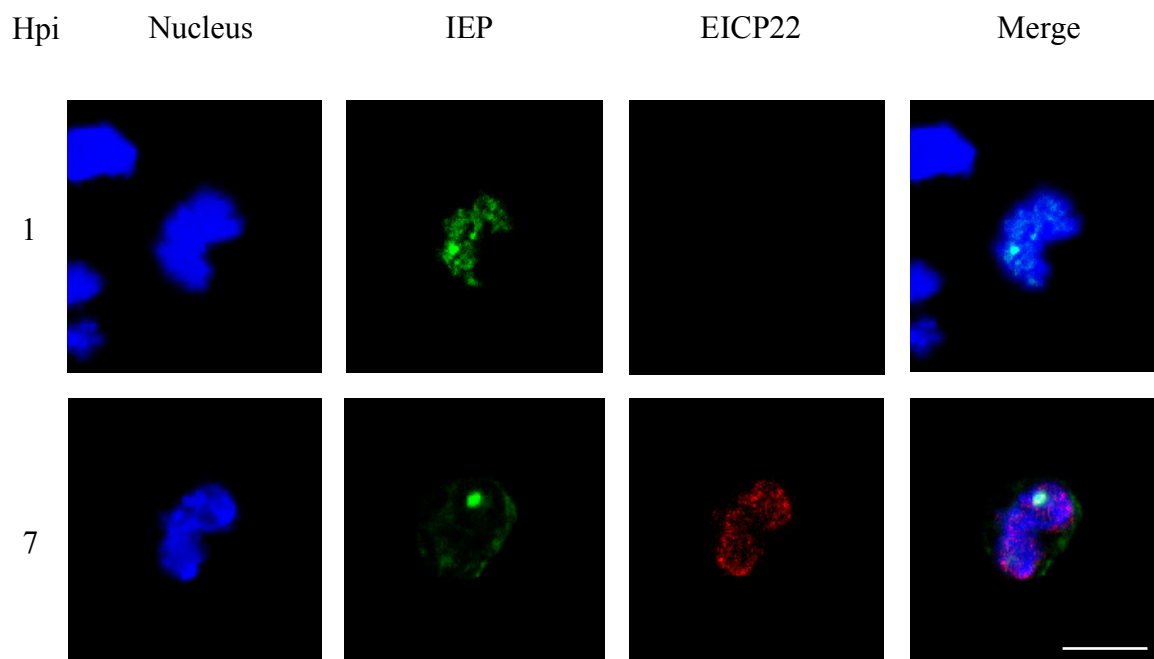


Figure 10: Double immunofluorescence staining of EHV-1 IEP (green) and EICP22 (red) proteins in EHV-1-inoculated and Nabut-treated blood CD172a⁺ cells at 1 and 7 hpi. Nuclei are counterstained with Hoechst (blue). Each image represents a single section through a cell. Scale bar = 10 μ m.

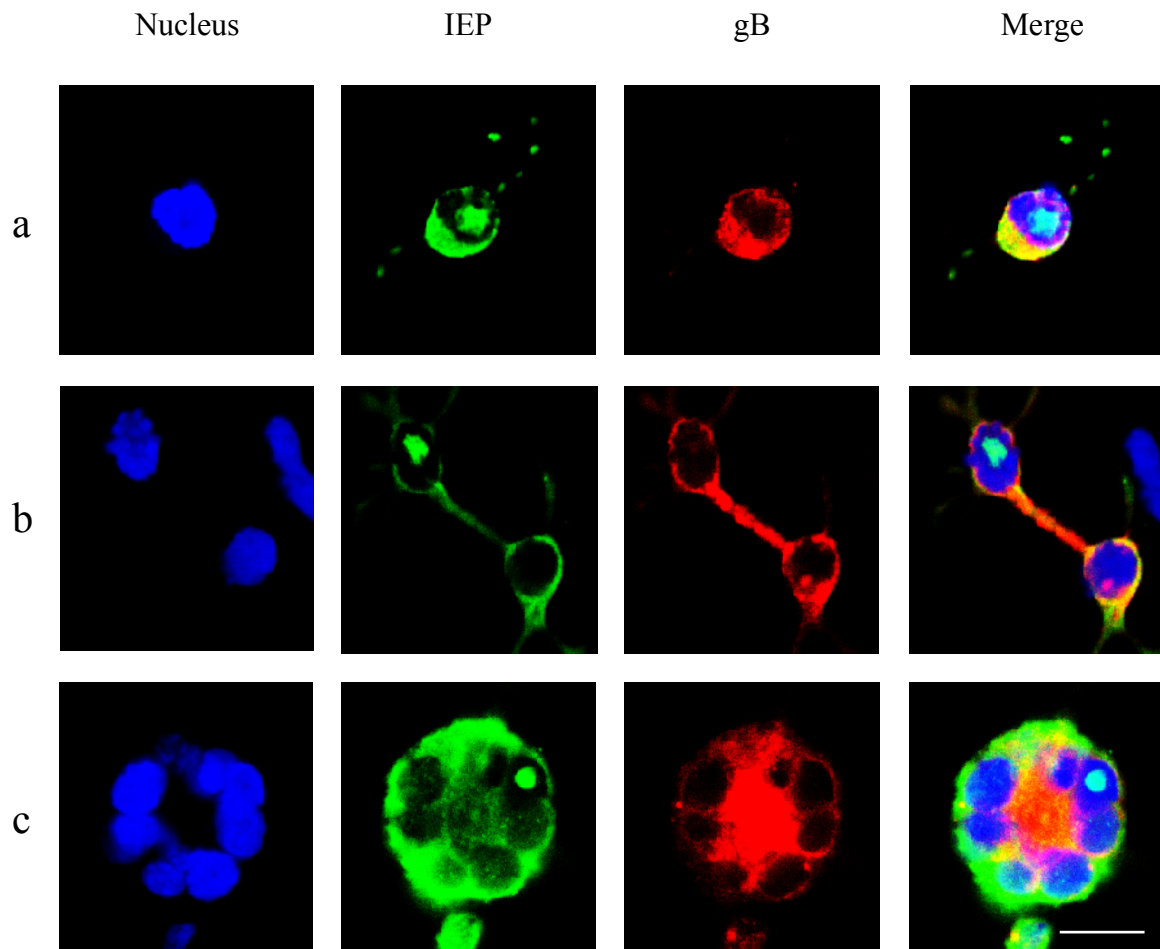


Figure 11: Double immunofluorescence staining of IEP (green) and gB (red) proteins expressed in EHV-1-inoculated and Nabut-treated blood CD172a⁺ cells at 12 hpi. Nuclei are counterstained with Hoechst (blue). (a) Formation of IEP-associated replicative compartment in the nucleus and cytoplasmic expression of gB in CD172a⁺ treated cells (b) Intercellular contact between one infected cell and a non-infected cell (c) 'Ring shaped' CD172a⁺ cell clusters of EHV-1 infection. Scale bar = 10 μ m.

Discussion

In the present study, we demonstrated that approximately 4% of equine nasal mucosal and blood CD172a⁺ cells, two target cells of EHV-1 were susceptible to EHV-1 infection *in vitro*. This is in agreement with van der Meulen *et al.* (2000), demonstrating that EHV-1 replication is highly restricted in equine PBMC. As little is known about the regulation of monocytic cell tropism and susceptibility to EHV-1, we hypothesize that a low efficiency of binding of EHV-1 to CD172a⁺ cells may be responsible for the restriction of EHV-1 replication in these cells, suggesting the presence of specific cell surface receptors with restricted expression patterns. Preliminary *in vitro* studies demonstrated significant differences in the binding efficiency of EHV-1 between CD172a⁺ and RK-13 cells, in terms of percentage of infected cells and number of virus particles bound per cell. The authors found that EHV-1 does not bind efficiently to CD172a⁺ cells compared to RK-13 cells and the low efficiency of EHV-1 binding to CD172a⁺ cells was not shown to be virus-dose dependent. This already indicates that a block at the virus entry level is partially responsible for the restricted viral replication in CD172a⁺ cells.

Next to the restriction of EHV-1 replication in CD172a⁺ cells, we demonstrated significant differences in the kinetics of EHV-1 protein expression between susceptible CD172a⁺ cells and control RK-13 cells at very early time of infection. We found that IEP was detected later in the nuclei of blood and nasal CD172a⁺ (5 hpi) than in the nuclei of control RK-13 cells (3 hpi). Similarly, EICP22 and late (gB and gC) protein expression were delayed in both CD172a⁺ cell types. Moreover, we observed that EICP22 and late gB and gC proteins accumulate slower in blood CD172a⁺ cells than IEP, suggesting a delay associated with a consecutive block of viral protein expression in CD172a⁺ cells. Besides, a progression of stages could be defined for the nuclear association of IEP with EICP22 during EHV-1 infection, as shown for HSV-1 infection (Knipe *et al.*, 1987; Taylor *et al.*, 2003; Chang *et al.*, 2011). In EHV-1-inoculated RK-13 cells, IEP showed a diffuse nuclear distribution and co-localized with EICP22 at early time of infection while both proteins presented a distinct nuclear distribution at later time of infection. By the use of PAA, an inhibitor of viral DNA polymerase, we confirmed that IEP-associated nuclear structures, so called replication compartments (RCs) were essential for viral DNA

replication (Quinlan *et al.*, 1984). Interestingly, we observed that IEP localized into RCs slower in CD172a⁺ (24 hpi) than in RK-13 cells (7 hpi). In addition, we found that the viral production and percentage of EHV-1 producing cells was significantly lower than in RK-13 cells. Taken together, we demonstrated that the replication of EHV-1 in CD172a⁺ cells was delayed at a very early time of infection, starting from the nuclear expression of IEP, which successively slowed down the replication machinery in these cells, thus leading to a non-productive infection. We believe that the restriction/delay of replication in CD172a⁺ cells is part of an immune evasive strategy employed by EHV-1. By slowing its replication down, EHV-1 allows a better survival of CD172a⁺ cells while the virus uses them as ‘Trojan horses’ to disseminate undetected throughout the body. However, we think that not all infected leukocytes will transfer the virus to endothelial cells, not every transfer will lead to effective spread to neighboring endothelial cells and not every successful infection of endothelial cells will cause infection of surrounding tissues. Indeed, studies demonstrated that EHV-1 viremia is detected in pregnant mares, which do not abort and only a minority of viremic horses (10%) develop neurological disorders during equine herpes myeloencephalopathy (EHM) outbreaks (Slater *et al.*, 2007; Goehring *et al.*, 2006). In addition, we are also convinced that the outcome of infections of leukocytes with neurovirulent and non-neurovirulent leukocytes are quite different based on preliminary *in vitro* experiments we performed demonstrating strikingly different outcomes in EHV-1 replication between the two strains in target CD172a⁺ cells.

Further, we showed that the delay of EHV-1 protein expression in susceptible blood CD172a⁺ cells could be relieved following treatment with TSA and NaBut, two histone deacetylase (HDAC) inhibitors. HDACs are known to condense the chromatin structure by deacetylation of histones in order to repress cellular and/or viral transcription. According to this, our results suggest that HDACs play a role in the silencing of gene expression in EHV-1 target cells. This was confirmed by the expression of EICP22 (nucleus) and gB (cytoplasm) starting from 5 and 7 hpi in HDAC inhibitor-treated CD172a⁺ cells, accompanied by the rapid formation of nuclear IEP-associated RC (7-9 hpi) and resulting in a productive EHV-1 infection in these cells. These results were consistent with recent work demonstrating the importance of protein acetylation and control of IE gene expression via HDACs

during herpesvirus infections (Meier, 2001; Everett *et al.*, 2009). In addition, we found that a higher number of CD172a⁺ cells (12-14%) were susceptible to EHV-1 infection following treatment with HDAC inhibitors compared to untreated cells (4%). This suggests that the permissiveness for EHV-1 infection might be linked to repression of IE promoter, as described for HCMV infection (Murphy *et al.*, 2002; Wright *et al.*, 2005). Besides, we found that treatment of cells with TSA or sodium butyrate selectively increased EHV-1 replication in target CD172a⁺ cells but not in control epithelial cells (RK-13). It is possible that the effectiveness of HDAC inhibitors treatment is cell-dependent or that RK-13 cells possess a low level of HDAC, which directly correlates with their increased EHV-1 permissiveness (Cody *et al.*, 2014). Further studies are still needed to determine which individual HDAC(s) might be the most critical for inhibition and which cellular and viral transcription factors are involved in the association of IE promoter with hypoacetylated histones and mediate silencing of EHV-1 genome within CD172a⁺ cells. Control of EHV-1 infection by specific HDAC inhibitors is likely to be a key step in the pathogenesis of EHV-1 *in vitro* and *in vivo*. We hypothesize that *in vivo*, acute EHV-1 infection promotes activation of blood monocytes that subsequently migrate into different tissues (pregnant uterus and/or central nervous system) and differentiate into permissive macrophages, as described for HCMV infection (Smith *et al.*, 2004). The changes in cellular differentiation from monocytes into macrophages may be directly correlated to the association of specific cellular chromatin remodeling factors around the IE promoter and could play a role in controlling EHV-1 infection. Thus, specific HDAC inhibitors may represent useful therapeutic tools, which could serve to reactivate EHV-1 latent virus and/or to clear virus from cellular reservoirs. To date, valproic acid (VPA), a carboxylate HDAC inhibitor, has been used to induce EBV lytic-phase gene expression and to sensitize EBV⁺ tumor cells to cytotoxicity in presence of the anti-herpesvirus drug ganciclovir, in the treatment of patients with EBV⁺ lymphomas (Feng & Kenney, 2006). Göttlicher *et al.*, 2001 demonstrated that VPA relieves repression of transcription factors, which recruit histone deacetylases, and causes hyperacetylation of the N-terminal tails of histone H3 and H4 *in vitro* and *in vivo*.

Finally, we observed the formation of clusters of CD172a⁺ cells composed of one cell containing a nuclear IEP-associated RC and surrounded by cells presenting cytoplasmic IEP and gB expression at a later stage of infection in HDAC-treated cells

(12-24 hpi). This result indicates that EHV-1 can efficiently spread by cell-to-cell contacts in productive infected CD172a⁺ cells and transfer cytoplasmic viral proteins to uninfected neighbor cells. This finding is partially in agreement with previous studies from Van der Meulen *et al.* (2000), which demonstrated the formation of intercellular contacts between EHV-1-inoculated PBMC within clusters after mitogen stimulation, thus facilitating transmission of virus between cells. As little is known about the mechanism underlying efficient EHV-1 cell-to-cell transmission in CD172a⁺ cells, we hypothesize that after productive viral infection in individual CD172a⁺ cells, EHV-1 recruits neighbor cells either by direct contact(s) via induction of membrane extensions (synapses) driven by cellular adhesion molecules (CAMs), or by indirect contact via a paracrine signaling, which induces cell migration to the developing focus of infection, similarly as in HIV infection (Nikolic *et al.*, 2011; Boomker *et al.*, 2005). Both contacts may result in the formation of cell clusters, which trigger cell-to-cell fusion events. These ‘microfusion’ events are likely to be responsible for infectious virus transfer and cytoplasmic transfer of viral material between an infected cell to uninfected surrounded cells, as described in the transmission of HCMV infection *in vitro* (Gerna *et al.*, 2000; Digel *et al.*, 2006). The adhesion molecules involved in cell-to-cell interactions as well as the exact composition of the cytoplasmic material transferred from an infected to uninfected CD172a⁺ cells still remain to be investigated. However, the results of direct cell-to-cell transmission of EHV-1 between CD172a⁺ cells, which undergo complete viral replication under HDAC inhibition, constitute relevant findings in light of the pathogenesis of EHV-1 *in vivo* and *in vitro*.

In conclusion, we have demonstrated that the replication of EHV-1 is restricted and delayed at the level of viral gene transcription in nasal and blood CD172a⁺ cells compared to RK-13 cells. These data substantiate the hypothesis that EHV-1 hijacks CD172a⁺ cells and silences its replication within the cells, acting like a ‘Trojan horse’, in order to survive in the blood circulation and reach target organs and to evade immunosurveillance. Silencing of EHV-1 gene expression in CD172a⁺ cells was shown to be tightly regulated by histone deacetylation events. A better understanding of the molecular details of chromatin regulation could give new insights into the pathogenesis of EHV-1, both *in vivo* and *in vitro*, and provide future therapeutic strategies.

Acknowledgements

We are grateful to Dr. O'Callaghan and Dr. Osterreider for supplying the antibodies against EHV-1 proteins. We also acknowledge Carine Boone, Lieve Sys and Zeger van den Abeele for their excellent technical support.

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*B. Replication of neurovirulent EHV-1 is not delayed in
CD172a⁺ monocytic cells*

Adapted from

Laval, K., Brown, I.K. and Nauwynck, H. J.

Short communication submitted to Virus Research

Abstract

Equine herpesvirus type 1 (EHV-1) is responsible for respiratory disorders, abortion and myeloencephalopathy (EHM) in horses. Two pathotypes of EHV-1 strains are circulating in the field: neurovirulent (N) and non-neurovirulent (NN). For both strains, CD172a⁺ monocytic cells are one of the main carrier cells of EHV-1 during primary infection, allowing the virus to invade the horse's body. Recently, we showed that NN EHV-1 strains showed a restricted and delayed replication in CD172a⁺ cells. Here we characterize the *in vitro* replication kinetics of two N EHV-1 strains in CD172a⁺ cells and investigate if the replication of the N EHV-1 strain is similarly silenced as shown for the NN EHV-1 strain. We found that N EHV-1 replication was restricted to 7-8% in CD172a⁺ cells compared to 100% in control RK-13 cells. N EHV-1 replication was not delayed in CD172a⁺ cells but virus production was significant lower ($10^{3.0}$ TCID₅₀/10⁵ inoculated cells) than in RK-13 cells ($10^{8.5}$ TCID₅₀/10⁵ inoculated cells). Approximately 0.04% of CD172a⁺ cells produced and transmitted infectious EHV-1 (0.04%) to neighbour cells compared to 65% of RK-13 cells. Unlike what we observed for the NN strain, pretreatment of CD172a⁺ cells with histone deacetylases inhibitors did not influence the replication of the N EHV-1 strain in these cells. Overall, these results show that the EHV-1 replication of N strain in CD172a⁺ cells differs from that observed for the NN strain, which may contribute to their different pathogeneses *in vivo*.

Introduction

Equine herpesvirus type 1 (EHV-1) is a major pathogen of horses worldwide. After primary replication in the epithelial cells of the upper respiratory tract (URT), EHV-1 disseminates through the body via a cell-associated viremia in PBMC to target organs such as the pregnant uterus or the central nervous system. Secondary replication in the endothelial cells lining the blood vessels of those organs can cause vasculitis and ischemic thrombosis, leading to severe symptoms such as abortion and/or equine myeloencephalopathy (EHM) (Edington *et al.*, 1991; Smith *et al.*, 1996; Wilson, 1997). Different EHV-1 strains circulating in the field have been characterized as neurovirulent (N) or non-neurovirulent (NN). It was demonstrated that a single nucleotide polymorphism (SNP) in the catalytic subunit of the viral DNA polymerase was associated with the neurovirulence of naturally occurring strains (Goodman *et al.*, 2007; Nugent *et al.*, 2006). CD172a⁺ monocytic cells were identified as one of the main carrier cells of EHV-1 in the URT mucosa and bloodstream (Gryspeerdts *et al.*, 2012; van der Meulen *et al.*, 2000). *In vivo* studies demonstrated that the number of infected CD172a⁺ cells in tissues of the URT was 3 to 7 times higher after inoculation with the Belgian EHV-1 N strain (03P37) than with the Belgian EHV-1 NN strain (97P70) (Gryspeerdts *et al.*, 2010). In addition, the expression of some late proteins in EHV-1-infected PBMC was hampered at early stages of infection and it was proposed that CD172a⁺ monocytic cells may serve as a ‘Trojan horse’ to facilitate the dissemination of EHV-1 to target organs (Laval *et al.*, 2015a; van der Meulen *et al.*, 2003b).

Recently, we demonstrated that the replication of EHV-1 was restricted and delayed at the level of viral gene transcription in CD172a⁺ cells compared to the RK-13 control cell line (Laval *et al.*, 2015a). We showed that EHV-1 gene expression in CD172a⁺ cells was silenced and that inhibition of histone deacetylases could relieve repression of viral gene expression. However, this study was carried out with the EHV-1 NN strain only. At present, it is not known whether EHV-1 N strain behave similarly. Knowledge of the replication kinetics with these strains is important, as different outcomes in EHV-1 replication in CD172a⁺ cells between the two strains may account for differences in the neuropathogenic vs abortigenic potential of EHV-1 *in vivo*. Thus, the main purpose of this study was to characterize *in vitro* the

replication kinetics of EHV-1 N strains in CD172a⁺ cells and to investigate if replication of the EHV-1 N strain is silenced similar as shown for EHV-1 NN.

Materials and methods

Virus

The Belgian EHV-1 N strains 95P105 and 03P37 were originally isolated in 1995 and 2003 from the blood of paralytic horses (Garré *et al.*, 2009; van der Meulen *et al.*, 2003a). The Belgian EHV-1 NN strain 97P70, which was isolated from an aborted fetus in 1997, was included in this study as a positive control for HDAC inhibition. Virus stocks used for inoculation were at the fourth passages in equine embryonic lung cells (EEL) and one subsequent passage in RK-13.

Cells

A. Isolation of equine blood CD172a⁺ cells

Blood CD172a⁺ cells were isolated as described by Laval *et al.* (2015a). Briefly, PBMC were isolated by density centrifugation on Ficoll-Paque, resuspended in leukocyte medium (LM) (RPMI supplemented with 5% FCS and antibiotics) and cultured for 12 h. Non-adhering lymphocytes were washed and the adherent cells consisted of >90% of viable CD172a⁺ cells, as assessed by flow cytometry.

B. Rabbit kidney epithelial (RK-13) cells

RK-13 cells were maintained in Modified Eagle's medium (MEM) supplemented with antibiotics and 5% FCS.

EHV-1 inoculation

Cell populations were inoculated *in vitro* with EHV-1 strains 03P37, 95P105 or 97P70 (control strain) at a MOI of 1 in 200µl leukocyte medium for 1 h at 37°C with 5% CO₂. Cells were gently washed twice with RPMI to remove the inoculum and further incubated with fresh medium. Mock inoculations were carried out in parallel. At 1, 3, 5, 7, 9, 12 and 24 hours post-inoculation (hpi), cells were collected for quantification of EHV-1 infected cells, viral production and production of infectious EHV-1 by immunofluorescence staining, virus titration and cocultivation assay,

respectively.

Histone deacetylase inhibition was done by pre-treating cells with 100 nM trichostatin A (TSA) or 0.5 mM of sodium butyrate (NaBut) for 2 h at 37°C prior to EHV-1 inoculation, as described previously by Laval et al. (2015a). HDAC inhibitors were maintained throughout the course of infection. All inhibitors were purchased from Sigma-Aldrich. The concentration of inhibitors used in this study did not decrease the cell viability (> 95%) determined by flow cytometry, as described above. CD172a⁺ cells pretreated or not with HDAC inhibitors and inoculated with EHV-1 NN (97P70) strain were included as a positive control in this study (data not shown). HDAC inhibitors treatment did not affect the kinetics of EHV-1 protein expression in inoculated RK-13 cells (data not shown).

Virus titration

To quantify EHV-1 replication, extracellular virus titers were determined at different hpi, as described previously by Laval et al. (2015a). Briefly, the supernatant containing the extracellular virus was collected, centrifuged at 400xg for 10 min at 4°C and stored at -70°C until titration. For the inactivation curve, EHV-1 was incubated for different times at 37°C.

Indirect immunofluorescence staining of EHV-1 proteins

After fixation with methanol, cells were washed with PBS and incubated with a rabbit polyclonal antibody anti-IEP (1:1000) to detect IEP expression and either a mouse monoclonal anti-EICP22 (K2 to IR4) (1:500) or anti-gB (4B6) (1:100) antibody against early (EICP22) and gB proteins expression, respectively. The IEP and EICP22 antibodies were kindly provided by Dr D. J. O'Callaghan (Louisiana State University, USA) and the 4B6 antibody by Dr N. Osterrieder (Freie Universität Berlin, Germany). Subsequently, samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:100) or Texas Red-conjugated goat anti-mouse IgG (1:100) antibodies (Molecular Probes). The percentage of viral antigen-positive cells was calculated based on 300 randomly selected cells. Samples were analyzed by confocal microscopy (Leica TCS SP2 Laser Scanning Spectral Confocal System; Leica Microsystems).

Cocultivation assay

A cocultivation assay was used to detect and quantify EHV-1 producing CD172a⁺ cells by co-cultivation of these EHV-1-inoculated CD172a⁺ cells with a permissive rabbit cell culture (RK-13), where a semi-solid overlay technique was applied, as described previously by Laval et al. (2015a).

Statistical analysis

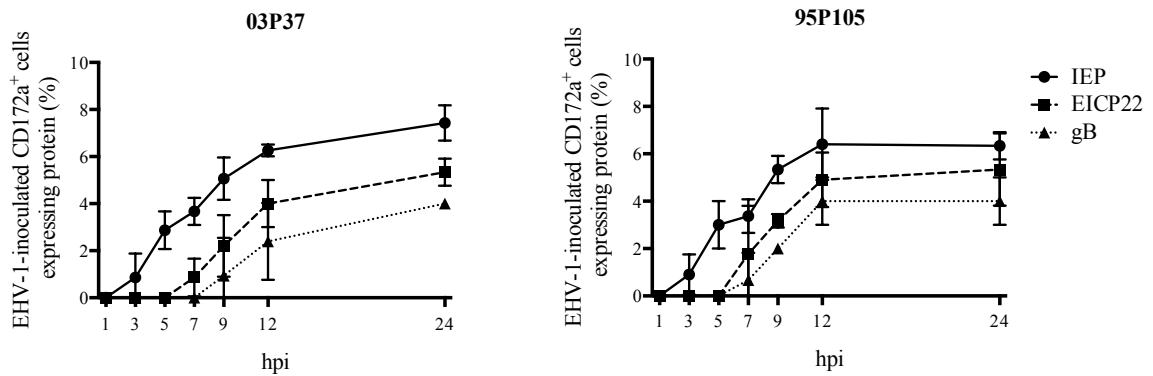
Data were analyzed with GraphPad Prism 5 software (GraphPad software Inc., San Diego, CA, USA). Analyzed data for statistical significance were subjected to a two-way analysis of variance (ANOVA). All results shown represent means and standard deviation (SD) of three independent experiments. Results with p -value ≤ 0.05 were considered statistically significant.

Results

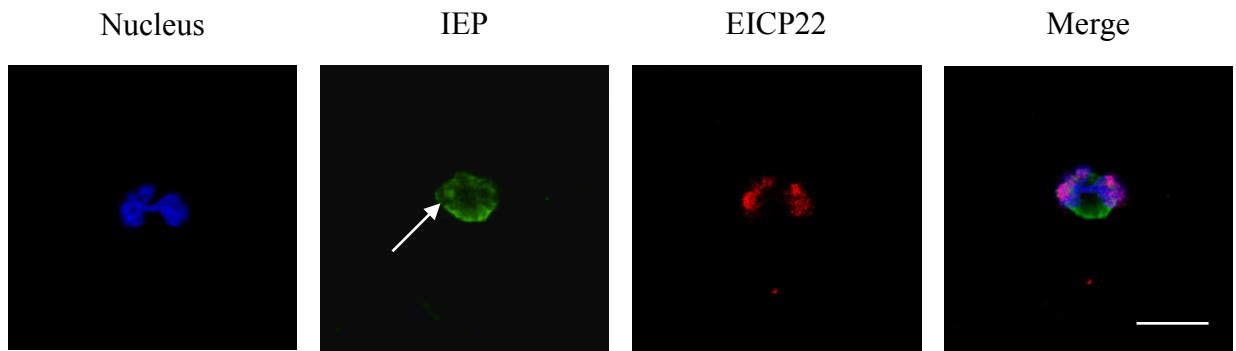
1. Replication of EHV-1 neurovirulent strain is restricted in CD172a⁺ cells compared to RK-13 cells

In CD172a⁺ cells inoculated with EHV-1 N strain (03P37), IEP, EICP22 and gB proteins were first detected at 3, 7, and 7-9 h post-inoculation (pi), respectively (Fig. 1(A), left panel). The kinetics of EHV-1 protein detection in CD172a⁺ cells were similar with those observed in RK-13 cells (data not shown). The percentage of IEP-, EICP22- and gB-positive CD172a⁺ and RK-13 cells increased over time pi. At 24 hpi, 100% of RK-13 cells were positive for all viral proteins (data not shown). In contrast, a maximum of $7.4 \pm 0.7\%$ IEP-positive, $5.3 \pm 0.5\%$ EICP22-positive and 4% of gB-positive CD172a⁺ cells was reached at 24 hpi. Similar results were observed for CD172a⁺ cells inoculated with EHV-1 N strain (95P105) (Fig. 1(A), right panel). IEP was expressed in the nucleus of CD172a⁺ cells within replicative compartments while IEP signal was diffuse throughout the cytoplasm (Fig. 1(B)). EHV-1 gB protein was exclusively expressed in the cytoplasm of CD172a⁺ cells, as previously described by Laval et al. (2015b) (data not shown). The use of a higher MOI (10) did not alter the number of IEP-positive cells (Fig. 1(C)). To quantify EHV-1 replication, extracellular virus titers were determined at 1, 12 and 24 hpi in CD172a⁺ cells and compared to virus titers in control RK-13 cells. As shown in Fig. 1(D), no significant increase in EHV-1 extracellular virus titers was observed in CD172a⁺ cells (approx. $10^{3.0}$ TCID₅₀/10⁵ inoculated cells) over time post-inoculation. A significant lower virus titer was observed in CD172a⁺ compared to RK-13 cells ($10^{8.5 \pm 0.4}$ TCID₅₀/10⁵ inoculated cells) at 24 hpi ($p < 0.0001$). A cocultivation assay was used to detect and quantify EHV-1 producing CD172a⁺ cells by co-cultivation of these EHV-1-inoculated CD172a⁺ cells with RK-13 cells. We found that the number of EHV-1-transmitting CD172a⁺ cells (0.04%) was significantly lower than the number of EHV-1-transmitting RK-13 cells (65%) ($p < 0.0001$). These results showed that the replication of EHV-1 N strains are highly restricted in CD172a⁺ cells compared to RK-13 cells.

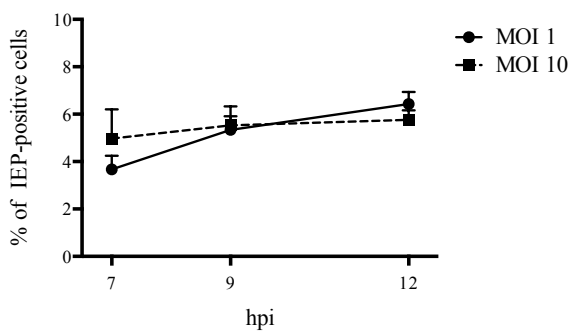
A



B



C



D

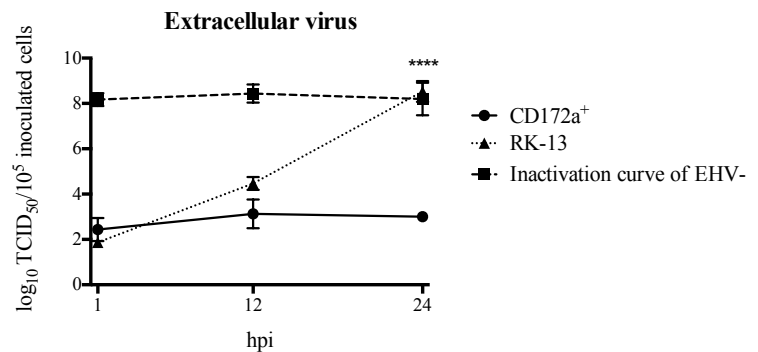
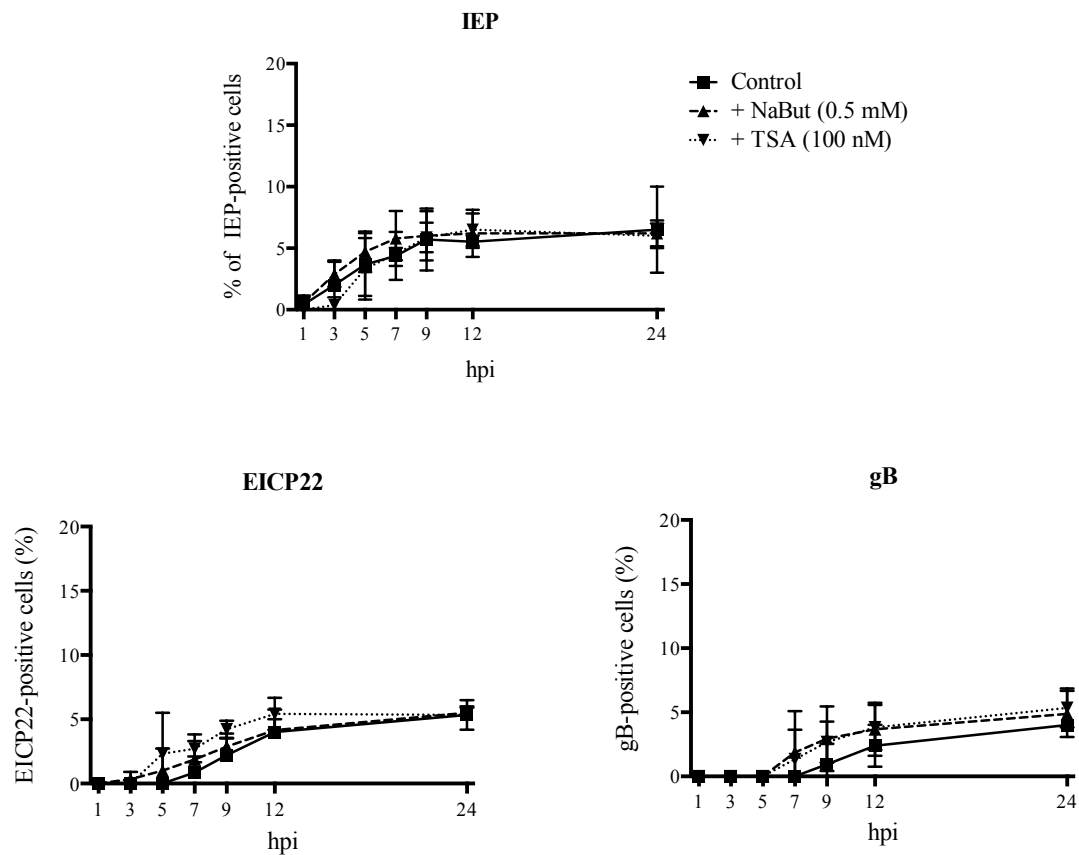


Figure 1: Expression kinetics of EHV-1 immediate-early (IEP), early (EICP22) and late gB proteins in CD172a⁺ cells inoculated with neurovirulent EHV-1 strains 03P37 and 95P105. (B) Double immunofluorescence of EHV-1 (03P37) IEP (green) and EICP22 (red) proteins in CD172a⁺ cells. Nuclei are counterstained with Hoechst (blue). Each image represents a single optical section through a cell. Arrow indicates the formation of IEP-associated replicative compartment. Scale bar = 10 μ m. (C) Expression kinetics of IEP in CD172a⁺ cells inoculated with EHV-1 (03P37) at a MOI of 1 or 10. No statistical differences were observed between the two groups by a two-way ANOVA test. (D) Kinetics of EHV-1 production in CD172a⁺ cells compared to RK-13 cells. Experiments were performed in triplicate and data are represented as means \pm SD. A two-way ANOVA test was performed to evaluate significant differences between CD172a⁺ and RK-13 cells (ns = not significant and **** = $p < 0.0001$).

2. The restricted replication of EHV-1 neurovirulent strain in CD172a⁺ cells is not controlled by histone deacetylases

Since histone deacetylation events were shown to play a crucial role in the silencing of EHV-1 gene expression in CD172a⁺ cells inoculated with a EHV-1 NN strain (Laval *et al.*, 2015a), we investigated whether histone deacetylases (HDACs) were similarly involved in the restricted replication of a EHV-1 N strain in CD172a⁺ cells. TSA- or NaBut treatment did not affect the percentage of IEP- and EICP22-positive CD172a⁺ cells for EHV-1 N strain (03P37). A slight but not significant increase in gB-positive CD172a⁺ cells was observed starting from 7 hpi (Fig. 2(A)). In addition, the titration of supernatant of NaBut- and TSA- treated cells did not show a significant rise in virus titer compared to non-treated cells at 12 and 24 hpi, respectively (Fig. 2(B)).

A



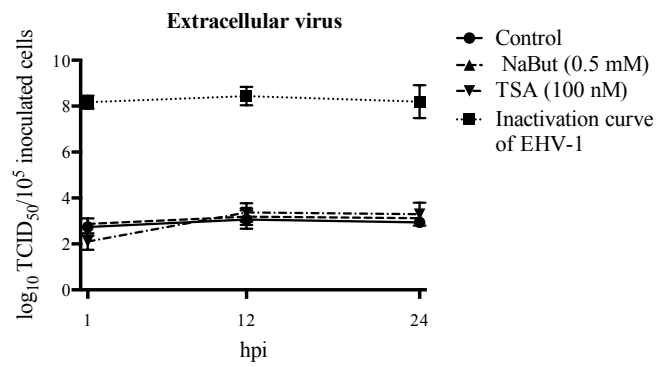
B

Figure 2: Replication of EHV-1 (03P37) in CD172a⁺ cells following treatment with HDACi, trichostatin A (TSA) and sodium butyrate (Nabut). (A) Comparison of the expression kinetics of IEP, EICP22 and gB in HDACi-treated and non-treated CD172a⁺ cells. (B) EHV-1 extracellular virus titer in NaBut- and TSA-treated CD172a⁺ cells compared to untreated cells at 1, 12 and 24 hpi. Experiments were performed in triplicate. Error bars show \pm SD. No statistical differences were observed between HDACi-treated and non-treated cells by a two-way ANOVA test.

Discussion

Taken together, we showed that the replication of two EHV-1 neurovirulent (N) strains was restricted to 7-8% of CD172a⁺ cells compared to 100% of RK-13 cells. This is in agreement with a study from van der Meulen *et al.* (2000), demonstrating that EHV-1 replication was highly restricted in equine PBMC. We reported previously that only a maximum of 4% of cells were susceptible to infection with non-neurovirulent strains (Laval *et al.*, 2015a). This is in line with the *in vivo* work from Gryspeerdt *et al.* (2012) that showed that a significantly higher percentage of CD172a⁺ cells became infected with EHV-1 N versus EHV-1 NN strains. Recently, we demonstrated that EHV-1 only binds to 15-20% of CD172a⁺ cells compared to 70% of RK-13 cells after 1 h inoculation and independent of the EHV-1 strains used (N and NN) (Laval *et al.*, 2015c). We showed that the narrow tropism of EHV-1 among CD172a⁺ cells was predominantly determined by the presence of specific cellular receptors. This study confirmed that a block at the virus binding level was partially responsible for the restricted replication of both EHV-1 N and NN strains in CD172a⁺ cells. However, our results suggest that additional block(s) exist in the replication cycle of EHV-1 N and NN strains in CD172a⁺ cells, at the level of virus DNA replication, assembly and/or egress, which may contribute to the immune evasive strategies of EHV-1.

We found that the spatio-temporal distribution of EHV-1 proteins was comparable between CD172a⁺ cells and control RK-13 cells after inoculation with EHV-1 N strains, in striking contrast with our previous findings on EHV-1 NN strains. In the current study, we could demonstrate that treatment of CD172a⁺ cells with TSA and NaBut, two HDAC inhibitors, did not significantly influence the replication of an EHV-1 N in these cells. In contrast, HDAC inhibitors treatment increased and unblocked replication of an EHV-1 NN in CD172a⁺ cells (Laval *et al.*, 2015a). These data showed that HDAC activity in the control of EHV-1 replication in target CD172a⁺ cells is likely to be strain dependent. Thus, we believe that the differential control of HDACs in EHV-1-infected CD172a⁺ cells may partially contribute to different EHV1 pathogenesises *in vivo*, in addition to the single nucleotide polymorphism in the viral DNA polymerase associated with N EHV-1 (Goodman *et al.*, 2007). Indeed, we found that the number IEP-positive CD172a⁺ cells expressing

gB at 24 hpi was significantly higher upon inoculation of cells with EHV-1 N (75%) versus EHV-1 NN (20%) strains, as reported in our previous work. We hypothesize that a delay in the replication associated with HDAC activity early in infection of CD172a⁺ cells with EHV-1 NN strain may slow down the expression of some glycoproteins at the cell surface. This may allow EHV-1 NN strain to behave as a ‘stealth virus’ persisting longer in CD172a⁺ cells and functionally hiding itself from the immune system in a better way than EHV-1 N strain. It is possible that EHV-1 NN strains have evolved from EHV-1 N strains and developed additional immune evasive strategies, such as the manipulation of HDAC activity in target CD172a⁺ cells. However, the fact that both EHV-1 pathotypes can still cause a viremia in the presence of virus-neutralizing antibodies and CTL precursors indicates that EHV-1 has evolved multiple strategies to bypass detection by the immune system. Future studies will sequence and compare the complete genome sequence of the used EHV-1 N and NN strains. We think that differences in the coding sequences of some EHV-1 regulatory proteins between the 2 strains might directly affect their functional interaction with HDACs. In addition, future studies should include more EHV-1 N and NN strains in order to draw any firm conclusions on the regulation of EHV-1 replication by HDACs.

Overall, we can conclude that the replication of EHV-1 N strains in CD172a⁺ cells is restricted compared to RK-13 cells. We found that the replication of the tested EHV-1 N strain is not delayed or controlled by HDAC, in contrast to previous observations using an EHV-1 NN strain. We believe that different outcomes in EHV-1 replication between N and NN strains may contribute to differences in the neuropathogenic vs abortigenic potential of EHV-1 *in vivo*. Further study of these mechanisms may contribute to the development of new therapeutic strategies to prevent EHV-1 related diseases.

Acknowledgements

This research was supported by the Concerted Research Action 01G01311 of the Research Council of Ghent University, Belgium. Kathlyn Laval and Hans J. Nauwynck are members of BELVIR consortium (IAP, phase VII) sponsored by Belgian Science Policy (BELSPO). The authors are grateful to Dr. O'Callaghan and Dr. Osterreider for supplying the antibodies against EHV-1 proteins. The authors also acknowledge Carine Boone and Lieve Sys for their excellent technical support.

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Chapter 4.

Entry of EHV-1 into CD172a⁺ monocytic cells

Adapted from

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Journal of General Virology (2015) (In press)

Abstract

Equine herpesvirus type 1 (EHV-1) causes respiratory disease, abortion and neurological disorders in horses. Cells from the myeloid lineage (CD172a⁺) are one of the main target cells of EHV-1 during primary infection. Recently, we showed that EHV-1 restricts and delays its replication in CD172a⁺ cells, as part of an immune evasive strategy to disseminate to target organs. Here, we hypothesize that a low efficiency of EHV-1 binding to and entry in CD172a⁺ cells is responsible for this restriction. Thus, we characterized EHV-1 binding and entry into CD172a⁺ cells and showed that EHV-1 only bound to 15-20% of CD172a⁺ cells compared to 70% of RK-13 control cells. Enzymatic removal of heparan sulphate (HS) did not reduce EHV-1 infection, suggesting that EHV-1 does not use HS to bind and enter CD172a⁺ cells. In contrast, we found that treatment of cells with neuraminidase (NA) reduced infection by 85-100% compared to untreated cells while NA-treatment of virus had no effect on infection. This shows that sialic acids residues present on CD172a⁺ cells are essential in the initiation of EHV-1 infection. We found that $\alpha_v\beta_3$ integrins are involved in the post-binding stage of CD172a⁺ cell infection. Using pharmacological inhibitors, we showed that EHV-1 does not enter CD172a⁺ cells in a clathrin- or caveolae-dependent endocytic pathway, nor by macropinocytosis, but requires cholesterol, tyrosine kinase, actin, dynamin and endosomal acidification, pointing towards a phagocytic mechanism. Overall, these results show that the narrow tropism of EHV-1 among CD172a⁺ cells is determined by the presence of specific cellular receptors.

Introduction

The entry of enveloped animal viruses into their host cells is mediated either by direct fusion of the viral envelope with the plasma membrane or by endocytosis, which can be pH-independent or -dependent (Marsh & Helenius, 2006). The entry of the prototype *Alphaherpesvirus*, herpesvirus simplex virus type 1 (HSV-1), into permissive cells can be mediated by both mechanisms and has been shown to be cell-type dependent (Nicola *et al.*, 2003; Wittels & Spears, 1991; Nicola *et al.*, 2005).

Equine herpesvirus type 1 (EHV-1), a member of the subfamily *Alphaherpesvirinae*, is a major pathogen of horses worldwide. EHV-1 is one of the main causes of respiratory disease, abortion and encephalomyelopathy (Allen & Bryans, 1986; Patel & Heldens, 2005). Like HSV-1, EHV-1 can enter cells, including rabbit kidney epithelial cells (RK-13) and equine dermal cells (ED) via direct fusion with the plasma membrane (Frampton *et al.*, 2007). The initial binding of EHV-1 to target cells is mediated via an interaction between virus glycoproteins gB and/or gC and cellular heparan sulphate (Neubauer *et al.*, 1997; Osterrieder *et al.*, 1999). The interaction of glycoprotein gD with a putative receptor (MHC-1) followed by the activation of a gH/gL complex, in combination with gB and gD, are required to complete the fusion between the viral envelope and the cell membrane (Kurtz *et al.*, 2010; Sasiki *et al.*, 2011; Atanasiu *et al.*, 2007). Van de Walle *et al.* (2008) demonstrated that EHV-1 enters via the endocytic/phagocytic pathway into CHO-K1 and PBMC and that α_v integrins are involved. In addition, Hasebe *et al.* (2009) indicated that EHV-1 entry pathways are cell-type dependent.

CD172a⁺ monocytic cells were identified as one of the main target cells of EHV-1 in the upper respiratory tract and the bloodstream during primary infection (van der Meulen *et al.*, 2000; Vandekerckhove *et al.*, 2010; Gryspeerdt *et al.*, 2012). EHV-1 replication was found to be restricted and delayed in CD172a⁺ cells at a very early stage of infection (Laval *et al.*, 2015a). It was proposed that CD172a⁺ cells may serve as a 'Trojan horse' to facilitate the spread of EHV-1 to target organs and evade immune-surveillance. However, until now, little is known about the regulation of the CD172a⁺ cell tropism. Here, we investigated the binding and entry of EHV-1 into CD172a⁺ cells. Using Dio-labeled virus particles, we compared the kinetics of EHV-1

binding to CD172a⁺ cells and to RK-13 cells, a cell line known to be fully susceptible to EHV-1 infection. Using pharmacological inhibitors and confocal microscopy, we investigated the entry mechanism(s) of EHV-1 into CD172a⁺ cells.

Material and methods

Virus

Two Belgian EHV-1 strains were included in this study. The neurovirulent strain 03P37 was originally isolated in 2003 from the blood of a paralytic horse (Garré *et al.*, 2009; van der Meulen *et al.*, 2003a), and the non-neurovirulent strain 97P70 was first isolated in 1997 from the lungs of an aborted fetus (van der Meulen *et al.*, 2000).

Cells

A. Isolation of equine blood CD172a⁺ cells

Blood CD172a⁺ cells were isolated as described by Laval *et al.*, 2015. Briefly, PBMC were isolated by density centrifugation on Ficoll-Paque, resuspended in leukocyte medium (RPMI supplemented with 5% FCS and antibiotics) and cultured for 12 h. Non-adhering lymphocytes were washed and the adherent cells consisted of >90% of CD172a⁺ cells, as assessed by flow cytometry.

B. Rabbit kidney epithelial (RK-13) cells

RK-13 cells were used as a control cell line for blood CD172a⁺ cells and were maintained in MEM supplemented with antibiotics and 5% FCS.

C. Cell viability

Cell viability was determined by flow cytometry prior to virus inoculation, using 1 µg ml⁻¹ propidium iodide and was > 90% in all cell populations.

EHV-1 purification and Dio-labeling

Viral purification was performed as described by Yang *et al.*, 2014. Briefly, culture fluids of EHV-1-infected (03P37 and 97P70) RK-13 cells were clarified and virus was collected after centrifugation. The virus suspension was brought on a discontinuous Optiprep gradient containing 10-30% (w/v) of iodixanol and centrifuged. The visible opalescent virus bands were harvested separately. The buffer was exchanged to HNE

(5mM HEPES, 150 mM NaCl, 0.1mM EDTA, pH 7.4) buffer by the use of a 50kDa filter device. Purified EHV-1 in solution was labeled with 3,3'-Diiodoacetylcarboxyanine perchlorate (Dio) dissolved in DMSO (Molecular Probes). Dio-labeling did not significantly decrease virus infectivity. The purity of EHV-1 suspensions was evaluated by lipophilic labeling and immunofluorescence staining of EHV-1 gB as similarly described by Yang et al. (2014). The staining was analyzed with confocal microscope by randomly selecting 10 regions. The ratio of EHV-1 gB-positive particles (virions) versus Dio-positive particles was calculated and referred to as the degree of viral purity (>90%).

Kinetics of EHV-1 attachment

Cells were cooled down to 4°C and were washed with ice-cold RPMI and inoculated at a MOI of 1 or 10 with Dio-labeled EHV-1 (03P37 or 97P70) for 1 h on ice. Cells were washed 2X with cold RPMI to remove unbound virus particles and were further incubated on ice for 0, 5, 10, 15, 30, and 60 min. Cells were fixed with 1% PFA for 10 min. Cell nuclei were stained with Hoechst 33342 (10 µg ml⁻¹; Molecular Probes) for 10 min at 37°C. Finally, coverslips were mounted on microscope slides and analysed by confocal microscopy.

The percentage of EHV-1 positive cells was calculated based on the number of cells with viral particles bound on the plasma membrane of 300 randomly selected cells. The average number of virus particles attached per cell was calculated based on the number of particles attached at the plasma membrane of 10 randomly selected EHV-1 positive cells. For each cell, the entire plasma membrane was screened for the presence of virus particles by generating a Z-stack.

Antibody-blocking assay

CD172a⁺ cells were pre-incubated with 10 or 50 µg/ml of neutralizing antibodies directed against CD29 (clone TDM29, IgG1, Merck Millipore), CD49d (clone 9F10, IgG2b, BD Pharmingen), CD11a/CD18 (clone CVS9, IgG1, AbD Serotec), CD51/61 (clone 23C6, IgG1, BioLegend), or with isotype-matched control antibodies for 30 min at 37°C. Cells were washed twice with RPMI and inoculated with EHV-1 (03P37

and 97P70, MOI 1) for 1 h and further incubated for 12 h at 37°C. Cells were fixed with methanol and incubated with a rabbit polyclonal antibody anti-IEP (1:1000) (kindly provided by Dr. D. O'Callaghan, Louisiana State University, USA) to detect immediate-early protein (IEP) expression, followed by a goat anti-rabbit IgG FITC (1:100) antibody. The nuclei were counterstained with Hoechst and the percentage of IEP-positive cells was determined as previously described.

To determine the role of $\alpha_v\beta_3$ (CD51/61) integrin in EHV-1 binding to CD172a⁺ cells, CD172a⁺ cells were pre-incubated with 10 $\mu\text{g/ml}$ of CD51/61 or with isotype-matched control antibodies for 30 min on ice. Cells were inoculated with Dio-labeled EHV-1 (03P37 or 97P70) particles (MOI 1) for 1 h on ice. Cells were fixed with PFA 1% and immunofluorescence staining was performed as described above.

Enzymatic treatment

For enzymatic removal of cell surface heparan sulphate or sialic acids, CD172a⁺ cells were incubated with 1 and 10 U/ml of heparinase I and III from *Flavobacterium heparinum* (Sigma) or 50 mU/ml neuraminidase (NA) from *Vibrio cholera* (Roche Diagnostics) diluted in DPBS for 1 h at 37°C. The concentration of heparinase and neuraminidase used in this study did not decrease the cell viability (> 90%). After washing, untreated and enzymatic-treated cells were inoculated with EHV-1 03P37 or 97P70 strains at a MOI of 1 for 1 h at 37°C. Cells were washed and further incubated for 12 h at 37°C.

To remove sialic acids from the virus, EHV-1 03P37 and 97P70 suspensions were incubated on a shaker for 1 h at 37°C with 50 mU/ml Glycocleave NA (*Vibrio cholera*) enzyme beads (GALAB Technologies) diluted in DPBS. Beads were washed two times with DPBS before incubation with the virus to remove buffers. Before inoculation, NA beads were separated from the virus by centrifugation (200 x g, 10 min, 4°C). Untreated virions were incubated in DPBS without beads and underwent the same manipulation as the NA-treated virus. Untreated cells were inoculated with either NA-treated or untreated virus at a MOI of 1 for 1 h at 37°C. Cells were washed and further incubated for 12h. The percentage of IEP-positive cells was determined as previously described.

Effect of different entry inhibitors on EHV-1 infection

Cells were pre-treated for 30 min at 37°C with inhibitory compounds: 10 mM methyl- β -cyclodextrin (m β CD) dissolved in PBS, 5 μ g/ml filipin III (fil) dissolved in DMSO, 10 μ g/ml genistein in DMSO, 500 μ M amantadine in PBS, 1 μ g/ml chlorpromazine in PBS, 100 μ M amiloride in DMSO, 100 μ M dynasore in DMSO and 20 μ M latrunculin B in DMSO. Then, cells were inoculated with EHV-1 03P37 or 97P70 strains (MOI 1) in the presence of inhibitors and incubated for 1 h at 37°C. After washing, cells were incubated for 12 h in the absence of inhibitors. Only m β CD and fil were removed at the time of virus inoculation in order to avoid any effect on cholesterol in the viral envelope. The maximal concentration of inhibitors was optimized to ensure that viability of cells was always over 85-90%. The percentage of IEP-positive cells was determined as previously described.

To assess the effectiveness of chlorpromazine, amantadine and amiloride on endocytosis inhibition, CD172a⁺ cells (untreated or pre-treated with 1 μ g/ml chlorpromazine, 500 μ M amantadine or 100 μ M amiloride) were incubated with Alexa Fluor 647-labeled transferrin (100 μ g/ml) and FITC-labeled dextran (1 mg/ml) for 1h at 37°C in the presence of the relevant inhibitor or medium. Alexa Fluor 647-labeled transferrin and FITC-labeled dextran, used as positive controls for clathrin-mediated endocytosis and macropinocytosis, were obtained from Thermo Fischer and Sigma-Aldrich, respectively. After 1h, cells were washed with PBS and the uptake of transferrin or dextran in CD172a⁺ cells was analysed by flow cytometry. The internalization of fluorescent marker was calculated as the mean fluorescence intensity (MFI) and expressed as a percentage of uptake. The 100% uptake was determined from CD172a⁺ cells, which have not been exposed to any inhibitor and have been incubated with the marker.

Colocalization with clathrin or caveolin-1

CD172a⁺ cells were inoculated with Dio-labelled EHV-1 (03P37 or 97P70) (MOI 1) for 0, 15 and 60 min at 37°C, washed and fixed with methanol. Cells were incubated with a rabbit polyclonal antibody anti-clathrin (1:200) (Biorbyt) or a goat polyclonal antibody anti-caveolin-1 (1:200) (Abcam), followed by a goat anti-rabbit IgG Texas-

Red[®] (1:200) or rabbit anti-goat IgG Texas-Red[®] (1:200) antibodies. The nuclei were counterstained with Hoechst. As a negative control, mock-inoculated cells were stained following the aforementioned protocols.

Effect of acidotropic agents on EHV-1 infection

Cells were treated with several acidotropic agents: ammonium chloride (NH₄Cl), chloroquine, or monensin. Cells were pre-incubated with different concentrations of NH₄Cl (0, 10, 20 or 30 mM) dissolved in DMSO, chloroquine (0, 1, 10 or 50 μM) in PBS or monensin (0, 1, 10 or 50 μM) in DMSO, for 2 h at 37°C. Untreated and treated cells were inoculated with EHV-1 (MOI 1) for 1 h in the presence of the drugs. After washing, cells were incubated in presence of the drugs for 12 h at 37°C. The inhibitor concentrations used did not decrease the cell viability (>90%). The percentage of IEP-positive cells was determined as previously described.

Statistical analysis

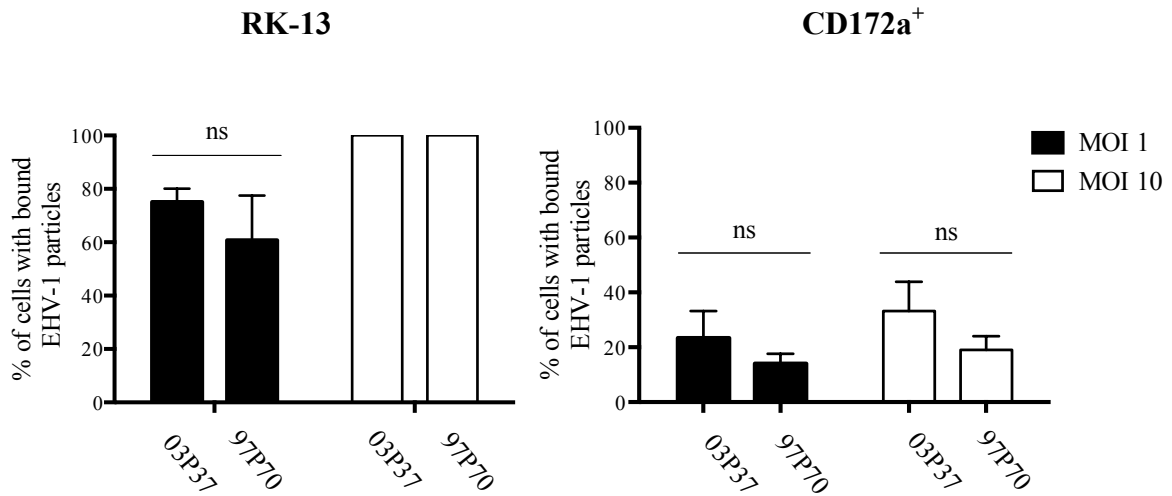
Data were analyzed with GraphPad Prism 6 software (GraphPad software Inc., San Diego, CA, USA). Data analyzed for statistical significance were subjected to a two-way ANOVA. Prior to statistical analyses, all variables were checked for assumptions of ANOVA (equal variances and normal distribution of the standardized residuals) by Levene's test. When the variables were not equal, a Kruskal–Wallis' test followed by pairwise comparisons were performed. All results shown represent means and standard deviation (SD) of three independent experiments. Results with *P*-value ≤ 0.05 were statistically significant.

Results

1. EHV-1 does not bind efficiently to CD172a⁺ cells compared to RK-13 cells

To characterize the attachment of EHV-1 to target CD172a⁺ cells, we compared the binding kinetics of Dio-labeled EHV-1 particles to CD172a⁺ cells and RK-13 cells. EHV-1 particles were observed on the plasma membrane of approximately 70% of RK-13 cells with a MOI of 1 at 60 min post-binding and this percentage was independent of the EHV-1 (03P37 or 97P70) strains used. At a MOI of 10, 100% of RK-13 cells showed virus particles bound on their surface (Fig. 1(A), left panel). In contrast, EHV-1 97P70 particles were observed on the plasma membrane of 14% of CD172a⁺ cells with a MOI of 1 and this percentage was not significantly higher in cells incubated with EHV-1 03P37 particles (23 %) or with a higher MOI (10) (Fig. 1(A), right panel). The number of EHV-1 particles attached to RK-13 cells significantly increased from 15 to 60 min ($p < 0.001$) (Fig. 1(B), left panel and Fig. 2(A)). The use of a higher MOI (10) increased the number of virus particles attached per RK-13 cell and made the counting of individual EHV-1 particles impossible. The binding of EHV-1 particles to CD172a⁺ cells increased from 1 to 60 min with a maximum of 3 ± 1 EHV-1 03P37 particles and 1.9 ± 0.1 EHV-1 97P70 particles bound per CD172a⁺ cell at 60 min post-binding at a MOI of 1 (Fig. 1(B), right panel and Fig. 2(B)). Taken together, these data demonstrate that EHV-1 does not bind efficiently to CD172a⁺ cells compared to RK-13 cells. The low percentage of CD172a⁺ cells with bound EHV-1 particles may suggest that EHV-1 particles attach to specific receptors on the surface of a restricted population of CD172a⁺ cells.

A



B

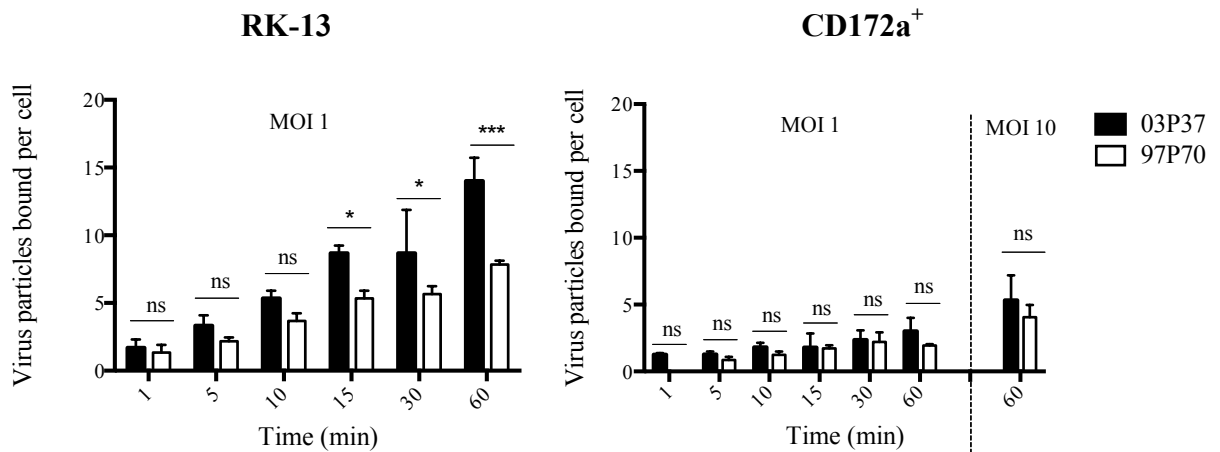


Figure 1: EHV-1 binding to RK-13 cells and CD172a⁺ cells. Cells were inoculated with Dio-labelled EHV-1 (03P37) or (97P70) particles (MOI 1 or 10) and incubated on ice for different durations. (A) Percentage of EHV-1 positive cells at 60 min post-binding. (B) Time-course of EHV-1 particles binding per cell. Three independent experiments were performed and data are represented as means + SD, ns = not significant; * = $p < 0.05$; *** = $p < 0.001$.

A

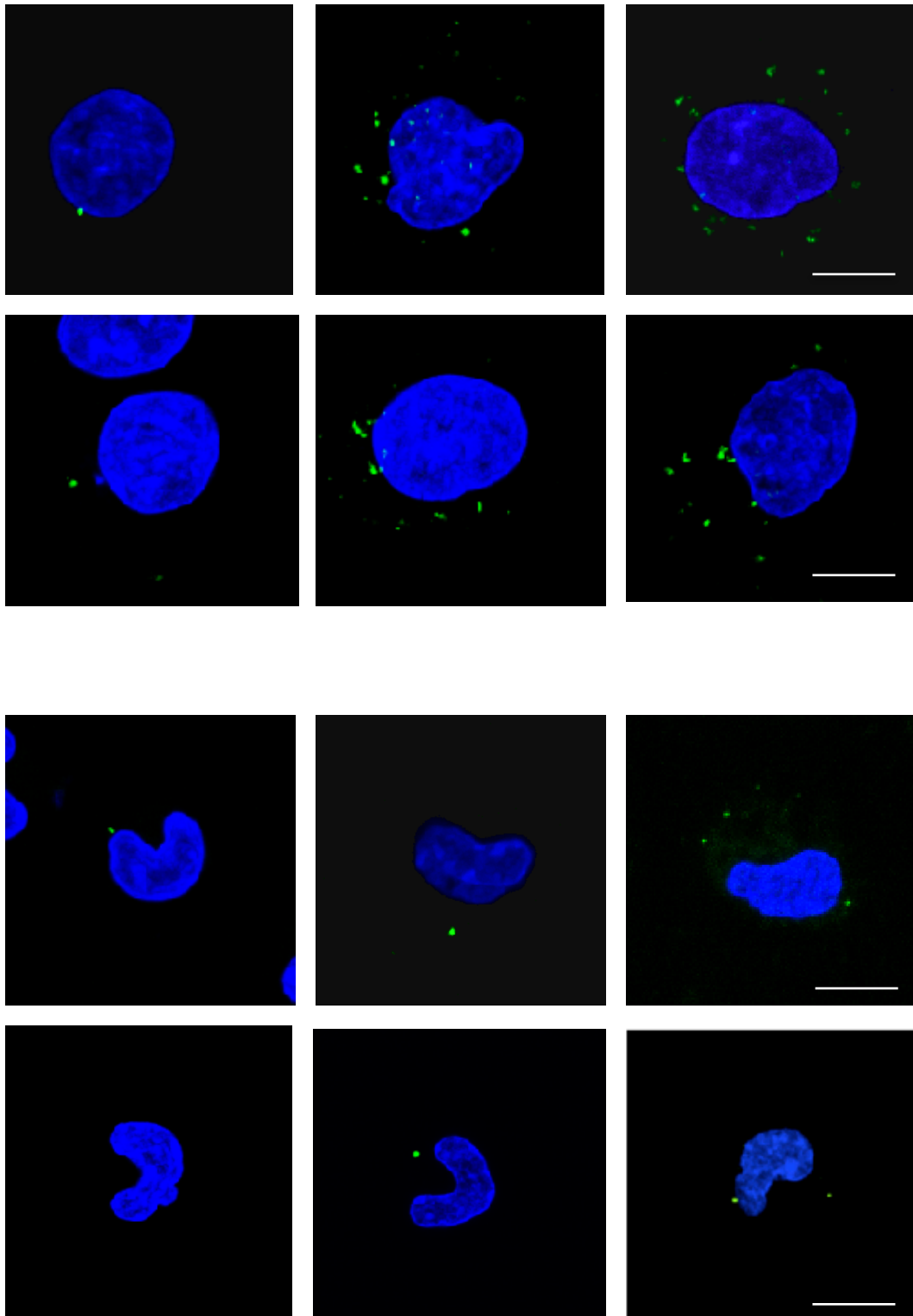


Figure 2: Confocal images of EHV-1 binding to RK-13 (A) and CD172a⁺ cells (B). EHV-1 particles (green) were visualized at the plasma membrane at 1, 15 and 60 min post-binding. Nuclei were counterstained with Hoechst (blue). All confocal images shown represent merged Z-stacks. Scale bar, 10 μ m.

2. EHV-1 infection of CD172a⁺ cells depends on $\alpha_v\beta_3$ integrin

Since α_v integrins have been reported to play a role in the entry of EHV-1 into PBMC (van de Walle *et al.*, 2008) and $\alpha_v\beta_3$ integrin was found to be expressed in only 17% of CD172a⁺ cells (Laval *et al.*, 2015b), we investigated whether $\alpha_v\beta_3$ integrin could be a potential cellular receptor involved in the restricted binding and/or entry of EHV-1 into CD172a⁺ cells.

We first performed infection-blocking experiments using a function-blocking antibody against $\alpha_v\beta_3$ integrin. Blocking antibodies against $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins, which are found expressed on 10-14% and 94% of CD172a⁺ cells (Laval *et al.*, 2015) were also included in this study to rule out their involvement in EHV-1 infection. Pre-treatment of EHV-1 inoculated CD172a⁺ cells with anti- $\alpha_v\beta_3$ integrin (CD51/61) antibody resulted in a dose-dependent significant decrease of EHV-1 infection in CD172a⁺ cells, compared to isotype control ($p < 0.01$). When used at a concentration of 50 $\mu\text{g/ml}$, we found that the anti- $\alpha_v\beta_3$ integrin antibody inhibited EHV-1 infection levels by 70 and 80% in CD172a⁺ cells inoculated with EHV-1 03P37 and 97P70 strains, respectively ($p < 0.001$) (Fig. 3). In contrast, no significant decrease of EHV-1 infection was observed in CD172a⁺ cells after pre-incubation with $\alpha_4\beta_1$ (CD49d/CD29) or $\alpha_L\beta_2$ (CD11a/CD18) blocking antibodies at a concentration of either 10 or 50 $\mu\text{g/ml}$.

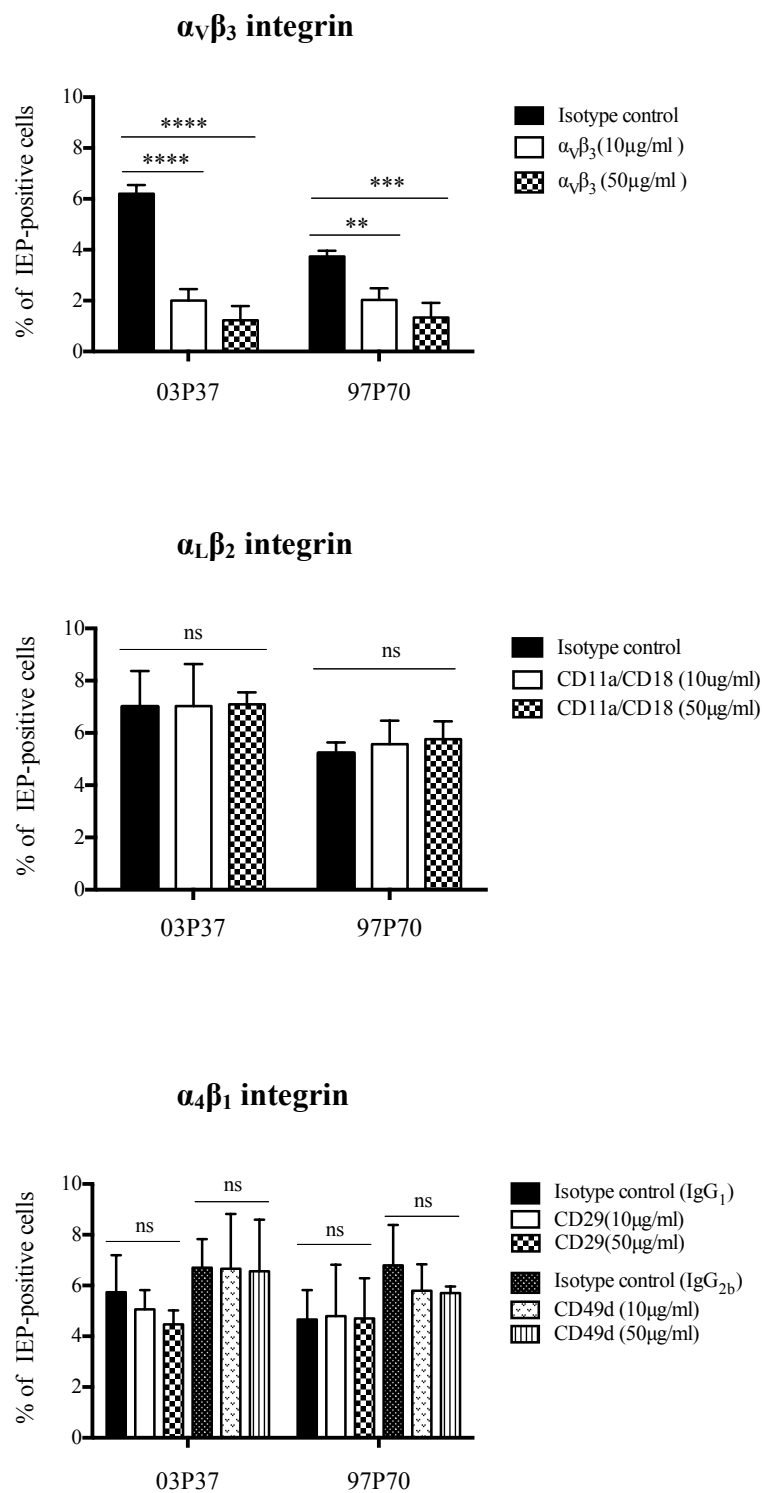


Figure 3: $\alpha_V\beta_3$ integrin-blocking antibody inhibits EHV-1 infection of CD172a⁺ cells. Cells were pre-incubated with $\alpha_4\beta_1$ (CD49d/CD29), $\alpha_L\beta_2$ (CD11a/CD18) and $\alpha_V\beta_3$ (CD51/CD61) antibodies or isotype-controls followed by EHV-1 inoculation (03P37 or 97P70 (MOI 1)). The percentage of immediate-early protein (IEP) positive cells was determined at 12h. Error bars show + SD, ns = not significant; ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$.

To determine whether the interaction of EHV-1 with $\alpha_v\beta_3$ integrin occurred during binding or at a post-binding step, we carried out EHV-1 binding-blocking experiments with blocking antibody to $\alpha_v\beta_3$ integrin. After pre-incubation with 10 $\mu\text{g/ml}$ of $\alpha_v\beta_3$ (CD51/61) integrin or specific isotype control, CD172a⁺ cells were inoculated with Dio-labeled EHV-1 (03P37 or 97P70) particles for 1 h on ice. The percentage of cells with bound virus particles was analyzed as previously described. As shown in Fig. 4, no significant decrease of EHV-1 03P37 or 97P70 binding to CD172a⁺ cells was observed after pre-incubation of cells with $\alpha_v\beta_3$ integrin-blocking antibody, compared to isotype control. We could not visualize $\alpha_v\beta_3$ integrin expressed at the plasma membrane of CD172a⁺ cells. It is possible that CD172a⁺ cells do not express high levels of $\alpha_v\beta_3$ integrin on their surface and confocal microscopy is not as sensitive as flow cytometry to detect low expression levels of $\alpha_v\beta_3$ integrin. Taken together, these results suggest that $\alpha_v\beta_3$ integrin is not used by EHV-1 for its initial attachment to the CD172a⁺ cell surface but is rather involved at a post-binding step.

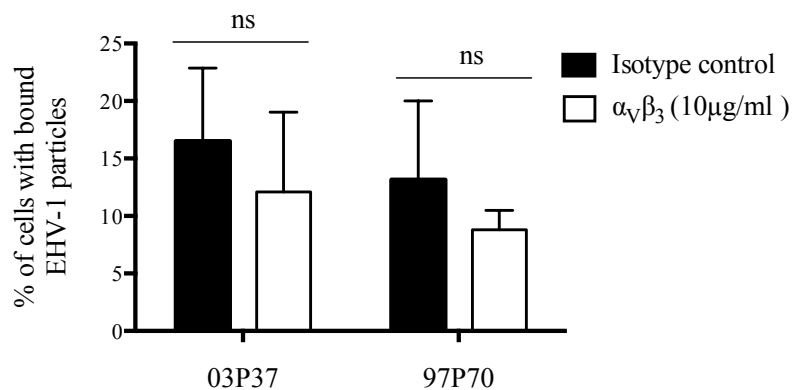


Figure 4: $\alpha_v\beta_3$ integrin-blocking antibody does not inhibit EHV-1 binding to CD172a⁺ cells. Cells were pre-incubated with $\alpha_v\beta_3$ (CD51/CD61) integrin-blocking antibody or isotype control for 30 min at 4°C, followed by inoculation with Dio-labeled EHV-1 03P37 or 97P70 strains (MOI 1) at 4°C. Cells were washed, fixed with 1% PFA without permeabilization and stained with Hoechst. The percentage of EHV-1 positive CD172a⁺ cells was determined at 60 min post-binding. Experiments were performed three times. Error bars show + SD. No statistical differences (ns) were observed from isotype control by a two-way ANOVA test.

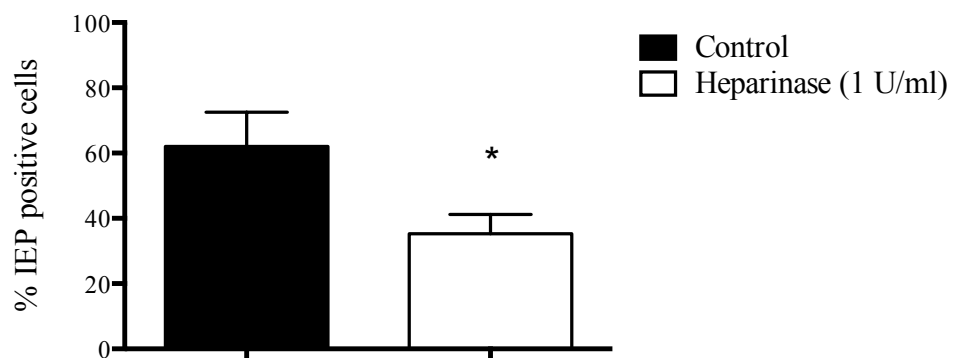
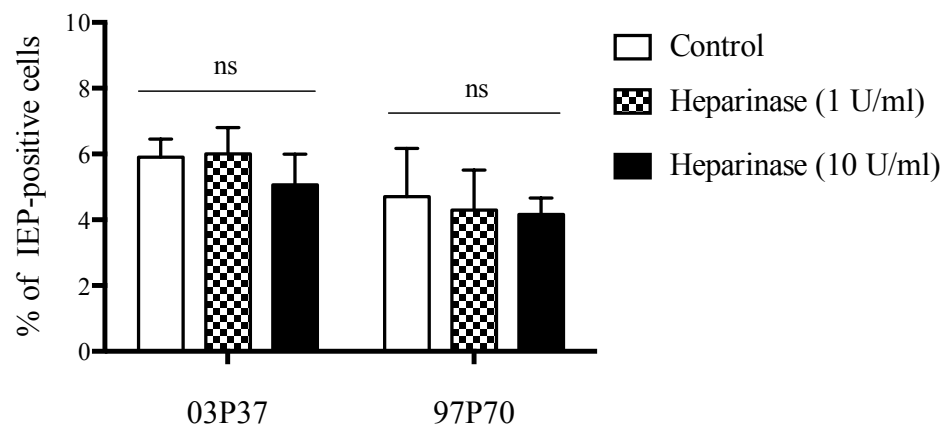
3. Sialic acids, but not cellular heparan sulphate, play a role in EHV-1 infection of CD172a⁺ cells

Heparan sulphate (HS) moieties are known to play a role in the binding step of several herpesviruses, including HSV-1, PRV and CMV (Skula & Spears, 2001, Shieh *et al.*, 1992; Trybala *et al.*, 1998; Compton *et al.*, 1993). However, the role of HS in EHV-1 entry into target CD172a⁺ cells as an attachment receptor has not been confirmed yet. To determine whether the interaction of EHV-1 with CD172a⁺ cells also involves HS molecules, cells were pre-treated with 1 or 10 U/ml of heparinase I and III, prior to inoculation with EHV-1 (03P37 or 97P70). As a positive control, RK-13 cells were pre-treated with 1U/ml of this enzyme before EHV-1 inoculation. As shown in Fig. 5(A), a significant reduction of EHV-1 infection was observed in heparinase-treated RK-13 cells compared to non-treated cells ($p < 0.05$). In contrast, we found that enzymatic removal of cell surface HS did not significantly reduce EHV-1 infection in CD172a⁺ cells compared to non-treated cells (Fig. 5(B)). This suggests that HS does not play a role in EHV-1 entry into CD172a⁺ cells.

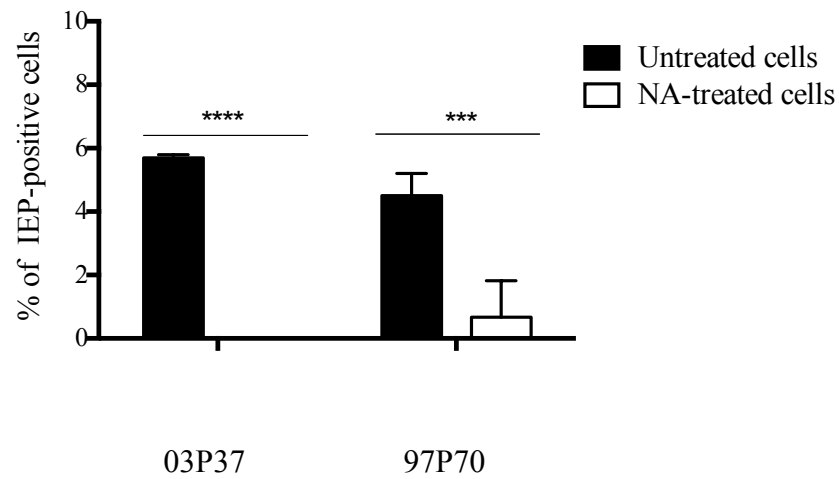
To further investigate the nature of the cellular receptor involved in the restricted attachment of EHV-1 to CD172a⁺ cells, we examined the role of sialic acids in EHV-1 infection of CD172a⁺ cells. Sialic acids constitute another group of carbohydrates other than glycosaminoglycans, and are known to serve as receptors for several viruses, such as influenza virus, respiratory syncytial virus or polyomaviruses (Gamblin *et al.*, 2004; Baretto *et al.*, 2003; Cahan *et al.*, 1983). Attachment to sialic acids present on cells is usually mediated by receptor binding proteins that are constituents of viral envelopes, such as the hemagglutinin protein for influenza virus (Skehel & Wiley, 2000). In addition, sialic acid binding immunoglobulin (Ig)-like lectins (siglecs) that are expressed on immune cells can recognize sialic acid moieties on the virus, as shown for HIV and PRSSV infections (Zou *et al.*, 2011; Delputte & Nauwynck, 2004).

To assess the role of sialic acids in EHV-1 infection on CD172a⁺ cells, cells were pre-treated with 50 mU/ml NA prior to inoculation with EHV-1. We found that EHV-1 infection was reduced by 100% and 85% in NA-treated CD172a⁺ cells inoculated with EHV-1 03P37 and 97P70, respectively, compared to untreated cells ($p < 0.001$) (Fig. 5(C)). As the presence of sialic acids on the virion surface has been shown to be

important in HSV-1 infection (Teuton *et al.*, 2007), we examined whether sialic acids on EHV-1 were involved in infection of CD172a⁺ cells by enzymatic removal of sialic acids from the surface of EHV-1. As shown in Fig. 5(D), no significant decrease in EHV-1 infection of untreated CD172a⁺ cells was observed between NA-treated and untreated virus. Taken together, these results indicate that EHV-1 depends on terminal sialic acid residues present on the cell surface for the initiation of its infection.

A**B**

C



D

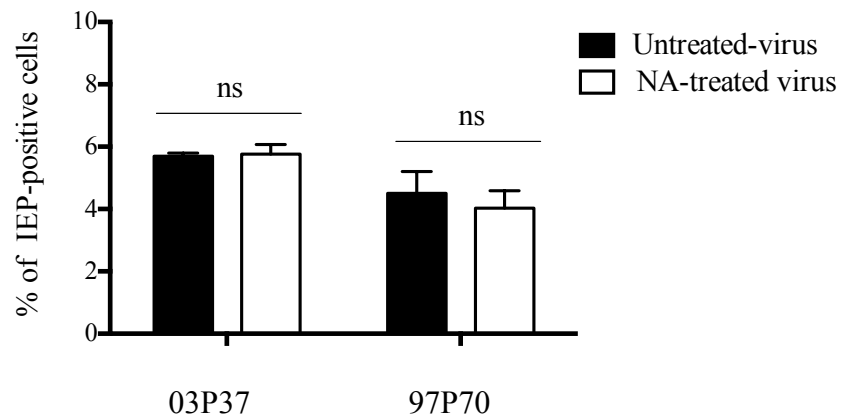
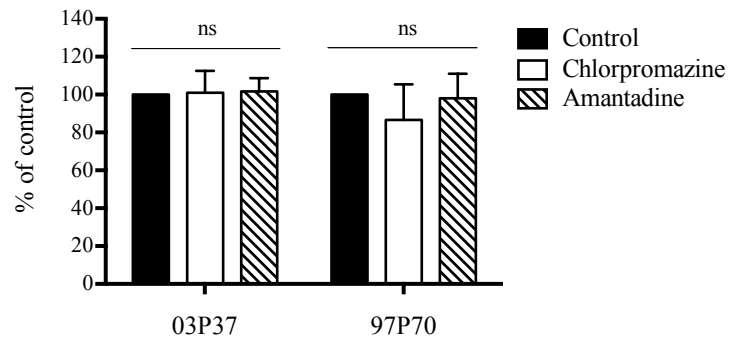
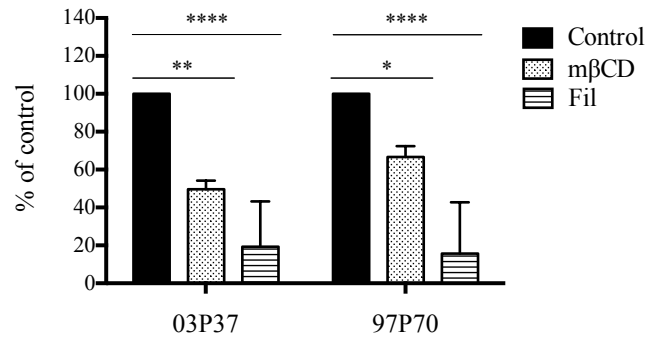
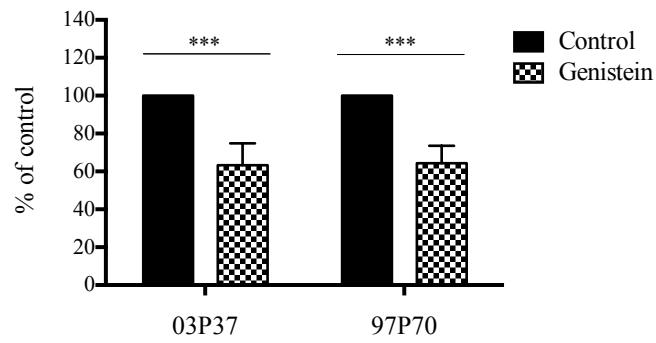
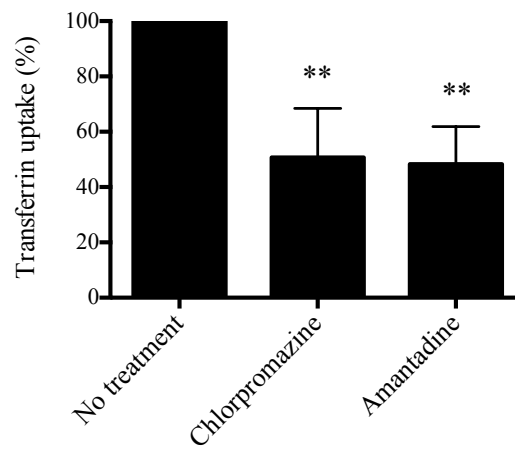


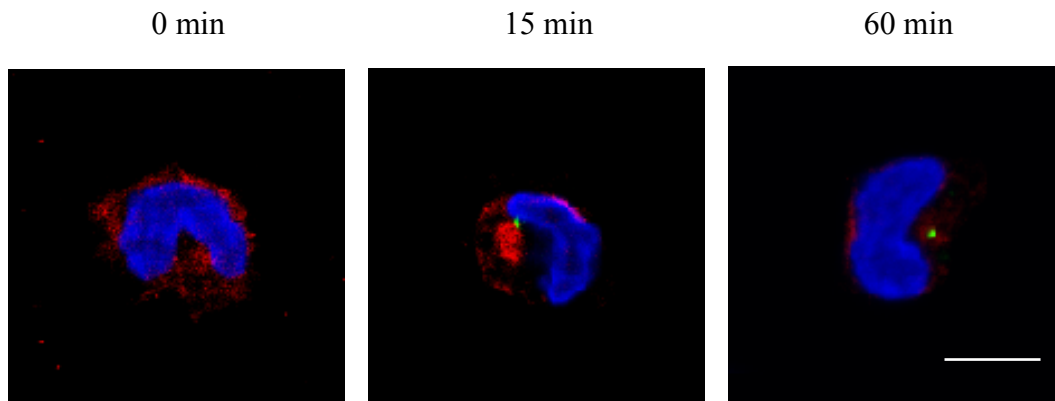
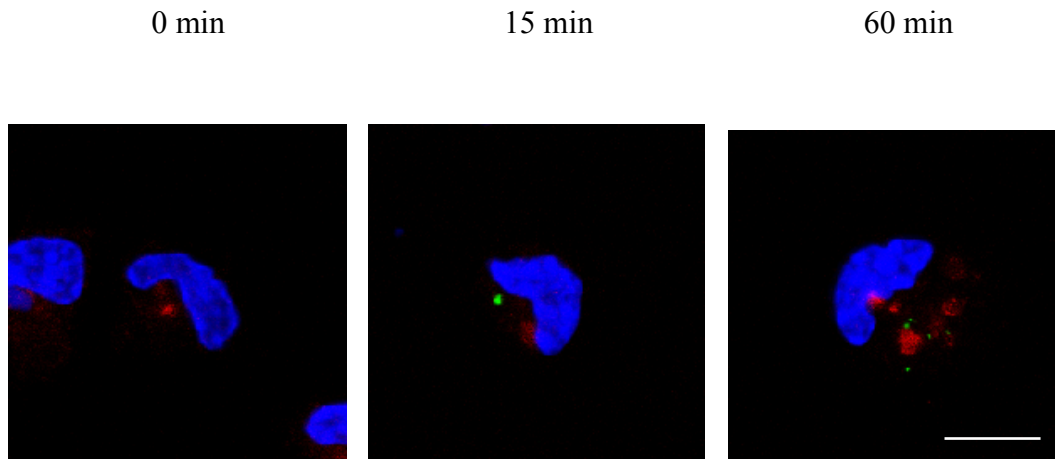
Figure 5: Removal of sialic acids but not heparan sulfate from CD172a⁺ cells reduces EHV-1 infection. (A) To confirm the activity of heparinase I and III, RK-13 cells were pre-treated or not with 1 U/ml of this enzyme prior to EHV-1 (03P37) inoculation. CD172a⁺ cells were pre-treated with heparinase I and III (B) or neuraminidase (C) prior to EHV-1 inoculation (03P37 or 97P70 (MOI 1)). Cells were further incubated for 12 h. (D) Effect of NA treatment of EHV-1 on the replication in untreated cells. Virus suspensions were pre-treated with Glycocleave NA beads before addition on CD172a⁺ cells. The percentage of immediate-early protein (IEP) positive cells was determined at 12h. Error bars show + SD, * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$ and ns = not significant.

4. EHV-1 entry into CD172a⁺ cells depends on cholesterol-rich lipid rafts but does not occur via clathrin- or caveolae-mediated endocytosis

We characterized the endocytic route of EHV-1 entry into CD172a⁺ cells using pharmacological/chemical inhibitors and co-localization assays. Pre-treatment of CD172a⁺ cells with chlorpromazine and amantadine, two specific inhibitors of clathrin-mediated endocytosis did not result in a significant decrease of EHV-1 infection compared to non-treated cells (control) and was independent of the EHV-1 strain used (Fig. 6(A)). To control the effectiveness of these inhibitors, CD172a⁺ cells untreated or pre-treated with chlorpromazine or amantadine were analysed for transferrin uptake, a marker for clathrin-dependent endocytosis. Chlorpromazine and amantadine caused a significant reduction in the uptake of transferrin in CD172a⁺ cells compared to untreated cells ($p < 0.01$) (Fig. 6(D)). Moreover, no clear signal of co-localization between clathrin molecules and EHV-1 particles was detected in CD172a⁺ cells at 0, 15 and 60 min post-inoculation (Fig. 6(E)).

The lipid raft/caveolae-mediated pathway is characterized by the formation of primary endocytic vesicles dependent on cholesterol, lipid rafts, cellular tyrosine kinase and/or phosphatase activities (Pelkmans, 2005). To disrupt lipid rafts and determine their role in EHV-1 entry into CD172a⁺ cells, cells were pre-treated with filipin (Fil), a sterol-binding drug that sequesters cholesterol within the membrane or with methyl- β -cyclodextrin (m β CD) that removes cholesterol from the plasma membrane. As shown in Fig. 6(B), EHV-1 infection was significantly reduced by 80% in CD172a⁺ cells cultured in the presence of Fil ($p < 0.0001$) and by 40-50% in cells cultured in the presence of m β CD ($p < 0.05$), independent of the EHV-1 strain used. In addition, pre-treatment of cells with the tyrosine kinase inhibitor genistein reduced EHV-1 infection by 40% in CD172a⁺ cells ($p < 0.001$) (Fig. 6(C)). However, no clear signal of co-localization between caveolin-1 molecules and EHV-1 particles was detected in CD172a⁺ cells at 0, 15 and 60 min post-inoculation (Fig. 6(F)). Taken together, we concluded that the entry route of EHV-1 into CD172a⁺ cells is not via a clathrin or caveolae-dependent mechanism. Moreover, these results suggest that cholesterol-rich lipid raft domains at the plasma membrane and cellular tyrosine kinase activity contribute to EHV-1 infection of CD172a⁺ cells.

A**B****C****D**

E**F**

*Figure 6: EHV-1 enters CD172a⁺ cells in clathrin- and caveolae-independent pathways. (A) Cells were pre-treated with chlorpromazine (1 $\mu\text{g/ml}$), amantadine (500 μM) or (B) with two sterol-binding drugs (m β CD (10 mM) and fil (5 $\mu\text{g/ml}$)) or (C) genistein (10 $\mu\text{g/ml}$) prior to EHV-1 inoculation (03P37 and 97P70 (MOI 1)). Cells were further incubated for 12 h. The percentage of IEP-positive cells was calculated as a percentage relative to that obtained in the control. (D) CD172a⁺ cells (untreated or pre-treated with 1 $\mu\text{g/ml}$ chlorpromazine or 500 μM amantadine) were incubated with Alexa Fluor 647-labeled transferrin (100 $\mu\text{g/ml}$) for 1 h at 37°C in the presence of the relevant inhibitor or medium. After 1 h, cells were washed and the uptake of transferrin (%) was analyzed by flow cytometry. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$; ns = not significant. Visualization of EHV-1 03P37 particles (green) and (E) clathrin protein (red) or (F) caveolin-1 protein (red) in CD172a⁺ cells. Nuclei were counterstained with Hoechst (blue). All confocal images represent a single section through a cell. Scale bar, 10 μm .*

5. EHV-1 infection of CD172a⁺ cells requires actin and dynamin but does not occur via macropinocytosis.

Previous studies showed a possible role for macropinocytosis in HSV-1 infection (Nicola *et al.*, 2003; Nicola *et al.*, 2005). To test whether macropinocytosis is involved in EHV-1 infection of CD172a⁺ cells, cells were pre-treated with amiloride, a specific inhibitor of macropinocytosis and latrunculin B, an inhibitor of actin polymerization. As shown in Fig. 7(A), EHV-1 infection was not reduced in CD172a⁺ cells treated with amiloride. In contrast, EHV-1 infection was significantly reduced by 55-65% in cells treated with latrunculin B ($p < 0.0001$). To control the effectiveness of amiloride, CD172a⁺ cells untreated or pre-treated with amiloride were analyzed for dextran uptake, a marker for macropinocytosis. Pre-treatment of CD172a⁺ cells with amiloride caused a significant reduction in the uptake of dextran compared to untreated cells ($p < 0.0001$) (Fig. 7(B)).

The GTPase dynamin is a key component of clathrin- and caveolae-mediated endocytosis and phagocytosis (Mettlen *et al.*, 2009; Nabi and Le, 2003; Gold *et al.*, 1999). Interestingly, the inhibition of uptake by dynamin constitutes a major difference between phagocytosis and macropinocytosis (Pelkmans & Helenius, 2003). To test whether EHV-1 infection of CD172a⁺ cells requires dynamin activity, cells were pre-treated with dynasore, a specific inhibitor of dynamin-1 and dynamin-2, which blocks dynamin-dependent endocytosis (Macia *et al.*, 2006). We found that EHV-1 (03P37 or 97P70) infection was significantly reduced in CD172a⁺ cells treated with dynasore compared to untreated cells ($p < 0.0001$) (Fig. 7(C)). Taken together, we concluded that EHV-1 infection is dependent on actin and dynamin but does not occur via macropinocytosis.

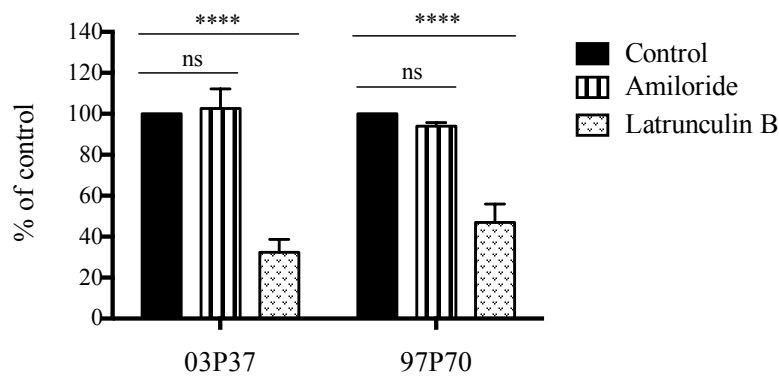
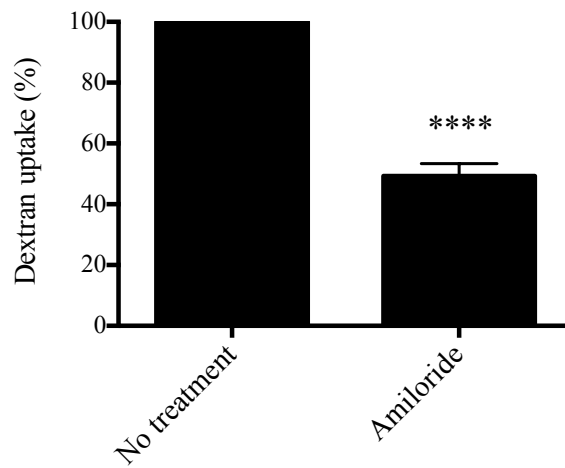
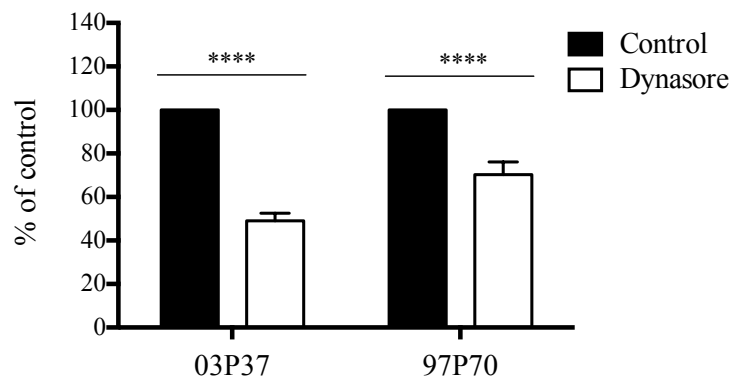
A**B****C**

Figure 7: EHV-1 infection of CD172a⁺ cells is dependent on actin and dynamin but does not require macropinocytosis. (A) Cells were pre-treated with amiloride (100 μ M), latrunculin B (20 μ M) or dynasore (100 μ M) (C), prior to EHV-1 inoculation (03P37 or 97P70 (MOI 1). Cells were further incubated for 12 h. The percentage of IEP-positive cells is expressed as percentage of control. (B) CD172a⁺ cells (untreated or pre-treated with 100 μ M amiloride) were incubated with FITC-dextran (1 mg/ml) for 1h at 37°C in the presence of the inhibitor or medium. After 1h, cells were washed and the uptake of dextran (%) was analyzed by flow cytometry. Error bars show + SD, **** = $p < 0.0001$ and ns = not significant.

6. Entry of EHV-1 into CD172a⁺ cells requires a low-pH step

To determine whether a pH drop was a crucial step in the infectious entry of EHV-1 into CD172a⁺ cells, we examined the effects of acidotropic agents: ammonium chloride, chloroquine and monensin on EHV-1 infection. Pre-treatment of CD172a⁺ cells with increased concentrations of these inhibitors resulted in a dose-dependent significant decrease of EHV-1 infection compared to non-treated cells ($p < 0.01$) and was independent of the EHV-1 strain used (Fig. 8). EHV-1 infection was reduced by 90% in CD172a⁺ cells treated with 30 mM ammonium chloride or 50 μ M chloroquine and was completely blocked in cells treated with 50 μ M monensin. No significant difference in the efficiency of inhibition of EHV-1 infection was observed in CD172a⁺ cells treated with inhibitors before inoculation, at the time of virus inoculation or 1 h post-inoculation, indicating that these agents did not affect binding of EHV-1 to cells but rather blocked EHV-1 infection at a post-binding step (data not shown). Taken together, these results show that EHV-1 enters CD172a⁺ cells in a low-pH dependent manner.

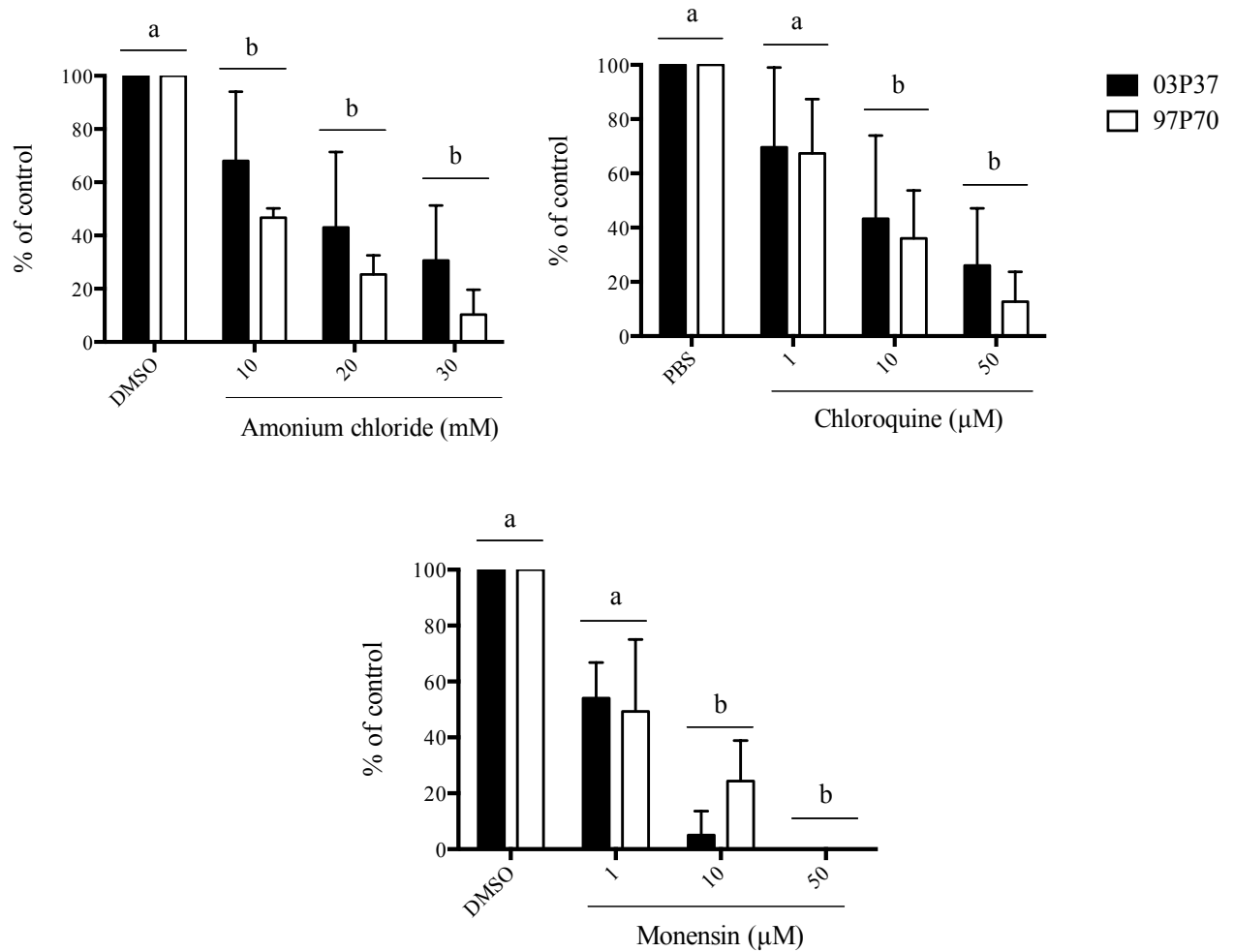


Figure 8: Acidotropic agents block EHV-1 infection of CD172a⁺ cells. Cells were pre-incubated with ammonium chloride, chloroquine and monensin prior to EHV-1 inoculation (03P37 or 97P70 (MOI 1)). The percentage of IEP-positive cells was calculated as a percentage of control. Error bars show +SD, different letters indicate significant differences ($p < 0.05$).

Discussion

In this study, we observed that EHV-1 only bound to a limited number of CD172a⁺ cells (15-20%, compared to 70% of RK-13 cells at 1 h post inoculation). These results suggest that EHV-1 binds to specific receptors on the cell surface of a small population of CD172a⁺ cells. Recently, we demonstrated that EHV-1 replication was highly restricted in CD172a⁺ cells *in vitro* (Laval *et al.*, 2015). We can confirm now that a block at the virus binding level is partially responsible for the restricted viral replication in CD172a⁺ cells.

To date, equine major histocompatibility complex class I (MHC-I) molecules have been identified as a unique gD receptor for EHV-1 entry into equine cells, including endothelial cells of the central nervous system (CNS) (Kurtz *et al.*, 2010, Sasaki *et al.*, 2011). As MHC-I molecules are expressed on the cell surface of all nucleated cells, MHC-I cannot explain the restricted EHV-1 attachment and entry into a relatively small subset of CD172a⁺ cells. In line with this, it was shown that MHC-I antibodies did not efficiently block EHV-1 entry into PBMC, suggesting the presence of a different receptor(s) (Azab *et al.*, 2012). Here we could demonstrate that $\alpha_v\beta_3$ integrins play a role in the post-binding stage of EHV-1 infection in CD172a⁺ cells. This result correlates with a study by van de Walle *et al.*, 2008, which showed that the RSD integrin motif, present in EHV-1 gD can bind to $\alpha_v\beta_3$ integrins found on PBMC and facilitates EHV-1 entry via endocytosis. Virus binding and entry are distinct processes involving different receptors. It is often assumed that viruses can bind to target cells to specific receptors and may require additional receptors to enter the cells. Cellular integrins have been shown to serve as receptors and/or co-receptors for several herpesviruses, including HSV, HCMV, Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) (Gianni *et al.*, 2010; Wang *et al.*, 2005; Chesnokova *et al.*, 2009; Garrigues *et al.*, 2008). Based on these findings, it is our understanding now that $\alpha_v\beta_3$ integrin is not used as the cellular receptor, crucial for the binding, but rather as a co-receptor for EHV-1 entry into CD172a⁺ cells. Although speculative at this point, the interaction of EHV-1 with $\alpha_v\beta_3$ integrin may induce conformational changes of the cellular receptor and initiate signaling cascades that trigger uptake of EHV-1 within the cell. EHV-1 may use $\alpha_v\beta_3$ integrin to route its entry via endocytosis into CD172a⁺ cells in order to shorten its exposure at the cell

surface. Thus, we believe that the restricted expression of $\alpha_V\beta_3$ integrins on CD172a⁺ cells may be an important determinant of EHV-1 tropism. Taken together, we suggest that co-receptor tropism on CD172a⁺ cells might have direct implications in the pathogenesis on EHV-1, as is the case for example in HIV infections (Goodenow & Collman, 2006).

In this study, we could rule out that HS is involved in EHV-1 infection of CD172a⁺ cells. The lack of involvement of HS in herpesvirus entry is rather exceptional. However, a recent study from Garrigues et al. (2014) showed that binding of the gammaherpesvirus KSHV is not dependent on HS and that receptors other than HS can mediate attachment to cells. To further examine the nature of the main receptor involved in the binding of EHV-1 to CD172a⁺ cells, we focused our research on the role of sialic acids in EHV-1 infection of CD172a⁺ cells. To date, several viruses such as influenza virus or polyomaviruses have been shown to bind specifically to sialic acids present on the surface of target cells, while its possible role in EHV-1 infection has not yet been described (Matrosovich *et al.*, 2013). We found that removal of sialic acids from cells inhibited EHV-1 infection of CD172a⁺ cells by 90-100%. In contrast, neuraminidase treatment of EHV-1 had no effect on infection of untreated CD172a⁺ cells. These results suggest that EHV-1 may interact with a sialic acid-containing cell-surface receptor on CD172a⁺ cells. So far, influenza virus is the best study example of sialic acids involvement in viral entry. Influenza virus uses hemagglutinin (HA) as an attachment receptor to bind to sialic acids on the cell surface (Wu *et al.*, 2004). The type of linkage between terminal sialic acid and the second sugar is one of the major determinants of influenza virus tropism and host specificity (Connor *et al.*, 1994; Matrosovich *et al.*, 2000). Further work will determine which sialylated glycans are present on CD172a⁺ cells and which are involved in EHV-1 binding and entry. The identity of EHV-1 envelope glycoprotein(s) involved as viral glycan-binding proteins should also be further investigated. Recent work from Andoh et al. (2014), demonstrated that EHV-1 gC protein had hemagglutination activity against equine red blood cells and that this activity was not inhibited by heparin. These results may suggest a potential role of EHV-1 gC as a viral lectin, involved in the binding of sialic acids residues on CD172a⁺ cells.

Following binding to cell-surface receptors, EHV-1 must be internalized to initiate infection. EHV-1 can enter target cells via direct fusion with the plasma membrane or via an endocytic/phagocytic entry pathway, which has been proposed to be the choice

of entry of EHV-1 into PBMC (Frampton *et al.*, 2007; van de Walle *et al.*, 2008). Based on this published information, the last part of our study further defined the endocytic mechanism(s) of EHV-1 entry into CD172a⁺ cells using pharmacological inhibitors and co-localization assays. We demonstrated that EHV-1 enters into CD172a⁺ cells via an endocytic pathway that is independent of clathrin and caveolae but is dependent on cholesterol, tyrosine kinase, actin, dynamin and requires a low-pH step. In addition, we found that treatment of CD172a⁺ cells with amiloride, a commonly used inhibitor of macropinocytosis, had no effect on EHV-1 infection. Hence, our data indicate that EHV-1 does not enter CD172a⁺ cells by clathrin- or caveolae-dependent endocytosis or macropinocytosis, and suggest that EHV-1 enters CD172a⁺ cells via phagocytosis or via a non-classical endocytic pathway. Several indications point towards a phagocytic mechanism for EHV-1 in CD172a⁺ cells. Phagocytosis is triggered by the recognition of large particles by cell surface phagocyte receptors, which leads to the polymerization of actin underneath the contact zone and the formation of phagocytic “cup” around the ingested particle (Mercer *et al.*, 2009). This is usually a property of specialized cell types including macrophages and dendritic cells. Phagocytosis is sensitive to the depletion of cholesterol, implying the involvement of lipid rafts. However, it has been proposed that phagosomes are not formed entirely of lipid rafts but rather contain both lipid raft and non-raft regions of the membrane, called mixed membrane domains (Kannan *et al.*, 2008; El-Sayed & Harashima, 2013). Phagocytosis is also dependent on dynamin, a coordinating molecule that regulates the formation of membrane extensions around the particle (Di *et al.*, 2003). Finally, maturation of phagosomes is associated with a pH decrease (Flannagan *et al.*, 2012), in line with our observation that EHV-1 entry in CD172a⁺ cells requires a pH drop. Phagocytosis-like uptake of alphaherpesviruses has been reported before in a study by Clement *et al.* (2006) on HSV entry, which further supports the possibility that EHV-1 might enter CD172a⁺ cells via phagocytosis. One argument against this entry route is the fact that EHV-1 virions are smaller (approximately 200 nm) than the generally accepted size limit for phagocytosis (approximately 500 nm). In any case, further studies are required to elucidate whether phagocytosis is the main mode of EHV-1 entry into CD172a⁺ cells, a task hampered by a lack of specific pharmacological inhibitors.

Surprisingly, we found that both neurovirulent and non-neurovirulent EHV-1 particles bound and entered CD172a⁺ cells in a similar way. These findings indicate that the mechanism of EHV-1 entry into CD172a⁺ cells may not account for differences in the neuropathogenic vs abortigenic potential of EHV-1 *in vivo* but may rather reflect differences in EHV-1 replication between the two strains. However, it is clear now that several block(s) exist in the replication cycle of EHV-1 in CD172a⁺ cells, which all may make part of an immune evasive strategy. While a small fraction of CD172a⁺ cells show EHV-1 particles bound to their surface, we think that not all viral particles will be internalized and not all viral genomes will be delivered to the nucleus and initiate efficient viral replication. Indeed, among 15-20% of CD172a⁺ cells with virus particles attached, we previously found that only 4 to 8% of cells were susceptible to EHV-1 infection, dependent of the EHV-1 strain used (Laval *et al.*, 2015a; Laval *et al.*, manuscript in preparation).

In conclusion, we showed that EHV-1 binds to a restricted number of CD172a⁺ cells, indicating that EHV-1 may use specific receptor molecules. Based on our data, we propose that EHV-1 interacts with a sialic acid-containing cell-surface receptor on CD172a⁺ cells and that $\alpha_v\beta_3$ integrin acts as a co-receptor, which may explain the restricted tropism of the virus to particular CD172a⁺ cells. In addition, our data suggest a phagocytosis-like uptake mechanism for EHV-1 in CD172a⁺ cells. Further unravelling of this entry route of EHV-1 may provide new insights into the pathogenesis of EHV-1, both *in vivo* and *in vitro*, and may contribute to the development of new therapeutic strategies.

Acknowledgements

This research was supported by the Concerted Research Action 01G01311 of the Research Council of Ghent University, Belgium. Kathlyn Laval and Hans J. Nauwynck are members of BELVIR consortium (IAP, phase VII) sponsored by Belgian Science Policy (BELSPO). Jolien Van Cleemput is a researcher funded by the Research Foundation Flanders (FWO) (11Y5415N). Katrien C.K. Poelaert is a researcher funded by the Institute for the promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) (141627). The authors are grateful to Dr. O'Callaghan for supplying the antibodies against EHV-1 proteins. The authors also acknowledge Carine Boone and Lieve Sys for their excellent technical support.

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Chapter 5.

EHV-1 enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells

Adapted from

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Journal of Virology (2015) 89 (10912-10923)

Abstract

Equine herpesvirus type 1 (EHV-1) is a main cause of respiratory disease, abortion and encephalomyelopathy in horses. Monocytic cells (CD172a⁺) are the main carrier cells of EHV-1 during primary infection and are proposed to serve as a ‘Trojan horse’ to facilitate the dissemination of EHV-1 to target organs. However, the mechanism by which EHV-1 is transferred from CD172a⁺ cells to endothelial cells (EC) remains unclear. The aim of this study was to investigate EHV-1 transmission between these two cell types. We hypothesized that EHV-1 employs specific strategies to promote the adhesion of infected CD172a⁺ cells to EC to facilitate EHV-1 spread. Here we demonstrated that EHV-1 infection of CD172a⁺ cells resulted in a 3 to 5-fold increase in adhesion to EC. Antibody-blocking experiments indicated that $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_V\beta_3$ integrins mediated adhesion of infected CD172a⁺ cells to EC. We showed that integrin-mediated PI(3)K and ERK/MAPK signaling pathways were involved in EHV-1-induced CD172a⁺ cell adhesion at early time of infection. EHV-1 replication was enhanced in adherent CD172a⁺ cells, which correlates with the production of TNF- α . In the presence of neutralizing antibodies, approximately 20% of infected CD172a⁺ cells transferred cytoplasmic material to uninfected EC and 0.01% of infected CD172a⁺ cells transmitted infectious virus to neighbouring cells. Our results demonstrated that EHV-1 infection induces adhesion of CD172a⁺ cells to EC, which enhances viral replication, but that transfer of viral material from CD172a⁺ cells to EC is a very specific and rare event. These findings give new insights in the complex pathogenesis of EHV1.

Introduction

Equine herpesvirus type 1 (EHV-1), a member of the sub-family *Alphaherpesvirinae*, is a ubiquitous pathogen in horses, causing serious economic losses in horse industry. Primary EHV-1 infection usually results in the establishment of a lifelong latent infection within the first months of life with subsequent viral reactivation causing clinical disease and viral shedding during periods of stress (Allen *et al.*, 2004; Foote *et al.*, 2004). EHV-1 infection is characterized by upper respiratory disease, neurological disorders, abortion or neonatal death (Allen & Bryans, 1986; Patel & Heldens, 2005). The virus first replicates in the epithelial cells of the upper respiratory tract and disseminates through the body via a cell-associated viremia in peripheral blood mononuclear cells (PBMC) to target organs such as the pregnant uterus or central nervous system. Secondary replication in the endothelial cells (EC) lining the blood vessels of those organs can cause vasculitis and ischemic thrombosis and may lead to severe symptoms such as abortion and/or neurological disorders (Edington *et al.*, 1991; Smith *et al.*, 1996; Wilson, 1997).

Monocytic cells (CD172a⁺) have been shown to be the main carrier cell type of EHV-1 during primary infection (Gryspeerd *et al.*, 2012; van der Meulen *et al.*, 2000; Vandekerckhove *et al.*, 2010). We previously reported that CD172a⁺ cells serve as a ‘Trojan horse’ to facilitate the spread of EHV-1 to target organs and evade immunosurveillance (Laval *et al.*, 2015). This may contribute to the observation that current vaccines do not provide full protection against severe symptoms, as EHV-1 can cause a viremia despite the presence of a virus-specific immune response in the horse (Kydd *et al.*, 2006; Mumford *et al.*, 1987; O'Neill *et al.*, 1999).

EC are actively involved in a wide variety of pathological processes such as thrombosis and vasculitis, and endothelial cell-monocyte interactions are known to play a central role in the pathogenesis of herpesviruses infections. For instance, human cytomegalovirus (HCMV) infection of EC has been shown to promote naïve monocytes adhesion to and migration through the endothelium and viral-mediated cellular activation was found to be responsible for HCMV-induced monocyte migration (Bentz *et al.*, 2006; Smith *et al.*, 2007). The mechanism of HCMV dissemination to host tissue is thought to be associated with HCMV-induced vascular diseases.

So far, several *in vitro* systems have demonstrated the potential utility of cultured EC in the study of the pathogenesis of EHV-1 (Chiam *et al.*, 2006; Tearle *et al.*, 2003). Studies showed that the infection of EC located in the vasculature of the late-gravid uterus or CNS was mediated by cell-to-cell contacts between infected PBMC and EC and occurred even in the presence of virus neutralizing antibodies (Goehring *et al.*, 2011; Smith *et al.*, 2001). In addition, Smith *et al.* (2001) provided evidence that activation of EC adhesion molecules may be involved in the transfer of virus from infected PBMC to EC and may determine the restricted tissue specificity of EHV-1. However, the precise mechanism underlying the transmission of EHV-1 from monocytic cells to EC is still unclear.

Given the importance of the interactions between monocytic cells and EC in the pathogenesis of EHV-1 infections, we studied the ability of EHV-1 inoculated CD172a⁺ cells to adhere and subsequently transmit EHV-1 infection to equine venous EC. We examined the contributions of specific cell adhesion molecules and the cellular signal transduction pathways involved in the adhesion process *in vitro*. Furthermore, we studied the replication kinetics of EHV-1 in CD172a⁺ cells upon adhesion to EC.

Material and methods

Virus

Two Belgian EHV-1 strains were included in this study. The neurovirulent strain 03P37 was originally isolated in 2003 from the blood of a paralytic horse (Garré *et al.*, 2009; van der Meulen *et al.*, 2003a) and non-neurovirulent strain 97P70 was first isolated in 1997 from the lungs of an aborted fetus (van der Meulen *et al.*, 2000). Virus stocks used for inoculation were at sixth passage, with five passages in equine embryonic lung cells (EEL) and one passage in RK-13 cells for strain 97P70, and four passages in EEL and one passage in RK-13 cells for strain 03P37. For virus inactivation, a thin layer of viral suspension was exposed to short-wave UV light for 10 min. Absence of viral infectivity was checked by virus titration on RK-13.

Cells

A. Isolation of equine blood CD172a⁺ cells

Healthy horses between 8 to 10 years old were used as blood donors. Horses were seropositive for EHV-1. The collection of blood was approved by the ethical committee of Ghent University (EC2013/17). Blood was collected by jugular venipuncture on heparin (15U ml⁻¹) (Leo) and diluted in an equal volume of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Gibco). PBMC were isolated by density centrifugation on Ficoll-Paque (d = 1.077g ml⁻¹) (GE Healthcare, Life Sciences) at 800 x g for 30 min at 18°C. The interphase cells, containing the PBMC, were collected and washed three times with DPBS. Cells were resuspended in leukocyte medium (LM) based on Roswell Park Memorial Institute (RPMI, Gibco) supplemented with 5% fetal calf serum (FCS) (Grainer), 1% penicillin, 1% streptomycin, 0.5% gentamycin (Gibco). Afterwards, cells were seeded in 6-well plates (Nunc A/S) at a concentration of 10⁶ cells per ml and cultivated at 37°C with 5 % CO₂. After 12 h, non-adhering lymphocytes were removed by washing cells three times with RPMI. The adherent cells consisted of > 90% monocytic cells, as assessed by flow cytometry after indirect immunofluorescence staining with a mouse monoclonal (mAb) anti-CD172a (VMRD, clone DH59B, 1:100, IgG1) directed against cells from myeloid lineage, followed by goat anti-mouse IgG FITC

(Molecular probes, 1:200).

B. Isolation of equine venous endothelial cells

Equine endothelial cells were obtained from the *vena cava* of a healthy horse at the slaughterhouse. The *vena cava* was harvested in a bottle containing Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 1% penicillin, 1% streptomycin, 0.5% gentamycin and 0.1% fungizone. One end of the vessel was closed using a hemostatic clamp. Pre-warmed enzyme mixture of 0.1% type I collagenase (Invitrogen) and 0.12% dispase (Sigma-Aldrich) in DMEM was infused into the segment until there was moderate distention of the vessel. After closing the segment with a second hemostatic clamp, the vessel was incubated during 30 - 40 min at 37°C. Then, one of the hemostatic clamps was opened. The loose endothelial cells were collected by flushing the vessel with warm DMEM. The effluent was collected with sterile syringes and transferred into chilled centrifuge tubes with FCS. Cells were pelleted by centrifugation at 200 x g at 4°C for 10 min. The supernatant was discarded and the cell pellet was resuspended in endothelial growth medium based on DMEM supplemented with 5% FCS, 1% penicillin, 1% streptomycin, 0.5% gentamycin, 1% sodium pyruvate, 1% non-essential amino acids 100X (Gibco), 50 ug/ml endothelial cell growth supplement (ECGS; Biomedical Technologies Inc.). Cells were plated on 0.5% gelatin-coated plasticware (Nunc A/S) and incubated at 37°C with 5% CO₂. After overnight incubation, non-adherent cells were removed by washing with pre-warmed DMEM and fresh endothelial growth medium was added. Thereafter, culture medium was refreshed every 2 - 3 days.

C. Immortalization of endothelial cells

Endothelial cell lines were immortalized by transduction of the genes encoding the Simian virus 40 large T-antigen (SV40LT) and human telomerase reverse transcriptase (hTERT). 50% confluent, primary endothelial cells were subsequently exposed to either a recombinant lentiviral vector containing the gene encoding SV40LT transforming protein or a lentiviral vector carrying the gene encoding hTERT or a combination of both (Applied Biological Materials Inc.). All primary EC were incubated in the presence of 8µg/ml polybrene (Applied Biological Materials Inc.). To avoid cytotoxicity, the viral supernatant was diluted after 30 min with heparin free EC growth medium (1:1) and further incubated overnight. The medium

was refreshed the next day, and then every other day until cells reached confluence. At 72 h post-transduction, cells were incubated with EC growth medium supplemented with 10 μ g/ml puromycin (Applied Biological Materials Inc.) to select for stable transduced cells. Selection continued until the cultures consisted of resistant surviving cells only. The latter were further expanded in standard medium and routinely passaged at a 1:3 split ratio using 10% trypsin (Sigma-Aldrich), 1% versene (Vel chemicals) in PBS. IEC were analyzed by indirect immunofluorescence for the expression of hTERT and SV40LT antigen.

D. Purity of primary and immortalized EC

The purity of primary and immortalized EC was analyzed by fluorescence staining with 1,1-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine-perchlorate-acetylated low density lipoprotein (DiI-Ac-LDL; Biomedical Technologies Inc.) and was always > 90%.

E. Cell viability

Cell viability was determined by flow cytometry prior to virus inoculation, using 1 μ g ml⁻¹ propidium iodide (Sigma-Aldrich), and was > 90% in all cell populations.

EHV-1 inoculation

Cells were inoculated *in vitro* with both replication competent and UV-inactivated EHV-1 (UV-EHV-1) strains 03P37 and 97P70 at a MOI of 1 in 1 ml of LM for 1 h at 37°C with 5% CO₂. Cells were gently washed twice with RPMI to remove the inoculum and further incubated with fresh medium. Mock inoculations were carried out in parallel.

Inhibition of the MEK/ERK signaling pathway was performed by using the MEK/ERK signaling inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) (Cell Signaling). The stock solution of U0126 was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. U0126 interferes with MEK1 and MEK2 directly by inhibiting the catalytic activity of the active enzyme, and consequently blocks the phosphorylation and activation of ERK1 and ERK2. Inhibition of the PI(3)K signaling pathway was performed by using the PI(3)K inhibitor LY294002 (Invivogen). The stock solution of LY294002 was

prepared in DMSO at a concentration of 50 mM. Cells were pre-incubated with U0126 (1 and 10 μ M), LY294002 (25 and 50 μ M) or DMSO-based diluent, used as a solvent control for 30 min at 37°C before EHV-1 inoculation. MEK/ERK and PI(3)K inhibitors were maintained in the culture media throughout the course of the experiment. The concentration of inhibitors used in this study and the DMSO-based diluent did not decrease the cell viability.

Where indicated, 10 ng/ml of equine recombinant TNF- α or 10 ng/ml of TNF- α neutralizing polyclonal antibody (R&D Systems) were added at the time of inoculation and maintained in the medium throughout the course of inoculation. TNF- α was reconstituted in PBS and diluted in LM.

Adhesion assay

After virus inoculation, cells were incubated with EHV-1 neutralizing IgG antibodies for 1 h at 37°C, 5% CO₂. Mock, EHV-1 or UV-EHV-1-inoculated CD172a⁺ cells were then detached using accutase solution (Sigma-Aldrich), counted and resuspended in LM supplemented with 10 μ g ml⁻¹ polymyxin B (LMPB) (Sigma-Aldrich). Polymyxin B is an antibiotic used to neutralize circulating LPS in the medium, and thus used in this study to prevent LPS-mediated adhesion of CD172a⁺ cells to endothelial cell monolayers. One hundred thousand CD172a⁺ cells were added to each well of EC, which were grown on Lab-Tek chamber slides with eight compartments (VWR). The monocyte/endothelial cell ratio in co-cultures was 1:3. CD172a⁺ cells adherent to the plastic plate were included as a control. At different time points of co-cultivation (2, 4, 6 and 12 h), adherent cells were fixed with 1 ml of methanol at -20°C for 20 min. Non-adherent cells were harvested, fixed with 1 ml of PFA 1% for 10 min at RT, permeabilized with 1 ml of 0.1% Triton X-100 and cytospinned on a slide. After 2, 4, 6 and 12 h of co-cultivation, both adherent and non-adherent cells were incubated for 1 h at 37°C with a rabbit polyclonal Ab anti-IEP (1:1000) to detect immediate early protein (IEP) expression. At 12 h of co-cultivation, cells were also double-stained with a rabbit polyclonal Ab anti-IEP (1:1000) and a mouse monoclonal anti-gB (4B6) (1:100) against late gB protein expression. The IEP and 4B6 antibodies were kindly provided by Dr. D. O'Callaghan (Louisiana State University, USA) and Dr. N. Osterrieder (Freie Universität Berlin, Germany), respectively. Where indicated, cells were fixed with 1 ml of PFA 1% for 10 min at

RT, washed twice with PBS and stained with a horse polyclonal antibody against EHV-1 late proteins (1:100). The horse polyclonal anti-EHV-1 antibody was obtained by hyper-immunization of a horse (van der Meulen *et al.*, 2003b). The polyclonal antibody was purified on a protein G column and subsequently biotinylated (Amersham International, Buckinghamshire, UK). This antibody could recognize the late proteins of EHV-1, as described in (9). Subsequently, samples were incubated for 50 min at 37°C with goat anti-rabbit IgG FITC (1:200) and goat anti-mouse IgG Texas-Red[®] (1:200) or Streptavidin-FITC (1:200) antibodies (Molecular probes). All antibodies were diluted in DPBS. The nuclei were counterstained with Hoechst 33342 (10 µg ml⁻¹; Molecular Probes) for 10 min at 37°C. As a negative control, mock-inoculated cells were stained following the aforementioned protocols. In addition, appropriate isotype-matched controls were included.

For each time point, the number of IEP- and gB-positive adherent CD172a⁺ cells was counted based on the total number of adherent CD172a⁺ cells counted in 5 randomly selected microscopic fields (5x 0.2mm²) using a fluorescent microscope (x40 objective) (Leica Microsystems DMRBE Wetzlar, Germany). Results were shown as number of cells per mm² and were also expressed as a percentage. In the non-adherent cell fraction, the number of IEP-positive CD172a⁺ cells was calculated based on three hundred cells counted in distinct fields and results were expressed as a percentage. Samples were analyzed by confocal microscopy (Leica TCS SP2 Laser scanning spectral confocal system, Leica microsystems GmbH, Germany).

Adhesion blocking assay

To determine the role of $\alpha_4\beta_1$ (CD29/CD49d), $\alpha_L\beta_2$ (CD11a/CD18) and $\alpha_V\beta_3$ (CD51/61) integrins in EHV-1-induced adhesion to endothelial cells, adhesion assays were performed as described above with some modifications. Prior to addition of CD172a⁺ cells to endothelial cells, EHV-inoculated CD172a⁺ cells were incubated with LMPB supplemented with 10 µg/ml of neutralizing monoclonal antibodies directed against CD29 (clone TDM29, IgG1, Merck Millipore), CD49d (clone 9F10, IgG2b, BD Pharmingen), CD11a/CD18 (clone CVS9, IgG1, AbD Serotec), CD51/61 (clone 23C6, IgG1, BioLegend), a mixture of CD29, CD49d, CD11a/18 and CD51/61 or with isotype-matched control antibodies for 30 min at 4°C. After washing, EHV-1-inoculated CD172a⁺ cells were added to wells of IEC. Cells were fixed after 2 h of

co-cultivation and stained for IEP expression, as previously described.

Flow cytometry

To evaluate integrin expression on CD172a⁺ cells, cells were incubated with 10 µg/ml of the anti-integrin monoclonal antibodies CD29, CD49d, CD11a/CD18 and CD51/61 or with isotype-matched control antibodies for 30 min at 4°C. Cells were washed twice in PBS and incubated with Alexa Fluor 647-labeled goat anti-mouse IgG (1:200 dilution; Molecular Probes) for 30 min at 4°C. After a final wash in PBS, 10,000 cells were analyzed with a FACSCanto flow cytometer equipped with a FACSDiva software (BD Biosciences, Mountain View, California USA).

Western Blot analysis

One million CD172a⁺ cells were mock-inoculated or inoculated with EHV-1 03P37 or 97P70 strains (MOI 1) and harvested at 0, 5 and 15 min post-inoculation. Cells were resuspended in ice-cold lysis buffer (TNE buffer, 10% NP40, 1 mM Na₃VO₄, 10 mM NaF, protease inhibitor cocktail) and incubated for 20 min at 4°C. Then, samples were boiled for 10 min. SDS-PAGE and Western blotting were performed as described previously (32). Protein concentrations of 20 to 30 µg were used for all experiments. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific) according to the manufacturer's instructions. Blots were blocked in 5% nonfat dry milk in PBS-Tween 20 for 1 h at RT and incubated with primary antibodies for 1 h or overnight (according to the manufacturer's instructions). After several washes in 0.1% Tris-buffered saline (TBS)-Tween 20, blots were incubated with HRP-conjugated secondary antibodies for 1 h at RT and developed using enhanced chemiluminescence. Phospho-specific ERK1/2 antibody (1:1000; Cell Signaling) and Phospho-Akt antibody (1:1000; Cell Signaling) signals were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Total ERK1/2 antibody (1:1000; Cell Signaling) and Total Akt (1:1000; LSBio) signals were detected with Pierce ECL (Thermo Scientific). As a control for loading, total β-actin (Abcam) levels were assessed.

ELISA

Equine TNF- α content in culture supernatants of mock and EHV-1-inoculated CD172a⁺ cells cultured on plastic or on top of IEC was measured with an enzyme-linked immunosorbent assay (ELISA) kit obtained from Thermo Scientific. The assay was performed according to the manufacturer's instructions. Recombinant equine TNF- α was used as a standard, and the assay sensitivity was 16 pg/ml.

Co-cultivation assay

A co-cultivation assay was used to detect and quantify EHV-1 producing CD172a⁺ cells by co-cultivation of these EHV-1-inoculated CD172a⁺ cells with EC, where a semi-solid overlay technique was applied. Briefly, EHV-1-inoculated and mock-inoculated cells were harvested at 12 hpi. One hundred thousand cells per ml were tenfold-diluted in LM and 0.5 ml of each dilution was added on EC monolayers in a 6-well plate and overlaid with a 0.94% carboxymethylcellulose medium (Sigma-Aldrich) prepared in RPMI-2X and centrifuged at 800 x g for 30 min at 18°C, as previously described (8). Cells were further incubated for 5 days at 37°C, 5% CO₂. The cell monolayers were then stained with 5% crystal violet and the number of plaques was counted. The percentage of infected cells producing infectious EHV-1 was calculated based on the number of plaques counted and the number of cells seeded per ml according to the volume plated. This experiment was performed three times.

Statistical analysis

Data were analyzed with GraphPad Prism 6 software (GraphPad software Inc.). Data analyzed for statistical significance were subjected to a two-way analysis of variance (ANOVA). All results shown represent means and standard deviation (SD) of three independent experiments. Results with p -value ≤ 0.05 were considered statistically significant.

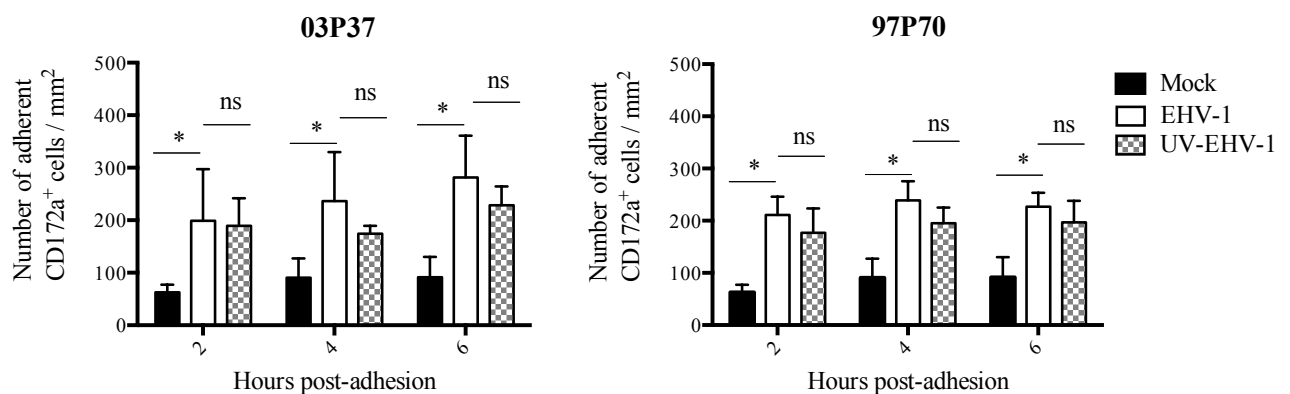
Results

1. EHV-1 infection induces adhesion of CD172a⁺ cells to EC

To determine whether EHV-1 infection may enhance adhesion of CD172a⁺ cells to EC, we compared the adhesion kinetics of mock-, UV-inactivated EHV-1- and EHV-1-inoculated CD172a⁺ cells to EC monolayers (Fig.1).

EHV-1 infection resulted in a significant increase ($p < 0.001$) in adhesion of CD172a⁺ cells to primary and immortalized venous EC monolayers and was independent of the EHV-1 strain used (03P37 or 97P70) (Figs. 1A, 1B and 1C). A maximum of 200-230 adherent cells per mm² was reached with EHV-1-inoculated CD172a⁺ cells after 2 h of co-culture compared to approximately 50 adherent cells per mm² with mock-inoculated CD172a⁺ cells. In addition, no significant difference in the adhesion kinetics of CD172a⁺ cells to EC monolayers was observed between UV-EHV-1- and EHV-1-treated cells. These data indicate that active EHV-1 gene expression was not required to induce adhesion of CD172a⁺ cells to EC monolayers and thus, viral entry was sufficient to activate CD172a⁺ cell adhesion to EC. As the kinetics of CD172a⁺ cell adhesion were similar in primary EC cultures and immortalized EC lines, the following experiments were performed using immortalized EC only.

A



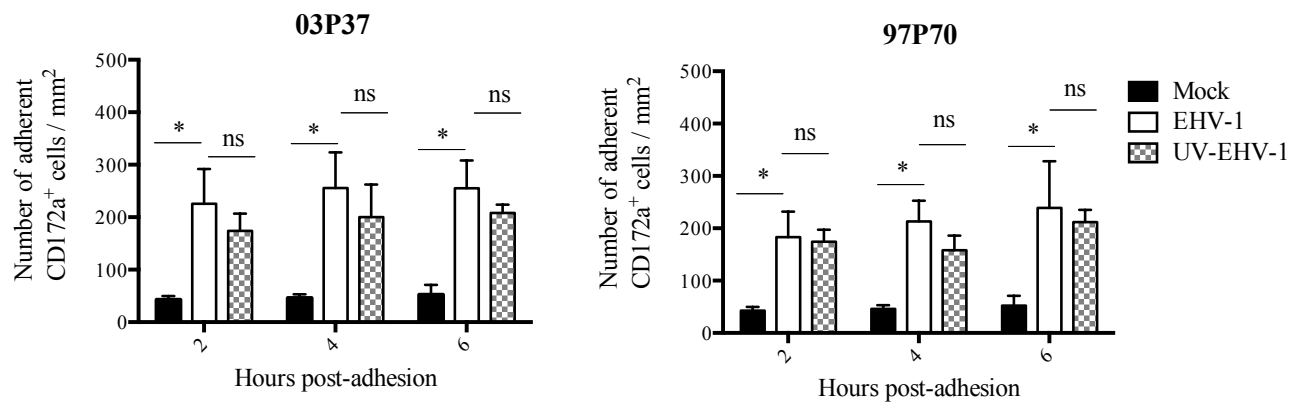
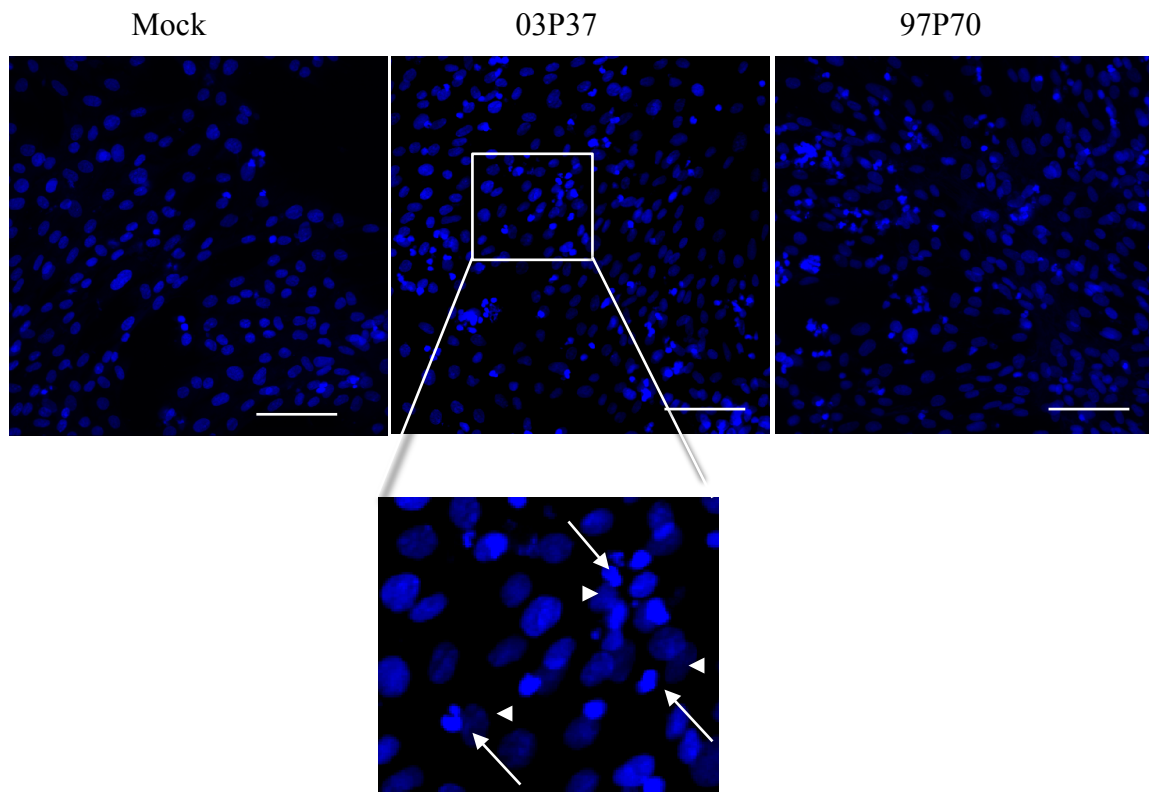
B**C**

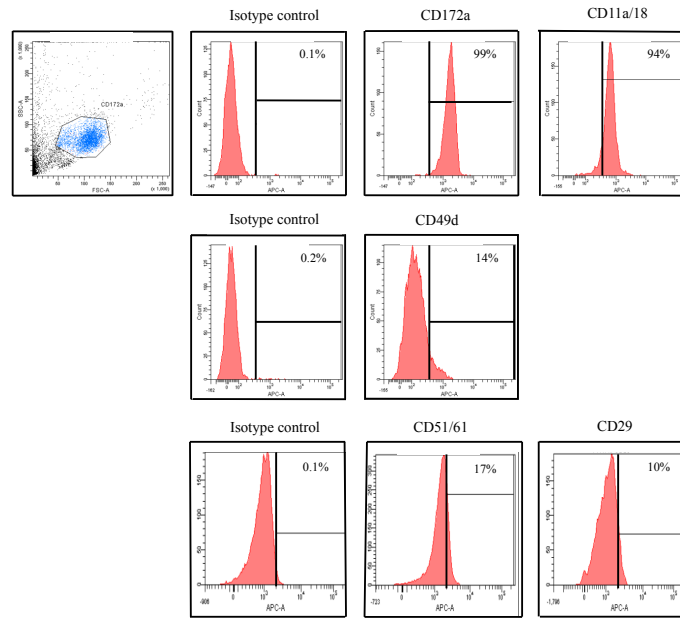
Figure 1: Adhesion kinetics of mock-inoculated or EHV-1-inoculated blood CD172a⁺ cells to equine endothelial cells (EC). Cells were inoculated with replication competent EHV-1 or UV-inactivated EHV-1 (UV-EHV-1) strains 03P37 or 97P70 (MOI 1) and directly co-cultured with primary (A) or immortalized (B) monolayers for 2, 4 and 6 h. The number of adherent CD172a⁺ cells was calculated per mm². Experiments were performed three times. Error bars show + SD and a two-way ANOVA test was performed to evaluate significant differences from EHV-1 to mock and UV-inactivated EHV-1 samples, (ns = not significant and * = $P < 0.001$). (C) Low magnification, confocal images of blood CD172a⁺ cell adhesion to EC after 2 h co-culture (merged Z-stacks). Nuclei were counterstained with Hoechst (blue). Arrows point at adhering monocytic cells, arrowheads indicate EC. Scale bar, 100 μ m.

2. EHV-1-mediated CD172a⁺ cell adhesion to EC depends on cellular adhesion molecules

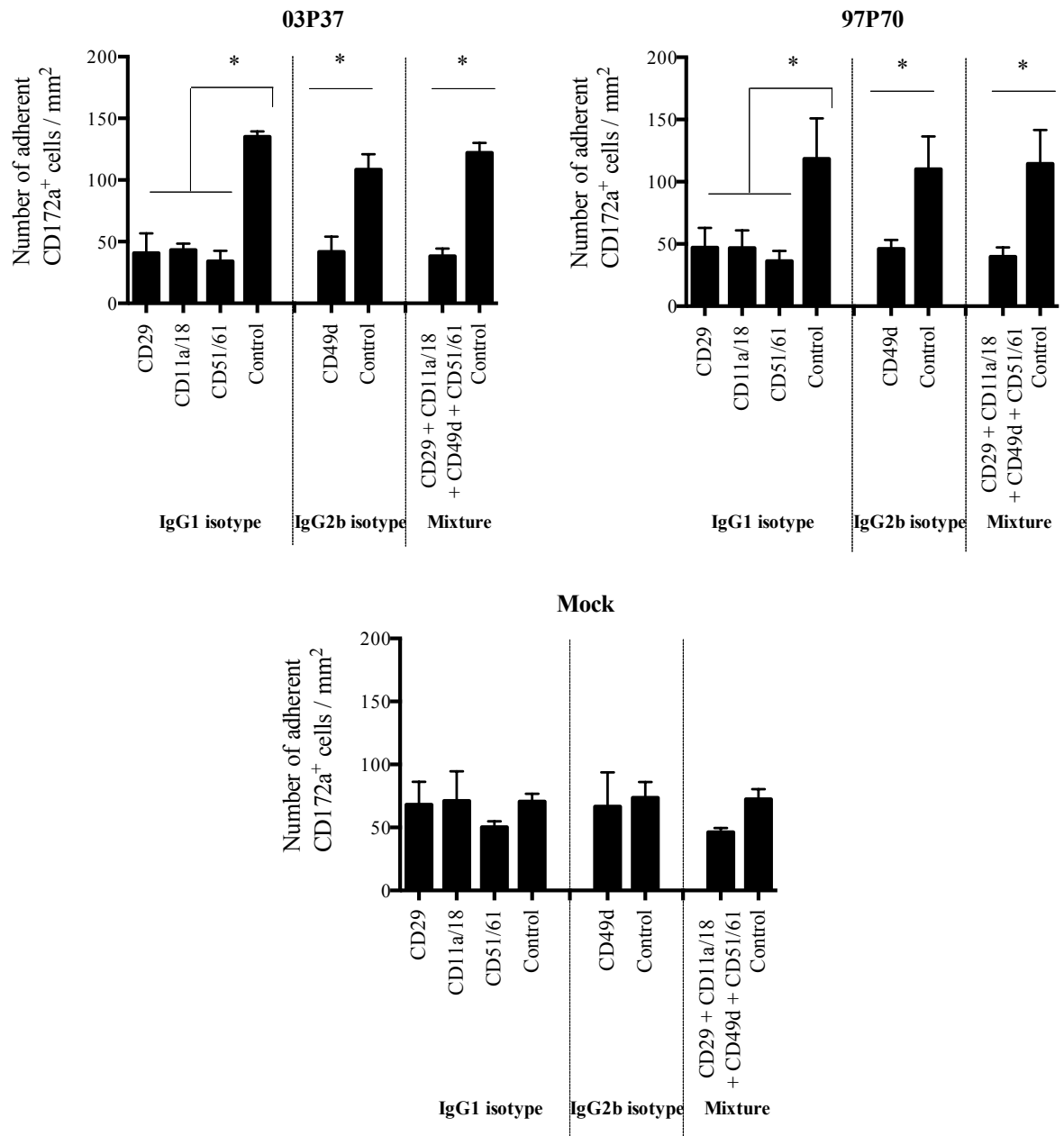
To determine the contribution of specific cellular adhesion molecules in EHV-1-induced adhesion of activated CD172a⁺ cells to EC, we performed adhesion-blocking experiments using blocking antibodies against $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_V\beta_3$ integrins. These integrins are known to play a role in mediating firm adhesion of monocytes to the endothelium (Elangbam *et al.*, 1997; Mazzone & Ricevuti, 1995; Weerasinghe *et al.*, 1998). The presence of $\alpha_4\beta_1$ (CD49d/CD29), $\alpha_L\beta_2$ (CD11a/CD18) and $\alpha_V\beta_3$ (CD51/CD61) integrins on CD172a⁺ cells was confirmed by flow cytometry (Fig. 2A) but it was not possible to compare the expression levels between mock- and EHV-1-infected CD172a⁺ cells due to the low percentage of infected cells detected at early stages of infection (data not shown).

Pre-treatment of EHV-1-inoculated CD172a⁺ cells with blocking antibodies resulted in a 3-fold decrease in adhesion to EC after 2 h of co-culture, compared to isotype controls ($p < 0.001$) (Figs. 2B and 2C). After pre-incubation with blocking antibodies, the number of adherent CD172a⁺ cells per mm² in EHV-1-inoculated cells decreased to levels at or slightly below those of mock-inoculated cells (± 50 adherent cells per mm²). Pre-treating cells with a mixture of blocking antibodies had no cumulative effect on decreasing CD172a⁺ cell adhesion to EC, compared to treatment of cells with a single antibody. Interestingly, no significant decrease in cell adhesion was observed with mock-inoculated CD172a⁺ cells. Taken together, these results show that interactions between EHV-1-inoculated CD172a⁺ cells and EC are in part mediated by integrins.

A



B



C

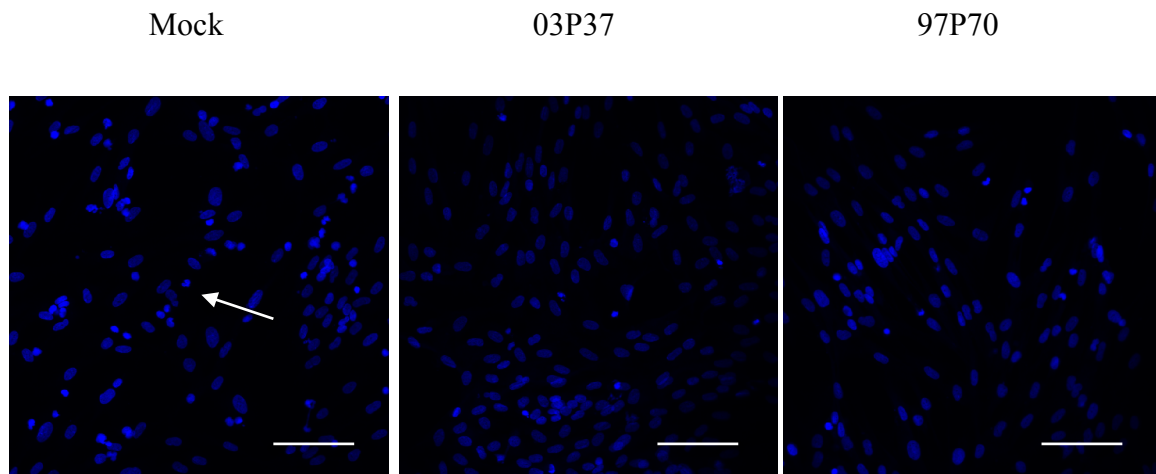


Figure 2: Adhesion of mock-inoculated or EHV-1 (03P37 or 97P70)-inoculated blood CD172a⁺ cells to EC monolayers after pre-incubation of monoclonal antibodies against $\alpha_4\beta_1$ (CD49d/CD29), $\alpha_1\beta_2$ (CD11a/CD18) and $\alpha_v\beta_3$ (CD51/CD61) integrins. (A) Flow cytometric analyses of the expression of CD49d, CD29, CD11a/CD18 and CD51/CD61 integrins on CD172a⁺ cells. (B) Cells were mock-inoculated or inoculated with EHV-1 strains 03P37 or 97P70 (MOI 1), pre-incubated with 10 $\mu\text{g}/\text{ml}$ of specific blocking antibodies for 30 min at 4°C and co-cultured with immortalized EC for 2 h at 37°C. As a control, cells were pre-incubated with isotype-matched antibodies. The number of adherent CD172a⁺ cells was calculated per mm². Experiments were performed three times. Error bars show + SD and a two-way ANOVA test was performed to evaluate significant differences from controls, (* = $P < 0.001$). (C) Low magnification, confocal images of mock-inoculated or EHV-1 (03P37 or 97P70)-inoculated blood CD172a⁺ cells adherent to EC after pre-incubation with $\alpha_1\beta_2$ (CD11a/18) blocking antibody. Nuclei were counterstained with Hoechst (blue). The white arrow points at an adhering monocytic cell. All confocal images shown represent merged Z-stacks. Scale bar, 100 μm .

3. EHV-1-induced adhesion of CD172a⁺ cells to EC is dependent on integrin-mediated PI(3)K and ERK/MAPK signaling pathways

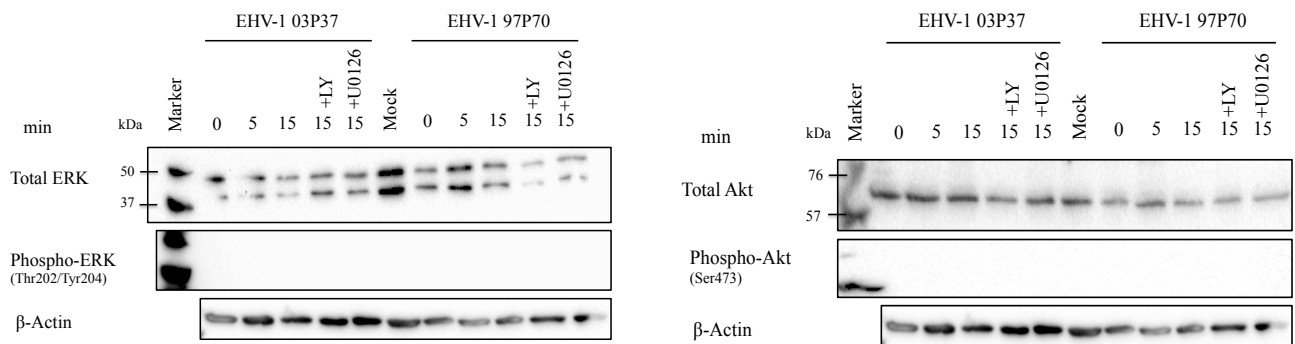
Next, we wanted to identify the mechanism by which EHV-1 induces adhesion of CD172a⁺ cells adhesion to EC. Binding of herpesviruses to integrins is known to activate cellular pathways, including PI(3)K/Akt (PI(3)k for phosphatidylinositol 3-kinase and Akt for protein kinase B), one of the early events of integrin-ligand interaction and the downstream ERK/MAPK (MAPK for mitogen-activated protein kinase and ERK for extracellular signal-regulated kinases). Both these pathways have been implicated in the manipulation of cellular functions such as adhesion and in the control of herpesviruses infection and replication in target cells (Chang *et al.*, 2012; Naranatt *et al.*, 2003; Smith *et al.*, 2007). Hence, we hypothesized that EHV-1 would also bind to cellular integrins present on CD172a⁺ cells and induce PI(3)K/Akt and ERK/MAPK activation, leading to increased adhesion of CD172a⁺ cells to EC.

We first tested whether EHV-1 infection up-regulates ERK/MAPK and PI(3)K activities in CD172a⁺ cells. We compared by Western blot analyses the levels of phospho-specific ERK1/2 and total ERK1/2 as well as the levels of phospho-specific Akt and total Akt, between mock and virus-inoculated CD172a⁺ cells in the presence or absence of the specific MEK/ERK non-competitive inhibitor U0126 and the PI(3)K reversible inhibitor LY294002. We found that mock- and EHV-1-inoculated CD172a⁺ cells showed similar levels of total ERK1/2 and total Akt expression at 0, 5 and 15 min post-inoculation. However, we failed to detect any levels of ERK1/2 and Akt phosphorylation, even by using a highly sensitive detection kit (Fig. 3A). It is possible that the concentration of cells used in this assay (10⁶ cells per ml) was not sufficient to detect any signal. Due to technical limitations, it was not possible to increase the cell concentration. Thus, we could not test whether EHV-1 infection up-regulates ERK/MAPK and PI(3)K activities in CD172a⁺ cells.

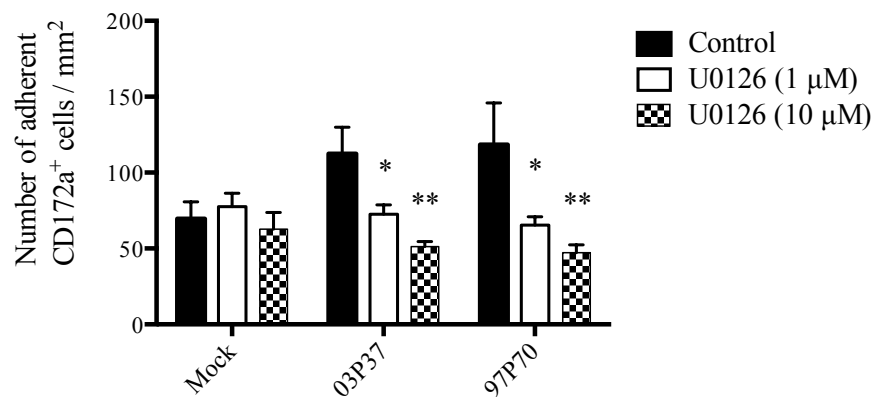
However, we could analyse the potential role of ERK/MAPK and PI(3)K activation in EHV-1-induced CD172a⁺ cells adhesion to EC, by pre-treating CD172a⁺ cells with U0126 (1 and 10 μ M), LY294002 (25 and 50 μ M) or with DMSO-based diluent as a control, for 30 min before EHV-1 inoculation. Treated CD172a⁺ cells were then added to immortalized EC monolayers and co-cultured for 2 h. Inhibition of the ERK/MAPK signaling pathway prior to EHV-1 inoculation resulted in a dose-dependent significant decrease ($p < 0.001$) in CD172a⁺ cell adhesion to mock-levels

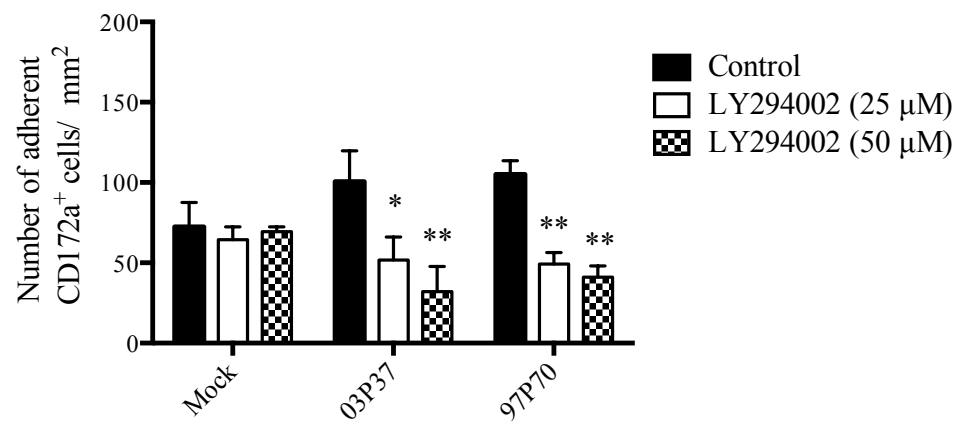
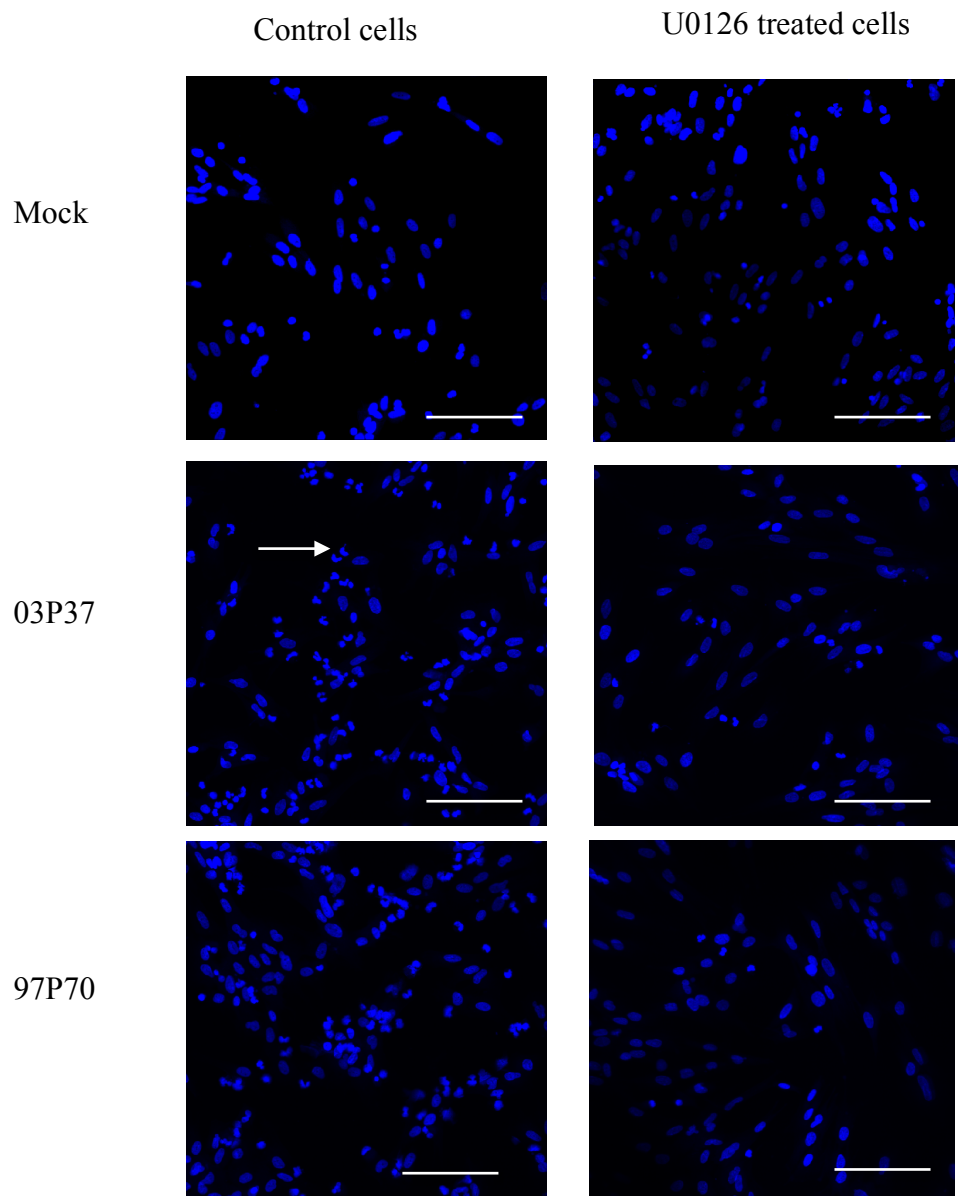
(Figs. 3B and 3C). When CD172a⁺ cells were pre-treated with 10 μ M of U0126, the EHV-1 (03P37 and 97P70) induced CD172a⁺ cell adhesion to EC monolayers was reduced by 55 and 61%, respectively. Confocal images of the reduced adhesion of blood CD172a⁺ cells to EC upon inhibition of the ERK/MAPK signaling pathway were depicted in Fig. 3D. Inhibition of PI(3)K activity prior to EHV-1 inoculation resulted in a similar decrease of CD172a⁺ cell adhesion. Pre-treating mock-inoculated cells with the inhibitors U0126 or LY294002 had no significant effect on CD172a⁺ cell adhesion. Besides, we found that inhibition of ERK/MAPK and PI(3)K pathways did not significantly decrease EHV-1 infection of CD172a⁺ cells compared to controls (Fig. 3E). Taken together, these data suggest that EHV-1 infection induces CD172a⁺ cell adhesion in a PI(3)K- and ERK/MAPK-dependent manner at a very early time of infection.

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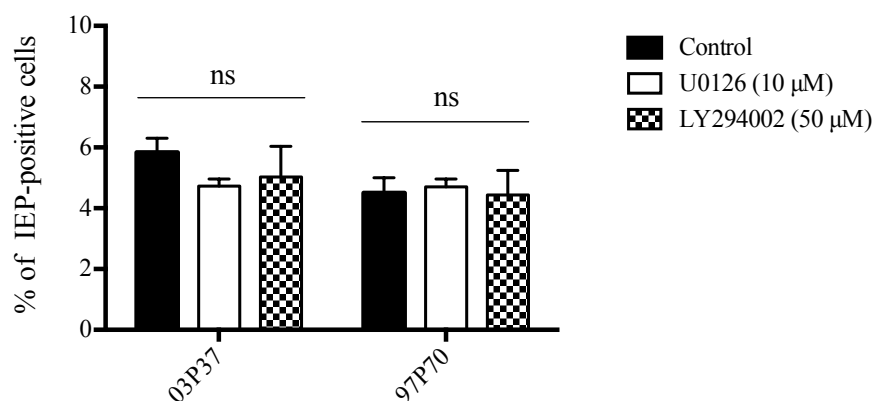


Figure 3: Effect of inhibition of the ERK/ MAPK and PI(3)K signaling pathways on EHV-1-induced CD172a⁺ cell adhesion to EC monolayers. (A) Western blot analyses of the levels of phospho-specific ERK1/2 and total ERK1/2 as well as the levels of phospho-specific Akt and total Akt, between mock- and EHV-1-inoculated CD172a⁺ cells in the presence or absence of ERK/MAPK and PI(3)K inhibitors. One million CD172a⁺ cells were pre-incubated with MEK/ERK inhibitor U0126 (10 μM), PI(3)K inhibitor LY294002 (50 μM) or DMSO-based diluent for 30 min at 37°C. Cells were (mock) inoculated with EHV-1 03P37 or 97P70 strains (MOI 1) and lysed at 0, 5 and 15 min post-inoculation. As a control for loading, total β-actin levels were assessed. (B) CD172a⁺ cells were pre-incubated with U0126 (1 or 10 μM) or LY294002 (25 or 50 μM) (C) for 30 min at 37°C. Cells were (mock) inoculated with EHV-1 strains 03P37 or 97P70 (MOI 1) and co-cultured for 2 h with immortalized EC. The number of adherent CD172a⁺ cells was calculated per mm². (D) Low magnification, confocal images of the reduced adhesion of blood CD172a⁺ cells to EC upon inhibition of the ERK/MAPK signaling pathway. Nuclei were counterstained with Hoechst (blue). The white arrow points at an adhering monocytic cell. All confocal images shown represent merged Z-stacks. Scale bar, 100 μm. (E) U0126 or LY294002 pre-treatments did not decrease EHV-1 infection of CD172a⁺ cells. CD172a⁺ cells adherent to plastic were pre-treated with U0126 (10 μM), LY294002 (25 μM) or DMSO-based diluent for 30 min at 37°C and inoculated with EHV-1 03P37 or 97P70 strains (MOI 1) for 1 h at 37°C in LM supplemented with inhibitors. Cells were further incubated in LM for 12 h at 37°C. The percentage of IEP-positive cells was calculated based on 300 cells counted in distinct fields. Experiments were performed three times. Error bars show + SD and a two-way ANOVA test was performed to evaluate significant differences from controls, (ns = not significant, * = $P \leq 0.001$ and ** = $P < 0.0001$).

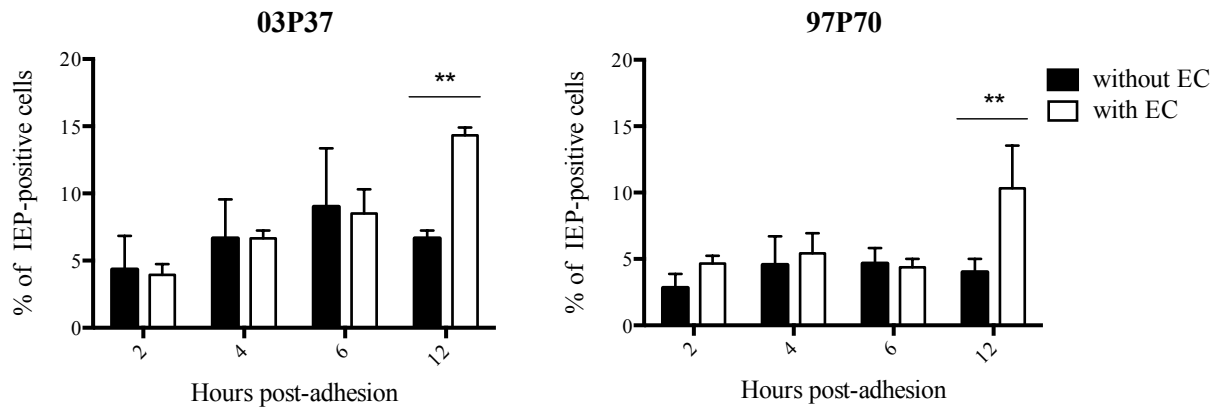
4. EHV-1 replication is enhanced in CD172a⁺ cells upon adhesion to EC

To determine whether EHV-1 replication was enhanced in CD172a⁺ cells upon adhesion to EC, we compared the expression kinetics of immediate-early protein (IEP) and gB protein in CD172a⁺ cells cultured on plastic (without EC) or on top of immortalized EC monolayers for 2, 4, 6 and 12 h.

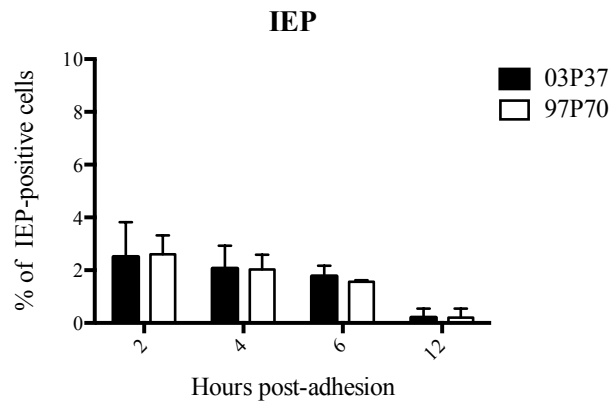
From 2 to 12 hours post-adhesion (hpa) on EC monolayers, the percentage of IEP-positive CD172a⁺ cells significantly increased from 4% to 14% for EHV-1 03P37 strain ($p < 0.01$). At 12 hpa, the percentage of IEP-positive CD172a⁺ cells was significantly higher in cells adherent to EC than cells adherent to plastic ($p < 0.01$) (Fig. 4A, left panel). In contrast, the percentage of IEP-positive CD172a⁺ cells remained stable from 2 to 6 hpa (4%) and significantly increased to 10% at 12 hpa, for EHV-1 97P70 strain ($p < 0.01$) (Fig. 4A, right panel). No significant difference in the percentage of IEP-positive cells was observed in CD172a⁺ cells inoculated with EHV-1 03P37 or 97P70 strains and cultured on plastic. The percentage of IEP-positive cells decreased in the non-adherent CD172a⁺ cell fraction over time post-adhesion, for both EHV-1 strains (Fig. 4B). No gB protein was detected in cells from 2 to 6 hpa. An upward trend and a significant increase in gB expression ($p < 0.05$) was observed in IEP-positive cells inoculated with EHV-1 03P37 and 97P70 strains, respectively and co-cultured for 12 h with EC monolayers, compared to cells cultured on plastic (Fig. 4C). Confocal images showed the expression of IEP and gB protein in EHV-1-infected CD172a⁺ cells after 12 h co-culture with immortalized EC (Fig. 4D). The expression of gB in IEP-positive CD172a⁺ cells upon adhesion to EC was comparable between the two strains (60%).

Taken together, these results show that the adhesion of EHV-1 inoculated CD172a⁺ cells to EC speeds up the viral replication process in CD172a⁺ cells inoculated with EHV-1 97P70 strain, but to a lesser extent in CD172a⁺ cells inoculated with EHV-1 03P37 strain, compared to EHV-1 inoculated CD172a⁺ cells adherent to plastic. Moreover, the enhancement of EHV-1 replication in CD172a⁺ cells upon adhesion to EC suggests that direct cell-to-cell contacts provide a favorable environment for EHV-1 replication in CD172a⁺ cells.

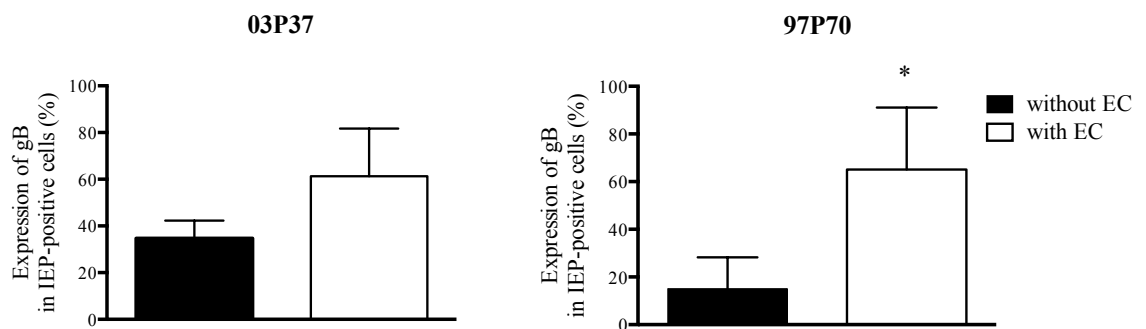
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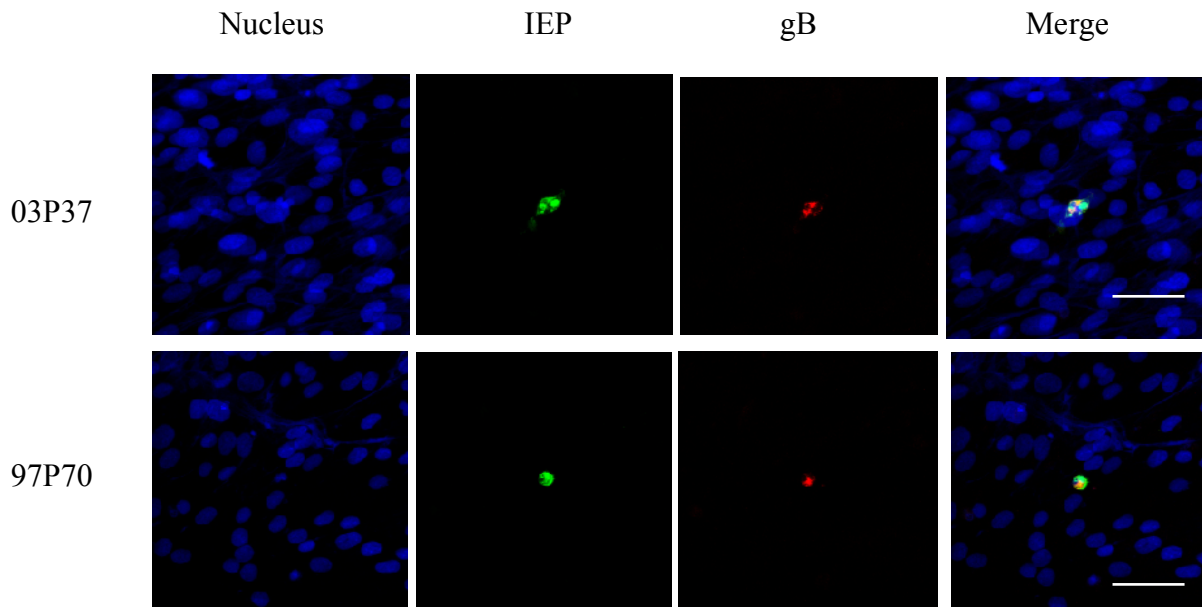
D

Figure 4: Expression kinetics of EHV-1 proteins in blood $CD172a^+$ cells upon adhesion to EC. (A) Expression kinetics of EHV-1 immediate-early (IEP) protein in adherent blood $CD172a^+$ cells. Cells were inoculated with EHV-1 03P37 or 97P70 strains (MOI 1) and co-cultured with immortalized EC monolayers for 2, 4, 6 and 12 h. As a control, EHV-1-inoculated $CD172a^+$ cells were incubated on plastic (without EC). (B) Expression kinetics of EHV-1 immediate-early (IEP) protein in the non-adherent $CD172a^+$ cell fraction after 2, 4, 6 and 12 h co-culture with immortalized EC. (C) Expression of EHV-1 gB protein in infected $CD172a^+$ cells after 12h co-culture with immortalized EC in the presence of neutralizing EHV-1 antibodies. Three independent experiments were performed and data are represented as means + SD. A two-way ANOVA test was performed to evaluate significant differences from control, (* = $P < 0.05$ and ** = $P < 0.01$). (D) Double immunofluorescence of EHV-1 immediate-early protein (IEP) (green) and gB (red) proteins in blood $CD172a^+$ cells adherent to immortalized EC for 12 h in the presence of neutralizing EHV-1 antibodies. Nuclei are counterstained with Hoechst (blue). All confocal images represent merged Z-stacks. Scale bar = 50 μm .

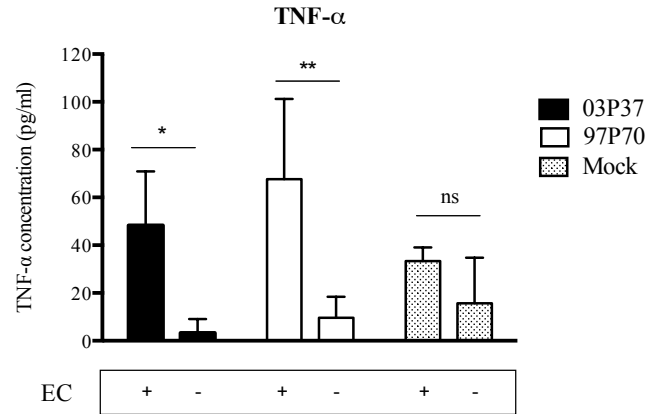
5. Increased EHV-1 replication in CD172a⁺ cells upon adhesion to EC correlates with the production of tumor necrosis factor alpha (TNF- α)

Since TNF- α has been described as a potent inducer of viral gene expression in monocytes/macrophages, we investigated whether TNF- α is produced during interaction of EHV-1-inoculated CD172a⁺ cells and EC and whether TNF- α enhances EHV-1 replication in CD172a⁺ cells.

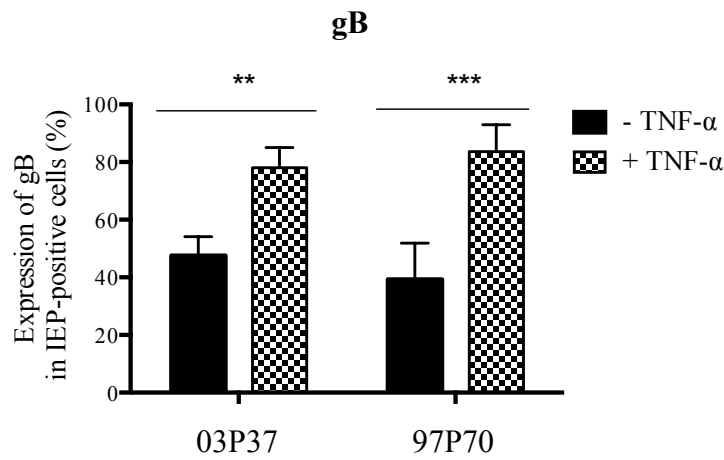
The concentration of TNF- α in supernatants of mock-inoculated and EHV-1-inoculated CD172a⁺ cells cultured on plastic or on top of immortalized EC monolayers was determined by ELISA. At 12 hpa, the concentration of TNF- α in supernatants of CD172a⁺ cells inoculated with EHV-1 03P37 or 97P70 strains and co-cultured with EC was significantly higher ($p < 0.05$ and $p < 0.001$, respectively) than the TNF- α concentration in supernatants of EHV-1-inoculated cells cultured on plastic (Fig. 5A). No significant difference was detected in TNF- α concentrations between supernatants of mock-inoculated CD172a⁺ cells cultured on plastic or on top of EC.

We also tested if the addition of exogenous TNF- α to CD172a⁺ cells at the time of virus inoculation affected EHV-1 replication. The expression of gB in CD172a⁺ cells cultured on plastic was compared in the absence or presence of 10 ng/ml recombinant equine TNF- α at 12 h post-inoculation (hpi). We found that the number of cells positive for gB among IEP-positive cells was significantly higher ($p < 0.001$) when stimulated with equine recombinant TNF- α (Fig. 5B). Moreover, we demonstrated that the addition of neutralizing TNF- α antibody to co-cultures of CD172a⁺ cells adherent to EC generated a trend, although not statistically significant, towards a decrease in the percentage of gB-positive cells (Fig. 5C). Taken together, these results indicate that TNF- α is an important soluble mediator enhancing EHV-1 replication in CD172a⁺ cells upon adhesion to EC.

A



B



C

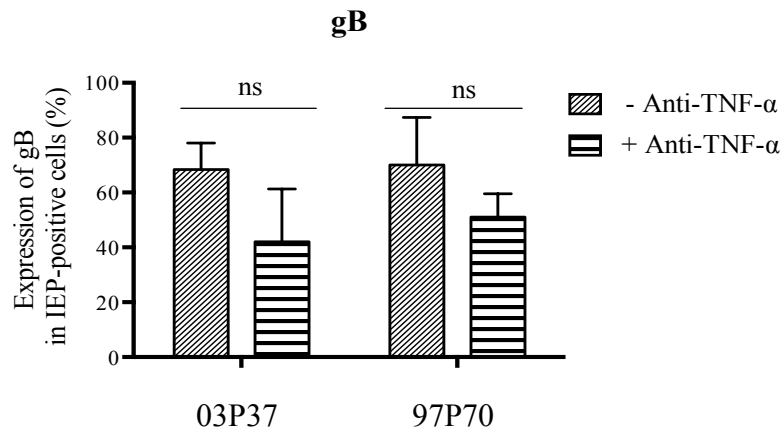


Figure 5: Effect of TNF- α in the enhancement of EHV-1 replication in CD172a⁺ cells upon adhesion to EC. (A) Concentration of TNF- α (pg/ml) in supernatants of CD172a⁺ cells mock-inoculated or inoculated with EHV-1 03P37 or 97P70 strains (MOI 1), cultured on plastic or on the top of immortalized EC for 12 h. (B) Expression of EHV-1 gB protein in CD172a⁺ cells adherent to plastic and cultured in LM supplemented or not with 10 ng/ml of equine recombinant TNF- α (+TNF- α) for 12 h. (C) Expression of EHV-1 gB protein in CD172a⁺ cells adherent to EC and cultured for 12 h in LM supplemented or not with 10 ng/ml of TNF- α neutralizing antibody (+ Anti-TNF- α). Data represent means + SD of three independent experiments and ns = not significant, * = $P < 0.05$, ** = $P < 0.001$ and *** = $P < 0.0001$ (by two-way ANOVA test).

6. Transfer of cytoplasmic EHV-1 material from infected CD172a⁺ cells to uninfected adjacent EC

To investigate whether CD172a⁺ cells transmit EHV-1 to EC, we analyzed the intracellular distribution dynamics of IEP and gB proteins in EHV-1 inoculated CD172a⁺ cells after 12 h co-culture with immortalized EC monolayers in the presence of neutralizing antibodies (Fig. 6). We found that IEP was expressed in nuclear replicative compartments and in the cytoplasm of infected CD172a⁺ cells adherent to EC. The gB protein was only expressed in the cytoplasm of these cells and no gB expression was detected at the plasma membrane of CD172a⁺ cells after 12 h co-culture with EC. However, when immunostaining was performed with a biotinylated horse polyclonal antibody that recognizes EHV-1 late proteins, some signal could be detected at the plasma membrane of 2 and 0.6% of CD172a⁺ cells adherent to EC monolayers and inoculated with EHV-1 03P37 and 97P70, respectively (Fig. 6A). With both EHV-1 strains, a cytoplasmic IEP positive 'bridge' was formed from an infected CD172a⁺ cell to an adjacent EC (Fig. 6B). Some cytoplasmic IEP staining was also observed in the adjacent EC. The late protein gB expressed a weak signal colocalized with IEP in some regions of both cytoplasmic bridge and cytoplasm of the EC. Eighteen and 27% of IEP/gB (03P37 and 97P70) positive CD172a⁺ cells showed cytoplasmic transfer of EHV-1 material to adjacent EC. However, IEP/gB-positive plaques could not be observed in EC, also at later time points post-inoculation, indicating that spread of infection from CD172a⁺ cells to EC did not automatically lead to productive infection in EC.

To address whether the enhanced EHV-1 replication in CD172a⁺ cells upon adhesion to EC led to a productive infection, we determined the percentage of CD172a⁺ cells that produced and transmitted infectious EHV-1 to neighbouring endothelial cells by co-cultivation assay. After 5 days co-culture of inoculated CD172a⁺ cells with EC monolayers, we showed that 0.1% and 0.05% of CD172a⁺ cells inoculated with EHV-1 03P37 and 97P70 strains, respectively, transmitted the infection to EC. Taken together, these results provide evidence for direct cytoplasmic transfer of EHV-1 material from late infected CD172a⁺ cells to adjacent EC, which typically lead to a non-productive infection in these cells.

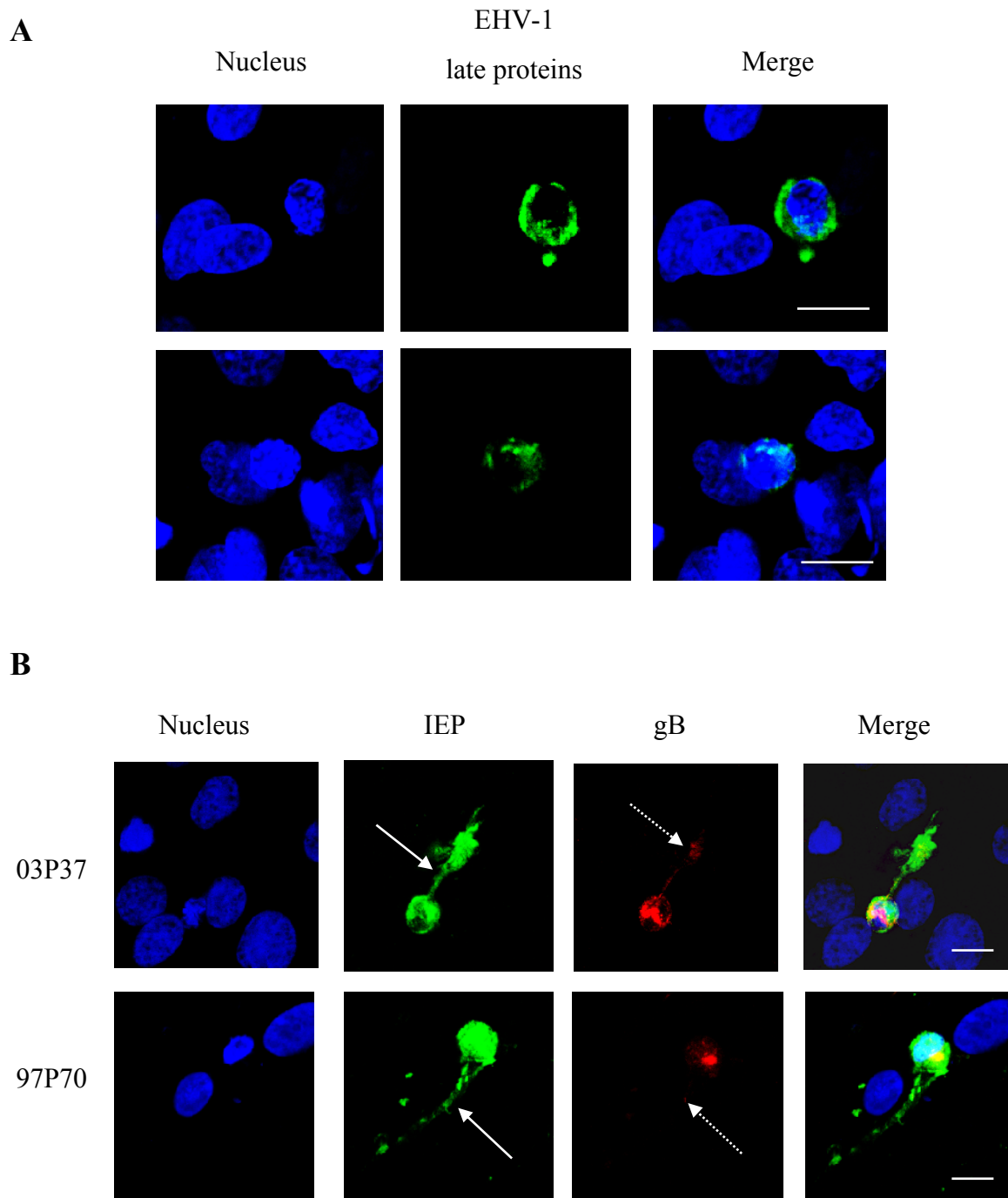


Figure 6: Transfer of cytoplasmic EHV-1 material from EHV-1-infected CD172a⁺ cell to uninfected EC. Cells were inoculated with EHV-1 03P37 and 97P70 strains (MOI 1) and co-cultured with immortalized EC for 12 h the presence of neutralizing EHV-1 antibodies. (A) Expression of EHV-1 late proteins at the plasma membrane of infected CD172a⁺ cells adherent to immortalized EC monolayers. Cells were fixed with PFA 1% without permeabilization and stained with a biotinylated horse polyclonal antibody directed against EHV-1 late proteins (green). (B) EHV-1 immediate-early protein (IEP) (green) and gB protein (red) were detected by indirect immunofluorescence. Arrows display cytoplasmic transfer of IEP (full arrow) and gB protein (dotted arrow), from late stage infected CD172a⁺ cell to one adjacent EC (arrowhead). Nuclei were counterstained with Hoechst (blue). All confocal images represent merged Z-stacks. Scale bar, 10 μ m.

Discussion

Monocytic cells are assumed to fulfill key role in EHV-1 spread from the blood to target organs, but the precise interactions between monocytic cells and EC have not been unraveled. In the present study, we demonstrated that EHV-1 infection increased adhesion of CD172a⁺ monocytic cells to EC *in vitro*. Significantly more EHV-1-inoculated CD172a⁺ cells adhered to EC than to mock-inoculated cells. UV-treatment of EHV-1 did not affect the EHV-1-triggered adhesion process, indicating that the binding of EHV-1 to CD172a⁺ cells was sufficient to induce their adhesion to EC. We hypothesized that viral binding induces cellular signaling pathways that trigger functional changes in CD172a⁺ cells, which subsequently promote their adhesion to EC. By the use of an *in vitro* flow system, Smith et al. (2001) suggested that activation of adhesion molecules present on both leukocytes and EC surfaces is a prerequisite for the transfer of EHV-1 from leukocytes to the endothelium. However, the authors did not characterize in detail which adhesion molecules were activated on the endothelium or examine the exact nature of the adhesion molecules present on the cell surface of the carrier cells. Monocytes constitutively express a selection of adhesion molecules, including selectins and integrins (Huang *et al.*, 1995; Lusinskas *et al.*, 1994). Here, we showed that blocking $\alpha_4\beta_1$ (VLA-4), $\alpha_L\beta_2$ (LFA-1), and $\alpha_V\beta_3$ integrins on EHV-1-inoculated CD172a⁺ cells significantly reduced adhesion to levels comparable to mock-inoculated cells. These results indicated that integrins mediated the adhesion of CD172a⁺ cells to EC. However, adhesion was not completely inhibited by the addition of blocking antibodies, suggesting that other adhesion molecules may be involved in this process. Besides integrins, L-selectins are expressed on myeloid cells and have been shown to play an important role in the initial interaction or tethering of monocytes with the endothelium (Giuffre *et al.*, 1997; Vestweber & Blanks, 1999). Unfortunately, there is no antibody available against L-selectin that cross-reacts specifically with equine monocytes to allow further experiments. Moreover, although we demonstrated that EHV-1 infection induces integrin dependent adhesion of CD172a⁺ cells to endothelial cells, at this stage, it is not clear whether EHV-1 infection induces activation or increased expression of specific integrins. Indeed, the antibodies used in this study do not discriminate between inactive or activated forms of the integrins and it was

technically impossible to assess any potential up-regulation of integrin expression on EHV-1 inoculated cells compared to mock, due to a low percentage of infected CD172a⁺ cells detected.

Next, we addressed the mechanism by which EHV-1 infection induces adhesion of CD172a⁺ cells to EC in the absence of viral gene expression. We demonstrated that EHV-1 infection induces CD172a⁺ cell adhesion in a PI(3)K and ERK/MAPK-dependent processes at a very early stage of infection. The specific MEK/ERK inhibitor U0126 and the PI(3)K inhibitor LY294002 were able to efficiently decrease CD172a⁺ cell adhesion to EC in a dose-dependent manner. Together, these data show that integrin engagement and the activation of integrin-mediated signaling pathways are essential in the induction of EHV-1-infected CD172a⁺ adhesion to EC. Several herpesviruses, such as pseudorabies virus (PRV), herpes simplex virus 1 (HSV-1), human herpesvirus 8 (HHV-8) and human cytomegalovirus (HCMV) have been reported to activate integrin-mediated ERK-MAPK and/or PI(3)K signaling pathways through the interactions of viral glycoproteins with putative receptors present at the plasma membrane of host cells (Naranatt *et al.*, 2003; Nogalski *et al.*, 2011; Reeves *et al.*, 2012; Setas Pontes *et al.*, 2015; Zheng *et al.*, 2014). To date, the EHV-1 envelope glycoprotein D (gD) has been shown to interact with α V integrins present on PBMC and to be critical for viral entry via endocytosis (Van de Walle *et al.*, 2008). Here we hypothesized that EHV-1 gD interacts with α V integrins on CD172a⁺ cells and subsequently activates integrin-mediated signaling pathways (PI(3)K and ERK/MAPK) (Fig.7.A). We believe that this activation may induce specific functional changes in CD172a⁺ cells, including the activation of integrins, which in turn may facilitate their adhesion to specific endothelial adhesion molecules (Carlos *et al.*, 1991) (Fig.7.B). This latter hypothesis is supported by the study of Chang *et al.* (2012) which demonstrated that PKC-dependent ERK activation contributes to monocyte adhesion and by studies of Smith *et al.* (2007 and 2004), demonstrating that PI(3)K activity is essential for HCMV-mediated induction of monocyte motility and firm adhesion to EC. However, further studies are still needed to decipher the precise mechanism of regulation of integrins by EHV-1 in CD172a⁺ cells and to directly address the potential relationship between integrin activation and cellular signal transduction pathways in cell adhesion.

In addition, we showed that EHV-1 replication was enhanced in CD172a⁺ cells upon adhesion to EC. The percentages of IEP- and gB-positive CD172a⁺ cells were higher

in cells adherent to EC than in cells adherent to plastic. These findings corroborate *in vitro* studies on HIV, demonstrating that contacts with EC enhanced HIV-1 replication in infected monocytes and macrophages (Fan *et al.*, 1994; Gilles *et al.*, 1995). Interestingly, we demonstrated that the percentage of IEP-positive CD172a⁺ cells significantly increased from 2 to 12 hpa for EHV-1 03P37 strain while it only increased at 12 hpa for EHV-1 (97P70). Moreover, we observed a higher increase in gB protein expression in IEP-positive cells inoculated with EHV-1 97P70 strain and co-cultured for 12 h with EC monolayers, compared to cells inoculated with EHV-1 03P37. Taken together, the authors believe that these results are due to differences in the replication kinetics of EHV-1 in target monocytic cells between the two strains. This hypothesis is based on a previous study from Laval *et al.* (2015) demonstrating that EHV-1 (97P70) replication was restricted and delayed in CD172a⁺ cells adherent to plastic and ongoing *in vitro* studies on the replication kinetics of EHV-1 (03P37) in monocytic cells, indicating strikingly different outcomes (unpublished data). We think that the adhesion of EHV-1 inoculated CD172a⁺ cells to EC speeds up the viral replication machinery in CD172a⁺ cells inoculated with EHV-1 97P70 strain, but to a lesser extent in CD172a⁺ cells inoculated with EHV-1 03P37 strain.

In addition, we showed that TNF- α was produced via direct interaction between CD172a⁺ cells and EC and that TNF- α triggered the enhanced EHV-1 replication observed in CD172a⁺ cells. This is in agreement with previous studies on HCMV and HIV infections, which demonstrated that the production of TNF- α induces cellular changes in monocytic cells, resulting in increased viral gene expression (Poli *et al.*, 1990; Soderberg-Naucler *et al.*, 1997). TNF- α secreted by EC was also found to be associated with increased HIV expression in monocytic cells (Borghi *et al.*, 2000). From our findings, we hypothesize that TNF- α is indirectly influencing IEP expression in CD172a⁺ cells by inducing differentiation of CD172a⁺ monocytic cells into EHV-1-permissive macrophages. TNF- α may induce the production of cellular factors important for EHV-1 replication in CD172a⁺ cells. However, further studies are still needed to determine the specific role played by TNF- α in the pathogenesis of EHV-1 infection.

We also demonstrated that the percentage of gB-positive CD172a⁺ cells adherent to EC was reduced following treatment with TNF- α neutralizing antibody. We considered that other cytokines besides TNF- α might also play a role in the enhanced EHV-1 expression observed in CD172a⁺ /EC co-cultures, as both monocytic cells and

EC are known to secrete a variety of inflammatory cytokines which are important in the regulation of viral replication. However, we failed to detect substantial amounts of IL-1 β and IFN- α produced upon co-culture of mock- or EHV-1-inoculated CD172a⁺ cells with ECs (data not shown). Taken together, our results indicate that the enhanced EHV-1 replication observed in CD172a⁺ cells is a complex process involving both cell contacts and the production of soluble factors, with major implications for EHV-1 spread to EC (Fig.7.C). Interestingly, EHV-1 neurovirulent and non-neurovirulent strains both enhanced EHV-1 replication in CD172a⁺ cells upon adhesion to EC. These findings suggest that additional factors account for the difference in pathogenesis of EHV-1 *in vivo*.

Finally, we demonstrated that approximately 20% of late infected CD172a⁺ cells were able to transfer cytoplasmic EHV-1 material to uninfected, adjacent EC in the presence of neutralizing antibodies (Fig.7.D). Our data are in line with the findings of Digel et al. (2006), showing direct transfer of HCMV cytoplasmic material from infected to neighboring uninfected cells. Although speculative at this point, we hypothesize that EHV-1 spreads from CD172a⁺ cells to EC through ‘microfusion’ events, as described for other herpesviruses (Gerna *et al.*, 2000; Van de Walle *et al.*, 2003). This strategy would allow the virus to spread from CD172a⁺ cells to the endothelium of target organs in the presence of neutralizing antibodies. In our experiments, transfer of EHV-1 from late infected CD172a⁺ cells to EC predominantly resulted in a non-productive infection in EC. These data suggest that: (1) only a minority of infected CD172a⁺ cells can efficiently transfer EHV-1 to EC and initiate an infection, (2) EHV-1 infection of EC does not automatically result in viral spread to the surrounding tissues.

Previous *in vivo* studies showed that EHV-1 infection of endothelial cells of the pregnant uterus or CNS resulted in damages of the microvasculature of target organs due to the initiation of an inflammatory cascade, vasculitis and microthrombosis (Edington *et al.*, 1986; Patel *et al.*, 1982; Smith *et al.*, 1992). EHV-1 infection of endothelial cells also induced extravasation of mononuclear cells, resulting in perivascular cuffing and local hemorrhages (Stierstorfer *et al.*, 2002). According to this, we believe that the adhesion and transfer of EHV-1 from CD172a⁺ monocytic cells to endothelial cells may contribute to clinical thrombosis in horses with EHV-1 infection. The use of specific anti-adhesive molecules may represent a valuable

therapeutic tool to reduce adhesion of infected CD172a⁺ cells and subsequent transfer of EHV-1 to EC of target organs.

In conclusion, our data suggest that EHV-1 uses specific cellular signaling pathways to trigger cellular changes in CD172a⁺ cells, which result in increased adhesion to EC. The interaction between EHV-1-infected CD172a⁺ cells and EC enhances EHV-1 replication in CD172a⁺ cells through cell-to-cell contacts and secretion of soluble factors, which subsequently facilitates viral transfer to EC.

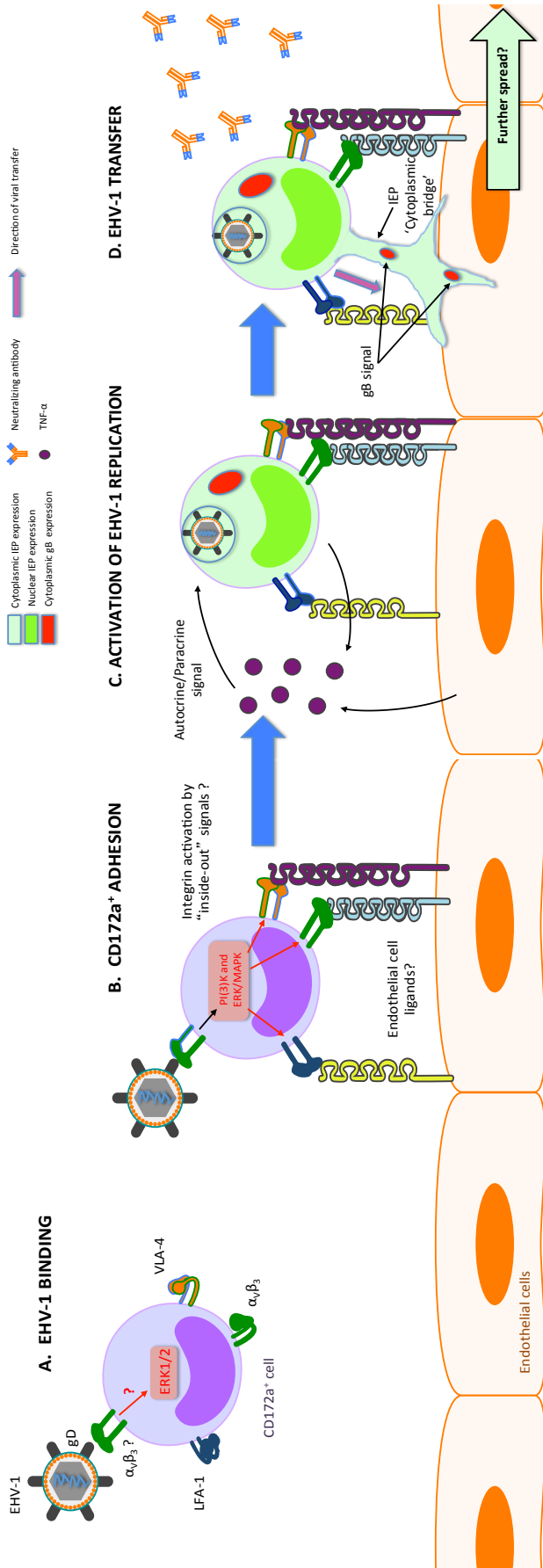


Figure 7: Hypothetical model of the transmission of EHV-1 from CD172a⁺ cell to EC. (A) Interaction of EHV-1 via gD with $\alpha_v\beta_3$ integrin on the surface of CD172a⁺ cell activates the intracellular integrin-mediated PI(3)K and ERK/MAPK signaling pathways. (B) This activation leads to the upregulation of LFA-1 ($\alpha_1\beta_2$), VLA-4 ($\alpha_4\beta_1$), and $\alpha_v\beta_3$ integrin expression on the surface of the infected CD172a⁺ cell. Thus, activated LFA-1, VLA-4, and $\alpha_v\beta_3$ integrins can interact with some of their respective endothelial ligands and mediate CD172a⁺ cell adhesion to EC. (C) Both cell-to-cell contacts and secretion of soluble factors such as TNF- α activate the replication of EHV-1 in CD172a⁺ cell. (D) These processes facilitate direct transfer of cytoplasmic EHV-1 material from infected CD172a⁺ cell to adjacent EC.

Acknowledgements

This research was supported by the Concerted Research Action 01G01311 of the Research Council of Ghent University, Belgium. Kathlyn Laval and Hans J. Nauwynck are members of BELVIR consortium (IAP, phase VII) sponsored by Belgian Science Policy (BELSPO). The authors are grateful to Dr. O'Callaghan and Dr. Osterreider for supplying the antibodies against EHV-1 proteins. The authors also acknowledge Carine Boone and Lieve Sys for their excellent technical support; Maria Pontes and Korneel Grauwet for their technical advice.

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Chapter 6.

General discussion

Viruses have evolved under pressure of their host's immune system over millions of years. During this co-evolution, viruses developed various strategies to subvert the host's antiviral responses to ensure their own replication and survival. These strategies are as diverse as the viruses themselves. While small RNA viruses mainly rely on antigenic variability to avoid host's immune responses, large DNA viruses such as herpesviruses have evolved complex immune evasive strategies to increase fitness. The hallmark of a herpesvirus infection is the capacity to establish lifelong latent infections in an immunocompetent host, generally without causing severe symptoms. The lifelong persistence of herpesviruses reflects the delicate balance these viruses establish with the host's immune system. It is generally not in the evolutionary interest of herpesviruses to kill or impair their host, as latent viruses require a host that stays alive and is healthy enough to interact with others. In addition, the persistence of herpesviruses is facilitated by a wide range of host innate and adaptive immune evasion mechanisms that the reactivated virus exploits to stay as long as possible in its host. Thus, it is not surprising that current vaccines and antiviral therapies against herpesvirus infections often are not fully protective.

Among animal herpesvirus members, equine herpesvirus type 1 (EHV-1) is one of the major pathogens affecting horses worldwide. The virus is responsible for respiratory disorders, abortion, neonatal foal death and myeloencephalopathy (EHM) (Allen & Bryans, 1986; Patel & Heldens, 2005). The interest in EHV-1 has increased during the last decade by frequent outbreaks of abortions and/or neurological disorders causing serious economic losses to the horse industry every year. It has been reported that the incidence of EHM during outbreaks can range from 10 to more than 30% and the incidence of abortion during outbreaks can exceed 50% (Goehring *et al.*, 2006; Lunn *et al.*, 2009). With a total global equine population estimated at 58 million, with 6 million in Europe, mortality rates of around 40% during EHM outbreaks raise concerns in the equine industry worldwide (Allen *et al.*, 2004; Lunn *et al.*, 2009). Despite many years of research, no fully protective vaccines against EHV-1 or efficient antiviral drugs have been developed. Current vaccines (inactivated and live-attenuated) do not provide full protection against EHV-1 associated diseases. These vaccines fail to completely prevent virus shedding and cell-associated viremia, and subsequently, fail to protect against severe EHV-1 symptoms. Indeed, EHV-1 can still

spread via a cell-associated viremia in vaccinated animals, indicating that EHV-1 is able to manipulate the host's immune system (O'Neill *et al.*, 1999; Kydd *et al.*, 2006). In addition, no efficacious antiviral treatment is available for EHV-1 infections and treatment is mainly limited to supportive therapy. It is clear that a radical paradigm shift in the design of control strategies against EHV-1 infection and herpesvirus infections in general is needed. This requires a breakthrough in the knowledge on the pathogenesis of these viruses and their immune evasion mechanisms. It is for this reason that our research group looked for new leads in the interactions between EHV-1 and its host cells.

Previous *in vivo* and *ex vivo* studies of the pathogenesis of EHV-1 demonstrated that EHV-1 misuses single immune cells from the URT to breach the BM into the connective tissues. These carrier immune cells were mainly identified as CD172a⁺ monocytic cells (Gryspeerd *et al.*, 2010; Vandekerckhove *et al.*, 2010). Interestingly, it was found that the expression of particular late proteins in EHV-1-infected carrier monocytic cells was hampered at early stages of infection (Gryspeerd *et al.*, 2012; van der Meulen *et al.*, 2000). These results suggested that an early block in the replication cycle of EHV-1 may protect infected monocytic cells from efficient recognition by the immune system and hence may allow these carrier cells to reach target organs. Monocytic cells are known to play crucial roles in the innate and adaptive immune responses during viral infections. In addition, monocytic cells are very motile cells and can migrate from the blood into specific tissues during homeostasis, inflammatory or immune responses (Shi & Pamer, 2011). Thus, it is not surprising that EHV-1 hijacks monocytic cells and uses them as immune-privileged vehicles for viral dissemination within the horse. Until now, the precise mechanism(s) by which EHV-1 modulates its replication within CD172a⁺ monocytic cells and how the virus is transmitted to target organs in the presence of neutralizing antibodies are still unknown and thus, this comprised the main focus of this study.

EHV-1 replication in CD172a⁺ monocytic cells: the Trojan Horse model

In the first part of this thesis (Chapter 3), we examined *in vitro* the replication kinetics of two neurovirulent and three non-neurovirulent EHV-1 strains in target CD172a⁺ monocytic cells and compared them with those in the RK-13 control cell line. We demonstrated that the replication of both EHV-1 strains was highly restricted in CD172a⁺ cells compared to RK-13 cells. However, we found that the spatio-temporal distribution dynamics of EHV-1 proteins within susceptible CD172a⁺ cells differ between the two strains. While the replication kinetics of the neurovirulent EHV-1 strain were comparable between susceptible CD172a⁺ and RK-13 cells, the non-neurovirulent EHV-1 strain delayed its replication in CD172a⁺ cells at a very early time of infection. In addition, we observed that both neurovirulent and non-neurovirulent EHV-1 infections were mainly non-productive in susceptible CD172a⁺ cells. Finally, we found that histone deacetylases (HDACs) regulate the silencing of gene expression of a non-neurovirulent EHV-1 strain in CD172a⁺ cells whereas they did not appear to influence the replication of a neurovirulent EHV-1 strain in these cells.

Chromatin remodeling by HDACs plays a key role in the regulation of viral gene expression during herpesvirus infections (Guise *et al.*, 2013). Thus, it is not surprising that EHV-1 has developed epigenetic strategies to regulate its own replication through HDACs within target CD172a⁺ cells. HDACs are known to condense chromatin structure by deacetylation of lysine residues on histone tails, resulting in repression of cellular and/or viral transcription (Sarkar *et al.*, 2006). HDACs perform their repressive functions as components of several multi-protein co-repressor complexes, such as the co-repressor of RE1 silencing transcription factor (CoRest) (You *et al.*, 2001). These are then targeted to specific genomic regions by interactions with DNA binding factors, such as transcription factors. However, herpesviruses, such as HSV-1, have developed various strategies to block HDAC repressor activity. Upon dissociation of HDAC-1 or -2 from the CoREST/REST/HDAC repressor complex by HSV-1 ICP0, HSV-1 Ser/Thr US3 kinases are then able to indirectly phosphorylate these HDACs, thus rendering them inactive (Gu *et al.*, 2005; Gu & Roizman, 2007; Walters *et al.*, 2010). Phosphorylation of HDACs results in increased levels of histone

acetylation and activation of viral gene expression in cells. From our observations, we postulate that the neurovirulent EHV-1 strain acts similarly to HSV-1, and blocks the repressor activity of HDAC at promoter sites, thus enhancing its replication in CD172a⁺ cells. In contrast, the non-neurovirulent EHV-1 strain appears to have adapted during evolution to misuse HDACs instead of blocking their activity. Silencing gene expression by HDACs might be a strategy for the virus to slow down its replication in CD172a⁺ cells, effectively allowing non-neurovirulent EHV-1 to behave as a ‘stealth virus’, persisting in CD172a⁺ cells and functionally hiding itself from the immune system longer compared to neurovirulent EHV-1.

Interestingly, HDACs have also been reported to limit CMV infection *in vitro*. It was shown that the relatively high levels of HDACs in monocytes and T2 cells were correlated with non-permissiveness for HCMV (Murphy *et al.*, 2002). Following treatment with HDAC inhibitors, non-permissive cells were rendered transiently permissive for HCMV, suggesting that HDACs played a role in the repression of viral replication. It was proposed that the chromatin structure around the IE promoter changes with cellular differentiation and thus may play a role in controlling HCMV latency and reactivation. Thus, we hypothesize that, *in vivo*, acute EHV-1 infection promotes activation of blood monocytes that subsequently migrate into different tissues (pregnant uterus and/or CNS) and differentiate into permissive macrophages. We speculate that this differentiation may decrease HDAC activity and increase histone acetylase levels at EHV-1 promoter sites, thus directly enhancing viral replication in macrophages.

Moreover, we suggest that differences in the genome sequences of neurovirulent and non-neurovirulent EHV-1, in addition to the single nucleotide polymorphism in the viral DNA polymerase, might be responsible for the differential control of HDAC activity in CD172a⁺ cells at very early time of infection *in vitro* and thus may contribute to their differing pathogenesises *in vivo*. We proposed that mutations in the coding sequences of some regulatory proteins of non-neurovirulent EHV-1 strain might alter their capacity to bind to and subsequently phosphorylate HDAC and/or HDAC-containing complexes at viral promoters in CD172a⁺ cells. This may result in increased levels of HDACs and repression of EHV-1 gene expression, and thus may indirectly delay viral replication in CD172a⁺ cells. However, the fact that both EHV-1 strains can still cause a viremia in vaccinated horses indicates that manipulation of

HDACs is only one part of EHV-1's strategy to bypass detection by the immune system. Overall, these results are giving new insights in how EHV-1 modulates its replication within CD172a⁺ monocytic cells and uses them as a 'Trojan horse' in order to evade immune surveillance and disseminate within the horse.

HDAC inhibitors: the Achilles heel of the Trojan horse?

In this context, the use of antiviral compounds that specifically inhibit the activity of HDACs may be an interesting strategy to modulate EHV-1 infections. Over the past decade, HDAC inhibitors have been mainly used as potent anti-proliferative agents in cancer therapies. Research has led to the development of HDAC inhibitors that modify the acetylation state of a large number of cellular proteins involved in oncogenic processes, resulting in antitumor effects (Atadja, 2011). In addition, HDAC inhibitors have been used as antiviral agents against HIV and EBV infections. For instance, valproic acid, a carboxylate HDAC inhibitor, has been combined with highly active anti-retroviral therapy (HAART) and used as an adjuvant therapy in clinical trials to purge latent reservoirs of HIV-1 in resting CD4⁺ T-cells (Demonte *et al.*, 2004; Matalon *et al.*, 2011). This enabled detection of HIV particles and their subsequent elimination by HAART. Similarly, HDAC inhibitors such as sodium butyrate and valproic acid have been used to activate EBV lytic-phase gene expression in cultured cells and mouse models (Ghosh *et al.*, 2012; Shin *et al.*, 2015). In addition, a pre-clinical trial with the HDAC inhibitor arginine butyrate combined with ganciclovir gave promising results in the treatment of EBV-lymphoma patients (Perrine *et al.*, 2007).

Based on these studies, we propose that the use of specific HDAC inhibitors in combination with antiherpes viral drugs and vaccination could be an interesting approach to treat and prevent EHV-1-induced disease in horses infected with EHV-1 non-neurovirulent strains. Specific HDAC inhibitors could be used to relieve the temporary block of non-neurovirulent EHV-1 gene expression and speed up the replication machinery in nasal and blood CD172a⁺ cells of infected horses. Then, treatment with antiherpes viral drugs could inhibit viral replication in these cells at the level of the URT and blood circulation and prevent viremia and/or subsequent viral

spread to the endothelial cells of target organs. The use of antiherpes viral drugs should still be taken with caution since no adequate antivirals against EHV-1 are currently available on the market. To maximize the chance of preventing EHV-1-induced severe symptoms, horses should also be vaccinated before treatment with HDAC inhibitors to ensure that they build up a strong immune response to clear the virus while forcing viral replication in CD172a⁺ cells. As cellular immunity mediated by CTLs is crucial in the protection against EHV-1 infection, the use of live-attenuated vaccines is preferred. So far, the current live-attenuated vaccines available in Europe can only reduce and not prevent viral shedding and clinical signs (Rosas *et al.*, 2006). Thus, a better strategy of vaccination at the URT should be developed to prevent viremia. An adequate live-attenuated vaccine should be designed to raise a local immunity at the level of the URT. This may allow EHV-1 infected nasal monocytic cells to become visible by the immune system and be eliminated by CTL before they cross the BM and reach the connective tissues and the blood circulation. This new vaccine should be both administered intranasally and intramuscularly to completely prevent viremia. For safety reasons, this EHV-1 vaccine strain should be able to replicate in the epithelial cells of the URT but no longer infect monocytic cells.

HDAC inhibitors may be of lesser use in horses showing EHV-1 induced neurological disorders, as we demonstrated that the replication of a neurovirulent strain was not tightly regulated by HDACs. Based on our hypothesis that neurovirulent EHV-1 strains are rapidly visible to the immune system in CD172a⁺ cells compared to non-neurovirulent EHV-1 strains, we speculate that the intranasal administration of a live-attenuated vaccine will induce a local humoral and cellular immunity in the horse and clear the virus at the URT, thus preventing cell-associated viremia, as described above. Still, future studies should include more neurovirulent versus non-neurovirulent strains to see whether this difference in HDAC regulation may be generalized or not.

The use of HDAC inhibitors as therapeutic tools to combat EHV-1 infections should still be carefully considered. As HDACs are involved in various cellular processes, including cell-cycle regulation, cell proliferation and differentiation, it is crucial to use highly selective and potent HDAC inhibitors to activate EHV-1 replication in CD172a⁺ cells in order not to impair essential cellular functions. Future studies should

determine which individual HDAC(s) are the most critical for inhibition and which cellular and viral transcription factors are involved in association of the EHV-1 promoter with hypoacetylated histones and mediate silencing of the non-neurovirulent EHV-1 genome within CD172a⁺ cells. Results should be compared between the two pathotypes. The efficacy of HDAC inhibitors should be tested both *in vitro* and *in vivo*. Clinical studies should determine the best route of administration, the therapeutic index of HDAC inhibitors and their adverse effects in horses. HDAC inhibitor-based therapy might also be considered as an interesting strategy to purge latent EHV-1 reservoirs in aviremic horses.

In addition to their function as an inhibitor of HDAC activity, short-chain fatty acids like (sodium) butyrate have been shown to play a role in the diet of the horse. Pasture forage is fermented by the equine colonic microbiota to short-chain fatty acids, including acetate, propionate and butyrate. Short-chain fatty acids provide a major source of energy for the horse and contribute up to 60-70% of its energy requirements upon absorption from the caecum and colon (Argenzio & Hintz, 1972; Argenzio *et al.*, 1974). Among short-chain fatty acids, butyrate has potent effects on a variety of colonic functions and plays a key role in the maintenance of the health of colonic tissue (Bugaut & Bentejac, 1993; Cuff *et al.*, 2005; Hamer *et al.*, 2008; Treem *et al.*, 1994). Butyrate is also involved in various cellular processes, including cell differentiation, cell-cycle arrest and apoptosis, and exerts its action in a concentration-dependent manner (Fung *et al.*, 2011; Li, 2011; Orchel *et al.*, 2005). A study from Li *et al.* (2008) showed that butyrate induces several changes in the expression of genes associated with regulatory pathways that are critical to cell growth, immune response and signal transduction. In addition, it was proposed that natural stimuli, such as stress and weaning might directly affect the intracellular concentration of butyrate, and thus influence its availability to modulate gene expression (Li & Elsasser, 2005). According to this, we postulate that nutritional environment and physiological factors (stress, pregnancy...) may influence the concentration of butyrate in the horse, which in turn may modulate cellular and viral gene expression, thus controlling EHV-1 infection *in vivo*. Future studies should examine the epigenetic effects of physiologic concentrations of butyrate in the control of EHV-1 infection in horses.

Entry of EHV-1 into CD172a⁺ cells: Boarding the Trojan horse

The next step of this thesis was to investigate the molecular mechanisms underlying the "restricted" infection of EHV-1 in CD172a⁺ cells. Virus attachment and entry are some of the most critical stages during virus infection and play an important role in cell tropism and pathogenesis of virus infections. However, little is known about the regulation of CD172a⁺ cell tropism and susceptibility to EHV-1 infection. In Chapter 4, we characterized the entry of EHV-1 into CD172a⁺ monocytic cells. Using diolabeled EHV-1 particles, we demonstrated that EHV-1 binds to a limited number of CD172a⁺ cells compared to RK-13 cells, suggesting the presence of a specific subset of CD172a⁺ monocytic cells that could differently support EHV-1 replication and contribute to the pathogenesis of EHV-1. In this study, we used the mouse monoclonal anti-CD172a antibody to identify cells of myeloid origin, but we could not differentiate between specific subsets, such as granulocytes, DCs, monocytes and macrophages. This task was mainly hampered by the lack of specific myeloid markers commercially available that cross-react with equine cells (Ibrahim *et al.*, 2007). In humans and other mammal species, blood monocytes can be divided in two distinct subsets: CD14⁺CD16⁻ cells, which are often called "classic monocytes" and CD14⁺CD16⁺ monocytes, suggested to resemble mature tissue macrophages (Passlick *et al.*, 1989). Interestingly, CD16⁺ cells constitute a small percentage of the total monocyte population and they are characterized as being more pro-inflammatory and having a more prominent role in infections. Previous *in vivo* and *in vitro* studies have shown that CD16⁺ monocytes are more permissive to productive HIV-1 infection than the majority of blood monocytes, and that this relative permissiveness is due to enhanced viral entry (Ellery *et al.*, 2007). A study from Williams *et al.* (2005) also demonstrated that the CD14⁺CD16⁺ cells were consistently positive for SIV DNA, suggesting that these cells may function as Trojan horses, which carry the virus into the brain. In the future, it will be interesting to further identify the specific subset of CD172a⁺ cells susceptible to EHV-1 infection *in vitro* and check whether CD14⁺CD16⁺ cells are similarly involved.

The ability of EHV-1 to bind to a limited set of CD172a⁺ cells also suggests the presence of specific receptor(s) at the surface of these cells. Previous studies have already shown that EHV-1 utilizes a cell surface receptor that is distinct from any of

the known alphaherpesvirus entry receptors (Frampton *et al.*, 2005). The Major Histocompatibility Complex Class I (MHC I) has been identified as a functional entry receptor for gD in equine dermal and equine brain microvascular endothelial cells (Kurtz *et al.*, 2010; Sasaki *et al.*, 2011). However, a study from Azab *et al.* (2012) demonstrated that MHC I antibodies did not efficiently block EHV-1 entry into PBMC, suggesting the presence of different receptors in equine leukocytes. Hence, we investigated the nature of the receptor(s) involved in the binding of EHV-1 to CD172a⁺ monocytic cells. We found that enzymatic removal of heparan sulfate did not affect EHV-1 infection, while removal of sialic acids from the cell surface by treatment with neuraminidase had a very strong effect in inhibiting EHV-1 infection of CD172a⁺ cells. These results showed that sialic acid residues on these cells and not heparan sulfate are involved in EHV-1 infection. Thus, we propose that EHV-1's narrow cellular tropism *in vitro* may be in part due to its interactions with specific sialic acid containing cell-surface receptor(s) on CD172a⁺ cells.

Sialic acid-binding viruses include important human pathogens, such as adenovirus, influenza virus and rotavirus (Arnberg *et al.*, 2002; Isa *et al.*, 2006; Matrosovich *et al.*, 2015). So far, influenza virus is the best-studied example of sialic acid involvement in viral entry. Influenza virus uses hemagglutinin (HA) as an attachment receptor to bind to sialic acids on the cell surface (Wu & Air, 2004). The type of linkage between terminal sialic acids and the second sugar is one of the major determinants of influenza virus tropism and host specificity (Imai & Kawaoka, 2012). Human influenza viruses preferentially bind to sialic acid attached to galactose by an α 2,6 linkage while avian and equine influenza viruses bind to α 2,3 galactose-linked sialic acids (Connor *et al.*, 1994; Matrosovich *et al.*, 2000). To date, sialic acid-containing lipids and polymers have been developed as entry blockers of influenza virus into cells (Guo *et al.*, 2002; Matrosovich & Klenk, 2003). As alternatives to sialic acids, HA binding peptides have also been considered promising candidates as novel antiviral drugs (Matsubara *et al.*, 2010). Similarly, the use of antivirals that specifically act as entry blockers of EHV-1 into target CD172a⁺ cells may be an interesting approach to prevent EHV-1 infection. However, further studies are still needed to determine which sialylated glycans are present on CD172a⁺ cells and which are involved in EHV-1 binding and entry. In addition, EHV-1 glycoprotein(s) that

may serve as viral glycan-binding proteins should be identified. Recently, it has been demonstrated that EHV-1 gC has hemagglutination activity against equine red blood cells (Andoh *et al.*, 2015). This suggests a potential role of gC as a viral lectin, involved in the binding of sialic acids residues on CD172a⁺ cells. EHV-1 gC may also be an interesting candidate in the design of sialic acid binding peptides that block EHV-1 binding to CD172a⁺ cells.

In this chapter, we also found that blockage of $\alpha_v\beta_3$ integrins by neutralizing antibodies significantly reduced EHV-1 entry at a post-binding step. We hypothesized that $\alpha_v\beta_3$ integrin is not used as a cellular receptor, involved in attachment of EHV-1 to cells, but rather as a co-receptor used to mediate post-binding entry of EHV-1 into CD172a⁺ cells. The sequential engagement of distinct receptors and co-receptors may allow the timing of key events in EHV-1 entry into CD172a⁺ cells. Interestingly, several studies have indicated that $\alpha_v\beta_3$ integrin serves as a co-receptor for various herpesviruses, including HSV and HCMV (Gianni & Campadelli-Fiume, 2012; Wang *et al.*, 2005). For instance, it has been shown that $\alpha_v\beta_3$ integrin acts as a co-receptor routing HSV to a pathway dependent on lipid rafts, dynamin-2 and acidic endosomes (Gianni *et al.*, 2010). For EHV-1, it has been demonstrated that the interaction between $\alpha_v\beta_3$ integrin present on PBMC and the arginine, serine, and aspartic acid (RSD) motif present in gD triggers EHV-1 entry via endocytosis potentially by inducing conformational changes of the receptor and initiating signaling cascades (Van de Walle *et al.*, 2008). EHV-1 may use $\alpha_v\beta_3$ integrin to route its entry via endocytosis into CD172a⁺ cells in order to shorten its exposure at the cell surface. The restricted expression of $\alpha_v\beta_3$ integrin on CD172a⁺ cells may also be an important determinant of EHV-1 tropism. Thus, $\alpha_v\beta_3$ integrin could be a viable target for antiviral therapies to prevent EHV-1 infection and in particular cell-associated viremia by interrupting the entry process of EHV-1 into susceptible CD172a⁺ cells.

Finally, we characterized the mode of entry of EHV-1 into CD172a⁺ cells by the use of pharmacological inhibitors and co-localization assays. We demonstrated that EHV-1 enters CD172a⁺ cells via an endocytic mechanism that requires cholesterol, tyrosine kinase activity, actin polymerization, dynamin activity and endosomal acidification. It is not surprising that EHV-1 enters target CD172a⁺ cells by endocytosis as this mechanism offers several advantages over entry by fusion. First of all, endocytosis

allows the virus to bypass obstacles associated with cytoplasmic crowding and the network of microfilaments (Sodeik, 2000). Secondly, a drop in pH within endocytic vesicles may allow viruses to sense their location in the cell and trigger the membrane/fusion penetration reactions that deliver the viral genetic material to the cytoplasm (Marsh & Helenius, 1989). Thirdly, viruses that are endocytosed avoid leaving evidence of their presence exposed at the plasma membrane and thus, this may delay their detection by the immune system. Many viruses including HSV-1 and HIV are capable to enter cells via fusion but often prefer to use endocytic pathways for productive entry (Daecke *et al.*, 2005; Miyauchi *et al.*, 2009; Nicola *et al.*, 2003). Here, we propose that EHV-1 may enter CD172a⁺ cells by phagocytosis. Phagocytosis is mainly used by professional phagocytes, such as monocytes and macrophages to interfere with microbial invasion and to remove dead cells (Flannagan *et al.*, 2012). In addition, studies reported that some human herpesviruses, including HSV-1 and CMV use phagocytosis-like uptake to gain entry into cells (Clement *et al.*, 2006; Tiwari & Shukla, 2012). Thus, we propose that EHV-1 exploits phagocytosis to promote its entry within CD172a⁺ monocytic cells. The formation of protrusions may facilitate uptake of EHV-1 into the cell and may protect the virus from neutralizing antibodies. Future studies are still needed to further examine the phagocytic uptake of EHV-1 into CD172a⁺ cells. A better understanding of the interactions between EHV-1 and membrane protrusions on CD172a⁺ cells may provide new insights in the development of therapeutics against EHV-1 infection in particular by preventing virus uptake.

In conclusion, a hypothetical model of EHV-1 entry into CD172a⁺ cells is provided in Figure 1. We postulate that EHV-1 initially binds to CD172a⁺ cells via an interaction between EHV-1 gC and sialic acid containing cell-surface receptor(s) present on CD172a⁺ cells (A). This interaction may initiate intracellular signaling cascades that activate $\alpha_v\beta_3$ integrin on the cell surface of CD172a⁺ cells and thus facilitate the second interaction of EHV-1 via gD with its $\alpha_v\beta_3$ integrin co-receptor (B). This second interaction may in turn activate integrin-mediated signaling pathways, which trigger rearrangements of the actin cytoskeleton in the cell, including the polymerization of actin underneath the contact zone, facilitating the formation of membrane protrusions at the surface of CD172a⁺ cells (C). This leads to the engulfment of EHV-1 particles

and clustering of $\alpha_v\beta_3$ integrins in phagosomes and transport within the cytoplasm (D). Here, we want to point out that the mechanism of EHV-1 binding and entry into CD172a⁺ cells was similar between EHV-1 non-neurovirulent and neurovirulent strains and thus may not account for differences in the neuropathogenic vs abortigenic potential of EHV-1 *in vivo*. However, we think that the development of antivirals that block several steps of EHV-1 entry into target CD172a⁺ cells could efficiently prevent cell-associated viremia and both EHV-1 induced abortion and neurological disorders in horses.

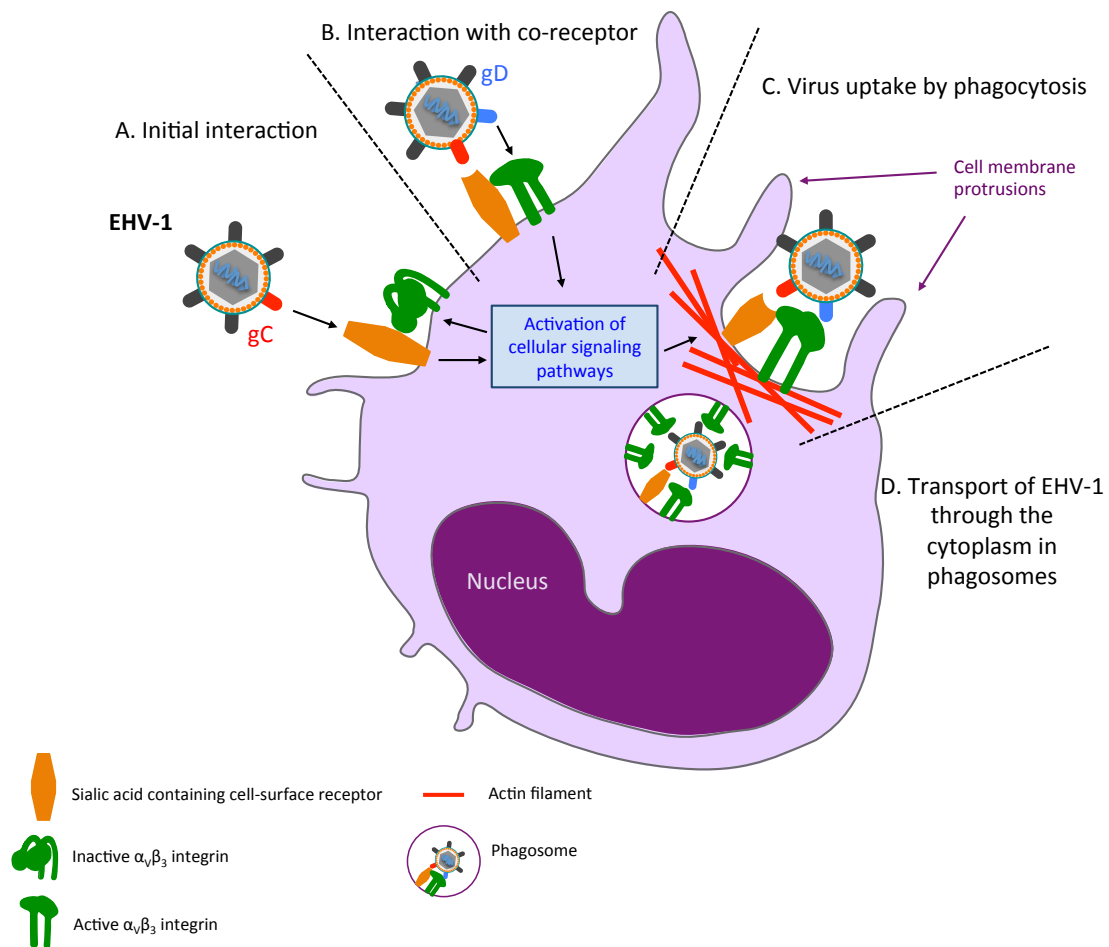


Figure 1: Hypothetical model of EHV-1 entry into a CD172a⁺ cell. (A) Initial attachment of EHV-1 may occur via binding of gC to a sialic acid containing cell-surface receptor and initiates intracellular signaling cascades that activate $\alpha_v\beta_3$ integrin on the cell surface of CD172a⁺ cells. (B) This may facilitate the second interaction of EHV-1 via gD with $\alpha_v\beta_3$ integrin co-receptor, which in turn activates cellular integrin-mediated signaling pathways. (C) This activation may induce rearrangements of the actin meshwork underneath the contact zone, clustering of $\alpha_v\beta_3$ integrins and the formation of membrane protrusions that facilitate uptake of EHV-1 into the cell. (D) EHV-1 is transported through the cytoplasm in phagosomes.

Transmission of EHV-1 from CD172a⁺ monocytic cells to endothelial cells (EC): unloading the Trojan horse

EHV-1-induced abortion and neurological disease are direct results of viral spread from infected leukocytes to the EC lining the blood vessels of the pregnant uterus and CNS, and their subsequent infection. In order to initiate a replication in the EC of these target organs, EHV-1-infected CD172a⁺ cells must employ specific strategies to adhere and subsequently transmit EHV-1 to the EC in the presence of neutralizing antibodies. Some studies highlighted the importance of cell-to-cell contacts between PBMC and EC in the transmission of EHV-1 and the involvement of adhesion molecules present on the cell surface of both cell types (Goehring *et al.*, 2011; Smith *et al.*, 2001). However, at the start of this thesis, it was still unclear how exactly CD172a⁺ cells adhere and transmit EHV-1 to EC.

In Chapter 5, we demonstrated that EHV-1 infection significantly increased adhesion of CD172a⁺ cells to EC. Antibody experiments showed that $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_v\beta_3$ integrins partially mediated the adhesion of EHV-1 infected CD172a⁺ cells to EC. We found that the activation of integrin-mediated signaling pathways, including PI(3)K and ERK/MAPK were also essential for this adhesion process. Based on these results, we proposed that the interaction of EHV-1 via gD with $\alpha_v\beta_3$ integrin on the surface of a CD172a⁺ cell activates the intracellular integrin-mediated PI(3)K and ERK/MAPK signaling pathways which in turn up-regulate $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_v\beta_3$ integrin expression on the surface of the infected CD172a⁺ cell. Thus, activated integrins can interact with some of their respective EC ligands and mediate CD172a⁺ cell adhesion to EC. The interaction of EHV-1 gD with $\alpha_v\beta_3$ integrin is likely to direct events that modulate both viral entry and cellular adhesion. As integrin activation is a complex and dynamic process, it is still unclear whether viral binding to $\alpha_v\beta_3$ integrin activates distinct and/or overlapping integrin-mediated signaling pathways that mediate these events. Integrins, including $\alpha_v\beta_3$ integrins, are well known to play various roles in cell adhesion, migration, angiogenesis and immune responses (Hynes, 2002; Weis & Cheresh, 2011). Therefore, it is not surprising that EHV-1 utilizes $\alpha_v\beta_3$ integrins as co-receptors to enter into target CD172a⁺ cells and as cellular receptors to facilitate viral

transmission. This suggests a very important role of $\alpha_v\beta_3$ integrin in the pathogenesis of EHV-1.

Binding of herpesviruses to integrins is known to activate specific cellular pathways that play a key role in the manipulation of biological functions of monocytes. For instance, it has been shown that integrin engagement and the subsequent activation of PI(3)K signaling pathway in HCMV-infected monocytes results in the production of pro-inflammatory mediators that drive newly infected monocytes from the blood circulation to host tissues and facilitate their adhesion to EC (Smith *et al.*, 2004). Similarly, we think that EHV-1 infection of CD172a⁺ cells may result in cellular activation, the induction of cellular motility, migration and secretion of inflammatory cytokines and chemokines. Although speculative at this stage, it is interesting to think that EHV-1 might use an inflammatory response to induce the migration and adhesion of infected CD172a⁺ cells to EC and the recruitment of additional monocytic cells that may serve as target for viral infection in the tissue. This could participate to the inflammatory cascade associated with EHV-1 infection of EC of the CNS and pregnant uterus, thus contributing to the development of EHV-1-induced disease.

In Chapter 5, we also found that EHV-1 replication was enhanced in CD172a⁺ cells upon adhesion to EC and correlated with the production of TNF- α . Based on this finding, we postulate that EHV-1 is able to manipulate the biology of infected CD172a⁺ monocytic cells by inducing their differentiation into macrophages and promoting their adhesion to EC. While short-lived monocytes that are less permissive to EHV-1 infection are used as a 'Trojan horse' to evade immune surveillance and disseminate via the blood circulation within the host, it appears that EHV-1 prefers to use long-lived permissive macrophages to spread to EC of target organs. As TNF- α is known to be primarily produced by macrophages and EC and to be central for the control of viral replication (Borghi *et al.*, 2000; Kodukula *et al.*, 1999; Paludan & Mogensen, 2001; Pavic *et al.*, 1993), it is likely that the production of TNF- α by both cell types may enhance EHV-1 replication in differentiated CD172a⁺ cells. Although we could only detect noticeable amounts of TNF- α in CD172a⁺-EC co-cultures, we believe that the secretion of other inflammatory cytokines and chemokines might be crucial in the regulation of EHV-1 replication in CD172a⁺ cells both *in vitro* and *in vivo*.

Following enhancement of EHV-1 replication in CD172a⁺ cells, we observed transfer of viral material from infected CD172a⁺ cells to neighbouring EC. We suggest that EHV-1 spreads from infected CD172a⁺ cells to neighbour EC through cell-cell fusions. Transmission of virus from monocytes to endothelial cells via similar ‘microfusion events’ has already been described for other herpesviruses, such as HCMV and PRV (Digel *et al.*, 2006; Van de Walle *et al.*, 2003). For instance, it has been shown that the adhesion and subsequent fusion of immune-masked porcine monocytes to EC result in efficient spread of PRV to EC. The fusion process was found to be virus-mediated since the presence of PRV gH was found indispensable for the fusion to occur. For EHV-1, a recent study from Spiesschaert *et al.* (2015) demonstrated that gB mediated EHV-1 transfer from PBMC to EC. Interestingly, they also demonstrated striking differences between EHV-1 and EHV-4-infected PBMC in their capacity to adhere and transfer the virus to EC, which might help to explain their differing pathogenesises *in vivo*. Like other herpesviruses, EHV-1 appears to use cell-to-cell contacts as an immune evasive mechanism to spread from infected CD172a⁺ cells to EC of target organs in the presence of neutralizing antibodies. However, one additional possible strategy might be that EHV-1-infected CD172a⁺ cells migrate through the endothelium of the pregnant uterus by diapedesis. Indeed, transendothelial migration of monocytes has been shown to be of particular interest in studies of the pathogenesis of HIV and HCMV infection (Bentz *et al.*, 2006; Westhorpe *et al.*, 2009). In the future, it will be interesting to examine the potential of EHV-1-infected CD172a⁺ cells to migrate through EC. This could be achieved by performing a transendothelial migration assay with the use of transwell chambers. The permeability of EC upon adhesion of EHV-1-infected CD172a⁺ cells should also be examined. Although EHV-1 is able to spread from CD172a⁺ cells to EC, this transfer mainly resulted in a non-productive infection. We think that not all infected leukocytes will transfer the virus to endothelial cells, not every transfer will lead to effective spread to neighboring endothelial cells and not every successful infection of endothelial cells will cause infection of surrounding tissues. Previous *in vivo* studies showed that EHV-1 infection of endothelial cells of the pregnant uterus or CNS resulted in damages of the microvasculature of target organs due to the initiation of an inflammatory cascade, vasculitis and microthrombosis (Edington *et al.*, 1986; Patel *et al.*, 1982; Smith *et al.*, 1992). Moreover, it has been reported that EHV-1 infection of endothelial cells

induced extravasation of mononuclear cells, resulting in perivascular cuffing and local hemorrhages (Stierstorfer *et al.*, 2002). Thus, we suggest that the adhesion and transfer of EHV-1 from CD172a⁺ monocytic cells to endothelial cells may contribute to clinical thrombosis in horses with EHV-1 infection. Therefore, the use of anti-adhesive therapies might be an interesting approach to prevent virus passing from infected CD172a⁺ cells to EC and thus interfere with EHV-1 pathogenesis. So far, anti-adhesive therapy in clinical trials has been widely studied for treatment of inflammatory disorders such as inflammatory bowel disease and Crohn's disease (CD) (van Assche, *et al.*, 2002). For instance, natalizumab, a humanized monoclonal antibody against human α_4 integrin has been approved for the treatment of patients with CD (van Assche, *et al.*, 2005). However, before using anti-adhesive therapies for treatment of EHV-1 infections, future studies are still needed to characterize the specific adhesion molecules present on both CD172a⁺ cells and EC that are involved in the multistep process of leukocyte recruitment to EC, a task hampered at the time of this thesis by the lack of antibodies that cross-react with equine cells.

In conclusion, the work in this thesis provides new insights in the interaction between EHV-1 and CD172a⁺ monocytic cells. Increasing evidence supports the concept that EHV-1 hijacks immune cells and uses them as a 'Trojan horse' to disseminate within the horse and evade immune surveillance (Figure 2). As this mechanism may be fundamental for the development of EHV-1-induced diseases in vaccinated horses, halting or uncovering the 'Trojan horse' may be of a particular interest in the development of new therapeutics against EHV-1 infections.

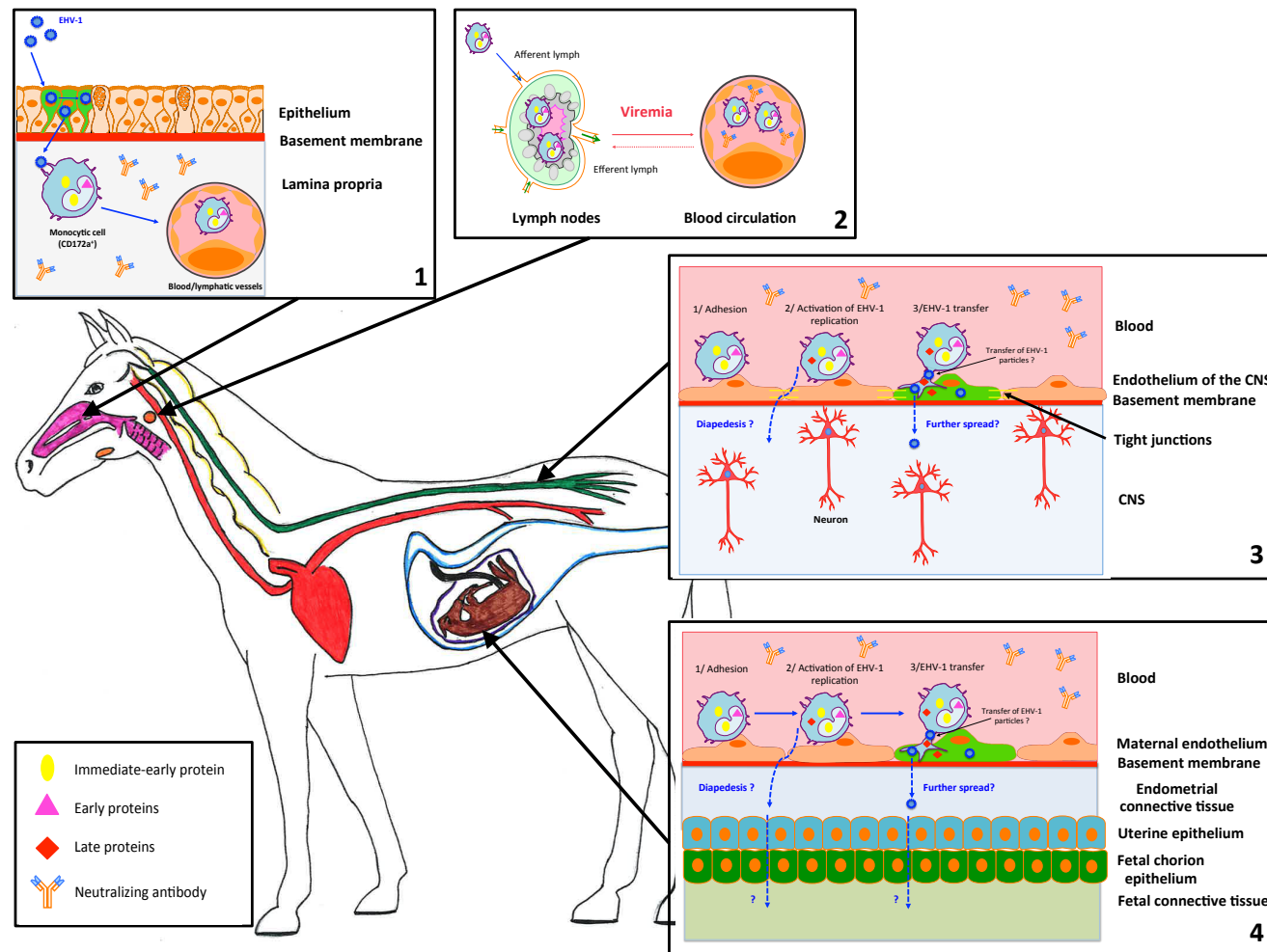


Figure 2: Hypothetical 'Trojan horse' model of EHV-1 dissemination in infected horses. (1) EHV-1 first replicates in the epithelial cells of the URT. Then, EHV-1 enters a small population of CD172a⁺ monocytic cells and uses these cells to cross the BM and reach the connective tissues. EHV-1 silences its replication within CD172a⁺ cells to avoid detection by the immune system while reaching the bloodstream and draining lymph nodes (2). Via the blood circulation, EHV-1 is transported undetected within CD172a⁺ cells to target organs such as the CNS (3) or pregnant uterus (4). There, EHV-1-infected CD172a⁺ cells adhere to EC lining the blood vessels of these organs. The adhesion process activates EHV-1 replication within CD172a⁺ cells and facilitates EHV-1 transfer to EC and their subsequent infection.

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Chapter 7.

Summary-Samenvatting

Summary

Equine herpesvirus type 1 (EHV-1) is one of the major pathogens affecting horses worldwide. After primary replication in the upper respiratory tract (URT), the virus spreads via a cell-associated viremia to the pregnant uterus and/or the central nervous system. Replication in the endothelial cells lining the blood vessels of these organs can lead to abortion or neurological disease. Current vaccines do not provide full protection against EHV-1-induced diseases. Indeed, EHV-1 can still spread via a cell-associated viremia in vaccinated animals, thus avoiding the host's immune response. Equine CD172a⁺ monocytic cells have been shown to be one of the main target cells of EHV-1 in the URT and in the blood and were proposed to serve as a 'Trojan horse' to facilitate the dissemination of EHV-1 to target organs. Therefore, the general aim of this thesis was to investigate in detail *in vitro* how EHV-1 hijacks specific CD172a⁺ monocytic cells and modulates its replication in order to disseminate within the host.

In Chapter 1, an overview of the current knowledge of EHV-1 was given with emphasis on the pathogenesis of EHV-1 and the known EHV-1 immune evasive strategies.

In Chapter 2, the aims of this thesis were formulated.

In Chapter 3, the replication kinetics of EHV-1 non-neurovirulent (Chapter 3.A) and neurovirulent (Chapter 3.B) strains in equine CD172a⁺ monocytic cells were examined and compared with replication kinetics in rabbit kidney epithelial cells (RK-13) control cell line. The number of cells supporting replication for both EHV-1 strains was highly restricted among CD172a⁺ cells (4-8%) compared to RK-13 cells (100%). However, the spatio-temporal distribution dynamics of EHV-1 proteins within the limited population of susceptible CD172a⁺ cells differed between the two strains. While the replication kinetics of neurovirulent EHV-1 were comparable between susceptible CD172a⁺ and RK-13 cells, non-neurovirulent EHV-1 delayed its replication in CD172a⁺ cells at a very early time of infection. In addition, both neurovirulent and non-neurovirulent EHV-1 infections were mainly non-productive in

susceptible CD172a⁺ cells. Finally, it was shown that histone deacetylases (HDACs) regulated the silencing of non-neurovirulent EHV-1 gene expression in CD172a⁺ cells whereas it did not influence the replication of neurovirulent EHV-1 in these cells.

In Chapter 4, the binding and entry of EHV-1 into CD172a⁺ monocytic cells was examined in order to get better insights in the regulation of CD172a⁺ cell tropism and susceptibility to EHV-1 infection. By the use of Dio-labeled EHV-1 particles, it was shown that EHV-1 only bound to 15-20% of CD172a⁺ cells compared to 70% of RK-13 control cells, suggesting the presence of specific receptor(s) at the surface of the cells. Enzymatic removal of heparan sulfate did not affect EHV-1 infection while removal of sialic acids from the cell's surface by treatment with neuraminidase significantly inhibited EHV-1 infection of CD172a⁺ cells, indicating that sialic acid residues on these cells and not heparan sulfate were essential for EHV-1 infection. Moreover, it was found that $\alpha_v\beta_3$ integrins were involved in the post-binding stage of CD172a⁺ cell infection. Finally, the mode of entry of EHV-1 into CD172a⁺ cells was characterized by the use of pharmacological inhibitors and co-localization assays. It was concluded that EHV-1 entered CD172a⁺ cells via an endocytic mechanism that requires cholesterol, tyrosine kinase activity, actin, dynamin activity and endosomal acidification, pointing towards a phagocytic mechanism.

Given the importance of the interactions between monocytic cells and endothelial cells (EC) in the pathogenesis of EHV-1, **Chapter 5** focused on the ability of EHV-1 inoculated CD172a⁺ cells to adhere and subsequently transmit EHV-1 infection to equine venous EC. It was shown that EHV-1 infection significantly increased adhesion of CD172a⁺ monocytic cells to EC *in vitro*. Antibody experiments showed that $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_v\beta_3$ integrins partially mediated the adhesion of EHV-1 infected CD172a⁺ cells to EC. In addition, it was demonstrated that the activation of integrin-mediated signaling pathways, including PI(3)K and ERK/MAPK were also essential for this adhesion process. EHV-1 replication was enhanced in CD172a⁺ cells upon adhesion to EC and correlated with the production of TNF- α . In the presence of neutralizing antibodies, it was observed that approximately 20% of infected CD172a⁺ cells transferred cytoplasmic material to uninfected EC, resulting mainly in a non-productive infection.

In **Chapter 6**, a general conclusion on the research data generated in this thesis was formulated in which the new insights in the interaction between EHV-1 and CD172a⁺ cells were summarized.

The main conclusions drawn in this thesis are:

- When entering into CD172a⁺ cells, EHV-1 modulates its replication, essentially hijacking the cells and using them as a ‘Trojan horse’. This mechanism allows the virus to pass through the blood circulation, evading immune surveillance and reaching the target organs. The differential control of HDACs in CD172a⁺ cells may potentially contribute to the differences in pathogenesis between neurovirulent and non-neurovirulent EHV-1 *in vivo*.
- The attachment and entry processes of EHV-1 into CD172a⁺ cells play determining roles in the narrow cell tropism and pathogenesis of EHV-1.
- EHV-1 uses cell-to-cell contacts as an immune evasive mechanism to spread from infected CD172a⁺ cells to EC *in vitro*. The adhesion and transfer of EHV-1 from CD172a⁺ monocytic cells to EC may possibly contribute to clinical thrombosis in horses with EHV-1 infection.

Samenvatting

Equine herpesvirus type 1 (EHV-1) is wereldwijd één van de belangrijkste pathogenen bij paarden. Na primaire vermeerdering in de bovenste luchtwegen verspreidt het virus zich via een cel-geassocieerde viremie naar de drachtige uterus en/of het centraal zenuwstelsel. Vermeerdering in de endotheelcellen in de bloedvaten ter hoogte van deze organen kan leiden tot abortus of neurologische ziekte. De bestaande vaccins bieden geen volledige bescherming tegen ziektes veroorzaakt door EHV-1. EHV-1 is namelijk in staat om zich te verspreiden via een cel-geassocieerde viremie in gevaccineerde dieren, waarbij het de immuunrespons van de gastheer ontwijkt. CD172a⁺ monocyttaire cellen van paarden zijn gekend als één van de belangrijkste doelwitten van EHV-1 in de bovenste luchtwegen en in het bloed, en zouden door EHV-1 gebruikt worden als “Trojaans paard” om uit te zaaien naar doelwitorganen. Op basis daarvan werd de algemene doelstelling van deze thesis: het uitvoeren van een gedetailleerd onderzoek naar de manier waarop EHV-1 specifieke CD172a⁺ monocyttaire cellen kaapt en hoe het virus zijn vermeerdering aanpast zodat het zich kan verspreiden in de gastheer.

In **hoofdstuk 1** werd een overzicht gegeven van de huidige kennis omtrent EHV-1, met nadruk op de pathogenese en de gekende immuun-ontwijkende strategieën.

In **hoofdstuk 2** werden de doelstellingen van deze thesis geformuleerd.

In **hoofdstuk 3** werden de vermeerderingskinetieken van niet-neurovirulente (hoofdstuk 3.A) en neurovirulente (hoofdstuk 3.B) EHV-1 stammen in CD172a⁺ monocyttaire cellen vergeleken met de vermeerderingskinetiek in konijnennier epitheelcellen (RK-13), een controle cellijn. Voor beide EHV-1 stammen was het aantal CD172a⁺ monocyttaire cellen dat vermeerdering toeliet sterk beperkt (4-8%) in vergelijking tot RK-13 cellen (100%). Anderzijds verschilde de tijdruimtelijke verdeling van de EHV-1 eiwitten binnen de populatie gevoelige CD172a⁺ cellen voor de 2 virusstammen. Terwijl de vermeerderingskinetiek van neurovirulente EHV-1 in gevoelige CD172a⁺ en RK-13 cellen vergelijkbaar was, zagen we dat de vermeerdering van niet-neurovirulente EHV-1 vertraagd was vanaf een zeer vroege

fase in de infectie. Bovendien waren infecties met neurovirulente en niet-neurovirulente virusstammen in gevoelige CD172a⁺ monocyttaire cellen hoofdzakelijk niet-productief. Tenslotte werd aangetoond dat histone deacetylases (HDACs) de onderdrukking van niet-neurovirulente EHV-1 genexpressie regelden in CD172a⁺ cellen, doch niet de vermeerdering van neurovirulente EHV-1 in deze cellen.

In **hoofdstuk 4** werden de binding en toegang van EHV-1 in CD172a⁺ monocyttaire cellen onderzocht om zo een beter inzicht te verwerven in de regulatie van CD172a⁺ celtropisme en gevoeligheid voor EHV-1 infectie. Door gebruik te maken van Diodigemerkte EHV-1 partikels kon worden aangetoond dat binding enkel optrad bij 15 à 20% van de CD172a⁺ cellen, in vergelijking tot 70% bij de RK-13 controle cellen, wat erop wijst dat specifieke receptoren aanwezig zijn op de cellen. Enzymatische verwijdering van heparaansulfaat had geen effect op de EHV-1 infectie, maar verwijderen van siaalzuren van het celoppervlak met neuraminidase gaf wel een significante inhibitie van de EHV-1 infectie in CD172a⁺ cellen, wat erop wees dat siaalzuren en niet heparaansulfaat essentieel zijn voor infecties met EHV-1. Verder werd vastgesteld dat $\alpha_v\beta_3$ integrines betrokken waren in het stadium na binding tijdens EHV-1 infectie in CD172a⁺ cellen. Tenslotte werd de wijze van binnenkomst van EHV-1 in CD172a⁺ cellen gekarakteriseerd door het gebruik van farmacologische inhibitoren en co-lokalisatie testen. Het besluit was dat EHV-1 CD172a⁺ cellen binnenkwam via een endocytose mechanisme waarvoor cholesterol, tyrosine kinase activiteit, actine, dynamine activiteit en endosomale verzuring nodig waren, wat in de richting wijst van een fagocytose mechanisme.

Gezien het belang van de interacties tussen monocyttaire en endotheliale cellen (EC) in de pathogenese van EHV-1, werd in **hoofdstuk 5** gefocust op het vermogen van EHV-1 geïnoculeerde CD172a⁺ cellen om zich aan te hechten en vervolgens het virus over te dragen aan equine veneuze EC. Er werd aangetoond dat EHV-1 infectie leidde tot significant meer aanhechting van CD172a⁺ monocyttaire cellen aan EC *in vitro*. Antistofexperimenten toonden aan dat $\alpha_4\beta_1$, $\alpha_L\beta_2$ en $\alpha_v\beta_3$ integrines gedeeltelijk de aanhechting van EHV-1 geïnfecteerde CD172a⁺ cellen aan EC medieerden. Ook werd aangetoond dat de activering van integrine-gemedieerde signaalroutes, waaronder PI(3)K and ERK/MAPK, essentieel waren voor dit aanhechtingsproces.

EHV-1 vermeerdering was toegenomen in CD172a⁺ cellen na adhesie aan EC en gecorreleerd met de productie van TNF- α .

Bij ongeveer 20% van de geïnfekteerde CD172a⁺ cellen werd, in de aanwezigheid van neutraliserende antistoffen, waargenomen dat cytoplasmatisch materiaal overgedragen werd naar ongeïnfekteerde EC, wat aanleiding gaf tot overwegend niet-productieve infectie.

In **hoofdstuk 6** werd op basis van de bekomen gegevens in deze thesis een algemene conclusie geformuleerd waarin de nieuwe inzichten in de interactie tussen EHV-1 en CD172a⁺ cellen werden opgesomd:

De belangrijkste conclusies van deze thesis zijn:

- Bij het intreden in CD172a⁺ cellen moduleert EHV-1 zijn vermeerdering, waarbij de cel in wezen gekidnapt wordt en als een “Trojaans paard” gebruikt wordt. Dit mechanisme stelt het virus in staat om door de bloedcirculatie te passeren, waarbij het de immunobewaking ontwijkt en de doelwitorganen kan bereiken. De verschillen in de controle over HDACs in CD172a⁺ cellen liggen mogelijk mee aan de basis van de verschillen in de pathogenese tussen neurovirulente en niet-neurovirulente EHV-1 stammen *in vivo*.
- De aanhechtings- en intredeprocessen van EHV-1 in CD172a⁺ cellen spelen een determinerende rol in het nauwe celtropisme en de pathogenese van dit virus.
- EHV-1 gebruikt celcontacten als een immuunsysteem ontwijkend mechanisme zodat het zich kan verspreiden van geïnfekteerde CD172a⁺ cellen naar EC in doelwitorganen. De aanhechting en overdracht van EHV-1 van CD172a⁺ monocytair cellen naar EC kan mogelijks bijdragen tot klinische trombose in paarden met EHV-1 infecties.

Curriculum Vitae

Kathlyn Laval was born on August 21st, 1987 in Saint Jean de Braye, France. In 2005, she graduated with honors from the private high school 'Saint Charles' in Orleans, France. After two years as a candidate of 'Classes Préparatoires Vétérinaire' in Orleans, she continued her studies at the University of Tours where she obtained her bachelor in Science of Biology with honors in June 2009. In September 2009, she started her first year of 'master Infectiologie: Microbiologie, Virologie, Immunologie (IMVI)' at the University Paris Diderot, France. During this year, she did her semester project at the Swiss Federal Institute of Technology (ETH) in Zurich under the supervision of Prof. Dr. Ari Helenius, studying the entry mechanisms of Bunyaviruses. In September 2011, she started her second year of Master 'Fundamental Virology' at Pasteur Institute in Paris. She did her master thesis at the Imperial College in London under the supervision of Prof. Dr. Geoffrey Smith, characterizing the mechanisms of entry and spread of vaccinia virus. Her master thesis resulted in a publication in Journal of General Virology in 2012. In September 2012, she joined the Laboratory of Virology, at the Faculty of Veterinary Medicine, at the University of Ghent, where she started her doctor of philosophy (PhD) on the immunopathogenesis of equine herpesvirus type 1 (EHV-1). She is the author and co-author of several publications in international peer-reviewed journals, and the results of her work were presented in several international conferences. During her PhD, she supervised the writing of master's and PhD projects and was a teaching assistant for equine viruses diagnosis for last year veterinary students. She mentored six master's students, of whom one won a prize and two received high-level competitive scholarships (Belgian FWO and IWT) to start a PhD in the laboratory on EHV-1.

Publications in peer-reviewed international journals

- **Laval, K.**, Brown, I.K., Nauwynck, H.J. Replication of neurovirulent EHV-1 in CD172a⁺ monocytic cells. *Submitted to Virus Research*.
- **Laval, K.**, Favoreel, H.W., Van Cleemput, J., Poelaert, K.C.K., Brown, I.K., Nauwynck, H.J (2015). Entry of equine herpesvirus type 1 (EHV-1) into CD172a⁺ cells. *J Gen Virol.*, in press, doi: 10.1099/jgv.0.000375.
- **Laval, K.**, Favoreel, H.W., Poelaert, K.C.K., Van Cleemput, J., Nauwynck, H.J. (2015). Equine herpesvirus type 1 (EHV-1) enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells. *J Virol* 89, 10912-10923.
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Oral presentations

- **Laval, K.**, Favoreel, H.W., Poelaert, K.C.K., Van Cleemput, J., Nauwynck, H.J. (2015). Equine herpesvirus type 1 (EHV-1) enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells. Proceedings of 10th international congress ESVV for veterinary virology, August 31st – September 3rd, Montpellier, France (2015).
- **Laval, K.**, Favoreel, H.W., Poelaert, K.C.K., Van Cleemput, J., Nauwynck, H.J. (2015). Equine herpesvirus type 1 (EHV-1) enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells. Proceedings of 40th annual International Herpesvirus Workshop, July 25th- 29th, Boise, Idaho, USA (2015).
- **Laval, K.**, Favoreel, H.W., Nauwynck, H.J (2015). Equine herpesvirus type 1 (EHV-1) replication is delayed in CD172a⁺ cells and controlled by histone deacetylases. Proceedings of 39th annual International Herpesvirus Workshop, July 19th- 23th, Kobe, Japan (2014).

Selected poster presentations

- **Laval, K.**, Favoreel, H.W., Poelaert, K.C.K., Van Cleemput, J., Nauwynck, H.J. (2015). Equine herpesvirus type 1 (EHV-1) enhances viral replication in CD172a⁺ monocytic cells upon adhesion to endothelial cells. Proceedings of 40th annual International Herpesvirus Workshop, July 25th- 29th, Boise, Idaho, USA (2015).
- **Laval, K.**, Favoreel, H.W., Nauwynck, H.J (2015). Equine herpesvirus type 1 (EHV-1) replication is delayed in CD172a⁺ cells and controlled by histone deacetylases. Proceedings of 39th annual International Herpesvirus Workshop, July 19th - 23th, Kobe, Japan (2014).

Acknowledgments

I would like to express my sincere gratitude to many people who supported and helped me during the past 4 years working on this thesis.

First, I would like to thank Prof. Dr. Hans Nauwynck for his excellent guidance and continuous scientific support. I am extremely grateful about the opportunity you gave me to start a scientific career in your laboratory, which I hope will last! You definitely taught me how to go over my limits in science!

I would also like to thank Prof. Dr. Herman Favoreel for his excellent guidance and help in the writing of my publications and reading of my thesis. His suggestions and corrections considerably improved the quality of the published papers. I also really appreciated our scientific discussions and brainstorming sessions during my PhD.

I am grateful to my reading and examination committee members, Dr. Lennert Steukers, Prof. Dr. Piet Deprez, Prof. Dr. Richard Ducatelle and Prof. Dr. Laurent Gillet for the suggestions and remarks, which have also improved the quality of this thesis.

I would like to thank my husband and 'shadow promoter' Dr. Mathias Corteel, who supported me in my research since the beginning of my thesis. Our daily scientific discussions for the last 4 years (yes, it's a lot of scientific investments in a relationship) helped me so much in the analysis of my results and the development of new hypotheses, in particular when I was too focused on my research and lost the big picture! Your guidance, knowledge, veterinary expertise, criticisms about my work and desire for perfection in my research considerably strengthen the quality of the work I presented in this thesis. You invested so much energy to help me in my thesis that I feel you completed a second PhD yourself! I am extremely proud of you and what you achieved in your life and it is not just because I love you! I am so lucky to have met you in this laboratory.

Next, I would like to thank the EHV-1 team: Jolien, Katrien, Ivy and Jing. It has been a real pleasure to work with you. Katrien and Jolien, I still remember the day you arrived in the lab to start a master thesis under my supervision. It has been a lot of fun since that time. Now, I do not consider you as students or co-workers but as real

friends. I hope this friendship will continue over skype and you are more than welcome to visit us for some shopping in New York ☺. You are both real strong scientists and I am sure you will publish new more findings on EHV-1 soon! My dear friend, Ivy, it has also been a great pleasure to have you working with me! Your American touch and craziness brought some amazing atmosphere in the group. When you go home to your family, you should definitely pass by our place. I m really gonna miss you all!

A picture, better than words, to illustrate our friendship and to remember the good old times ☺



I would like to thank Carine Boone, Lieve Sys and Zeger van der Abeele for their excellent technical support. Carine, it has been a great pleasure to work next to you at the flow, I really enjoyed our conversations and your cheerful mind! Thanks to Dirk Demeulenaere for solving IT problems and helping me in the layout adjustments of my thesis; Gert Verdonck and Mieke Godefroid for taking care of administrative

issues. I also acknowledge all my colleagues from the laboratory of Virology for their help.

I would like to thank my two families (French and Belgium) for their love and support through these years, in particular to my family in Belgium, Dirk and Rhie Corteel who welcomed me in their house and supported me during the good and bad times of my live and PhD. You have been 'fantastic parents' for both of us and I am so happy to have you in my life. I would also like to thank my friends in Belgium (Inna, Dries, Elsa, Ilias, Kate, Caroline and Steven...) for showing me that there is also a life during and after the PhD. I spent amazing times with you all here in Belgium!

Même si tu n'es plus là, je sais que tu aurais été fière de moi... C'est toi qui m'a donné la force de continuer dans les moments difficiles et de ne jamais lâcher prise! J'y suis enfin arrivée... Tu seras toujours dans mon coeur et dans mes pensées, je t'aime Pépé... Ton grand Courlis.

