Vakgroep Virologie, Parasitologie en Immunologie

Faculteit Diergeneeskunde

Universiteit Gent

Interaction of EHV1 with equine dendritic cells and mesenchymal stem cells

Christophe Claessen

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen, 2016

Dissertation submitted to fullfill the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences, 2016

Promotoren:

Prof. Dr. H. Favoreel

Prof. Dr. G. Van de Walle

About the cover

The cover shows the typical light microscopic view of mock-infected equine dendritic cells (left) and equine mesenchymal stem cells (right) along with an transmission electron microscopic view of an equine herpesvirus 1 virion (Paillot et al., 2008).

Table of content

List of abbreviations

ActD	actinomycin D
Ag	antigen
APC	antigen presenting cells
cDC	conventional dendritic cells
CHPZ	chlorpromazine
CHX	cycloheximide
CTL	cytotoxic T-lymphocytes
CXCL	CX chemokine ligand
CXCR	CX chemokine receptor
DC	dendritic cells
ECM	extracellular matrix
ED	equine dermal fibroblasts
eGFP	enhanced green fluorescent protein
EHM	equine herpes myeloencephalopathy
EHV	equine herpesvirus
EICP	EHV1' infected cell protein
EREC	equine respiratory epithelial cells
F	filamentous
GM-CSF	granulocyte macrophage colony-stimulating factor
HCMV	human cytomegalovirus
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
IEP	immediate early protein
IFN	interferon
IPA-3	inhibitor of group I p21-activated kinases
KSHV	Kaposi's sarcoma herpes virus
LFA	lymphocyte function associated antigen
MDDC	monocyte-derived dendritic cells
MHC	major histocompability complex
MLV	modified live vaccines
MOI	multiplicity of infection
MP	macropinocytosis
MSC	mesenchymal stem cells
NK	natural killer
ORF	open reading frame
PAK-I	group I p21-activated kinases
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cells
PRV	pseudorabies virus
RK	rabbit kidney
RT-PCR	reverse transcriptase polymerase chain reaction
SIRP	signal regulatory protein

TAP	transporter associated with antigen processing
TGF	transforming growth factor
TNF	tumor necrosis factor
VHS	virion host-shutoff
VN	virus-neutralizing
VZV	varicella zoster virus

Chapter 1: Introduction

1. General characteristics of equine herpesvirus 1

1.1. Classification

The order *Herpesvirales* (Figure 1) consists of large double stranded DNA viruses and is divided in three families: *Alloherpesviridae*, which can infect fish and frogs, *Malacoherpesviridae*, which infects mollusks, and *Herpesviridae*, which are able to infect mammals, birds and reptiles. Assignment to this order is based on virion morphology and four biological properties that all members have in common: **(i)** they encode an array of enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing, **(ii)** synthesis of viral DNA and assembly of the capsid occurs in the nucleus, **(iii)** production of infectious progeny virus is invariably associated with lysis of the infected cell and **(iv)** all herpesviruses known to date are able to establish a latent infection in their natural hosts. The family *Herpesviridae* is further subdivided in three subfamilies, the *α-, β-* and *γ-herpesvirinae* based on their host cell range, duration of the replication cycle, cytopathology, genome structure and site of latency. All α-herpesviruses have a broad host range, short replication cycle, fast spread in cell culture, efficient destruction of infected cells and are able to establish latent infection in sensory ganglia and potentially immune cells. The most restricted host range with the longest replication cycle and enlargement of infected cells (cytomegaly) is characteristic for infection with β-herpesviruses, which often establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues. All γ-herpesviruses have a limited host range, a variable replication rate, and will frequently establish latency in lymphoid tissue (Davison et al., 2009; Roizman & Pellet, 2001).

The subfamily *α-herpesvirinae* contains the genera *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus*. Herpes simplex virus (HSV) 1 and HSV2 belong to the genus *Simplexvirus*. Equine herpesvirus (EHV) 1 and EHV4 belong to the genus *Varicellovirus*, along with

pseudorabies virus (PRV), varicella zoster virus (VZV) and bovine herpesvirus (BHV) 1. Marek's disease virus (MDV) 1 and infectious laryngotracheitis virus (ILTV) are examples of α-herpesviruses that affect chickens and belong to the genera *Mardivirus* and *Iltovirus*, respectively.

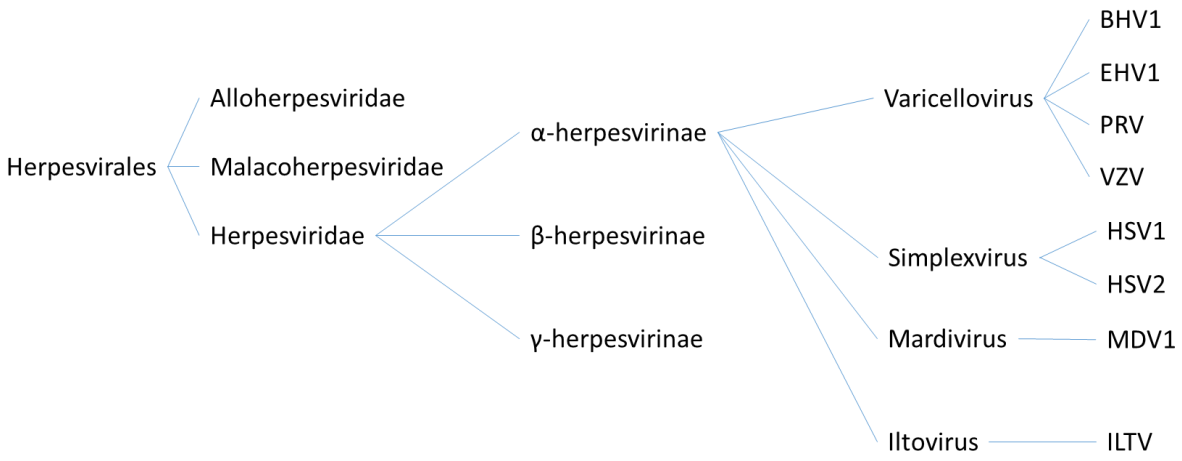


Figure 1. Schematic overview of the order Herpesvirales.

In equids, there are fourteen herpesviruses identified to date (Table 1) with EHV1 and EHV4 being the most clinically, epidemiologically and economically important (Slater, 2007).

Table 1: An overview of the equid herpesviruses identified to date.

Designation		*Host*	*Sub-family*	*Clinical signs*
EHV1	Equine abortion virus	Domestic horse	α	respiratory disease, abortion, neonatal death, equine herpes myeloencephalopathy
EHV2	Equine herpesvirus 2		γ	rhinitis, conjunctivitis, immunosuppression
EHV3	Equine coital exanthema virus		α	coital exanthema
EHV4	Equine rhinopneumonitis virus		α	respiratory disease, (abortion)
EHV5	Equine herpesvirus 5		γ	respiratory disease, multinodular pulmonary fibrosis
AHV1	Asinine herpesvirus 1	Donkey	α	lesions on external genitalia and udder
AHV2	Asinine herpesvirus 2		γ	?
AHV3	Asinine herpesvirus 3		α	respiratory disease
AHV4	Asinine herpesvirus 4		γ	Pneumonia
AHV5	Asinine herpesvirus 5		γ	Pneumonia, equine herpes myeloencephalopathy?
AHV6	Asinine herpesvirus 6		γ	Pneumonia
GHV	Gazelle herpesvirus	Gazelle	α	respiratory disease, encephalitis
ZHV	Zebra herpesvirus	Zebra	γ	?
WAHV	Wild ass herpesvirus	Wild ass	γ	?

References: domestic horse and gazelle (Davison et al., 2009), donkey (Kleiboeker et al., 2004, 2002; Vengust et al., 2008), zebra and wild ass (Ehlers et al., 1999)

1.2. Structure

The EHV1 virion consists of four distinct morphological components (Figure 2). A linear dsDNA **(i)** that is packaged in an icosahedral capsid **(ii)**. Those two structures form the nucleocapsid, which is surrounded by the tegument **(iii)**. The nucleocapsid and tegument is enclosed by an envelope in which multiple glycoproteins are embedded **(iv)** (Paillot et al., 2008).

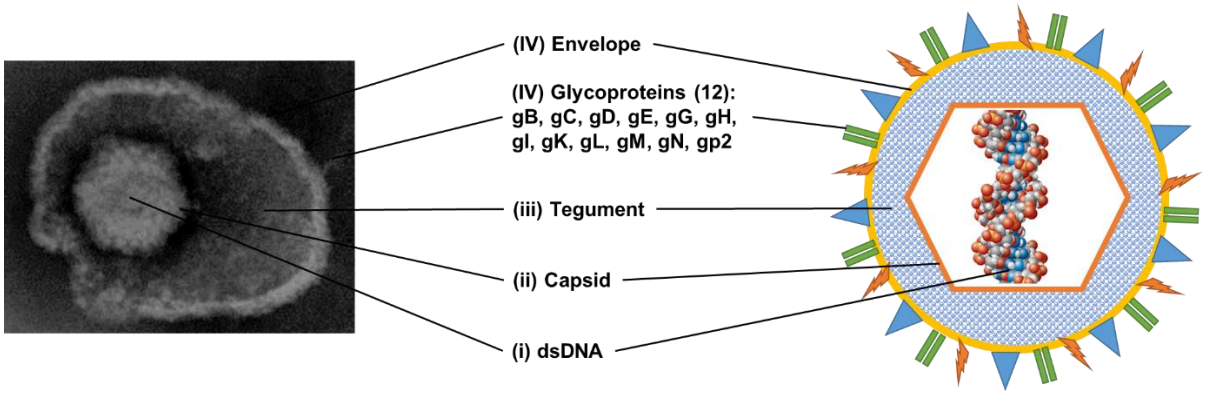


Figure 2. Structure of an equine herpesvirus 1 virion: Electron transmission microphotograph (left) and schematic representation (right) (adapted from Paillot et al., 2008).

The linear torus shaped dsDNA of EHV1 is about 150 kbp in length. Since four open reading frames (ORF) are duplicated in the 80 ORF containing EHV1 genome, at least 76 distinct proteins can be encoded with a potential for 77 distinct proteins due to alternative splicing of the immediate-early protein (IEP) encoding ORF64. The genome can be divided in unique long and unique short segment, with the latter flanked by an internal repeat and a terminal repeat (Figure 3) (Harty et al., 1989; Telford et al., 1992).

Figure 3. Schematic representation of the equine herpesvirus 1 genome: The unique short segment (US) is flanked by a terminal repeat (TR) and internal repeat (IR) and the latter divides it from the unique long segment (UL). The EHV1 genome encodes at least 76 different open reading frames (ORF).

The nucleocapsid, with an approximate diameter of 125 nm, is formed by enclosement of the viral genome within the icosahedral (20) capsid. The capsid consists of 12 pentameric and 150 hexameric capsomers (Roizman & Pellet, 2001; Wildy and Watson, 1962). The void

between the nucleocapsid and the envelope is filled with an electron dense protein matrix, the tegument (Davison et al., 2009). The envelope consists of a double phospholipid layer with twelve embedded glycoproteins. These envelope glycoproteins are transmembrane proteins with an external, internal and transmembrane segment and have several functions during attachment, penetration, egress, cell-cell spread, virulence and pathogenicity. For EHV1, eleven glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN) are homologues to envelope proteins found in most other α-herpesviruses (Slater, 2007; Turtinen and Allen, 1982). However, gp2 is only encoded by EHV1, EHV4 and asinine herpesvirus 3 (AHV3) (Smith et al., 2005). Furthermore, in addition to the secreted isoform of gG, EHV1 gG is also incorporated into the virion and this last is in contrast to other α-herpesviruses such as PRV (Drummer et al., 1998; Mettenleiter, 1999). An overview of the EHV1 glycoproteins and their functions can be found in Table 2.

Table 2: Overview of the 12 envelope glycoproteins of equine herpesvirus 1 and their known functions.

Glyco-protein	*kDa**	*Replication in vitro*	*Function*	*References*
gB (gp14)	138, 76	essential	penetration, cell-cell spread	(Neubauer et al., 1997; Spiesschaert et al., 2015b)
gC (gp13)	120	nonessential	attachment, egress, complement evasion	(Huemer et al., 1995; N Osterrieder, 1999)
gD (gP17/18 or gp60)	55	essential	entry, cell-cell spread	(Azab and Osterrieder, 2012; Sasaki et al., 2011a; Spear, 2004; Van de Walle et al., 2008b)
gE	ND*	nonessential	cell-cell spread as gE/gI	(Matsumura et al., 1998)
gG	68 yields 60 (s*), 12	nonessential	viral chemokine binding protein (vCKBP)	(Bryant, 2003; Thormann et al., 2012; Van de Walle et al., 2008c, 2007)
gH	125	essential	assist as gH/gL fusiogenic gB, route of entry	(Azab et al., 2013; Eisenberg et al., 2012)
gI	ND*	nonessential	cell-cell spread as gE/gI	(Matsumura et al., 1998)
gK	ND*	essential	penetration, egress, cell-cell spread	(Neubauer and Osterrieder, 2004)
gL	ND*	essential	gH processing, assist as gH/gL fusiogenic gB	(Eisenberg et al., 2012)
gM (gp21/22a or gp45)	250	nonessential	penetration, cell-cell spread	(Osterrieder et al., 1997)
gN (pUL49.5)	10	nonessential	gM processing, inhibition of the transporter associated with antigen presentation (TAP)	(Rudolph et al., 2002)
gp2 (gp300)	250	nonessential	attachment, egress, virulence	(Smith et al., 2005; Sun et al., 1996)

*** Abbreviations: kDa: apparent molecular weight on Western Blot, measured in kilo (1000) Dalton; ND: not determined; s: soluble gG isoform shed after splitting membrane-associated gG (mgG (68 kDa) → mgG (12 kDa) + sgG (60 kDa))**

1.3. Replication

The replication cycle of all herpesviruses can be divided in two general stages, namely the lytic and the latent phase. The lytic replication cycle is highly orchestrated and can be divided into three stages: **(i)** entry into the host cell, **(ii)** replication and **(iii)** virion assembly and

egress (Figure 4). Since the lytic replication cycle is quite similar for most herpesviruses, the replication cycle of EHV1 will be discussed based on data obtained from HSV1, the prototypical α-herpesvirus. During the latent phase, which usually lasts for the entire lifetime of the host, no infectious progeny virus is formed and only a limited set of viral genes are transcribed. Latently infected cells can be activated to enter the lytic phase which is characterized by production of infectious progeny virions (Lehman and Boehmer, 1999).

1.3.1. Entry

Before EHV1 can activate the host cell machinery for replication, the virus has to enter the cell. First, the virus will attach via gB and gC to heparan sulfate glycosaminoglycan moieties on the host cell surface (Nikolaus Osterrieder, 1999; Sugahara et al., 1997). Stabilization of this initial attachment is mediated by binding of gD to its respective entry receptor (Spear, 2004). While gD entry receptors for several α-herpesviruses such as HSV1, HSV2, PRV and BHV1, have been identified, studies on these entry receptors for EHV1 are still ongoing (Spear and Longnecker, 2003). EHV1 uses alternative entry receptors such as major histocompatibility complex (MHC) I for entry in equine dermal fibroblasts (ED) (NBL6) and equine brain microvascular endothelial cells, and αV integrins for entry into Chinese hamster ovary K1 cells and equine peripheral blood mononuclear cells (PBMC) (Kurtz et al., 2010; Sasaki et al., 2011a, 2011b; Van de Walle et al., 2008b). In addition, EHV1 can also enter PBMC through an MHCI- and integrin-independent mechanism (Azab et al., 2012). For HSV1 and HSV2, the gH/gL routes the binding of gD to its respective entry receptor and it is believed that conformational change in gD enables this glycoprotein to initiate complex interactions with gH/gL and gB, which will then lead to the activation of gB's fusiogenic activity (Spear and Longnecker, 2003). During EHV1 entry, the role of the gH/gL complex is less clear. However, a recent study demonstrated that gH acts as a router in equine epithelial

cells: if an SDI (serine, aspartic acid, isoleucine) motif in gH can interact with α4β1 integrins, then entry will occur via fusion, otherwise EHV1 will enter via endocytosis (Azab et al., 2013). Finally, during viral penetration, gB, gD, gM and gK are all essential to allow the delivery of the EHV1 nucleocapsid into the cytoplasm (Neubauer and Osterrieder, 2004; Osterrieder et al., 1997, 1996). Nucleocapsids will then be transported towards the nucleus along microtubules by the cellular motor protein dynein. During this retrograde transport towards the minus end of the microtubules, the activity of the cellular serine/threonine Rho-associated coiled-coil kinase 1 is essential and EHV1 will stabilize the microtubule network by inducing acetylation of tubulin (Frampton et al., 2010). Viral dsDNA will enter the nucleus through the nucleopore complex and leave empty capsids behind (Roizman & Pellet, 2001).

1.3.2. Replication

As for all herpesviruses, the EHV1 genome is transcribed by the host RNA polymerase II and transcription is tightly regulated in a cascade-like fashion: 1 immediate-early (α), 55 early (β) and 20 late (γ) viral mRNA. In addition, since viral proteins are synthesized on ribosomes in the cytoplasm and encapsidation of viral DNA occurs in the nucleus, there is an intense export of viral mRNA and import of viral proteins across the NPC. Regulation of the sequential cascade of expression of α, β and γ genes is regulated by six viral proteins acting as transcription activators or suppressors: the IE gene (IR1), four early genes (EICP0, 22, 27 and TR2) and the late gene α-trans inducing factor (Slater, 2007). The EHV1 homologue of the tegument protein α-trans inducing factor, also known as EHV1 virion protein 16, will initiate viral transcription of the only immediate-early gene IR1 (ORF64) (von Einem et al., 2006). Although EHV1 encodes only one IE mRNA, there are several IE polypeptides due to alternative splicing, from which the IE1 polypeptide is the vital regulatory protein that trans-activates expression of the early and late genes and subsequently trans-represses its own

expression (O'Callaghan et al., 1994; Smith et al., 1995, 1992). Early genes are expressed before viral DNA replication and encode proteins involved in nucleotide metabolism and replication of viral DNA (UL5, UL8, UL9, UL29, UL30, UL42, UL52) such as viral DNA polymerase (UL30) and other proteins such as thymidine kinase (UL38) (Zhang et al., 2014). Only after the initiation of viral DNA replication, expression of L genes will start and these genes encode mainly for structural proteins such as capsomers, tegument proteins and glycoproteins (Slater, 2007). Capsomers generated in the cytoplasm will enter the nucleus and form preassembled capsids. Viral progeny DNA will be packaged in these preassembled capsids, thereby yielding naked intranuclear nucleocapsids (Mettenleiter et al., 2009).

EHV1 is like its prototypical α-herpesviral counterpart HSV1 able to transform this lytic replication into latency, a non-productive phase characterized by a very limited transcription of only the latency associated transcripts such as ORF64 transcripts (IEP) (Baxi et al., 1995; Chesters et al., 1997). Although EHV1 will establish latency in about 50-80% of the adult horse population, the main sites of latency are still controversial (Allen, 2006; Allen et al., 2008; Edington et al., 1994). While some studies found that latency was mainly associated with lymphoid tissue and leukocytes (Carvalho et al., 2000; Chesters et al., 1997; Smith et al., 1998; Welch et al., 1992), other studies demonstrated that the neuronal perikarya in the trigeminal ganglion (TG) can act as a major latency site for EHV1 (Baxi et al., 1995; Slater et al., 1994).

1.3.3. Virion assembly and egress

Newly formed nucleocapsids undergo two envelopment processes. First, with the help of UL31, UL34 and US3 protein kinase, the intranuclear nucleocapsid buds into the perinuclear space through the inner nuclear membrane and thereby acquires its primary envelope. Fusion of these primary enveloped nucleocapsids with the outer nuclear membrane leads to de-

envelopment and subsequent release of naked nucleocapsid in the cytoplasm. Here, the naked intracytoplasmatic nucleocapsid will receive its full array of tegument proteins and final (secondary) envelope with glycoproteins via an intricate sequence of protein-protein interactions while travelling through the cytoplasm, the trans-Golgi network and the endosomal network. Mature virions are released by cell lysis or fusion of their transporting vesicles with the plasma membrane (Mettenleiter et al., 2009; Pomeranz et al., 2005).

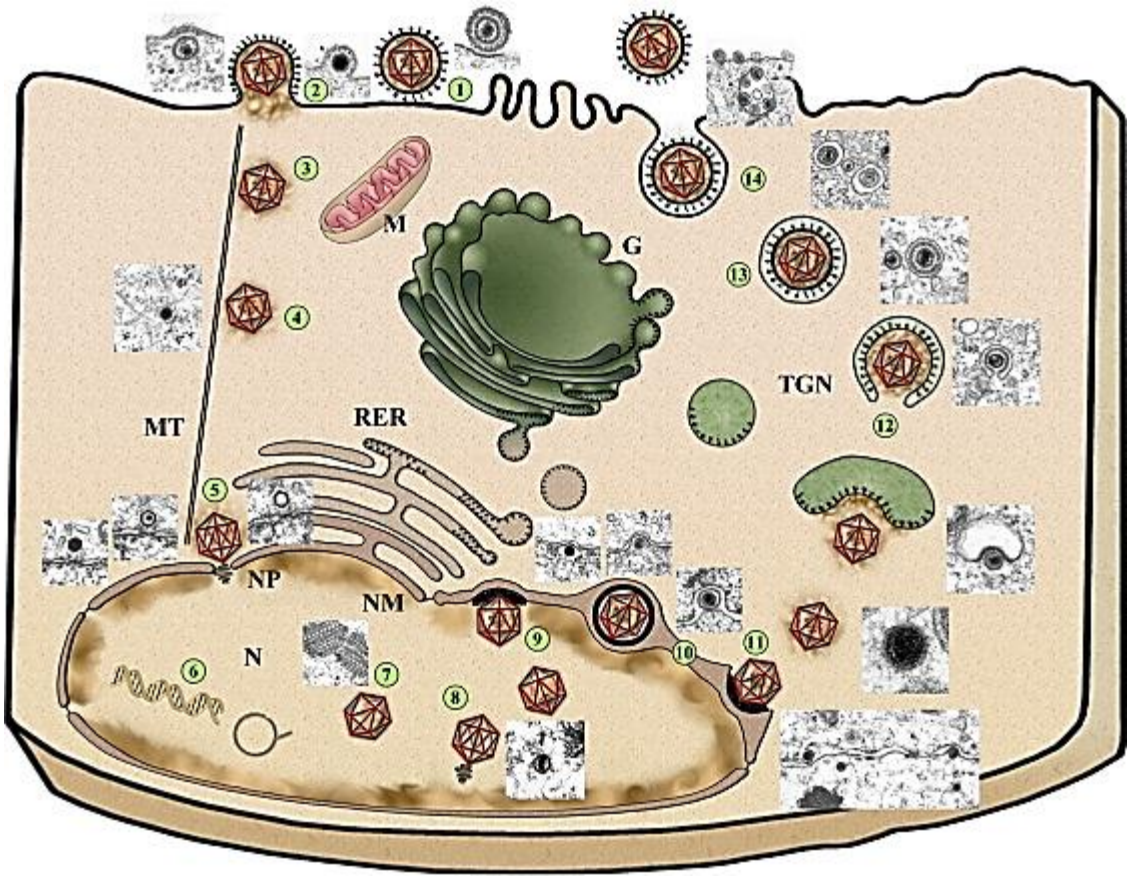


Figure 4: Schematic representation with electron microphotographs of the replication cycle of pseudorabies virus, a Varicellovirus, which is closely related to equine herpesvirus 1. After attachment (1) and penetration (2), naked nucleocapsids (3) are transported towards the nucleus along microtubules (4) where viral DNA will enter the nucleus through nuclear pores (5). Cleavage (7) and packaging of viral DNA into preformed capsids (8) occurs after viral transcription and replication (6). Naked nucleocapsids receive their primary envelope during budding (9) into perinuclear space (10). After fusion with the outer nuclear membrane, naked nucleocapsids (11) receive their tegument during transport towards the trans-Golgi network (TGN), where they will get their final (secondary) envelope with glycoproteins (12). The mature virion is transported within a vesicle towards the plasma membrane (13), where the virion is released from the cell (14) (Mettenleiter et al., 2009).

1.4. Epidemiology and pathogenesis

EHV1 is endemic in horse populations worldwide and most horses are probably subclinically infected during their first year of life (Allen and Bryans, 1986; Borchers et al., 2006; Pusterla, 2014). Transmission requires close, direct contact and can occur via secretions of acutely infected horses, via latently infected horses that reactivated, and via contaminated fomites and persons. Very high virus titers can be found in placentas and sometimes the aborted fetus. Foals probably contract EHV1 from their dams or from other infected foals (Gardiner et al., 2012; Gilkerson et al., 2015, 2000, 1999). EHV1 is shed via the respiratory secretions and although it was found present in semen, no venereal transmission has been reported (Tearle et al., 1996; Walter et al., 2012).

1.4.1. Primary replication

Upon contact of the virus with the respiratory mucosa (Figure 5), a primary replication cycle is initiated in the respiratory epithelium, which is mainly limited to the upper respiratory tract (URT) (nasal cavity, pharynx, trachea and bronchi) (Allen and Bryans, 1986; Allen and Murray, 1962).

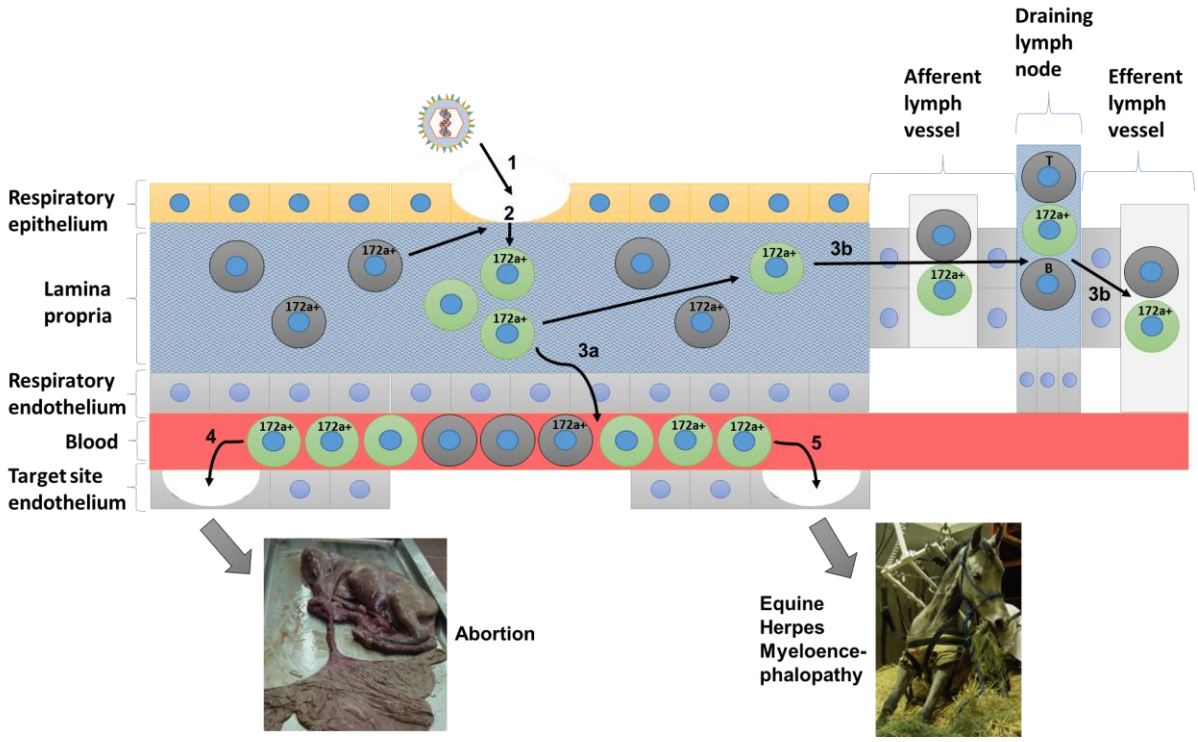


Figure 5: Schematic representation of the important steps in EHV1 pathogenesis. Upon contact with equine herpesvirus 1 (EHV1) (1), a primary replication is initiated in the respiratory epithelium. (2) EHV1 will pass the intact basal membrane and EHV1-infected (green) CD172a positive cells can be found within forming plaques and below the basal membrane. Cell-associated spread of EHV1, again mainly by CD172a positive cells, into the bloodstream (3a) and the lymph (3b) draining towards associated lymph nodes (B-/T-cells). Upon contact with endothelial cells at the pregnant uterus (4) or within the central nervous system (5), a secondary replication cycle induces endothelial cell damage which may lead to abortion or equine herpes myeloencephalopathy.

Recent studies have performed a time course analysis of EHV1 replication in the upper respiratory tract using an *in vitro* nasal mucosa explant system on the one hand and *in vivo* experimentally ponies on the other hand (Gryspeerdt et al., 2010; Vandekerckhove et al., 2011). It was found that after experimental infection with the neurovirulent (03P37) or abortigenic (97P70) Belgian EHV1 isolates that single EHV1-infected cells are present before the onset of plaque formation. At these sites, where later during infection plaques will be formed, the single EHV1-infected cells are either positive for pancytokeratin or the signal regulatory protein (SIRP) α (CD172a). At these early time points, 12 to 24 hpi, the virus spreads to cells in the underlying tissue without clear destruction of the basement membrane and this spread is generally faster for neurovirulent strains (Gryspeerdt et al., 2010;

Vandekerckhove et al., 2010). Unlike other α-herpesviruses, such as PRV (Glorieux et al., 2011), the plaques formed by EHV1 (apparent at 48 hpi) do not cross the basement membrane *in vivo* and *in vitro* (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). Interestingly, a more recent equine nasal explant study showed that EHV1 infection was associated with reduced integrin (α6) and increased collagen VII thickness below EHV1 plaques and the authors hypothesized that this modulation of the basement membrane could enhance leukocyte migration from apical to the sub-basal compartment (Bannazadeh Baghi and Nauwynck, 2016). EHV1 plaques consist mainly of epithelial cells with occasionally one or two cells positive for the cell surface marker CD172a. Although the basement membrane did not disappear, neurovirulent EHV1-infected cells, mainly CD172a but also CD5 positive, are observed in the connective tissue and draining lymph nodes at 24 to 48 hpi. In contrast to the neurovirulent strain where almost all EHV1-infected cells were CD172a positive, infection with the abortigenic strain resulted in a higher number of CD5 positive cells, although the majority of EHV1-infected cells still remained CD172a positive. Most of the infected cells in the lamina propria and draining lymph nodes are positive for the cell surface marker CD172a (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). A recent study with nasal explants showed that, irrespective of infection, EHV1-infected CD172a positive cells transmigrated from the apical to the basal side of the respiratory epithelium. At 24 h post addition of pre-infected CD172a positive cell types to equine nasal explants, only EHV1-infected monocyte-derived dendritic cells (MDDC) were present underneath the basement membrane. This is together with the unapparent degradation of the basement membrane and that EHV1-infected cells above and below the basement membrane were CD172a positive indicative for the important role that dendritic cells (DC) play in the spread of EHV1 (Baghi and Nauwynck, 2014).

1.4.2. Viremia

EHV1 infects mononuclear cells that can enter the bloodstream between 1 to 18 dpi (Figure 5) (Edington et al., 1986; Gibson et al., 1992; Gleeson and Coggins, 1980; Patel et al., 1982; Wilsterman et al., 2011). This cell-associated viremia is critical for the virus to spread to the endothelial cells of the pregnant uterus, the caudal spinal cord and rarely the eye (Allen and Bryans, 1986; Hussey et al., 2013; Pusterla, 2014; Slater et al., 1992).

There is still much debate about the exact subset(s) of PBMC, being T-lymphocytes, B-lymphocytes or monocytes, that are responsible for EHV1 cell-associated viremia (Gleeson and Coggins, 1980; Scott et al., 1983; Smith et al., 1998; Wilsterman et al., 2011). However, a recent *in vivo* study found that most EHV1-infected PBMC in the blood are positive for the cell surface marker CD172a (Gryspeerdt et al., 2010). Interestingly, *in vivo* infection of PBMC by EHV1 results most likely in an abortive and/or latent infection since no infectious EHV1 could be harvested from these cells (Gleeson and Coggins, 1980; Scott et al., 1983) which was later confirmed in another *in vivo* study where almost all EHV1-infected PBMC lacked the expression of late viral glycoproteins on their cell surface (van der Meulen et al., 2006).

1.4.3. Secondary replication

Despite the high endotheliotropism of EHV1, it is not clear which mechanism EHV1 uses to target endothelial cells at specific sites (Figure 5). However, some clues may be deduced from the following *in vitro* studies. Initial studies showed that EHV1 transfer from EHV1-infected PBMC to endothelium depends on the upregulation of putative adhesion molecules on both cell types and was not hampered by the presence of VN antibodies (Goehring et al., 2011; Smith et al., 2002). Later studies showed that transfer of EHV1 from PBMC to endothelial cells depends on expression of the viral gB and pUS3 proteins and, interestingly, that such

adhesion partly reversed the viral replication block in EHV1-infected PBMC (Laval et al., 2015; Spiesschaert et al., 2015a).

After the primary replication in the respiratory epithelium, a second replication cycle is initiated in the endothelial cells of the target organs and clinical signs during this stage of pathogenesis are caused by the consequences of endothelial cell damage such as vasculitis with perivascular oedema and inflammation, multifocal thrombosis, ischemia, microcotyledonary infarction and transplacental spread (Allen and Bryans, 1986; Allen and Murray, 1962; Gardiner et al., 2012; Smith et al., 1996; Smith and Borchers, 2001). Although EHV1 is able to infect neurons, no neurotropism as seen with other α-herpesviruses (HSV, PRV and BHV1) is exhibited by EHV1. The neurological deficits caused by EHV1 are believed to be the consequence of endothelial cell damage (Edington et al., 1986; Roizman & Pellet, 2001; Whitwell and Blunden, 1992).

1.4.4. Latency

Latency is an important epidemiological strategy of α-herpesviruses to ensure survival and spread within populations. Upon primary infection, latency is established in the draining lymph nodes of the respiratory system, CD5+/CD8+ lymphocytes and the trigeminal ganglion (Baxi et al., 1995; Chesters et al., 1997; Edington et al., 1994; Welch et al., 1992). As for other α-herpesviruses, trigeminal ganglion associated EHV1 latency is probably established by retrograde axonal transport of the virus from the sensory nerve endings in the nasal cavity to the neuronal perikarya which are located in the trigeminal ganglion (Zaichick et al., 2011). It is estimated that 80% of all horses are latently infected with EHV1 (Allen and Murray, 1962). During recrudescence, defined as reactivation of latent virus into the lytic replication cycle, latently infected horses will start shedding infectious virus again, presumably induced

by stimuli such as immunosuppression during stressful periods or pregnancy (Edington et al., 1985; Smith et al., 2010).

1.5. Clinical disease syndromes

The clinical signs associated with EHV1 are well known and consist in general of following clinical presentations: respiratory disorders, reproductive problems, central nervous system disorders and ocular disorders (Allen and Murray, 1962; Hussey et al., 2013; Slater et al., 1992).

It has been recognized that several EHV1 strains show a different biological phenotype (Gardiner et al., 2012; Slater, 2007; Smith et al., 2000; Tearle et al., 2003). High-virulence isolates such as Ab4 and Army183 are highly endotheliotropic and efficiently induce viremia, abortion and neurological disease. On the other hand, low-virulence isolates such as V592 are less endotheliotropic and only induce mild viremia and are seldom associated with abortion and neurological disease. By comparing several EHV1 isolates, including the reference strains Ab4 (neurovirulent) and V592 (non- neurovirulent), it was found that neurovirulent isolates were more associated with a single nucleotide polymorphism in ORF30. More specifically, the change of adenosine to guanosine at nucleotide position 2254 ($A_{2254} \rightarrow G_{2254}$) caused the substitution of the amino acid asparagine with the amino acid aspartatic acid ($A_{752} \rightarrow D_{752}$) in the viral DNA polymerase (Cuxson et al., 2014; Goodman et al., 2007; Nugent et al., 2006; Tewari et al., 2013; Van de Walle et al., 2009b). Although an *in vitro* study with nasal explants found no correlation between this single nucleotide polymorphism and enhanced plaque formation ability or a certain immune cell tropism, this D_{752} mutation did correlate with the increased invasion ability of EHV1 neurovirulent strains (Vandekerckhove et al., 2010). However, it is known that not all EHV1 isolates from neurological disease have the D_{752} phenotype and not all EHV1 isolates with the D_{752} phenotype cause neurological disease

(Perkins et al., 2009; Smith et al., 2010). In addition to the indication that virulence difference between isolates can also be associated with another single nucleotide polymorphism ($Adenosine_{2258} \rightarrow Cytidine_{2258}$) in ORF30 (Smith et al., 2010), it is plausible that virulence differences between isolates, in part, also depend on other parameters, such as the immune status of the host and expression of certain viral immune modulation factors.

Since EHV1 spreads rapidly in populations, outbreaks of abortion and EHM are not uncommon (Friday et al., 2000; McCartan et al., 1995; Pusterla, 2014). For example, abortion storms, where over 50% of the expected foal crop can be lost, have been reported worldwide (Carrigan et al., 1991). However, the number and severity of EHM outbreaks seem to increase in Europe and North America during recent years (Dunowska, 2016).

1.5.1. Respiratory disorders

Primary replication of EHV1 in the respiratory epithelium of the URT will usually result in a subclinical or mild, self-limiting URT infection. Sometimes clinical signs can develop such as nasal or ocular discharge, cough, fever, conjunctivitis, depression and anorexia. In neonates, immunocompromised and other naive young horses (< 2 year), a more severe respiratory syndrome can develop with following clinical signs: fever, serous to mucopurulent nasal discharge, red mucosa, swelling of draining lymph nodes, tachypnea, dyspnea and depression. These signs are the consequence of the viral pneumonitis and associated secondary bacterial bronchopneumonitis (Edington et al., 1986; Gibson et al., 1992; Kydd et al., 1994a; Patel et al., 1982). In sporadic cases, typically in young adults, EHV1 targets the pulmonary endothelium, thereby inducing severe pulmonary edema and death due to acute respiratory distress (Del Piero et al., 2000; Del Piero and Wilkins, 2001).

Presentation of symptoms, after experimental EHV1 infection, usually starts at 1 to 3 dpi (Gibson et al., 1992). However, under natural conditions, longer incubation periods have been reported, probably caused by differences in virulence, infectious dose and horse immunity (Kydd et al., 1996, 1994a). In previously infected horses with some residual adaptive immunity against EHV1, i.e. most of the adult horses, respiratory clinical signs may be minimal or absent and such infection can be followed by spontaneous abortion or EHM (Goehring et al., 2005).

The economic losses due to EHV1 respiratory disease, are not only caused by medical costs and lost practice days, but also by the poor performance syndrome in some horses that can be associated with nonspecific bronchial hypersensitivity and chronic obstructive pulmonary disease (Slater, 2007).

1.5.2. Reproductive disorders: abortion

Depending on the gestation time and degree of viral replication, three outcomes are possible (Allen and Bryans, 1986; van Maanen, 2002). First, a widespread infection of endometrial endothelium could result in abortion of a virus-negative fetus due to the extensive vasculitis and multifocal thrombosis. Second, more localized and less severe virus-induced vascular pathology may enable the virus to cross the epitheliochorial barrier and replicate in the fetus, possibly resulting in abortion of a virus-positive fetus. Third, transplacental transfer of EHV1 close to term may not result in abortion, but in the birth of a weak infected foal, that will die a couple of days later (Edington et al., 1991; Patel and Heldens, 2005).

(a) Abortion

EHV1 is one of the most important infectious causes of abortion in the horse (Gardiner et al., 2012). Almost all (95%) EHV1-associated abortions occur during the last four months of gestation (Allen and Bryans, 1986). Very rarely, EHV1 will induce abortion during early pregnancy (Dunowska, 2014a). Most EHV1-associated abortions are not preceded by clinical signs in the mare and result in expulsion of an infected or uninfected foal. In general, there is no reduced fertility of the mare if the dystocia did not cause uterine damage. The underlying mechanism for this higher susceptibility to EHV1-induced abortion in the last trimester of gestation is unknown, although upregulation at this time of putative adhesion molecules on the endothelial surface and PBMC could be involved (Smith et al., 2002, 2001).

(b) Neonatal foal death

Neonatal foal death is a rare consequence of EHV1 infection (Murray et al., 1998; Slater, 2007). It is not clear if virus transmission occurs *in utero* (transplacental) or *ex utero* (Hong et al., 1993; Patel and Heldens, 2005). Peripartum EHV1 infection results in apparently normal or weak foals that develop respiratory distress within one to two days, followed by icterus and subsequent death after one to two weeks (Mumford et al., 1987; Murray et al., 1998; Perkins et al., 1999).

1.5.3. Central nervous disorders: equine herpes myeloencephalopathy (EHM)

Secondary replication of EHV1 in the endothelial cells of the central nervous system is the first step in the development of EHM (Edington et al., 1986). EHM usually occurs six to ten days after infection, with exceptions as early as one day after infection, without clinical signs other than fever (~ four to nine days before the onset of EHM). The majority of EHM cases

appear to occur towards the end of the cell-associated viremia (Burgess et al., 2012; Jackson et al., 1977; van Maanen, 2002; van Maanen et al., 2001). EHM is mostly acute and clinical signs vary from mild ataxia to paralysis with recumbency leading to euthanasia. In most cases, the caudal spinal cord is most severely affected, resulting in hind limb weakness, paresis, paralysis, urinary and fecal incontinence and perianal sensory deficits (Crowhurst et al., 1981; Edington et al., 1986; Jackson et al., 1977). Sometimes other parts of the central nervous system are involved with clinical signs such as depression, recumbency, torticollis, blindness and quadriplegia (Friday et al., 2000; Greenwood and Simson, 1980).

1.5.4. Chorioretinopathy

EHV1 can induce choriorethinopathy, although this is rare. The vasculature of the equine ocular fundus is anatomically and physiologically (e.g. tight junctions) similar to the blood-brain barrier (Crispin et al., 1990; Hussey et al., 2013; Slater et al., 1992). While not regularly occurring, the clinical finding of chorioretinitis is an extra indication for (sub)clinical EHV1 infection. After experimental infection, lesions of the ocular vasculature manifest between three weeks and four months after infection. These lesions can be focal, multifocal and rarely diffuse and will induce permanent ischemic damage to the retina. Clinically, only diffuse lesions induce a significant loss of vision (Hussey et al., 2013).

1.6. Prevention and treatment

Despite many experimental attempts to develop a systemic antiviral to treat EHV1, there are no approved antivirals to date, nor has there been experimental evidence for their efficacy (Garré et al., 2009b). Therefore, since the sole treatment option after EHV1 infection is

supportive care with varying outcome, much attention should be paid towards preventive measurements such as management and vaccination.

1.6.1. Preventive therapy

Worldwide, disease control programs for EHV1 have three common goals: **(i)** prevent entry of disease into the population, **(ii)** diminish viral spread and clinical disease within the population and **(iii)** prevent spread of the disease to adjacent populations during an outbreak (Lunn et al., 2009a; Ostlund, 1993; Patel and Heldens, 2005; van Maanen, 2002). Since current vaccines against EHV1 do not reliably protect against viremia and subsequent abortion and EHM, the prevention of EHV1-associated disease is not only based on establishing a good vaccine-based herd immunity, but also on management to further lower environmental infection pressure (Dunowska, 2014a; Lunn et al., 2009a; Pusterla, 2014; Slater, 2007).

(a) Management

Outbreaks of EHM and abortion can often be linked to a source of exogenous virus (Ostlund, 1993; van Maanen, 2002). Prevention of EHV1 entry into a population is difficult since most horses are latently infected with EHV1 (Edington et al., 1994; Slater, 2007). Therefore, and ideally, new incoming horses should be vaccinated and geographically isolated and monitored for 21 days before mixing into the new herd (Allen and Bryans, 1986; L S Goehring et al., 2010; Kydd et al., 2012; Ostlund, 1993; Pusterla, 2014).

When a clinical case of EHV1 is suspected, the affected horse(s) should be immediately isolated and samples (nasal swabs and blood) submitted for diagnosis (Burgess et al., 2012; L S Goehring et al., 2010; Greenwood and Simson, 1980; Henninger et al., 2007; Pusterla,

2014). Quarantine of confirmed EHV1 cases and in-contact horses can be stopped when no horse has tested EHV1-positive for 21 days or if not a single horse showed EHV1-associated clinical signs for 28 consecutive days (Dunowska, 2014a; Slater, 2007).

EHV1 spread on a premise can be greatly reduced by stock management rules such as segregation of horses based on age and risk. Horses that are likely to shed virus, such as weaned foals and yearlings, should be separated from horses that are at high risk for severe EHV1 consequences such as pregnant mares (Dunowska, 2014a; Ostlund, 1993; Slater, 2007). Furthermore, indirect spread to other populations and premises can be prevented by extensive cleaning and disinfection of shared materials and vehicles. Although it was shown experimentally that EHV1 can remain infectious at room temperature for up to 7 days when dried on wood, paper and rope or up to 35 days when dried on burlap or horsehair, it is very unlikely that the virus remains infectious for more than 21 days after emptying the stables (Doll et al., 1959; Lunn et al., 2009a). Since EHV1 is an enveloped virus, it is relatively unstable in the environment and can easily be inactivated by heat, detergents, nonpolar solvents and common disinfectants. Premises and materials should be cleaned from organic materials with water and soap before chlorine-based disinfection with warm water (Tsujimura et al., 2015).

(b) Vaccination

Vaccination against EHV1 has two purposes: **(i)** reduce or prevent viral replication in the respiratory system and **(ii)** prevent the more severe sequelae of EHV1 infection such as abortion and EHM. Current vaccines, i.e. killed vaccines (inactivated) or modified live vaccines (MLV, attenuated), seem to succeed to meet the first objective, since nasal shedding of the virus and respiratory disease is significantly reduced if a horse becomes infected after vaccination. Therefore, these current vaccines can be used to shield individual horses against

EHV1 or to reduce the risk of spread of EHV1 during an outbreak within a horse population by vaccinating horses that do not show clinical signs, although the efficacy of the latter is controversial (Burrows et al., 1984; Dunowska, 2014a; Kydd et al., 2012). The second goal is far from reached with current vaccines, since they do not sufficiently and only variably protect against viremia and subsequent abortion or EHM (L S Goehring et al., 2010; Kydd et al., 2006b; Minke et al., 2004; Pusterla, 2014). In general, killed vaccines prime for mucosal antibody production and predominantly induce VN antibodies, capable of reducing nasal virus shedding and respiratory disease, but they are not effective against the cell-associated viremia and subsequent abortion or EHM. MLV induce a lower production of VN antibodies but a stronger cytotoxic T-lymphocyte (CTL) response when compared to killed vaccines, but even these vaccines also fail to fully protect against viremia and subsequent EHM and abortion (Allen, 2008, 2006). A possible concern for vaccination that has been described is the potential association between frequent vaccination and chance to develop EHM (Henninger et al., 2007). However, one has to interpret this with care since this association was primarily found in older horses that received frequent vaccination and an older age has been proposed a risk factor for EHM (Goehring et al., 2006). For MLV specifically, it is not advised to administer these vaccines to pregnant mares since MLV still may show residual virulence and could even revert to a higher virulence level which could have severe complications, especially in pregnant mares close to term (Minke et al., 2004).

Worldwide there are at least 12 commercially available vaccines against EHV1 (Slater, 2007), although in Belgium there is only one vaccine currently registered. This is an inactivated combination vaccine (Equip EHV 1/4; Zoetis) that claims to reduce nasal shedding of EHV1 and respiratory disease (Belgisch Centrum voor Farmacotherapeutische Informatie, 2015). Experimental studies have shown that vaccination with Duvaxyn EHV1/4 (also known as Equip EHV1/4) could reduce respiratory disease as well as the duration and amount of nasal

virus excretion, offered some protection against viremia and appeared to protect against EHV1 abortion. VN antibodies were associated with the shorter period of nasal virus excretion and complement fixing antibody titers were correlated with the improved clinical score (Heldens et al., 2001a; Minke et al., 2006). However, these results with Duvaxyn EHV1/4 were not fully translated under field conditions since EHV1 continues to circulate and only 50% of the foals and below 30% of the mares seroconverted after being administered the vaccine (Foote et al., 2006a, 2002).

Figure 6 gives a schematic overview of the different vaccine types established for EHV1, regardless of whether they are actually currently used in the clinic.

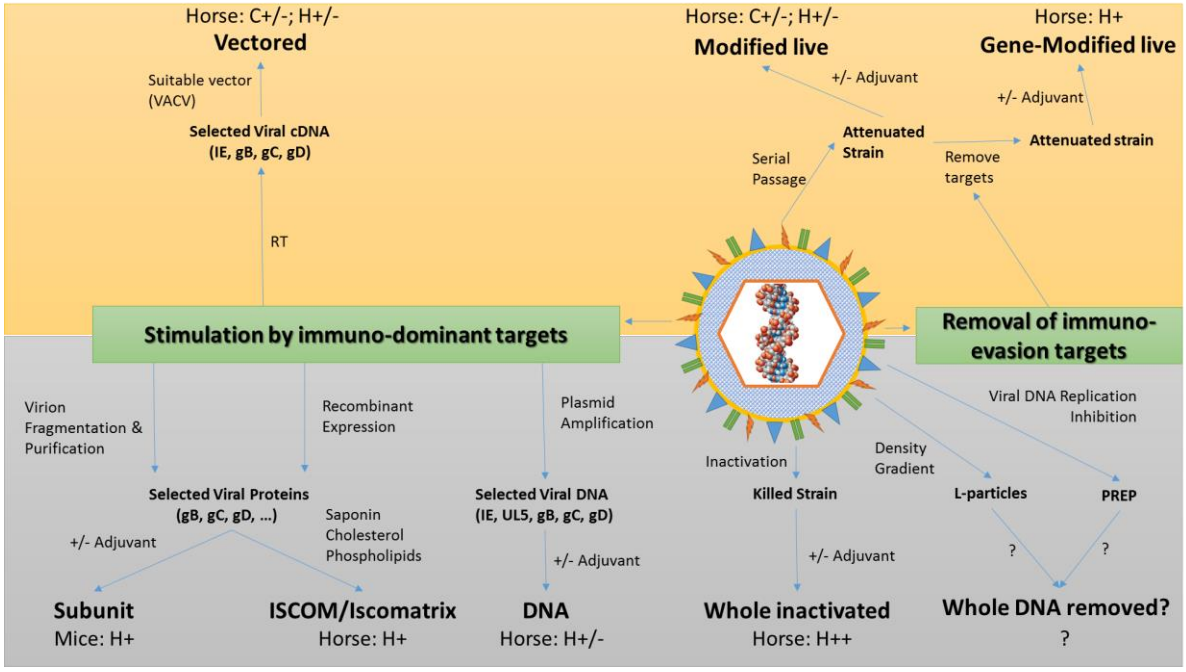


Figure 6: Overview of the different attenuated (orange) and inactivated/DNA (grey) vaccine types against EHV1 and their associated humoral (H) and cellular (C) immune responses. Abbreviations: ISCOM: immunostimulating complexes; L-particles: light particles; PREP: pre-viral replication DNA particles; RT: reverse transcriptase; VACV: vaccinia virus. References: subunit (Kukreja et al., 1998a; Osterrieder et al., 1995; Packiarajah et al., 1998; Stokes et al., 1996; Tewari et al., 1995, 1994), ISCOM (Foote et al., 2006b; Hannant et al., 1993), Iscomatrix (Foote et al., 2005), DNA (Minke et al., 2006; Ruitenberg et al., 2000; Soboll et al., 2006), whole inactivated (Breathnach et al., 2001; Foote et al., 2002; L. S. Goehring et al., 2010; Heldens et al., 2001a, 2001b; Holmes et al., 2006; Minke et al., 2006; van Maanen, 2002), whole DNA KO (Pardoe and Dargan, 2002), modified live (Bresgen et al., 2012; L. S. Goehring et al., 2010; Goodman et al., 2006), gene-modified live (Matsumura et al., 1998; Tsujimura et al., 2009; Van de Walle et al., 2010), vectored (Minke et al., 2006; Paillot et al., 2006; Soboll et al., 2010).

1.6.2. Curative therapy

Curative therapy is mainly centered around supportive care since no antiviral drugs are registered for use against EHV1 and experimental *in vivo* studies in which the efficacy of antivirals against EHV1 have been tested, yield contradictory results.

(a) Supportive care

The main treatment efforts are targeted towards easing clinical signs and preventing complications such as secondary bacterial infections (Edington et al., 1986; Kydd et al., 2012; Lunn et al., 2009a; Pusterla, 2014; van Maanen, 2002). Abortions occur suddenly with complete expulsion of the fetus and placenta and without clinical signs in the mare. Because of this and since further breeding capacities of the mare are not impaired unless the dystocia caused uterine damage, treatment of the mare is in general not necessary (Kydd et al., 2012; Ostlund, 1993; Pusterla, 2014; van Maanen, 2002). The main therapy for weak born foals is antibiotics and supportive care such as heat lamps, oxygen therapy, cardiovascular and nutritional support. Despite these attempts, most foals die within the first week postpartum (Allen and Bryans, 1986; Murray et al., 1998; van Maanen, 2002). Non-recumbent EHM horses should be constantly monitored and housed with minimal risk of injury. In cases of severe ataxia, the non-recumbent horse can be temporally assisted with a sling. If attempts to rise are made, the horse should be assisted with a head rope and immediately encouraged to hand-walk (Friday et al., 2000; Greenwood and Simson, 1980; McCartan et al., 1995; Pusterla, 2014; van Maanen et al., 2001). A recumbent horse should not be supported by a sling, but should be kept in a quiet and good ventilated stable in the sternal position with supply of intravenous hydration and antibiotics. Although unproven for their efficacy towards EHM, other drugs such as steroids can be supplied to reduce central nervous system inflammation, heparin to prevent thrombo-embolism and vitamin E as a free-radical scavenger (Edington et al., 1986; Goehring et al., 2005; Pusterla, 2014). The prognosis is

favorable for non-recumbent animals since they usually will fully recover. On the other hand, if the horse is recumbent for more than 24 hours, the prognosis is poor and euthanasia is usually indicated (Pusterla, 2014; van Maanen et al., 2001).

(b) Causative (antiviral) therapy

Acyclovir is a highly potent inhibitor of the replication of human α-herpesviruses like HSV1, HSV2 and VZV, with very few side-effects (Elion, 1982). However, efficacy of acyclovir during EHV1 pathogenesis remains to be conclusively confirmed by adequate experimental setups which are not affected by confounding factors (e.g. inadequate control groups) (Friday et al., 2000; Henninger et al., 2007). Furthermore, although the prodrug valacyclovir was able to induce plasma and nasal acyclovir concentrations that inhibited plaque formation *in vitro,* no significant differences between treated and untreated EHV1-infected horses could be demonstrated (Bentz et al., 2006; Garré et al., 2009a, 2009b, 2007; Maxwell et al., 2008). In addition, despite the promise shown in the murine model of EHV1 infection that siRNA could reduce viral replication and weight loss in treated animals (Fulton et al., 2009), this targeting of the essential viral proteins gB and the viral origin binding helicase was unable to significantly alter viral load, clinical signs and VN antibodies in the equine model of EHV1 infection (Brosnahan et al., 2010; Perkins et al., 2013).

2. Immunological response against equine herpesvirus 1

Natural or experimental infection with EHV1 will protect from reinfection during the next three to six months, i.e. it will prevent clinical signs, nasal virus excretion and viremia (Doll et al., 1955). Efficient protection against EHV1 infection and disease requires both humoral and cellular immune responses (Kydd et al., 2012; Lunn et al., 2009b). Briefly, innate

immunity is mainly mediated by neutrophils, macrophages and natural killer (NK)-cells and adaptive immunity is predominantly dependent on high levels of VN-antibodies and CTL-responses specifically generated against EHV1 epitopes (Bridges and Edington, 1986; Edington et al., 1989). However, high VN-antibody titers do not sufficiently prevent viremia, abortion and EHM (Breathnach et al., 2001; Hannant et al., 1993). Potential explanations for this can be found in studies where it was shown that **(i)** there is only a very low expression of late glycoproteins in infected PBMC during viremia, making it hard for VN antibodies to recognize these infected cells, and **(ii)** transfer of virus from PBMC to endothelial cells can still occur in the presence of VN antibodies (Goehring et al., 2011; Laval et al., 2015; van der Meulen et al., 2003). In contrast, there is a strong association between the presence of EHV1-specific CTL and protection against viremia, abortion and EHM (Allen et al., 1995; Allen, 2008; Kydd et al., 2003; O'Neill et al., 1999).

2.1. Humoral immunity

Because primary replication occurs in the respiratory system, the site of entry, a strong mucosal humoral immune response will be essential to prevent or severely impair EHV1 replication and subsequent viral spread. An experimental infection study with the non-neurovirulent Army183 strain demonstrated that the main mucosal antibody isotype raised upon EHV1 infection is the secreted immunoglobulin A. The virus-specificity of these short-lived (weeks) mucosal antibodies was demonstrated by the ability of nasal wash dilutions to reduce the plaque size of Army183 *in vitro* in a dose-dependent manner (Breathnach et al., 2001).

By sampling sera from horses before and after experimental or natural EHV1 infection, an elevation of VN (mainly IgG) and complement fixing (mainly IgM) antibody serum titers was observed starting around 14 dpi (Doll et al., 1953; Doll and Bryans, 1963; Thomson et al.,

1976). More specifically, an IgM response was detectable about 4-5 dpi with EHV1, which reached its maximum at 20-30 dpi and declined to its pre-infection level at 60-80 dpi. From 8-9 dpi, IgG isotype antibodies started to increase towards their maximum at 30-40 dpi and were shown to persist for about a year post infection (Mumford et al., 1987; Slater, 2007).

Convalescent sera from horses can recognize 11 EHV1 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, gp2) with gB, gC and gD being the most immunodominant (Crabb et al., 1991; Slater, 2007). Evaluation of these three glycoproteins as immunogenic targets in subunit, DNA, ISCOM and vectored vaccines have shown that they mainly induce VN antibodies and that this correlated with reduced respiratory disease and nasal virus shedding (Foote et al., 2005; Minke et al., 2006; Osterrieder et al., 1995; Ruitenberg et al., 2000; Soboll et al., 2006; Tewari et al., 1995). However, humoral immunity is not considered to be the most effective mechanism against abortion and EHM, since they cannot prevent cell-associated viremia (Allen and Bryans, 1986; Lunn et al., 2009b).

2.2. Interferon response

Interferons (IFN) are a family of anti-viral cytokines which can be induced upon viral infection by interaction of specific pathogen associated molecular patterns (e.g. viral dsDNA) with certain pattern recognition receptors which are localized intra- or extracellular. Virtually all virus-susceptible cells can secrete type I IFN (IFN-α/β) or type III IFN (IFN-λ), but type II IFN (IFN-γ) is only produced by certain immune cells such as NK-cells, T-lymphocytes and conventional dendritic cells (cDC). Amongst these IFN producing cells, plasmacytoid dendritic cells (pDC) are well known to produce substantial amount of type I and III IFN and are consequently termed natural interferon producing cells. After interaction of type I IFN with interferon-alpha/beta receptor 1/2, type II IFN with interferon gamma receptor 1/2 or type III IFN with IL-28Rα/IL-10 receptor β, a cascade of signaling pathways will lead to the

induction of multiple IFN stimulated genes whose products will exert the anti-viral effects of IFN (Iversen and Paludan, 2010; Samuel, 2001).

2.2.1. IFN-α/β

Viral replication within the respiratory epithelium induces release of type I interferon which initiates the innate immune response. These IFNs, together with IFN-γ, are believed to be strong stimulators of adaptive immunity, for example by contributing to the differentiation of CD8+ T-cells (Van de Walle et al., 2009a). IFN-α production by PBMC upon *in vitro* stimulation with EHV1 is independent of the type of EHV1 isolate used or the pre-existence of adaptive immunity against EHV1 (Goodman et al., 2012; Wagner et al., 2011). For horses, it remains to be determined if equine pDC will also produce massive amounts of type I and III IFN upon EHV1 infection, and as such, could be termed natural interferon producing cells similar to their human and murine counterparts (Van de Walle et al., 2009a).

2.2.2. IFN-γ

IFN-γ has a significant antiviral effect (Figure 7), not only by activating macrophages and lymphocytes, but also by reducing EHV1 replication *in vitro* (Sentsui et al., 2010). Synthesis of IFN-γ is also commonly used as a marker of cellular immunity since it induces upregulation of MHC-based presentation of viral peptides and promotes the development of a Th1 response leading to CTL-mediated lysis of infected cells (Allen, 2008; Paillot et al., 2008, 2006). Indeed, increased frequency of CD8+ IFN-γ producing cells could be associated with an increase in EHV1-specific CTL response (Paillot et al., 2006, 2005).

In contrast to type I and III IFN, type II IFN depend to a significant extend on the pre-existence of adaptive immunity, since it was previously shown that reduced clinical signs,

elevated IFN-γ, and lymphoproliferative responses, were significantly present only if these horses were immunized before the isolation and *in vitro* stimulation of PBMC with EHV1 (Coombs et al., 2006). Although in general only PBMC from horses > 4-6 years will yield significant IFN-γ responses upon *in vitro* stimulation with EHV1, such responses can be mimicked in yearlings (< 2 years) by experimental pre-immunization using either an inactivated vaccine or wild type EHV1 (Luce et al., 2007; Paillot et al., 2007, 2005). In addition, such age-dependent increase was also found for EHV1-specific CTLs, which are a marker for protection against EHM and abortion (Allen et al., 2008; Kydd et al., 2003; O'Neill et al., 1999). Furthermore, it was found that horses who suffered an EHV1 outbreak after MLV vaccination seemed to significantly enhance their IFN-γ response upon EHV1 stimulation of PBMC *in vitro*, and furthermore, it was shown that this was primarily mediated by CD4+ T-lymphocytes (Goodman et al., 2012).

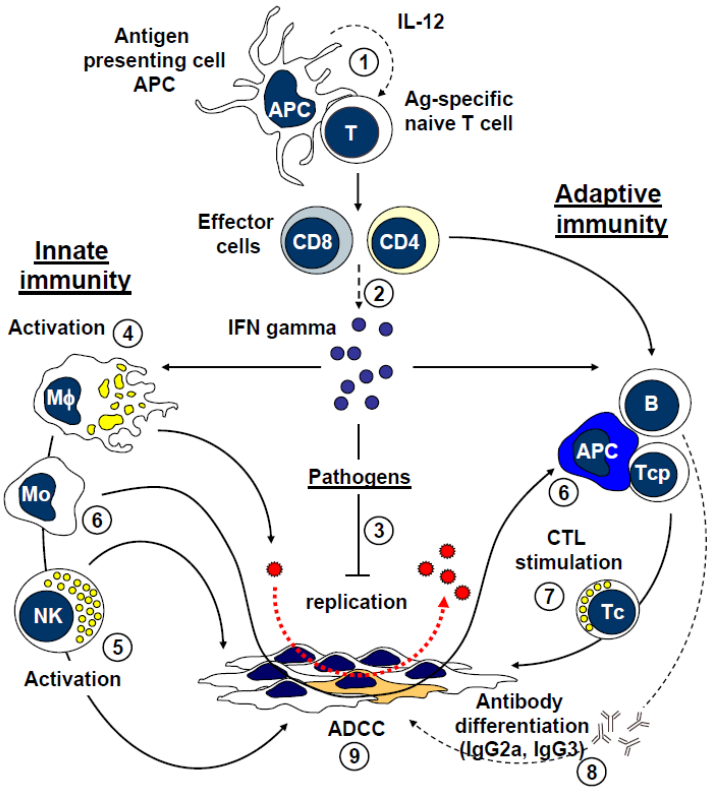


Figure 7: The effect of IFNγ on immune response and pathogens. The presentation of an antigen to a naive antigen-specific T lymphocyte (1) may induce its derivation to a type 1 effector cell synthesizing IFNγ (2). IFNγ can directly block pathogen replication (3), activate macro- phages (Mφ) for the pathogen elimination (4), and activate NK cells to lyse infected cells (5). IFNγ can up-regulate the expression of major histocompatibility complex (MHC) molecules on the monocyte (Mo) and Mφ surface (6), increasing the efficiency of antigen presentation to cells of the adaptive immune response (6). IFNγ is a strong immunomodulator of the adaptive immune response leading to the activation of CTL (7) or driving the antibody isotype (8) to favor antibody-dependent cell mediated cytotoxicity (ADCC) (9) (Paillot et al., 2008).

2.3. Cellular immunity

Blood leukocyte subpopulations change following EHV1 infection. Leukopenia consisting of neutropenia and lymphopenia (mainly CD8+ T cells) can be observed between 7 and 14 days after infection, after which a leukocytosis is seen that can persist for up to four weeks after infection (Slater, 2007). Lymphocyte proliferation is an essential part of the immune response, however, horse lymphocytes showed a suboptimal proliferation response towards UV-inactivated EHV1 or the mitogen phytohemagglutinin when collected between 2 and 10 weeks after experimental inoculation with UV-inactivated EHV1. After this period,

lymphocyte proliferation increased, but again declined to basal levels after 3 months. In contrast, no lymphocyte proliferation response could be observed upon *in vitro* incubation with live EHV1 if these horse lymphocytes were previously stimulated with live EHV1 *in vivo* or *in vitro* (Charan et al., 1997). This suggests immunodepression which could be mediated by mechanisms such as abortive replication in lymphocytes, function depression of EHV1-infected DC and downregulation of MHC-based viral antigen (Ag) presentation (see #3.1.3.) (Rappocciolo et al., 2003; Siedek et al., 1997; van der Meulen et al., 2001, 2000).

2.3.1. Importance of CTL

Within hours after initial infection of the respiratory epithelium, the virus will become intracellular and spread subsequently by cell-cell contact, thereby preventing virion neutralization by VN antibodies. Antibody-dependent cell cytotoxicity and NK cytotoxicity have been studied and although results suggested that these mechanisms have little importance during immunity against EHV1, further studies are necessary to substantiate this (Bridges and Edington, 1987; Chong et al., 1992; Stokes and Wardley, 1988). In contrast, experimental infection data do demonstrate a correlation between EHV1-specific CTL and protection against viremia, abortion and EHM (Allen, 2008; Kydd et al., 2003; O'Neill et al., 1999). First, CD8+ lymphocyte populations increase in the blood, lungs and URT-associated lymphoid tissues at 1 week after EHV1 inoculation (Breathnach et al., 2006). Second, the cytotoxic activity detected by chromium release assay and limited dilution assay confirmed that cytolytic activity during EHV1 re-stimulation of PBMC is mediated by CD8+ T-lymphocytes in a MHCI restricted fashion and not by lymphokine activated killer cells (Allen et al., 1995; O'Neill et al., 1999). Third, young ponies with low levels of EHV1-specific CTL precursors developed respiratory disease, viral nasopharyngeal shedding and viremia upon experimental EHV1 infection, whereas older ponies with high EHV1-specific CTL precursors

showed no or reduced respiratory disease, shedding and viremia (O'Neill et al., 1999). Moreover, experimental infection data further demonstrated a significant correlation between protection against viremia, higher frequency of EHV1-specific CTL and abortion or EHM (Allen, 2008; Kydd et al., 2003).

Because high frequency of EHV1-specific CTL after experimental infections is known to be correlated with reduced viremia and subsequent abortion and EHM (Allen, 2008), efforts have been made to characterize the major factors influencing such a response, predominantly to increase the vaccine efficacy towards abortion and EHM. Several studies were performed to evaluate the influence of the presented viral epitopes on certain MHCI haplotypes on the capacity to induce CTL responses, for example by evaluating CTL responses *in vitro* by using DC, that are transfected with specific EHV1 genes, as stimulator cells. Early studies in mice demonstrated that significant CTL responses could be induced by viral glycoproteins (i.e. gB, gC, gD) (Kukreja et al., 1998a, 1998b; Osterrieder et al., 1995; Packiarajah et al., 1998; Tewari et al., 1995; Whalley et al., 1995). However, equine studies demonstrated that it were not the viral glycoproteins such as gB, gC, gD that induced significant CTL responses, but the IEP (Kydd et al., 2014, 2006a, Soboll et al., 2010, 2003). In addition, it was found that induction of IEP-specific CTL responses were only consistently induced in horses with specific MHCI haplotypes, namely the equine leukocyte antigen A3.1 (B2 gene expressing) and A2 haplotype (Kydd et al., 2014; Soboll et al., 2003).

3. Immune evasion mechanisms of equine herpesvirus 1

During their co-evolution with the host, herpesviruses have developed several mechanisms to counteract immune surveillance and effectors from the immunocompetent host in order to persist in their host population, and in this regard, EHV1 is no exception (Loch and Tampé,

2005; Ma et al., 2013; Vossen et al., 2002). First, we will give a short general overview of EHV1-mediated modulation of humoral, cytokine and cellular immune responses. For the purpose of this dissertation, the virion host-shutoff (VHS) protein, EHV1' infected cell protein (EICP) 0, unique long protein (pUL) 56 and pUL49.5 and their reported immune evasion functions will be further discussed.

3.1. General overview

3.1.1. Evasion from humoral immunity

Productive EHV1 infection of monocytes and lymphocytes is very low *in vitro* and this was shown to be associated with a virtually complete absence of viral late envelope proteins on the cell surface of EHV1-infected PBMC (van der Meulen et al., 2003; van Der Meulen et al., 2000). Interestingly, viral replication was clearly blocked at an early phase of infection since no detectable gB or gM expression could be found in PBMC isolated from experimental infected ponies, despite expression of IEP and the early protein ICP22 in these infected cells (van der Meulen et al., 2006). More recently, Laval et al. (2014) confirmed the existence of an abortive replication in CD172a positive PBMC by showing that both virus production as well as the expression of IEP, ICP22 and gB was delayed in unstimulated CD172a-positive PBMC (Laval et al., 2014).

Although pre-stimulation of PBMC with mitogens could enhance expression of envelope proteins on the cell surface (and infection percentages) of PBMC *in vitro*, complement-mediated lysis of these EHV1-infected PBMC was still low (van der Meulen et al., 2003). In addition, EHV1 is also capable of interfering with the complement cascade via binding of gC, expressed on the cell surface of EHV1-infected cells, to complement factor C3b (Huemer et al., 1995).

3.1.2. Attenuation of the cytokine response

Chemokines are essential in mediating inflammatory responses during viral infection, and consequently, several viruses have devised mechanisms to disturb this complex signaling network, e.g. via the expression of viral chemokine binding proteins, either membrane-bound or secreted (Van de Walle et al., 2008a). For example, it was found that secreted gG, a broad viral chemokine binding protein (Bryant, 2003), could significantly inhibit IL-8 induced chemotaxis of equine neutrophils and murine macrophages *in vitro* and likewise, a significantly reduced number of these cell types could be recovered from the lungs of EHV1-infected mice when compared to mice infected with a gG-deletion mutant (Van de Walle et al., 2008c, 2007). More specifically, a specific amino acid domain (from AA301-340) in gG appeared to be critical for the viral chemokine binding protein activity of EHV1 gG (Thormann et al., 2012; Van de Walle et al., 2009c).

3.1.3. Evasion from CTL-based immunity

As previously mentioned, high frequencies of CD8+ CTL are associated with protection against viremia and subsequent abortion or EHM (Allen, 2008; Kydd et al., 2003; O'Neill et al., 1999). For the initiation of such virus-specific CTL response, professional antigen presenting cells (APC) such as DC need to present viral Ag via MHCI to naïve CTLs. Therefore, DC are essential to induce and maintain antiviral CTL responses by presenting these viral Ag in combination with sufficient co-stimulatory signals to naïve T-lymphocytes (Banchereau et al., 2000; Ludewig et al., 1999, 1998). DC are continuously patrolling interfaces with the outer world, including the respiratory mucosa, and upon recognition of a pathogen will start their maturation program, which is associated with migration towards secondary lymphoid organs. Because DC are one of the first immune cell types that will

encounter viruses at these entry sites and since these migratory professional APC are essential for the antiviral immunity, it may come as no surprise that α-herpesviruses have evolved several mechanisms to disturb DC functions, including viral antigen presentation towards CD8+ T cells. Indeed, infection of human DC with HSV1, HSV2 and VZV resulted in downregulation of cell surface molecules associated with Ag presentation (MHCI, CD1a), co-stimulation (CD40, CD80, CD83, CD86) and adhesion (CD54), but not MHCII and CD11c (Elboim et al., 2013; Heilingloh et al., 2014; Kobelt et al., 2003; Kruse et al., 2000a; Kummer et al., 2007; Lampen et al., 2010; Mikloska et al., 2001; M J Raftery et al., 2006; Salio et al., 1999; Samady et al., 2003). Since downregulation of cell surface markers on infected cells, including DC, has been (in part) attributed to the expression of several viral proteins, we will for the purpose of this dissertation discuss in more detail some of these viral proteins that we have further investigated in the current study.

3.2. Virion Host Shut-off (VHS): interference with CD86 and CD83 expression

3.2.1. CD86 and CD83

In general, CD86, also known as B7.2, is a monomeric type I transmembrane protein with a distal IgV domain and a IgC domain which is more proximal to the plasma membrane. Like the dimeric CD80 (B7.1), CD86 is also expressed on DC. Although both will interact with their ligand CD28 and CTLA-4 (CD152) on T-lymphocytes, there is a preferential interaction of CD80-CTLA-4 and CD86-CD28. Unlike CD80, CD86 is constitutively highly expressed on DC (Bhatia et al., 2006; Sansom et al., 2003). Although CD86 expression has already been found on the cell surface of equine MDDC, the expression of CD80 was only demonstrated at mRNA level (Durán et al., 2013; Flaminio et al., 2007; Moyo et al., 2013). In general, upon interaction of CD86 with CD28 on T-lymphocytes, a slow upregulation of CD80 expression on DC is induced with a subsequent elevated CTLA-4 based inhibitory T-signaling. Indeed,

during the initial DC-T-lymphocyte contact, a relatively weak CD86-CD28 interaction induces T-lymphocyte activation with subsequent upregulation of CD80 and CTLA-4. After this upregulation, the much stronger interaction of CD80-CTLA-4 will dissociate the activating CD86-CD28 interaction from the immunological synapse and leave CD80-CD28 to further modulate T-cell activation outcome. In addition to the CD86/CD80 mediated signaling towards T-lymphocytes, interaction of T-cell ligands with these DC co-stimulatory molecules will also redirect signaling towards DC (Bhatia et al., 2006; Sansom et al., 2003). For example, next to the CTLA-4-dependent induction of the tolerogenic program in DC, CD28 interaction with DC is able to induce cytokine responses of DC such as IL-6 and IFN-γ (Orabona et al., 2004). Also during the antiviral immune response, expression of CD86 is essential for the induction and maintenance of CD8+ CTL and CD4+ T-lymphocytes (Borowski et al., 2007; Duttagupta et al., 2009; Edelmann and Wilson, 2001).

In general, CD83, a structural and functional B7-like co-stimulatory molecule, is a highly glycosylated member of the Ig superfamily with an extracellular IgV-like domain. Next to its expression on activated lymphocytes, CD83 is also found at intermediate levels on blood and tissue DC (Prazma and Tedder, 2008). On equine MDDC a low to intermediate expression of cell surface CD83 has been demonstrated (Baghi et al., 2014; Dietze et al., 2008; Durán et al., 2013; Mauel et al., 2006; Moyo et al., 2013). It is generally accepted that CD83 is a marker for DC activation/maturation since activation of DC increases cell surface levels of CD83, together with MHCII and B7. Although the ligand for CD83 has not been identified, there is considerable evidence that this membrane bound form of CD83 significantly enhances T cell proliferation responses (Prazma and Tedder, 2008). For example, in addition to the reduction of DC-mediated (virus-specific) T-priming after knockdown of CD83, expansion of virus-specific primary and memory T-lymphocytes could only be accomplished if CD83 was co-

expressed on artificial created APC (Aerts-Toegaert et al., 2007; Hirano et al., 2006; Prechtel et al., 2007).

3.2.2. VHS: mode of action

Equine VHS is a 58 kDa tegument-associated viral protein encoded by ORF19 with mRNA-specific endonuclease activity (Feng et al., 1996). Because of the continuous battle for cellular resources to produce progeny virus, herpesviruses have developed several mechanisms to ensure and orchestrate viral gene expression. During HSV1 infection, VHS will reduce cellular mRNA translation and, although counterintuitive at first glance, will downregulate synthesis of certain viral proteins. With its mRNA-specific endonuclease HSV1 VHS will mainly target cellular mRNA for degradation and thereby free up the translation machinery for viral protein synthesis and thwart cellular early immune responses, such as IFN. However, to prevent overflow of the translation machinery with viral transcripts and to ensure that all necessary viral proteins are synthesized, HSV1 VHS will also target immediate early and early gene transcripts for degradation and will prevent functional exclusion of late mRNA transcripts for translation. At later times of HSV1 infection, VP16 and VP22 will downregulate VHS activity and subsequently facilitate its incorporation into forming virions. The dependence on VHS to ensure efficient viral replication depends on the cell type (Dauber et al., 2014). As a result of targeting cellular mRNA for degradation by VHS, the expression of cytokines and cell surface markers could be attenuated. This has already been described during HSV1 infection of MDDC, where deletion of VHS resulted not only in enhanced expression of IL-6 and tumor necrosis factor (TNF) α, but increased expression of the cell surface markers CD83 and CD86 (Samady et al., 2003).

In search for an Ag loading HSV1 vector that would not inactivate the ability of DC to mature and induce adaptive immune responses, the effects of VHS deletion in a multiple deletion

replication incompetent HSV1 construct was evaluated. The reason for including the deletion of VHS was based on the fact that a replication-incompetent HSV1 construct that did express VHS prevented viral- and/or LPS-mediated upregulation of CD83 and CD86 and hence, proper DC maturation. When human immature MDDC were infected with this VHSNULL construct, higher CD86 levels could be observed compared to the VHS-expressing HSV1 wild type construct. Upon LPS stimulation at the time of infection, levels of CD83 and CD86 were also higher when using VHSNULL versus wild type HSV1 construct (Samady et al., 2003).

For equine VHS, the evaluation of its mRNA endonuclease activity is limited to full protein blots or actin/tubulin mRNA blots in ED cells and rabbit kidney (RK) epithelial cells. Although these assays demonstrated only limited activity upon EHV1 infection of both cell types, so in a background, transfection of RK13 cells with an EHV1 VHS-expression plasmid demonstrated a high inhibition of reporter gene expression, similar to what has been reported for HSV1 VHS (Feng et al., 1996). The attenuation of cytokine and cell surface marker expression by EHV1 VHS has not been studied.

3.3. Infected cell protein 0 (ICP0): interference with CD83 expression

EICP0 is an early expressed (ORF63) tegument associated viral protein with an apparent molecular weight of 80-90 kDa (Everett et al., 2010). ICP0 of HSV1 is essential for efficient lytic infection and productive reactivation from latency. Like HSV1 ICP0 encoded by the immediate early gene, the EHV1 homolog EICP0 encoded by the early gene, is also characterized by an N-terminal E3 ubiquitin ligase (Everett et al., 2010). This E3 ubiquitin ligase activity is essential for HSV1 ICP0's ability to target cellular proteins for proteasome-dependent degradation by adding a ubiquitin tag to these proteins. The reported functions of ICP0 during HSV1 infection are diverse and include **(i)** induction of protein degradation, as

described above, **(ii)** interference with IFN signaling pathways, **(iii)** chromatin modifications and **(iv)** regulation of the DNA damage response. More particularly the HSV1 ICP0-mediated disruption of the nuclear domain 10 or the promyelocytic leukemia nuclear bodies, both of which are part of the intrinsic cellular defense against viral replication, is intensively being studied (Boutell and Everett, 2013).

During HSV1 infection of mature human MDDC, a downregulation of CD83 cell surface expression was observed starting from 4 hpi, accompanied by a clear reduction of total CD83 protein levels on Western Blot at 16 hpi (Kruse et al., 2000a; Kummer et al., 2007). In addition, Kummer et al. (2007) evaluated this CD83 downregulation in infected cells treated as follows: first, cycloheximide (CHX) was used, which allows only immediate early mRNA to be transcribed but not translated, followed by actinomycin D (ActD), which allows for translation but not transcription. This treatment resulted in inhibition of protein synthesis beyond immediate early protein expression and demonstrated that immediate early viral gene expression was sufficient to trigger downregulation of CD83. In addition, the authors found that an ICP0 HSV1 deletion mutant was partially impaired in downregulation of cell surface CD83 and they demonstrated the involvement of the E3 ubiquitin ligase activity in this CD83 downregulation (Kummer et al., 2007).

A later study by Heilingloh et al. (2014) confirmed the ability of ICP0 to downregulate CD83 on mature human MDDC. However, and in contrast to Kummer et al. (2007), they accumulated considerable evidence that ICP0-mediated CD83 downregulation and degradation was not dependent on the E3 ubiquitin ligase activity of ICP0 and suggested a less common ubiquitin-independent mechanism of targeting for proteasome-dependent degradation (Heilingloh et al., 2014).

No information is currently available on the role of EHV1 EICP0 in attenuating cell surface marker expression/degradation in EHV1-infected cells.

3.4. pUL56: interference with MHCI expression

3.4.1. Major Histocompatibility complex I (MHCI)

MHCI is a class of membrane expressed glycoproteins that present small peptides on the cell surface of nearly all nucleated cells. Whereas highly polymorphic classical MHCI molecules are involved in the presentation of cytoplasm-derived or even exogenous peptides to induce an immune response, the clearly less polymorphic non-classical MHCI are involved in the inhibition of NK-cells (David-Watine et al., 1990; Tallmadge et al., 2010). Of note, and similar to the human system, a non-classical lipid Ag presenting CD1 system, which looks like MHCI, but acts like MHCII, has also been identified in the horse immune system (Dossa et al., 2014). Classical MHCI-based presentation of non-self-peptides is an important means to destroy virus infected cells and is also an essential priming mechanism employed by APC to selectively activate and drive expansion of virus-specific lymphocytes (Banchereau and Steinman, 1998; Loch and Tampé, 2005). A simple overview of the intracellular Ag-derived MHCI-processing pathway is given below in Figure 8.

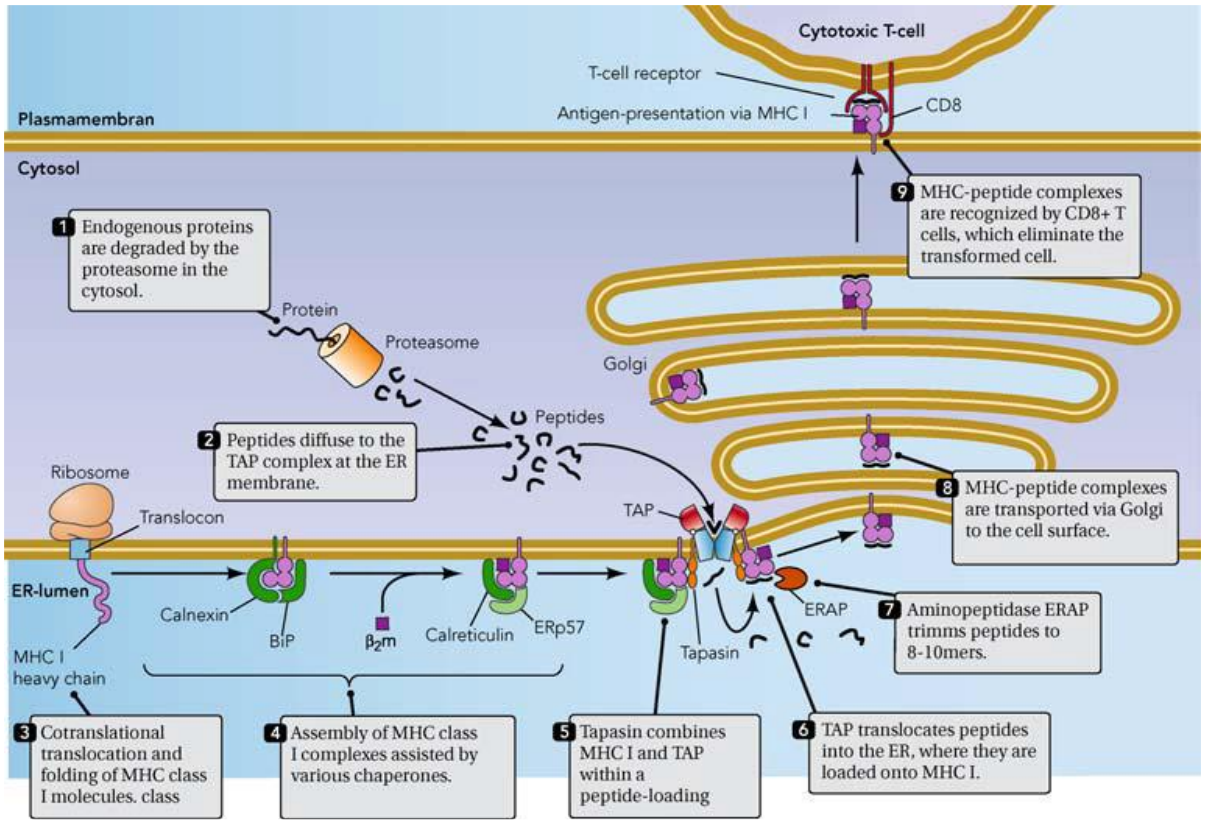


Figure 8: Overview of the intracellular derived Ag-processing pathway via MHCI (Loch and Tampé, 2005).

3.4.2. pUL56: mode of action

Equine pUL56 is an early expressed ORF1 encoded type II transmembrane phosphoprotein, which appears in different isoforms on Western Blot of infected ED cells starting at 2 hpi with a 22 kDa isoform, at 4 hpi a 28 kDa band appears and also two fainter bands of 20 and 25 kDa can be detected (Ma et al., 2012). EHV1 pUL56 has been shown to be involved for a variety of cells in the downregulation of cell surface markers such as MHCI (ED, equine respiratory tracheal epithelial cells (EREC)), MHCII (EREC) and CD46/CD63 (human epithelial carcinoma cells) (Huang et al., 2014; Ma et al., 2012; Soboll Hussey et al., 2014). In addition, pUL56 has also been shown to be involved in the attenuation of the cytokine response. More specifically, pUL56 reduced mRNA levels of IFN-α and increased mRNA levels of IL-10 in EHV1-infected EREC. Moreover, and although mRNA levels remained unchanged, protein

expression of IL-8 and monocyte chemotactic protein 1 was also downregulated in EREC in a pUL56-dependent manner (Soboll Hussey et al., 2014).

For the purpose of this thesis, we will further discuss the downregulation of cell surface MHCI by EHV1 pUL56 in more detail. Rappocciolo et al. (2003) was the first to demonstrate MHCI downregulation on NBL6 cells upon infection with EHV1. Furthermore, the authors proposed that an early viral protein could be responsible for this since cell surface MHCI was still downregulated when late viral protein synthesis was blocked with phosphonoacetic acid, but downregulation was abrogated when only immediate early protein synthesis was allowed. Furthermore, by using three antibodies recognizing different epitopes on MHCI, they suggested that EHV1 downregulated MHCI on ED cells in an allele/locus-specific manner since downregulation of MHCI was not as pronounced with the monomorphic epitope recognizing MHCI antibody when compared with the two other MHCI antibodies recognizing polymorphic conformation-dependent epitopes. Finally, the authors treated mock-infected ED cells with the protein transport inhibitor brefeldin A and proteins synthesis inhibitor CHX. The authors observed that such treatment did result in low degree of cell surface MHCI downregulation and that this inhibitor-induced downregulation of MHCI on mock-infected cells is lower than induced by EHV1 in untreated cells. Based on this, the authors suggested that the virus is doing more than just preventing MHCI transport to the cell surface and hypothesized that EHV1 probably enhances internalization of MHCI from the cell surface (Rappocciolo et al., 2003). Several years later, Ma et al. (2012) found that downregulation of MHCI on EHV1-infected ED cells was severely impaired when using the attenuated RacL11 strain compared to the Ab4 strain. Genome comparison of these two strains revealed that RacL11 lacked the majority of the ORF1 gene and the complete ORF2 gene. Using different ORF1/2 Ab4 deletion mutants, it was found that MHCI downregulation was dependent on ORF1, but not ORF2. Interestingly, pUL56, the protein encoded by ORF1, could only

downregulate MHCI on ED cells in a virus context and not by itself, indicating that this protein requires other viral (or cellular) factors for proper MHCI downregulation. Furthermore, the authors demonstrated that the transmembrane domain of the type II transmembrane protein UL56 is essential for cell surface MHCI downregulation and localization within the Golgi and vesicles in EHV1-infected ED cells (Ma et al., 2012). More recently, Huang et al. (2014) confirmed that MHCI is indeed internalized from the cell surface of EHV1-infected ED cells with subsequent lysosomal degradation, and demonstrated that this was dependent on tyrosine kinase activity, cholesterol, dynamin and the ubiquitination machinery, but not caveolae- or clathrin-mediated endocytosis (Huang et al., 2014; Rappocciolo et al., 2003). In a follow-up study, the early protein pUL43 was identified as the interaction partner of pUL56 for proper MHCI downregulation in EHV1-infected ED cells (Huang et al., 2015).

3.5. pUL49.5: interference with MHCI expression

This early-late type I transmembrane protein of about 10 kDa, also known as gN, is encoded by EHV1' ORF10 and forms a heterodimer complex with gM (Koppers-Lalic et al., 2008; Ma et al., 2012). Upon viral infection of the host cell, viral proteins and peptides in the cytoplasm will be degraded into small antigenic peptides by the proteasome. Before these proteasome-derived antigenic peptides can be presented on the cell surface via MHCI, they have to be translocated from the cytosol into the endoplasmic reticulum. This transport is an ATP-dependent process sustained by the transporter associated with antigen processing (TAP) (Figure 8). Viral antigenic peptides will be mounted on MHCI during a series of consecutive steps in the ER. This MHCI-Ag complex will then leave the endoplasmic reticulum and be transported through the Golgi network towards the plasma membrane where the viral Ag is presented by MHCI for recognition by specific CTL (Figure 8). Alphaherpesviruses, such as

PRV, HSV1, BHV1, EHV4 and EHV1, have developed several mechanisms to prevent the function of the TAP transporter, often via the UL49.5 protein. Although all these viruses encode pUL49.5, not all pUL49.5 orthologues inhibit the TAP (Koppers-Lalic et al., 2008). For example, HSV1 pUL49.5 does not inhibit TAP, but the HSV1 ICP47 inhibits TAP function by competing with the antigenic peptides for binding to TAP (Ahn et al., 1996). In contrast, the pUL49.5 proteins of PRV, BHV1, EHV4 and EHV1 are functional TAP inhibitors, although the individual mechanisms differ. For example, BHV1 and PRV pUL49.5 inhibit TAP by locking it in a translocation incompetent state. In addition, BHV1 pUL49.5 (but not PRV pUL49.5) also inhibits TAP by proteasome-dependent degradation. In contrast, TAP inhibition by EHV1 and EHV4 is mediated by preventing ATP binding to TAP (Koppers-Lalic et al., 2008).

Stable transfection of BHV1 pUL49.5 in human cells, including human MDDC, was shown sufficient for TAP inhibition (Lampen et al., 2010) and stable overexpression of EHV1 pUL49.5 in ED cells, either by transfection (EHV1) or retrovirus transduction (EHV4), was also capable to significantly downregulate peptide transport and cell surface MHCI expression. Nevertheless, deleting pUL49.5 encoding regions in EHV1 or EHV4, so in the virus background, was not sufficient to abolish MHCI downregulation, despite a significant upregulation of TAP-dependent peptide transport (Koppers-Lalic et al., 2008; Ma et al., 2012; Said et al., 2012).

4. CD172a positive cells and their role during herpesvirus infections

Recently, it was demonstrated that most of the EHV1-infected cells in the respiratory tissues, draining lymph nodes and blood are positive for the cell surface marker CD172a, indicating the importance of CD172a-positive cells in the pathogenesis of EHV1 (see also #1.4.)

(Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). The current dissertation is focused on two CD172a-positive cell types, namely DC and mesenchymal stem cells (MSC), and so these two cell types are discussed in more detail below.

4.1. General

In general, CD172a, also known as SIRP-α or SHPS-1, belongs to the SIRP family which are transmembrane proteins with closely related extracellular Ig-like domains and differing intracellular signaling tails. This diverse multigene family of immune receptors consists of inhibitory α, activating β, non-signaling γ and soluble δ members (van Beek et al., 2005). The first and best characterized member of this family, SIRP-α, is relatively ubiquitously expressed on myeloid cell such as macrophages, granulocytes and myeloid DC (including equine MDDC) (Baghi et al., 2014; Flaminio et al., 2007), but also on human and rat MSC (Rooney et al., 2008; Vogel et al., 2003). Generally, upon ligand binding, the four immunoreceptor tyrosine-based inhibitory motifs in the cytoplasmic tails of SIRP-α will become phosphorylated, thereby attracting and subsequently activating tyrosine phosphatases (e.g. SHP-1/2). These activated tyrosine phosphatases will in turn dephosphorylate target proteins which results in mostly negative regulation of cell function. Viruses may exploit the negative regulation by SIRP-α. For example, infection of rabbits with a myxomavirus unable to express the M128L protein, which is a viral homolog of the SIRP-α ligand CD47, resulted in increased survival and higher levels of inducible nitric oxide synthase in macrophages (Cameron et al., 2005). However, SIRP-α can also trigger activating signaling resulting in positive regulation of cell function. For example, an *in vitro* rat study demonstrated that CD47 ligation on macrophages induces nitric oxide production (Alblas et al., 2005). Although that most reports describe a negative signaling upon SIRP-α engagement, the study by Ablas et al. (2005) did describe a positive signaling after SIRP-α binding (Alblas et al., 2005), possibly

through a still to be identified mechanism where the two proline rich regions found in the cytoplasmic tail of human SIRP-α could be involved. These two proline rich regions could form docking sites for the SH3 domains of other tyrosine kinases and adaptor molecules (van Beek et al., 2005).

4.2. Dendritic cells (DC)

4.2.1. Major DC subsets in the respiratory system

DC are present in the respiratory epithelium and the lamina propria of the URT and lungs, where their dendrites will reach into the respiratory lumen. These DC have a high turnover under normal steady-state conditions and are swiftly recruited after exposure to pathogens. Mucosal tissue of the URT, such as in the nasopharynx, harbors the nasopharynx-associated lymphoid tissue where immature DC will contact B-lymphocytes (Holt and Stumbles, 2000; Lee and Iwasaki, 2007). DC found in these mucosal tissues are typically cDC of the migratory type and are derived from monocytes or common/macrophage DC precursors. In addition, pDC are scarcely present in the mucosa during steady-state conditions and are primarily found in the blood, but still in low numbers. Migratory cDC can also be found within the blood, but only in low numbers. Next to the migratory cDC, high numbers of non-migratory cDC are found within lymphoid tissues such as the draining lymph nodes. While sampling, processing and Ag presentation are the main functions of both cDC types, only migratory cDC are characterized by a high motile life style which is significantly enhanced upon encountering an activation stimulus such as a respiratory virus. Quite contrary, the main response of pDC upon stimulation, for example by a respiratory virus, is not Ag presentation but secretion of large amounts of IFN type I and III. During the early innate antiviral immune response, these natural interferon producing cells are crucial in activating innate immune effectors such as NK cells and macrophages. In contrast, although cDC also mediate the

innate antiviral immune response by secreting cytokines such as IFN-α, their main function remains that of an APC and as such to induce virus-specific adaptive immune responses such as generation of virus-specific CTL (Van de Walle et al., 2009a).

In men and mice, different DC subsets have been described and studied based on the isolation of these cells from lymphoid organs and the blood. In horses, however, the general accepted model for myeloid DC studies are MDDC and these are typically generated by culturing equine monocytes with IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF). After several days, MDDC have a typical dendrite/veiled morphology and are further characterized by their MHCI^{high}, MHCII^{high}, CD11a/CD18^{high}, CD172a^{high}, CD206$^{med-high}$, CD86$^{med-high}$ and CD83$^{low-med}$ immunophenotype (Baghi et al., 2014; Cavatorta et al., 2012; Dietze et al., 2008; Durán et al., 2013; Flaminio et al., 2007; Hammond et al., 1999; Mauel et al., 2006; Moyo et al., 2013; Steinbach et al., 2009). Such differentiation from monocytes to immature MDDC also occurs *in vivo* and the vast majority of human studies also have employed MDDC as a model to evaluate the interaction of human viruses with myeloid DC (Pollara et al., 2005).

4.2.2. Maturation response of mucosal DC

In the human and murine system, mucosal migratory cDC are proposed to be the first DC subtype to encounter the invading virus during the initial stages of viral mucosal infection. Although a small proportion of resident pDC will also make viral contact, most pDC encounter the virus at later stages of infection, e.g. upon arrival to the infection site by inflammation-induced migration, during viremia or in the secondary lymphoid organs (Pollara et al., 2005). Viral replication in the epithelial cells results in the release of the CX chemokine ligands (CXCL) 9-12 which attracts chemokine receptor (CXCR) 3 and CXCR4 positive immature migratory cDC, including those cDC present in the blood. DC can be activated by

interaction of pathogen associated molecular patterns with pattern recognition receptors such as toll-like receptor 3 (Steinman and Banchereau, 2007) and rapid maturation of the immature endocytic-active migratory cDC is typically initiated upon encountering the virus in the mucosa. Maturation is characterized by the rapid loss of endocytic capacity, increased expression and lower turn-over of MHCI and MHCII based Ag presentation on the cell surface, increased expression of adhesion (e.g. CD54) and co-stimulatory molecules (e.g. CD40, CD80, CD83, CD86) and increased motility and secretion of pro-inflammatory cytokines and chemokines (Banchereau and Steinman, 1998; Van de Walle et al., 2009a). Upon activation, maturing cDC and pDC will follow the CC chemokine ligand 19/21 gradient towards the draining lymph nodes by downregulating the CXCR4 receptor and upregulating the CC chemokine receptor 7 receptor on their cell surface. Freshly activated maturing migratory cDC will release a first wave of chemokines such as CXCL1-3, CXCL7-8 and CXCL16, thereby attracting CXCR2+ neutrophils and CXCR6+ Th1 cells. Upon initiation of migration, maturing cDC and pDC release several cytokines such as IL-1 and IL-2 or type I and III IFN, respectively, which mediate influx of innate effectors such as neutrophils, NK-cells and macrophages. Next, during migration, cDC release a second wave of chemokines, including CCL3-5, CCL8 and CXCL9, recruiting monocytes and CCR5+ memory T cells. Mucosal cDC will reach the draining lymph nodes mainly through the afferent lymphatics, while pDC mainly enter draining lymph nodes from the blood. Upon arrival in the draining lymph nodes, the Ag presenting cDC will release a third wave of chemokines, namely the Th2 and Treg attracting CCL22, the CCR7+-naïve-T attracting CCL19 and CXCR5+-naïve-B attracting CXCL13. Upon contact with naïve T cells, cDC will undergo terminal maturation and initiate the adaptive immune response by selective priming and clonal expansion of specific naïve T cells, which are critical to generate adaptive immune effectors such as CTL and plasma cells (Van de Walle et al., 2009a).

4.2.3. Relevance during viral infection

It is commonly accepted that DC play an essential role during viral infection (Banchereau et al., 2000; Banchereau and Steinman, 1998; Steinman and Banchereau, 2007). Their essential role in antiviral immunity was clearly demonstrated *in vivo* by inducing and maintaining CTL responses and protection from lethal challenge with lymphocytic choriomeningitis virus after intravenous administration of lymphocytic choriomeningitis virus presenting DC in mouse (Ludewig et al., 1999, 1998).

Herpesvirus infections are mainly controlled by CD4+ and CD8+ T effector cells, through the release of IFN-γ and generation of virus specific cytotoxicity (Banchereau et al., 2000). Also for the prototypical α-herpesvirus HSV1, the pivotal role for DC in antiviral immunity became apparent when depleting epidermal DC, known as Langerhans cell, from the footpad of mice resulted in enhanced virulence of both pathogenic and apathogenic HSV1 strains (Sprecher and Becker, 1986). In addition, depletion of Langerhans cell from footpads abolished *in vitro* HSV1-specific lymphoproliferative responses (Sprecher and Becker, 1989). Also for HSV1, DC were found to be the most potent inducers of CTL responses since drastically higher frequency of the CTL precursors where found *in vitro* when semi-purified splenic DC where used as stimulator cells, compared to raw splenic or peritoneal exudate stimulator cells (Hengel et al., 1987). Later on, it was demonstrated that migration of skin DC, predominantly of dermal versus epidermal origin, towards draining lymph nodes is essential for adequate HSV1 specific CTL generation. However, these migratory skin DC did not make contact with CD8+ T-cells and only presented viral Ag via MHCII. The major APC presenting HSV1 Ag via MHCI to CD8+ CTL in these draining lymph nodes where the draining lymph nodes resident DC and, interestingly, could only do so when migration of skin DC towards the draining lymph nodes was not blocked (Allan et al., 2006). In contrast, when

HSV1 is applied to mucosal surfaces such as the vaginal mucosa, migratory DC did make direct contact with T-cells and where found to be the main CD4+ or CD8+ T-cell priming APC in the draining lymph nodes (Lee et al., 2009).

In a recent *in vitro* study, mock- or EHV1-infected equine blood monocytes, nasal mucosal monocytic cells or MDDC were applied onto the apical side of nasal mucosal explants and the results showed that EHV1 infection did not alter the migration patterns of these CD172a-positive cells and that only MDDC were present below the basement membrane at 24h after applying these cells to the explants (Baghi and Nauwynck, 2014).

4.3. Mesenchymal stem cells (MSC)

4.3.1. Definition and differentiation capabilities of MSC

Stem cells have three properties in common: the **(i)** ability of self-renewal, the **(ii)** ability to differentiate in multiple cell types and the **(iii)** ability to reconstitute a given tissue *in vivo*. Based on differentiation capabilities, stem cells can be classified as totipotent, pluripotent, multipotent or unipotent stem cells. Cells isolated from the embryo up to the eight cell-stage morula are termed totipotent as they can differentiate in all cells of the extra-embryonal and embryonal tissues, including adult stem cells such as MSC. MSC display self-renewal properties and the ability to differentiate into mesodermal cells such as fibroblasts, chondrocytes, osteocytes, adipocytes and myocytes, and are therefore termed multipotent (Lakshmipathy and Verfaillie, 2005).

The acronym MSC should be *strictu sensu* reserved for mesenchymal stem cells that show **(i)** long term survival *in vivo*, display **(ii)** self-renewal *in vivo* and are able **(iii)** to reconstitute tissues *in vivo* based on their differentiation capabilities (Horwitz et al., 2005). In contrast, the International Society of Cellular Therapy postulated that human cells that show **(i)** self-

renewal *in vitro*, the **(ii)** ability to adhere to plastic surfaces *in vitro*, **(iii)** tri-lineage differentiation capabilities *in vitro* and the **(iv)** expression of a specific set of cell surface markers ($CD73^{pos}$, $CD90^{pos}$, $CD105^{pos}$, $CD14^{neg}$, $CD34^{neg}$, $CD45^{neg}$, $CD79\alpha^{neg}$, $MHCII^{neg}$) *in vitro*, should be referred to as mesenchymal stromal cells (Dominici et al., 2006). Since the equine mesenchymal cells used in this thesis were characterized based on *in vitro* properties, they should be termed equine mesenchymal stromal cells. However to minimize confusion, we will refer to this cell population using the acronym MSC and as "mesenchymal stem cells" throughout this PhD.

In general, equine MSC are characterized based on the guidelines of the International Society of Cellular Therapy for human MSC and so they should **(i)** display the typical spindle-shaped morphology, **(ii)** be plastic adherent , **(iii)** express a specific panel of surface markers and **(iv)** be able to perform a tri-lineage differentiation towards adipocytes, chondrocytes and osteocytes. The immunophenotypical profile of equine MSC, typically evaluated by flow cytometry, should be positive for CD29, CD44, CD90 and CD105, and negative for CD45, CD79α and MHCII (De Schauwer et al., 2012; Spaas et al., 2013). An overview of these typical cell surface markers expressed on equine MSC can be found below in Table 3.

Table 3: Typical cell surface markers expressed by equine MSC.

Cell surface markers	***Function***	***References***
CD29 (β1 integrin)	Adhesion, migration, signaling	(Ip et al., 2007; Miyamoto et al., 1995; Rüster et al., 2006; Steingen et al., 2008)
CD44	Migration, signaling, differentiation, proliferation, survival	(Herrera et al., 2007; Ponta et al., 2003; Poulsom, 2007)
CD90 (Thy-1)	Adhesion, migration, signaling, apoptosis, nerve regeneration, inflammation, HLA-G mediated immune suppression	(Campioni et al., 2009; Rege and Hagood, 2006)
CD105 (endoglin)	Migration, signaling, differentiation	(Anderson et al., 2013; Jaganathan et al., 2007; Qi et al., 2011; Romieu-Mourez et al., 2007; Sanz-Rodriguez et al., 2004)

4.3.2. MSC in the respiratory system

Human MSC can be isolated from nearly all adult tissues including the nose, trachea, bronchi, lung and peripheral blood. In addition to isolation from tissue, MSC can also be isolated from broncho-alveolar lavage fluid (da Silva Meirelles et al., 2006; Hennrick et al., 2007; Jakob et al., 2010; Lama et al., 2007; Popova et al., 2010; Ricciardi et al., 2012; Rolandsson et al., 2014; Sabatini et al., 2005; Sinclair et al., 2013). Furthermore, several authors have proposed that MSC are also part of the perivascular compartment in fetal and adult tissues (Caplan, 2008; Crisan et al., 2008; da Silva Meirelles et al., 2006; Murray and Péault, 2015; Rolandsson et al., 2014; Wong et al., 2015). These MSC, also named pericytes, showed a significant chemoattraction towards extracellular matrix (ECM) digest *in vitro* and migration of these MSC from their perivascular niche into tissues, including the lung, has already been demonstrated *in vivo* (Birbrair et al., 2015; Crisan et al., 2008; Wong et al., 2015). Also equine MSC can be isolated from a wide range of tissues and these include the bone marrow, fat, amniotic fluid, gingiva, periodontal ligament, umbilical cord blood and the peripheral blood. Isolation from this last source is technically less challenging and less invasive than most other isolation procedures (Braun et al., 2010; De Schauwer et al., 2012, 2011; Lovati et al., 2011; Mensing et al., 2011; Spaas et al., 2013)

4.3.3. Homing

It has been well described that MSC will migrate towards and accumulate within injured tissues and organs (Sohni and Verfaillie, 2013). MSC express several adhesion molecules (e.g. CD44), integrins (e.g. CD29), matrix metalloproteinases and chemokine receptors such as CCR1, CCR4, CCR7, CCR10 , CXCR4, CXCR5, CXCR6 which are involved in migration and homing (Farini et al., 2014; Herrera et al., 2007; Sohni and Verfaillie, 2013; Wei et al., 2013). For example, recruitment and healing of CXCL12 expressing injured tissues was

severely impaired by inhibition of CXCR4 signaling and vice versa greatly enhanced if MSC overexpressed CXCR4 (Eggenhofer et al., 2014; Farini et al., 2014; Rüster et al., 2006; Wong et al., 2015).

4.3.4. Immunomodulation capabilities of MSC

Due to their proposed immunosuppressive properties, MSC are attractive therapeutic candidates and have been validated for their effect on several diseases such as stroke, graft versus host disease and acute lung injury in humans (Wei et al., 2013). Although MSC were originally defined as immuno-privileged, which refers to their reduced rejection because of MHC mismatch, more recent studies are fueling the standpoint that MSC are not immune-privileged, but immunosuppressive. Indeed, and although administration of allogeneic MHC mismatched MSC induces cellular and humoral immune responses *in vivo*, including innate immune responses, the rejection of such mismatched MSC is in general slower than observed for other allogeneic cell types such as hematopoietic stem cells (Ankrum et al., 2014). However, MSC are not only capable to suppress, but also to enhance immune responses *in vivo* depending on the stimuli provided from the micro-environment, a process known as licensing. For example, pro-inflammatory cytokines such as IFN-γ and TNF-α can stimulate the MSC to release immunosuppressive factors (Krampera, 2011). Moreover, human MSC are able to perform Ag presentation functions and to display anti-microbial activity, which is mainly documented for bacteria and to a lesser extent fungi (Balan et al., 2014).

The available information on the immunological properties of equine MSC is much more limited compared to human or murine MSC, but for what is known thus far, appears to be similar between these 3 species. Equine MSC, just like human and murine MSC, express MHCI at intermediate levels and do not express MHCII on their cell surface. It is currently unknown if equine MSC express T-cell costimulatory proteins on their cell surface, although

mRNA expression of CD40 and CD80 has been demonstrated (De Schauwer et al., 2014). In men and mouse, it was shown that T-cell proliferation was not enhanced but rather suppressed by co-incubation with MSC, and this could be linked to the production of soluble mediators by MSC such as indoleamine 2,3-dioxygenase, nitric oxide, IL-6, transforming growth factor (TGF) β1, prostaglandin E2 and IL-10 (Kyurkchiev, 2014). In the horse, quantitative reverse transcriptase polymerase chain reaction analyses demonstrated that unstimulated MSC from umbilical cord or peripheral blood expressed TGF-β, CD40 and CD80 but not indoleamine 2,3-dioxygenase, TNF-α or CD86 (De Schauwer et al., 2014). Carrade et al. (2012) demonstrated that allogene- or phytohemagglutinin-stimulated, but not unstimulated, equine MSC could significantly inhibit T-cell proliferation. A model was proposed to explain these observations as follows: unstimulated equine MSC first become activated by IFN-γ and TNF-α that is secreted by stimulated and proliferating T-lymphocytes. These activated equine MSC will then secrete factors such as IL-6, prostaglandin E_2 and nitric oxide which in turn, either directly or indirectly, will downregulate T-cell proliferation, resulting in a lower IFN-γ and TNF-α release and hence, MSC activation (Carrade et al., 2012).

4.3.5. Interaction between herpesviruses and MSC

To date, only a handful of studies have evaluated the interaction between MSC and herpesviruses, including the human α-herpesvirus HSV1, the β-herpesviruses human cytomegalovirus (HCMV) and murine cytomegalovirus, and the γ-herpesviruses Kaposi's sarcoma-associated herpesvirus (KSHV), bovine herpesvirus 4 and Epstein-barr virus (Choudhary et al., 2011; Donofrio et al., 2005; Parsons et al., 2004; Smirnov et al., 2007).

The α-herpesvirus HSV1 was shown to productively infect human MSC, through a mechanism that is dependent on actin cycling and/or gD interaction with its receptors (Choudhary et al., 2011). The human β-herpesvirus HCMV was also shown capable of

productively infecting human MSC and this infection resulted in a drastic reduction of MHCI and CD90, and a strong reduction of CD29, CD44, CD73 and CD105. Interestingly, HCMV infection induced a significant upregulation of intercellular adhesion molecule (ICAM) 1 (CD54) and lymphocyte function associated antigen (LFA) 3 (CD58). ICAM-1 could also be upregulated by UV-inactivated HCMV and HCMV supernatants, although to a lesser extent when compared to live HCMV. This drastic attenuation of the cell surface profile in HCMV-infected MSC resulted in an increased adhesion capacity to several hematopoiesis-like cell lines like U937 (pro-monocytic cell line) and Jurkat (T-lymphoblast cell line) cells, and was shown to be partially dependent on ICAM-1. Osteogenic and adipogenic differentiation capability of MSC were also significantly reduced by HCMV infection (Smirnov et al., 2007).

Although the γ-herpesvirus KSHV could infect human MSC, such infection was not productive and resulted in a latent infection that was partially sensitive to epigenetic inhibitors (Parsons et al., 2004). In addition, inoculation with the γ-herpesvirus Epstein-barr virus did not yield productive infection of human MSC although viral DNA was detectable. In contrast, human MSC were found susceptible to productive infection by CMV and HSV1 (Sundin et al., 2006). The bovine γ-herpesvirus BHV4 could productively infect bovine MSC, yielding titers that were even higher than observed in cells normally used for virus stock generation (Donofrio et al., 2005).

Chapter 2: Aims

Equine herpesvirus (EHV) 1 is a ubiquitous α-herpesvirus in horse populations worldwide and can cause respiratory disease, abortion, equine herpes myeloencephalopathy (EHM) and even chorioretinopathy. After primary replication in respiratory epithelial cells, the virus will spread cell-associated, without destruction of the basement membrane, into the respiratory connective tissue, blood and draining lymph nodes. During the cell-associated viremia, a replication cycle will be initiated in the endothelial cells of targets organs. Interestingly, recent *in vivo* and *in vitro* nasal explant studies have demonstrated that the majority of these EHV1-infected cells are positive for the cell surface marker CD172a (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010).

Interestingly, migratory CD172a-positive equine dendritic cells (DC) and human and rat CD172a-positive mesenchymal stem cells (MSC) are present in the respiratory system, lymph nodes and blood of these species (Baghi et al., 2014; Holt and Stumbles, 2000; Lee and Iwasaki, 2007; Lim et al., 2010; Rooney et al., 2008; Sinclair et al., 2013; Spaas et al., 2013; Vogel et al., 2003). It has been previously demonstrated that human MSC are susceptible to the human α-herpesvirus herpes simplex virus type 1 (HSV1) and the β-herpesvirus human cytomegalovirus (HCMV) (Choudhary et al., 2011; Smirnov et al., 2007). HCMV-infected human MSC showed an altered expression profile of cell surface proteins (Smirnov et al., 2007). Furthermore, it has been shown that human α-herpesviruses such as HSV and varicella zoster virus (VZV) disturb the capacity of CD172a-positive DC to support T-proliferation, and this coincided with a reduced cell surface expression of markers associated with antigen presentation, co-stimulation and adhesion (Elboim et al., 2013; Kobelt et al., 2003; Kruse et al., 2000b; Kummer et al., 2007; Lampen et al., 2010; Mikloska et al., 2001; Morrow et al., 2003; Martin J Raftery et al., 2006; Salio et al., 1999; Samady et al., 2003). For EHV1 it has also been reported that infection impairs equine DC to support T-proliferation (Siedek et al., 1999, 1997), but the consequences of EHV1 infection, and as such the possible effect of viral

proteins on the expression of functionally important DC cell surface markers has not been explored to date.

Therefore the aims of this thesis were:

- To evaluate the consequences of EHV1 infection on the immunophenotypical profile of equine monocyte-derived dendritic cells (MDDC), specifically those proteins associated with antigen presentation, co-stimulation and adhesion. In addition, we aimed to determine the responsible viral factor(s) mediating the modification of these cell surface markers **(Chapter 3)**.
- To start exploring the cellular mechanisms used by EHV1 to induce downregulation of cell surface markers in equine MDDC, focusing on the downregulation of major histocompatibility complex (MHC) I, an antigen-presenting protein essential for the elimination of virus-infected cells by cytotoxic T cells (CTL) **(Chapter 4)**.
- To evaluate if equine blood-derived MSC express the cell surface marker CD172a and are susceptible to EHV1 infection. In addition, to study the consequences of EHV1 infection on the expression of cell surface proteins, generally used to immunophenotype MSC. Finally, we aimed to identify the underlying viral factor(s) responsible for such attenuation of the MSC cell surface marker profile **(Chapter 5)**.

Chapter 3: EHV1 induces alterations in the immunophenotypic profile of equine monocyte-derived dendritic cells.

Adapted from:

Christophe Claessen, Valérie De Lange, Teng Huang, Guanggang Ma, Nikolaus Osterrieder, Herman Favoreel, Gerlinde R. Van de Walle

The Veterinary Journal 210 (2016) 85–88

1. Abstract

Equine herpesvirus (EHV) 1 is an α-herpesvirus, ubiquitous in horse populations worldwide, that can cause respiratory, reproductive and central nervous disorders. EHV1 can infect a variety of different cells *in vitro* and *in vivo*, including dendritic cells (DC). DC are essential in the immune response against EHV1 since they induce the generation of cytotoxic T-lymphocytes (CTL). The present study shows, using monocyte-derived DC (MDDC), that EHV1 infection of equine MDDC downregulates cell surface expression of major histocompatibility complex (MHC) I, CD83, CD86, CD206, CD29 and CD172a, but not of CD11a/CD18 and MHCII . Unlike for other α-herpesviruses, this downregulation was not mediated by the virion host-shutoff (VHS) protein or the transporter associated with antigen processing (TAP)-inhibitor unique long protein (pUL) 49.5, nor did it depend on virus-induced secretory factors. Interestingly, downregulation of CD83 and CD86, but not the other cell surface markers, was in part mediated by the early viral protein pUL56. Taken together, these data indicate that EHV1 employs different and still unresolved mechanisms to induce downregulation of several functionally important cell surface proteins on equine DC.

2. Introduction

The Herpesviridae family consists of the three subfamilies α-, β-, γ-herpesvirinae, which can cause disease in humans and animals. These large DNA viruses are known for their efficient immune evasion strategies and consequently, encode a variety of viral proteins that antagonize the host immune system (Hewitt, 2003; Loch and Tampé, 2005; Vossen et al., 2002).

An adequate immune response against herpesviruses requires the following factors: (i) innate natural killer (NK) cell-mediated cell lysis, (ii) production of virus-neutralizing antibodies by B-lymphocytes and, most importantly, (iii) generation of cytotoxic T-lymphocytes (CTL) (Minke et al., 2004). In contrast to B-lymphocytes, T-lymphocytes cannot directly recognize premature antigens, unless they are primed by antigen presenting cells (APC) that mediate MHC-dependent antigen presentation. Dendritic cells (DC) are a subset of APC that play an essential role in the control of viral infections due to their capacity of eliciting CTL responses and maintaining protective antiviral immunity (Ludewig et al., 1999, 1998). They patrol sites of pathogen invasion such as the respiratory mucosa and, upon uptake of pathogens, migrate towards the secondary lymphoid organs to present the processed antigens to naïve CD4+ and CD8+ T-lymphocytes, resulting in activation of these T-lymphocytes and eventually clearance of viral infection (Banchereau and Steinman, 1998; Holt and Stumbles, 2000; Lambrecht and Hammad, 2012).

Since DC are one of the first immune cells to encounter α-herpesviruses at the infection site and they are crucial in mounting an adequate immune response, α-herpesviruses have evolved several mechanisms to thwart and even exploit DC function to enhance spread and prevent elimination by the host's immune system (Bedoui and Greyer, 2014; Novak and Peng, 2005; Van de Walle et al., 2009a).

Infection of human DC by the human α-herpesviruses herpes simplex (HSV) 1 and 2 and varicella zoster virus (VZV) impaired the capacity of DC to support T-lymphocyte proliferation (Kruse et al., 2000b; Morrow et al., 2003; Peretti et al., 2005). In addition, infection of human DC resulted in a selective, and viral protein-mediated, downregulation of cell surface molecules associated with adhesion (CD54), antigen presentation (MHCI, CD1a) and co-stimulation (CD40, CD80, CD83, CD86), but did not lowered cell surface expression of MHCII and CD11c (Elboim et al., 2013; Kobelt et al., 2003; Kruse et al., 2000a; Kummer et al., 2007; Lampen et al., 2010; Mikloska et al., 2001; Morrow et al., 2003; Martin J Raftery et al., 2006; Salio et al., 1999; Samady et al., 2003).

EHV1 is a ubiquitous α-herpesvirus in horse populations worldwide and continues to cause significant economic losses due to symptoms such as respiratory disease, abortion, neonatal death and equine herpesvirus myeloencephalopathy (EHM). Following inhalation, the virus initiates a primary replication within the upper respiratory epithelium. Subsequently, the virus spreads via the bloodstream and infects the endothelial cells of important target organs such as the pregnant uterus and the central nervous system (Allen and Bryans, 1986; Dunowska, 2014b; Lunn et al., 2009a). Similar to other α-herpesviruses, equine DC are susceptible to EHV1 infection and such infection did reduce their capacity to support T-lymphocyte proliferation (Siedek et al., 1999, 1997). The consequences of EHV1 infection on cell surface protein expression of equine DC, however, has never been examined to date. Therefore, we hypothesized that EHV1 also downregulates several cell surface markers on equine MDDC.

Here, we report that EHV1 induces a downregulation of MHCI, CD83, CD86, CD172a, CD206 and CD29, but not of CD11a/CD18 and MHCII on equine MDDC. Surprisingly, and in contrast to the findings for other α-herpesviruses, downregulation did not depend on expression of the viral proteins VHS or pUL49.5, nor did it depend on viral-induced secretory

factors. Interestingly, downregulation of the co-stimulatory B7 protein CD86 and the B7-like CD83 was in part mediated by the early viral protein pUL56.

3. Materials and methods

3.1. Cells and viruses

Rabbit kidney (RK13) cells were maintained in Dulbecco's Minimum Essential Medium (DMEM low glucose; Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/mL penicillin and 0.1 mg/mL streptomycin (1% Pen/Strep; Invitrogen), at 5% CO_2 and 37 °C. The enhanced green fluorescent protein (eGFP) expressing wildtype Ab4G and mutant viruses were propagated in RK13 cells and were described previously. Isogenic mutant viruses used in this study were Ab4GΔ1, Ab4GΔ10, Ab4G Δ19 and Ab4G Δ63 which lacked the pUL56-encoding open reading frame (ORF) 1, the pUL49.5-encoding ORF10, the VHS-encoding ORF19 and the EICP0-encoding ORF63, respectively (Goodman et al., 2007; Huang et al., 2015; Ma et al., 2012).

Equine MDDC were generated as previously described, with minor modifications (Baghi et al., 2014; Dietze et al., 2008; Hammond et al., 1999). Briefly, equine heparinized peripheral venous blood was collected after approval and under the guidelines of the institutional Ethical Committee (EC2010/147). After collection, blood was diluted 1:1 with $Ca2^{+}$/$Mg2^{+}$ free phosphate buffered saline (CMF-PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) (VWR) at room temperature. Diluted blood was layered onto Ficoll-Paque Plus (GE, 1.077 g/mL) and centrifuged at 18 °C for 30 min at 950 g, without brake. The peripheral blood mononuclear cell (PBMC) layer was collected, washed twice in CMF-PBS and once in RPMI-1640 (L-glutamine, Invitrogen), all at 4 °C. PBMC were then resuspended in monocyte isolation medium (MO-CM: RPMI-1640, 1% Pen/Strep) and seeded in 6-well culture dishes

(Thermo Scientific, Nunclon Delta Surface). After 2 h at 5% CO_2 and 37 °C, non-adherent cells were removed by washing with RPMI-1640 and the adherent cells were maintained in MO-CM with 5% FCS overnight at 5% CO_2 and 37 °C. After removing non-adherent cells by washing again, MDDC were generated by maintaining adherent cells for 3 days in MO-CM supplemented with 10% FCS, 10 ng/mL recombinant equine interleukin 4 (IL-4) (R&D) and 20 ng/mL recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Kingfisher Biotech).

3.2. Antibodies

Cells were labeled for the following cell surface markers: CD11a/CD18 (Serotec, CVS9), CD29 (Chemicon, TDM29), CD83 (Biolegend, HB15e), CD86 (Biolegend, IT2.2), CD172a (VMRD, DH59B), CD206 (Biolegend, 15-2), MHCI (VMRD, PT85A), MHCII (Novus, CVS20). Secondary antibodies were conjugated with Cy5 (Invitrogen). Isotype controls were IgG2b (Biolegend, MPC-11) and in-house made IgG1 (anti-porcine reproductive and respiratory syndrome virus) and IgG2a (anti-porcine circovirus type 2) antibodies.

3.3. Flow cytometry

MDDC were mock-infected or infected with the various eGFP-expressing viruses at a multiplicity of infection (MOI) of 1, unless otherwise indicated. In a separate set of experiments, MDDC were incubated with supernatants from mock-infected MDDC or MDDC infected for 16 h with Ab4G. Cells and virions were cleared from supernatants by centrifugation at 2,000 g and ultracentrifugation at 30,000 g, respectively. At 16 h post inoculation (hpi) or post supernatants addition, cells were detached with accutase (Invitrogen)

and subsequently washed once in CMF-PBS-5%FCS before labeling with primary antibodies, diluted in CMF-PBS-20%FCS, for 30 min on ice. Cells were washed with CMF-PBS-5%FCS and incubated with Cy5-conjugated secondary antibody for 30 min on ice in the dark. After washing with CMF-PBS-5%FCS, cell viability was assessed with 7-amino-actinomycin D (7AAD, Invitrogen). For each sample, at least 10,000 cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser. Only 7AAD negative (live) mock cells or 7AAD negative (live) and eGFP positive (infected) inoculated cells were evaluated. Isotype controls were included to correct for non-specific binding and spectral overlap between used fluorochromes was compensated with an automatic calibration technique (FACSDiva software, Becton Dickinson). The expression levels of surface markers were presented as percentage of positive cells compared to the isotype control or as mean fluorescence intensities normalized to the isotype control (mean fluorescence intensity ratio, MFIR). All data were analyzed using FACSDiva software (Becton Dickinson) and expressed as mean ± standard error of the mean (SEM).

3.4. Statistical analysis

Statistical analysis was performed with Prism (GraphPad) for at least three independent repeats on at least two different horses. Results are expressed as the mean ± SEM. A two-sided Student's t test for paired observations was used to evaluate statistical differences between mock(_SN)- and Ab4G(_SN) incubated MDDC at $P < 0.01$. One-way repeated measures ANOVA analysis with the Tukey post-test (95% confidence interval) was performed for all other comparisons at $P < 0.01$.

4. Results

4.1. Mock phenotype of equine MDDC

Equine MDDC were generated as previously described and characterized morphologically (Figure 1a), as well as by immunoprofiling using flow cytometry (Table 1). For the latter, cells were immunophenotyped as MHCI^{high}, MHCII^{high}, CD11a/CD18^{high}, CD172a^{high}, CD206$^{med-high}$, CD86$^{med-high}$ and CD83$^{low-med}$, which is in agreement with previous studies (Baghi et al., 2014; Dietze et al., 2008; Hammond et al., 1999). Furthermore, we explored the expression of CD29 on equine MDDC, a β1 integrin that is expressed on human blood DC (de Andrés et al., 2012). Equine MDDC were found to be CD29^{high} (Table 1), indicating that this cell surface marker can also be used to immunophenotype equine DC.

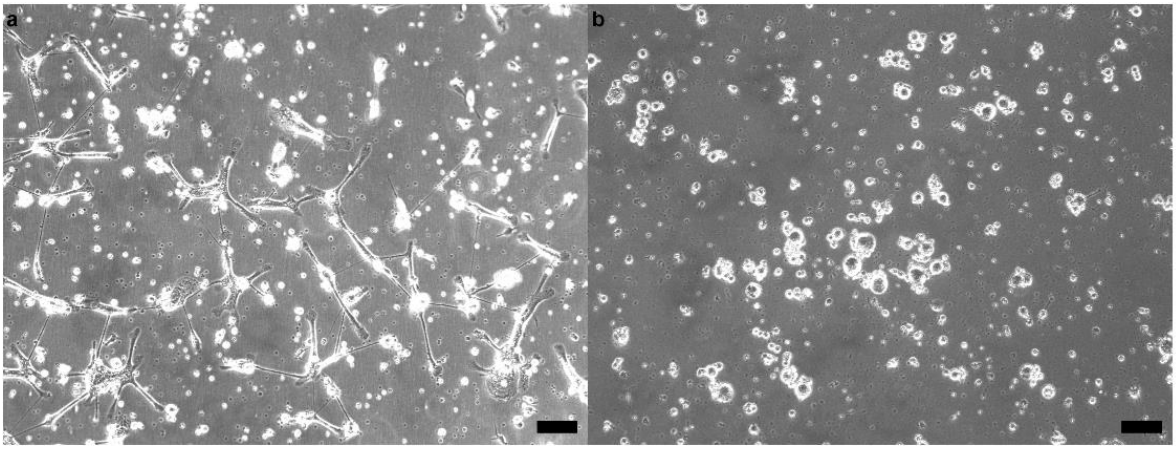


Figure 1: Morphology of equine monocyte-derived dendritic cells (MDDC). At day 1, equine PBMC were incubated with recombinant equine GM-CSF and IL-4. Photomicrographs of 10x magnification fields at day 4 from mock-inoculated MDDC (a) and MDDC inoculated for 16 h with the eGFP-expressing EHV1 strain Ab4G at MOI 10 (b). Scale bar = 100 µm

Table 1: Mock immunophenotype of equine monocyte-derived dendritic cells at day 4 post cultivation.

Cell surface protein	Mean % positive cells	SEM	Role in immunity
MHCI (HLA-ABC)	99.84	0.03	Endogenous Ag presentation to CD8+ (Banchereau and Steinman, 1998; Loch and Tampé, 2005; Steinman and Banchereau, 2007)
MHCII (HLA-DR)	95.07	0.79	Exogenous Ag presentation to CD4+ T (Banchereau and Steinman, 1998; Steinman and Banchereau, 2007; Zuo and Rowe, 2012)
CD11a/CD18 (LFA-1, αL/β2 integrin)	98.75	0.29	Trans-endothelial migration, immunological synapse and DC-T contact duration (Balkow et al., 2010; Bleijs et al., 2001; Borgman et al., 2014; Mittelbrunn et al., 2004; Rouzaut et al., 2010; Theodoridis et al., 2011)
CD29 (β1 integrin)	93.25	1.19	Adhesion, migration and signaling (Brown et al., 1997; de Andrés et al., 2012)
CD83 (BL11)	25.24	1.33	B7-like function (enhances T-cell priming) (Kruse et al., 2000a; Prechtel et al., 2007; Scholler et al., 2001)
CD86 (B7.2)	57.13	3.41	Binds CD28 for co-stimulatory signal upon MHC-TCR interaction (Banchereau and Steinman, 1998; Steinman and Banchereau, 2007)
CD172a (SIRP-α)	95.49	0.87	Migration, negative regulation of phagocytosis and bilateral DC-T signaling (van Beek et al., 2005)
CD206 (MMR)	70.99	3.17	Receptor-mediated endocytosis & antigen presentation, intracellular signaling (Martinez-Pomares, 2012; Novak and Peng, 2005)

4.2. EHV1 infection downregulates expression of proteins involved in antigen presentation and co-stimulation on equine MDDC

Based on previous reports showing that HSV and VZV infection of DC resulted in downregulation of cell surface markers associated with antigen presentation and co-stimulation, we investigated whether EHV1 infection of equine MDDC also resulted in alterations of these cell surface markers (Elboim et al., 2013; Kruse et al., 2000b; Mikloska et al., 2001; Samady et al., 2003). At 16 hpi with the eGFP-expressing wild type EHV1 strain Ab4G (Figure 1b), EHV1-infected MDDC showed a significant decrease (% reduction) in cell surface levels of CD83 (81%), MHCI (63%), CD29 (44%), CD86 (43%), CD206 (46%) and CD172a (26%). In contrast, there was no significant reduction of cell surface levels of CD11a/CD18 or MHCII (Figure 2).

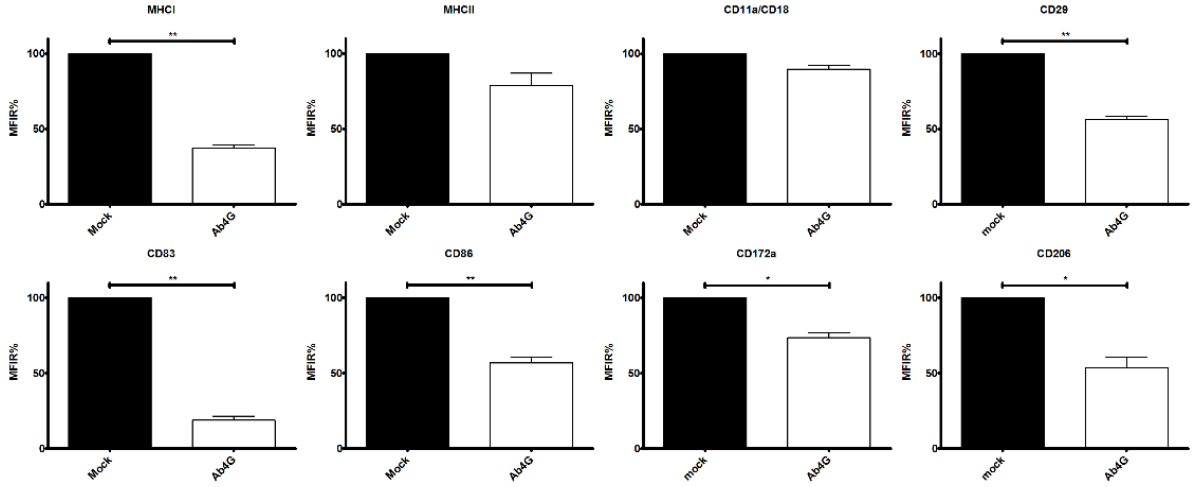


Figure 2: Selective downregulation on equine EHV1-infected equine monocyte-derived dendritic cells (MDDC). Day 3 old MDDC were mock-inoculated or inoculated with the eGFP-expressing EHV1 strain Ab4G at MOI 10. After 16 hours, the cell surface immunophenotype was analyzed on live/eGFP+ cells by flow cytometry. The mean fluorescent intensity ± SEM of at least three independent experiments of at least two different horses is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio, MFIR%). Two-sided Student's t-test for paired observations determined significant differences (* = P < 0.01; ** = P < 0.001).

One hypothesis to explain this downregulation is the involvement of anti-inflammatory cytokines that can be secreted by EHV1-infected cells which in turn could downregulate cell surface markers on human MDDC (Arrode et al., 2002; Chang et al., 2004; Raftery et al., 2004; Soboll Hussey et al., 2014). Equine MDDC were incubated with virus-free supernatant which was collected from Ab4G infected MDDC at 16 hpi (Ab4G_SN). Supernatant was confirmed free of virions by absence of eGFP signal from Ab4G_SN incubated equine MDDC (data not shown). Compared to MDDC incubated with supernatant collected from mock-inoculated MDDC (mock_SN), only for MHCI there was a very small and probably biological irrelevant, yet statistically significant, difference (6% upregulation) on Ab4G_SN inoculated MDDC. However, for all other tested proteins, there was no statistical significant alteration of cell surface expression between Ab4G_SN and mock_SN (Figure 3). Since these data did not provide indications that secreted factors could play a role in the observed

downregulation in EHV1-infected equine MDDC, the potential role of viral proteins was explored in more detail in a next set of experiments.

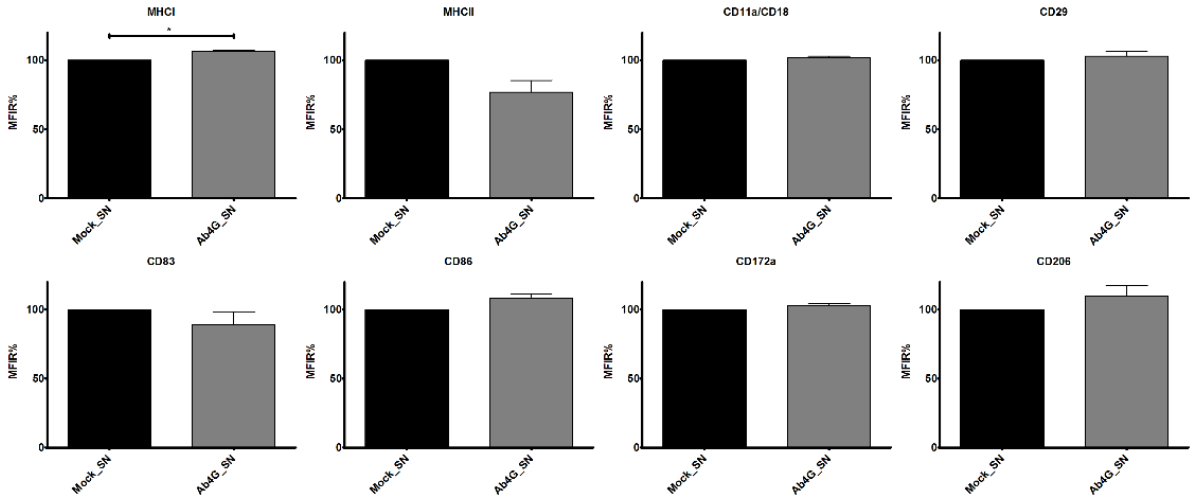


Figure 3: Immunophenotypic profile after incubation with virus-free supernatant. Day 3 old equine monocyte-derived dendritic cells (MDDC) were mock-inoculated or inoculated with virus-free supernatant from 16 h Ab4G inoculated MDDC (MOI 10). After 16 hours, the cell surface immunophenotype was analyzed for live MDDC by flow cytometry. The mean fluorescent intensity ± SEM of at least three independent experiments of two different horses is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio, MFIR%). Two-sided Student's t-test for paired observations determined significant differences (* = P < 0.01).

4.3. EHV1-mediated downregulation of cell surface markers on infected equine MDDC does not depend on the VHS protein

VHS was shown to be involved in the downregulation of CD83 and CD86 on HSV1 human infected MDDC (Samady et al., 2003). Therefore, we evaluated the involvement of EHV1-encoded VHS in the downregulation of cell surface proteins on EHV1-infected equine MDDC, using an eGFP-expressing (Ab4G) mutant lacking the VHS-encoding ORF19 region (Ab4G_Δ19). Briefly, we found no statistical significant difference between Ab4G and Ab4G_Δ19 infected MDDC at 16 hpi, indicating that downregulation of MHCI, CD29, CD83, CD86, CD172a and CD206 is not mediated by the VHS protein (Figure 4).

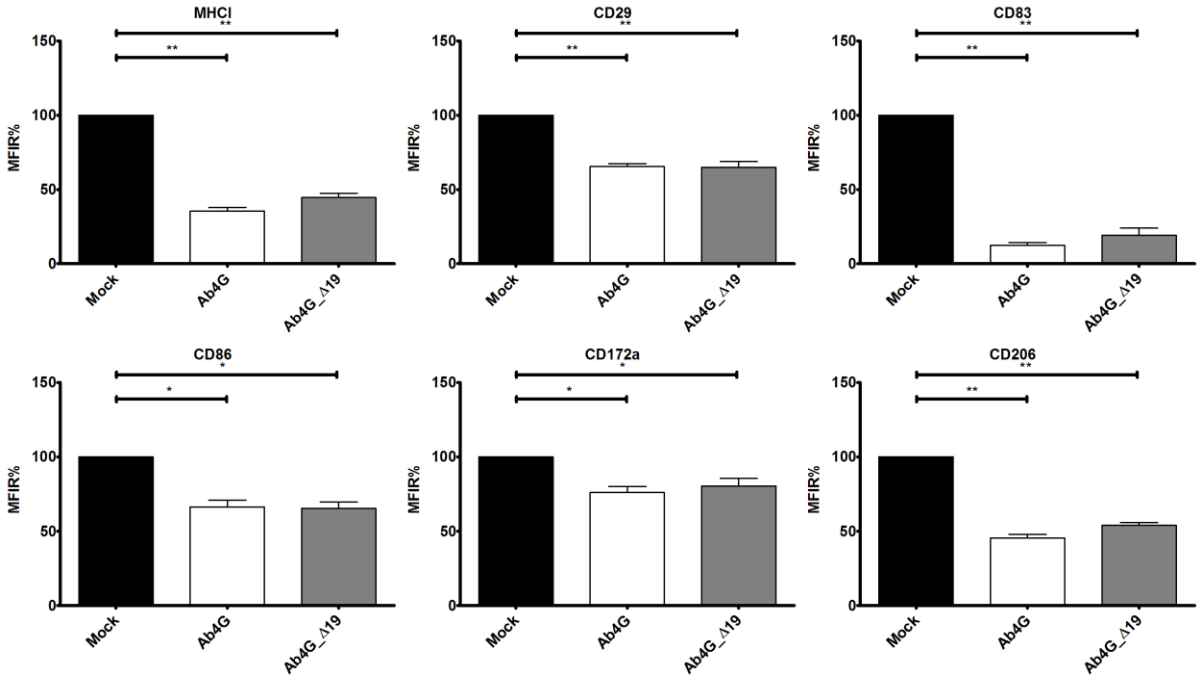


Figure 4: VHS is not involved in downregulation of cell surface markers on EHV1-infected equine monocyte-derived dendritic cells (MDDC). Day 3 old equine MDDC were mock-inoculated or inoculated with the eGFP-expressing EHV1 strain Ab4G or its isogenic deletion mutant which lacks the VHS encoding ORF19 region (Ab4G_Δ19). After 16 hours, the cell surface immunophenotype was analyzed on live/eGFP+ cells by flow cytometry. The mean fluorescent intensity ± SEM of at least three independent experiments of four different horses is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio, MFIR%). One-way repeated measures ANOVA analysis determined statistical differences (* = P < 0.01; ** = P < 0.001).

4.4. EHV1-mediated downregulation of CD83 and CD86 on infected equine MDDC partially depends on pUL56

Both the pUL49.5 and pUL56 protein play important roles in EHV1-mediated MHCI downregulation on NBL6 cells by inhibiting TAP and enhancing MHCI endocytosis, respectively (Huang et al., 2014; Koppers-Lalic et al., 2008; Ma et al., 2012). In addition, we and others found that EHV1 pUL56 can mediate downregulation of other cell surface proteins, besides MHCI, on equine mesenchymal stem cells (MSC) (CD29, CD105 and CD172a) and human epithelial carcinoma cells (CD46 and CD63) (Claessen et al., 2015; Huang et al., 2014).

Since we found that EHV1 induced a significant downregulation of MHCI and several other cell surface proteins on MDDC, we investigated the possible involvement of pUL49.5 and pUL56 in this process. To this end, equine MDDC were infected with eGFP-expressing wildtype EHV1 (Ab4G) or its isogenic deletion mutants lacking the pUL49.5-encoding ORF10 (Ab4G_Δ10) or the pUL56-encoding ORF1 (Ab4G_Δ1) regions. At 16 hpi, EHV1-infected MDDC were evaluated for downregulation of the different cell surface proteins. Upon comparison of Ab4G_Δ10 versus Ab4G infected MDDC, we found no significant difference in downregulation for any of the investigated cell surface proteins, including MHCI (Figure 5a).

In contrast, several observations were made when evaluating Ab4G_Δ1-infected MDDCs (Figure 5b). First, Ab4G_Δ1 did not impair downregulation of MHCI, CD172a and CD206. Second, we observed a very marginal and probably biologically irrelevant, yet statistically significant, difference (7%) in CD29 downregulation when comparing Ab4G_Δ1- and Ab4G-infected MDDCs. Third, Ab4G_Δ1-infected MDDC showed a moderately, but statistically significant, increase (%) in CD83 (16%) and CD86 (21%) expression when compared to Ab4G-infected MDDC (Figure 5b).

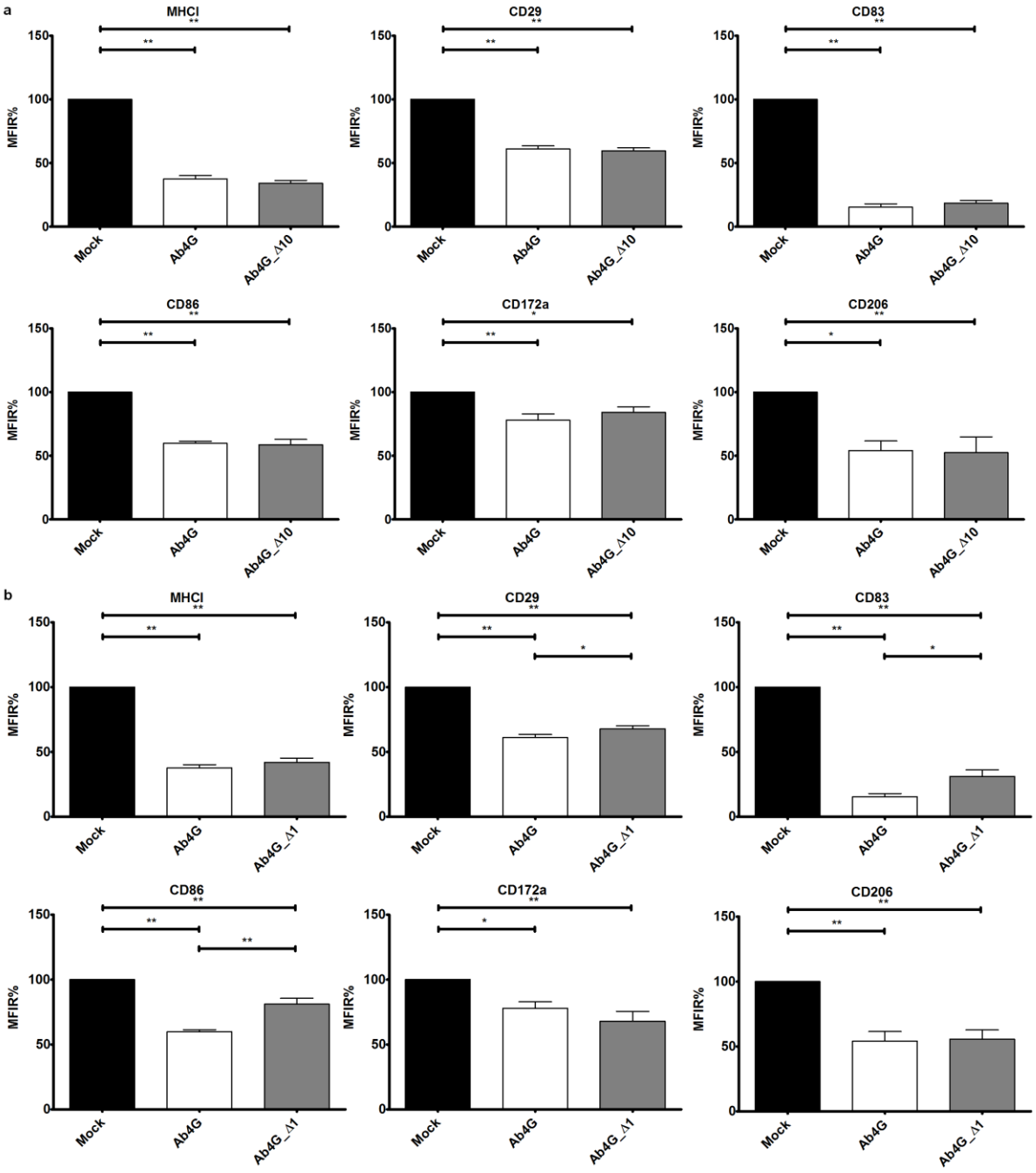


Figure 5: Evaluation of the role of pUL49.5 and pUL56 in the downregulation of cell surface markers on EHV1-infected equine monocyte-derived dendritic cells (MDDC). Day 3 old equine MDDC were mock-inoculated, inoculated with the eGFP-expressing EHV1 strain Ab4G or inoculated with the isogenic deletion mutants which are lacking the pUL49.5 encoding ORF10 region (a, Ab4G_Δ10) or the pUL56 encoding ORF1 region (b, Ab4G_Δ1). After 16 hours, the cell surface immunophenotype was analyzed on live/eGFP+ cells by flow cytometry. The mean fluorescent intensity ± SEM of at least three independent experiments of two different horses is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio, MFIR%). One-way repeated measures ANOVA analysis determined statistical differences (* = P < 0.01; ** = P < 0.001).

4.5. Downregulation of CD83 on EHV1-infected MDDC does not depend on EICP0

For HSV1, ICP0 has been reported to mediate CD83 downregulation on human MDDC (Heilingloh et al., 2014; Kummer et al., 2007). To evaluate if the EHV1' infected cell protein (EICP) 0 could possibly be involved in the downregulation of CD83, we infected equine MDDC with eGFP-expressing wildtype EHV1 (Ab4G) or its isogenic deletion mutant lacking the EICP0-encoding ORF63 region (Ab4G_Δ63). After 16 h, we found a similar downregulation of CD83 on wildtype and EICP0null (Ab4G_Δ63) infected cells (Fig. 6).

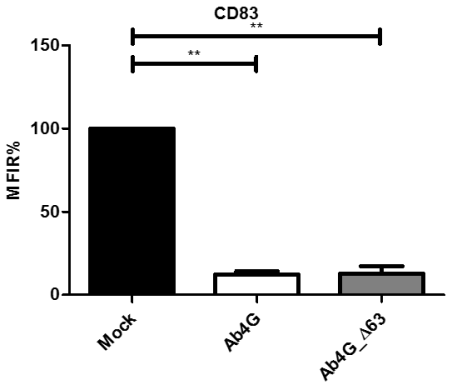


Figure 6: Downregulation of CD83 by equine herpesvirus 1 is not mediated by EICP0. Day 3 old equine monocyte-derived dendritic cells were mock-inoculated or inoculated with the eGFP-expressing EHV1 strain Ab4G or its isogenic deletion mutant lacking the EICP0 encoding ORF63 region (Ab4G_Δ63). After 16 hours, the cell surface immunophenotype was flowcytometrically analyzed on eGFP+ cells. The mean fluorescent intensity ± SEM of at least three independent experiments of four different horses is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio). One-way repeated measures ANOVA analysis determined statistical differences (= P < 0.001).**

5. Discussion

The present study is the first to report on the downregulation of several cell surface markers on equine MDDC upon infection with EHV1. Until now, such attenuation of cell surface markers on DC was only evaluated for the human α-herpesviruses HSV and VZV, and in line with these reports we similarly found a significant and robust downregulation of CD83 and CD86, but not of MHCII (Elboim et al., 2013; Kobelt et al., 2003; Kruse et al., 2000a;

Kummer et al., 2007; Mikloska et al., 2001; Morrow et al., 2003; Martin J Raftery et al., 2006; Salio et al., 1999; Samady et al., 2003). We also found a drastic downregulation of MHCI on EHV1-infected equine MDDC, which is in agreement with previous reports on HSV1-, HSV2- and VZV-infected MDDC (Elboim et al., 2013; Morrow et al., 2003; Salio et al., 1999), but in contrast to one other study on HSV1-infected MDDC (Mikloska et al., 2001). In addition, we also demonstrated a significant downregulation of CD29, CD206 and CD172a, but not of CD11a/CD18, which, to our knowledge, has not been previously reported for α-herpesvirus-infected MDDC.

Cell surface marker downregulation on viral-infected cells can be caused directly through expression of viral proteins or indirectly via the viral induction of cellular and/or viral secreted proteins (Loch and Tampé, 2005; Vossen et al., 2002). The latter mechanism is most likely not involved since we observed no downregulation upon incubation with virion-cleared supernatants collected from EHV1-infected MDDC. To investigate whether viral proteins induced our observed downregulation, we used recombinant EHV1 mutant viruses devoid in viral proteins that have been described previously to be involved in downregulation of cell surface markers on human DC expressing α-herpesvirus VHS or pUL49.5, and equine cells expressing EHV1 pUL49.5 or pUL56 (Claessen et al., 2015; Huang et al., 2014; Koppers-Lalic et al., 2008; Lampen et al., 2010; Samady et al., 2003).

Although we found that the early viral protein pUL56 was partially responsible for the downregulation of CD83 and CD86, we did not found any involvement of pUL56 regarding the other evaluated cell surface markers, including MHCI and CD29. This was unexpected, since we and others had previously found that downregulation of these markers on other cell types like equine fibroblasts (MHCI) or equine blood-derived MSC (MHCI, CD29) was pUL56-dependent (Claessen et al., 2015; Ma et al., 2012). Both VHS and pUL49.5 were not

involved in downregulation of any of the studied cell surface proteins on equine MDDC. Although VHS mediated CD83 and CD86 downregulation on HSV1-infected human MDDC, we found no VHS involvement in cell surface marker downregulation on equine MDDC (Samady et al., 2003). However, and in line with our findings, a later study reported that HSV1 VHS was not responsible for CD83 downregulation (Kummer et al., 2007). A potential explanation for this discrepancy in HSV1 VHS-induced downregulation, could be that the original study by Samady et al. (2003) used a multiple gene knockout VHS mutant where additional deletions could have caused the observed VHS-mediated downregulation of CD83 and CD86. Although pUL49.5, a TAP inhibitor, induced MHCI downregulation on transfected equine fibroblasts (EHV1) and human MDDC (BHV1) (Koppers-Lalic et al., 2008; Lampen et al., 2010), we did not found any such involvement for MHCI and other cell surface markers on equine MDDC. Possibly in line with our findings, the TAP inhibitor of HSV (ICP47) is not involved in downregulation of MHCI on human MDDC (Hill et al., 1995). Expression of CD83 has a high turn-over rate on human MDDC and was virtually absent after 24h infection with HSV1 (Klein et al., 2005; Kruse et al., 2000a). Furthermore, independent of its E3 ubiquitin ligase activity, EICP0 in part mediated CD83 downregulation and it was proposed that EICP0 mediates this by degradation of CD83 during normal recycling (Heilingloh et al., 2014; Kummer et al., 2007). Although CD83 on equine MDDC also exhibited a high turn-over rate (data not shown), we did not found an impaired downregulation of CD83 upon deletion of EICP0.

Since EHV1 seems to employ other immune evasive α-herpesvirus proteins for cell surface marker downregulation on equine MDDC then those described in other equine cells and human α-herpesvirus-infected human DC, it is interesting to note that the viral protein US3 mediates downregulation of MHCI on VZV-, pseudorabies virus (PRV)- and HSV-infected

cells (Deruelle et al., 2009; Eisfeld et al., 2007; Imai et al., 2013). However this viral protein seems a less likely candidate for MHCI downregulation on equine MDDC, since MHCI downregulation was not impaired in unique short protein (pUS) 3 null EHV1-infected equine fibroblasts (Ma et al., 2012). It was recently demonstrated that pUL56 needs pUL43 for MHCI downregulation in equine fibroblasts (Huang et al., 2015). Therefore, it would be of interest to evaluate whether this protein is also necessary for the downregulation of CD83 and CD86 on EHV1-infected equine MDDC, as this could explain the partial involvement of pUL56 in the downregulation of these cell surface markers. In addition, functional redundancy is common for α-herpesvirus proteins, where lack of one particular viral protein may be functionally complemented by other viral proteins (Mettenleiter et al., 2009). Such redundancy has been suggested regarding the anti-apoptotic activity of pUS3 and pUS5. The prevention of CTL-induced apoptosis of HSV1-infected fibroblasts could only be abolished if both viral proteins were deleted simultaneously (Aubert et al., 2006). Therefore, in order to conclusively rule out the involvement of EHV1 VHS, pUL56, pUL49.5 or others, experiments will need to be repeated with recombinant double or triple mutant viruses. A sufficient combination of immune evasion genes could certainly be useful for future development of vaccines, since current modified live or inactivated vaccines do not provide enough protection against viremia, abortion and EHM (Bürki et al., 1990; Burrows et al., 1984; Goodman et al., 2012; Kydd et al., 2006b).

6. Conclusions

We identified CD29 as a novel marker on equine MDDC and for the first time describe downregulation of the cell surface markers MHCI, CD29, CD83, CD86, CD172a and CD206 on EHV1-infected MDDC. This selective downregulation was not mediated by viral-induced

secretory factors or the viral proteins VHS, pUL49.5 or EICP0. Downregulation of CD83 and CD86 was in part mediated by the early viral protein pUL56.

Acknowledgements

This work was funded by a Concerted Research Action (GOA) of the Ghent University (#01G01311). We like to thank Chantal Vanmaerke for generating the various virus stocks.

Chapter 4: Exploring the cellular mechanism of MHCI downregulation on equine dendritic cells during EHV1 infection

1. Abstract

A major part of the antiviral immune response depends on the destruction of infected cells by cytotoxic T-lymphocytes (CTL). The capacity of dendritic cells (DC) to present viral antigens in the context of major histocompatibility complex (MHC) I is crucial for the generation of these CTLs. However, herpesviruses like equine herpesvirus (EHV) 1 have been shown to downregulate MHCI expression on the cell surface of infected cells. As reported in **Chapter 3**, we found that downregulation of MHCI also occurs on EHV1-infected equine monocyte-derived DC (MDDC) and in the current **Chapter**, we aimed to explore the underlying cellular mechanism responsible for this downregulation. Our salient findings were as follows. Firstly, MHCI downregulation on equine MDDC does not appear to be a direct consequence of EHV1 entry. Second, EHV1-induced MHCI downregulation most likely occurs via enhanced internalization of MHCI from the cell surface. Third, the ability of EHV1 to induce downregulation of MHCI on equine MDDC appears to involve clathrin, dynamin and group I p21-activated kinases, but not F-actin cycling.

2. Introduction

During viral infection, a major part of the immune response depends on the presentation of viral antigens (Ag) via MHCI. As such, viral infected cells can be recognized and destroyed by CTL. Crucial for the generation of these CTL are DC which, upon encountering a virus, will become activated and start migrating towards the draining lymph nodes (Banchereau et al., 2000; Ludewig et al., 1999, 1998). Upon arrival in the lymph nodes, DC fully maturate and will select and stimulate proliferation of Ag-specific T-lymphocytes by their presentation of viral Ags on MHCI (Van de Walle et al., 2009a). However, viruses, and herpesviruses in particular, have evolved several immune evasion strategies including those

aimed at disturbing the CTL-based immunity. For example, herpesviruses have evolved several mechanisms to disturb the MHCI presentation pathway at multiple levels such as interference with Ag loading on MHCI, disturbing MHCI trafficking towards the plasma membrane and inducing internalization of cell surface MHCI (Loch and Tampé, 2005; Vossen et al., 2002).

In line with this, we and others found that infection with EHV1 also results in downregulation of cell surface MHCI in a variety of cell types, including equine respiratory epithelial cells (EREC), equine dermal fibroblasts (ED), equine mesenchymal stem cells (MSC) and human epithelial carcinoma cells (Claessen et al., 2015; Huang et al., 2014; Ma et al., 2012; Soboll Hussey et al., 2014). In ED (NBL6) cells, EHV1-mediated downregulation of MHCI was found to occur through an enhanced internalization of this molecule (Huang et al., 2014), and moreover, to largely depend on the expression of the early viral protein UL56 and the early-late pUL43 (Huang et al., 2015). In line with this, we recently demonstrated that downregulation of MHCI on EHV1-infected equine MSC also depends on the expression of pUL56 (Claessen et al., 2015).

In **Chapter 3**, we demonstrated that EHV1 infection of equine monocyte-derived DC (MDDC) also results in downregulation of MHCI, along with downregulation of several other cell surface markers. In addition, and somewhat unexpectedly, we found that unique long protein (pUL) 56, pUL49.5, VHS and EICPO were not involved in downregulating MHCI on equine MDDC. Furthermore, it is not known which cellular mechanisms are involved in EHV1-mediated downregulation of MHCI on equine MDDC. Therefore, the aim of this study was to begin to explore potential cellular mechanisms underlying the downregulation of MHCI cell surface expression on equine MDDC.

3. Materials and Methods

3.1. Cells and viruses

Rabbit kidney (RK13) cells were maintained in Dulbecco's Minimum Essential Medium (DMEM low glucose; Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/mL penicillin and 0.1 mg/mL streptomycin (1% Pen/Strep; Invitrogen), at 5% CO_2 and 37 °C. The enhanced green fluorescent protein (eGFP) expressing wild type Ab4G was propagated in RK13 cells as previously described (Goodman et al., 2007; Ma et al., 2012).

Equine MDDC were generated as previously described in **Chapter 3** (Hammond et al., 1999; Dietze et al., 2008; Baghi et al., 2014). Briefly, equine heparinized peripheral venous blood was collected after approval and under the guidelines of the institutional Ethical Committee (EC2010/147). After collection, density-gradient separated PBMC were seeded in 6- or 24-well culture dishes. After removing non-adherent cells by washing, MDDC were generated by maintaining adherent cells for three days in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF).

3.2. Antibodies

Cells were labeled for the following cell surface markers: CD11a/CD18 (Serotec, CVS9), CD29 (Chemicon, TDM29), CD83 (Biolegend, HB15e), CD86 (Biolegend, IT2.2), CD172a (VMRD, DH59B), CD206 (Biolegend, 15-2), MHCI (VMRD, PT85A), MHCII (Novus, CVS20). As secondary antibody, a Cy-5 goat-anti-mouse IgG was used (Invitrogen). Isotype controls were IgG2b (Biolegend, MPC-11) and in-house made IgG1 (anti-porcine reproductive and respiratory syndrome virus) and IgG2a (anti-porcine circovirus type 2) antibodies.

3.3. Entry studies

After two days with IL-4 and GM-CSF, MDDC were mock-pre-treated or pre-treated with CXH. CHX treatment was continued until collection of the cells. After 30 minutes, MDDC were either mock-inoculated or inoculated with the eGFP-expressing live or UV-inactivated Ab4G strain at the indicated multiplicity of infection (MOI). At 4 or 16 h post infection (hpi), MDDC were collected for cell surface MHCI analysis by flow cytometric analysis of live (all conditions) or live/eGFP+ (untreated live Ab4G-inoculated) MDDC.

3.4. Internalization assay

See Figure 2a for a schematic overview of the experimental set-up. After two days incubation with IL-4 and GM-CSF, MDDC were mock-inoculated or inoculated with eGFP-expressing Ab4G at an MOI of 10. After allowing virus adherence for two hours at 4 °C, MDDC were washed and subsequently incubated for one hour with their old culture medium containing none or an excess amount of monoclonal MHCI antibody (PT85A) on a slow-tilting shaker at 4 °C. After washing, MDDC received their old culture medium and the 24-well culture dishes were shifted to 37 °C. At 16 hpi, all four MDDC samples were collected and only the mock- or EHV1-inoculated samples that were not labeled with MHCI (PT85A) antibody before collection at 16 hpi, were incubated with this MHCI (PT85A) antibody after collection. Subsequently, all four samples, either the condition labeled with MHCI antibody before collection or the condition labeled after collection, were stained with the Cy5-conjugated secondary antibody and the cell viability dye 7-amino-actinomycin D (7AAD) according to the below described flow cytometry protocol.

3.5. MHCI endocytosis inhibition studies

After two days incubation with IL-4 and GM-CSF, MDDC were mock-inoculated or inoculated with eGFP-expressing Ab4G at a multiplicity of infection 10. Following endocytosis inhibitors were present from 2 hpi onwards at the indicated concentrations: amiloride, rottlerin, cytochalasin D, chlorpromazine (all Sigma), IPA-3 (Tocris) and dynasore (Abcam). In a separate set of experiments, the MP inhibitors amiloride and rottlerin were added 30 min before inoculation with EHV1. At 16 hpi, MDDC were collected for flow cytometric analysis of live (mock Ab4G inoculated) or live/eGFP+ (Ab4G inoculated) MDDC.

3.6. Flow cytometry

MDDC were mock-infected or infected with the various eGFP-expressing viruses at a MOI 10, unless otherwise indicated. At 4 or 16 h post inoculation (hpi), cells were detached with accutase (Invitrogen) and subsequently washed once in Ca^{2+}/Mg^{2+} free phosphate buffered saline supplemented with 5 % fetal calf serum (CMF-PBS-5%FCS). Except for the samples pre-labelled with MHCI antibody (PT85A), other samples were incubated for 30 min on ice with their respective primary antibodies which were diluted in CMF-PBS-20%FCS. All samples were washed with CMF-PBS-5%FCS and subsequently incubated with CMF-PBS-10%goat serum for 15 min on ice, before labelling with Cy5-conjugated secondary antibody for 30 min on ice in the dark. Cells were washed with CMF-PBS-5%FCS and stained with the cell viability dye 7AAD (Invitrogen). For each sample, at least 2000 (internalization assay) or 10000 (all other assays) cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser. Only 7AAD negative (live) mock cells or 7AAD negative (live) and eGFP positive (infected) inoculated cells were evaluated. Isotype controls were included to

correct for non-specific binding and spectral overlap between used fluorochromes was compensated with an automatic calibration technique (FACSDiva software, Becton Dickinson). The expression levels of surface markers were presented as mean fluorescence intensities normalized to the isotype control (mean fluorescence intensity ratio, MFIR). All data were analyzed using FACSDiva software (Becton Dickinson) and expressed as mean ± standard error of the mean (SEM).

3.7. Statistical analysis

Statistical analysis was performed with Prism (GraphPad) for at least three independent repeats on at least two different horses. Results are expressed as the mean ± SEM in a bar graph or shown as independent repeats with the mean in a data point map. For two group comparisons, a two-sided Student's t test for paired observations was used to evaluate statistical differences at $P < 0.05$. A one-way repeated measures ANOVA analysis with the Tukey post-test (95% confidence interval) was performed for all other comparisons at $P < 0.05$.

4. Results and Discussion

4.1. Downregulation of MHCI on equine MDDC during EHV1 infection is not directly associated with viral entry

It was previously shown that EHV1 can enter susceptible cells via fusion or endocytosis, depending on the cell type (Frampton et al., 2007; Van de Walle et al., 2008b). MHCI appears to serve as an important viral entry receptor since blocking cell surface MHCI expression in equine brain microvascular endothelial cells, equine peripheral blood monocytes (PBMC) and on ED cells could significantly suppress entry of EHV1 (Kurtz et al.,

2010; Sasaki et al., 2011a). Because **(i)** we recently found that EHV1 induces downregulation of MHCI on equine MDDC (**Chapter 3**) and **(ii)** it has been reported that entry of some α-herpesviruses is associated with downregulation of entry receptors (Stiles et al., 2010; Stiles and Krummenacher, 2010), we aimed to evaluate whether MHCI downregulation is a direct consequence of EHV1 entry. If this would be the case, it is conceivable to expect that a higher multiplicity of infection (MOI) would induce a more pronounced downregulation of MHCI. To test this, equine MDDC were infected with EHV1 at an MOI of 1 or 10 and MHCI expression was determined using flow cytometry 4h later. We did not find a statistically significant difference in the percentage of MHCI downregulation at 4h post inoculation (pi) between MOI 1 versus 10 (Figure 1a). We did find that both MOI conditions induced a significant downregulation of cell surface MHCI at this relatively early time point (4 hpi) compared to mock-inoculated equine MDDC, but that downregulation of MHCI was substantially more pronounced at 16 hpi (Figure 1a), which further indicates that MHCI downregulation (mainly) occurs at post-entry stages of infection. To formally rule out a role of viral entry in MHCI downregulation, we repeated experiments using UV-inactivated EHV1 (MOI 10), which allows virus to still enter cells but does not lead to viral replication and subsequent viral gene expression. When compared to mock-inoculated cells, only live but not UV-inactivated EHV1 could induce a significant downregulation of cell surface MHCI (Figure 1b), further arguing against downregulation of MHCI as a direct consequence of EHV1 entry. To confirm the need for viral gene expression to downregulate MHCI, we pre-treated equine MDDC with the translation inhibitor cycloheximide (CHX). CHX treatment of both mock- and EHV1-inoculated equine MDDC induced a significant MHCI downregulation when compared to mock-treated mock-inoculated cells (Figure 1c), indicating that MHCI may have a relatively rapid turn-over on equine MDDC, similar to what has been reported for human immature MDDC (Ackerman and Cresswell, 2003; Zehn et al., 2004). However, no

statistically significant difference in MHCI cell surface expression was found between CHX-treated mock- versus EHV1-inoculated cells (Figure 1c), indicating that EHV1-mediated MHCI downregulation indeed does require new viral protein synthesis. In summary, these three different experimental approaches provide evidence that EHV1-induced downregulation of MHCI on equine MDDC is not a direct consequence of viral entry. These experiments do not rule out the possibility that MHCI may act as a receptor for EHV1 entry in equine MDDC. Indeed, in ED cells, EHV1 entry depends on MHCI, but, in line with our current findings, UV-inactivated EHV1 did not induce significant MHCI downregulation (Kurtz et al., 2010; Rappocciolo et al., 2003; Sasaki et al., 2011a).

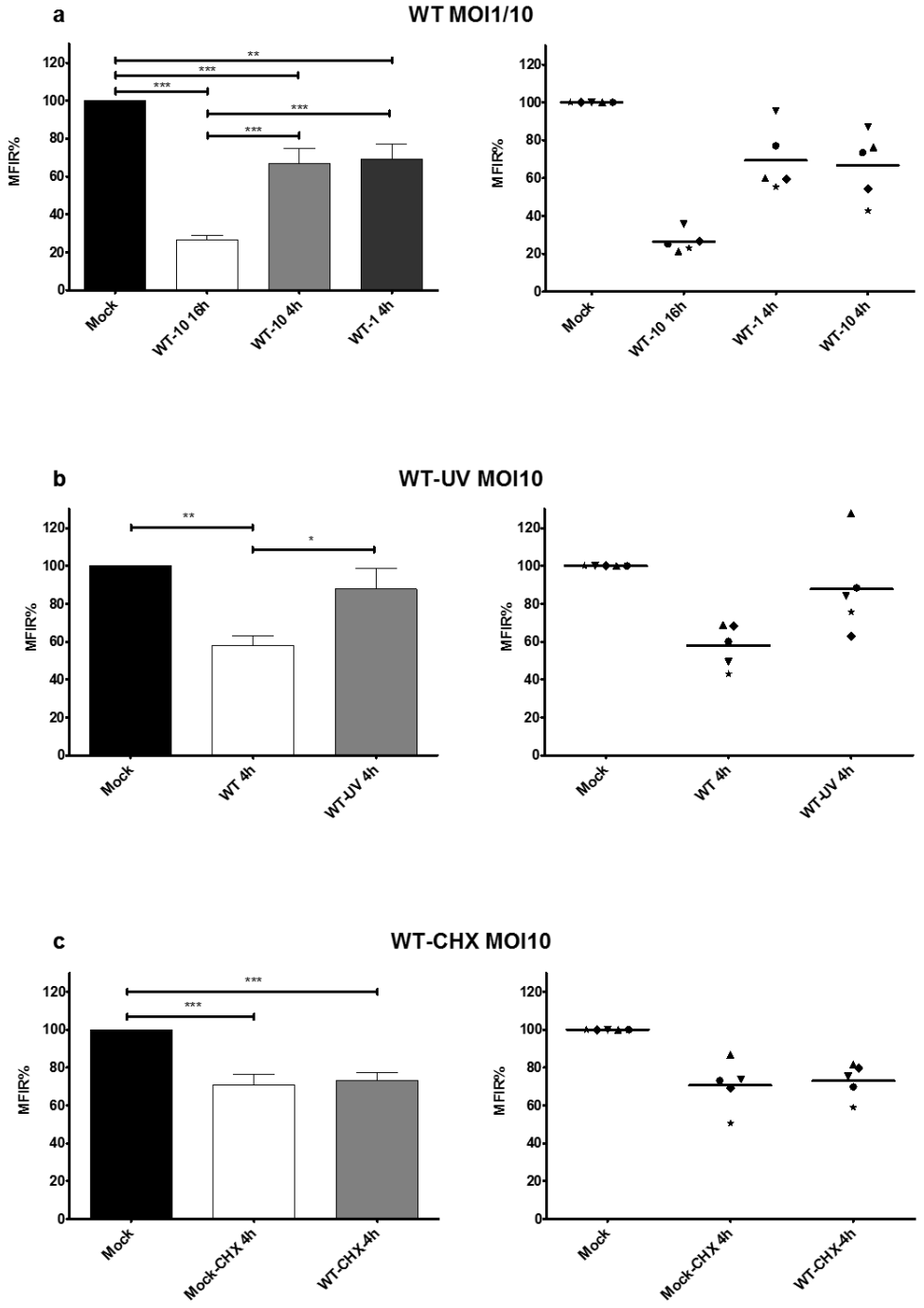


Figure 1: Downregulation of MHCI on equine dendritic cells during EHV1 infection is not directly associated with viral entry. Equine monocyte-derived dendritic cells (MDDC) were either mock-treated (a-b) or pre-treated for 30 minutes with cycloheximide (CHX) (c). Subsequently, MDDC were mock-inoculated or inoculated with live (a-c) or UV-inactivated (b) EHV1 strain Ab4G (WT) at a multiplicity of infection of 1 (a) or 10 (a-c). After 4 or 16 h, the cell surface immunophenotype was analyzed by flow cytometry. The mean fluorescence intensity ± SEM of five independent repeats (based on four horses) was normalized to the isotype control and expressed relative to mock-infected MDDC (mean fluorescence intensity ratio, MFIR%). One-way repeated measures ANOVA analysis with the Tukey post-test (95% confidence

interval) was used to determine statistically significant differences (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

4.2. EHV1-mediated downregulation of MHCI on equine MDDC occurs via enhanced internalization

In general, downregulation of MHCI expressed on the cell surface can be accomplished by either preventing delivery of MHCI to the cell surface or by enhancing removal of MHCI from the cell surface. Examples for both mechanisms have been described for herpesviruses. Preventing delivery of MHCI to the cell surface generally involves the prevention of Ag-MHCI complex formation (e.g. herpes simplex virus (HSV) 1) or delaying the transport of these MHCI complexes from the ER or Golgi towards the cell surface (e.g. varicella zoster virus (VZV)) (Loch and Tampé, 2005). Enhancing the removal of MHCI from the cell surface by enhanced internalization has been reported for Kaposi's sarcoma-associated herpes virus (KSHV) as well as EHV1, at least in EHV1-infected ED cells (Coscoy et al., 2001; Coscoy and Ganem, 2000; Huang et al., 2014). To evaluate whether prevention of cell surface delivery or enhanced internalization could be the underlying mechanism for the observed MHCI downregulation on equine MDDC, a flow cytometry-based internalization assay was performed, as schematically shown in Figure 2a. Briefly, EHV1-infected equine MDDC were incubated with or without MHCI antibodies directly after virus adhesion (i.e. 2h after adding the virus-containing supernatants). All samples were collected after 16h and (**i**) the samples that did not receive MHCI antibodies directly after virus adhesion were incubated with MHCI antibodies, followed by incubation with fluorochrome-conjugated secondary antibodies, whereas (**ii**) the samples that were labelled with MHCI antibodies directly after virus adhesion were incubated with the fluorochrome-conjugated secondary antibodies. Expression of MHCI on EHV1-infected equine MDDC was always compared to the MHCI expression on the corresponding mock-inoculated samples which were labeled in parallel as described above and served as controls (see also Figure 2a). The rationale for this was as

follows: if no significant difference in fluorescence would be observed between EHV1-inoculated versus mock-inoculated samples that where incubated with MHCI antibodies directly after virus adhesion, then this would be indicative for EHV1 preventing transport of new MHCI molecules to the cell surface (Figure 2a). In contrast, if a significant difference in fluorescence would be observed between these EHV1-inoculated versus mock-inoculated samples, this would suggest that at least some cell surface MHCI are downregulated by enhanced internalization from the cell surface (Figure 2a). The results from these experiments showed no significant difference in the expression of cell surface MHCI on EHV-1 infected MDDC, labeled with MHCI antibodies directly after virus adhesion, compared to EHV-1 infected MDDC that were labeled with MHCI antibodies 16 hpi (Figure 2b). Taken together, these results indicate that the downregulation of cell surface MHCI molecules during EHV1 infection occurs via an EHV1-enhanced internalization from the cell surface, similar to what has previously been reported for MHCI downregulation in EHV1-infected ED cells (Huang et al., 2014). Future experiments to confirm that an enhanced internalization of MHCI on infected equine MDDC is responsible for the observed MHCI downregulation could include the visualization of internalized MHCI in infected equine MDDC and/or co-localization experiments with certain cellular organelle markers (e.g. early and late endosomes). In addition, the underlying cellular degradation mechanisms could be identified by evaluating total MHCI protein levels on Western blot after treatment with inhibitors of endosome-lysosomal acidification (e.g. NH_4CL, chloroquine, bafilomycin A1) or the proteasome machinery (e.g. MG-132, epoxomicin) (Huang et al., 2014).

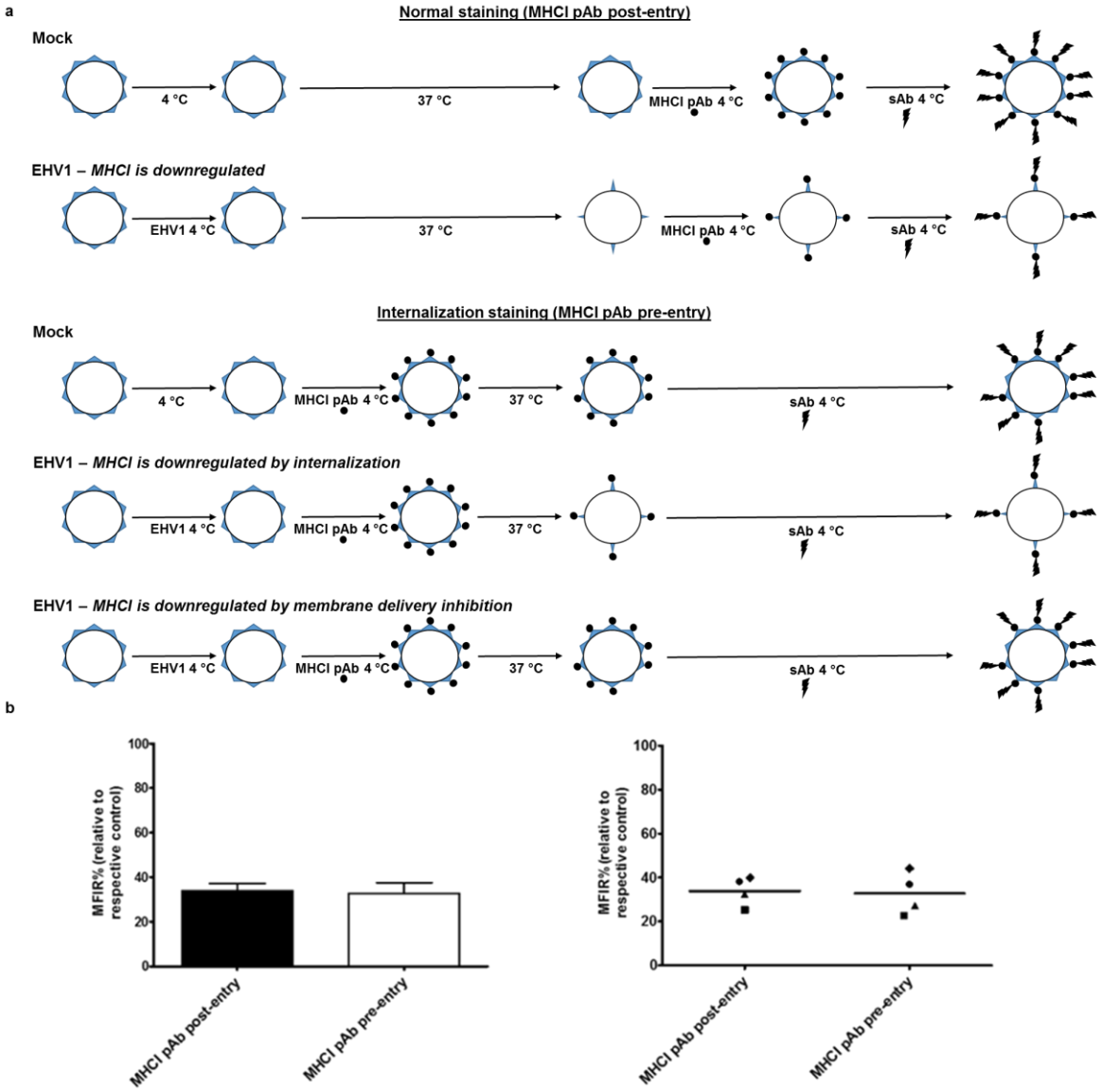


Figure 2: EHV1 induces internalization of MHCI on equine dendritic cells. (a) Overview of experimental set-up (see also Materials and Methods). EHV1-infected equine MDDC were always compared to mock-infected equine MDDC. (b) Equine monocyte-derived dendritic cells were collected after 16 h at 37 °C for analysis of cell surface MHCI. Primary anti-MHCI antibodies were added either directly after virus infection (2hpi, 'pre-replication') or at 16hpi ('post-replication'). The mean fluorescence intensity ± SEM of four independent repeats from four horses was normalized to the isotype control and expressed relative to their respective mock-infected MDDC (mean fluorescence intensity ratio, MFIR%). A two-sided Student's t test for paired observations was used to determine potential significant differences ($P < 0.05$).

4.3. Effect of specific endocytosis inhibitors on EHV1-induced MHCI downregulation in equine MDDC

The enhanced MHCI internalization mechanism responsible for MHCI downregulation in EHV1-infected ED cells was shown to depend on dynamin, cholesterol, receptor tyrosine kinases and ubiquitination, but not clathrin or caveolin (Huang et al., 2014). To evaluate whether MHCI downregulation in EHV1-infected MDDC occurs via similar or different mechanisms, experiments were performed to evaluate MHCI downregulation in the presence of several inhibitors of these cellular factors. In addition, and since DC employ a variety of endocytosis mechanisms such as pinocytosis, phagocytosis and macropinocytosis (MP) (Mercer and Greber, 2013), inhibitors of MP were also included. More specifically, equine MDDC were treated at 2 hpi with the following selection of endocytosis inhibitors: chlorpromazine (CHPZ, interferes with clathrin-mediated endocytosis), dynasore (interferes with dynamin), cytochalasin D (inhibits F-actin polymerization), IPA-3 (inhibits class I p21-activated kinases, central regulators in actin-controlling RhoGTPase signaling pathways), amiloride (inhibits MP) and rottlerin (inhibits MP). First, we performed control experiments to ensure that these inhibitors did not negatively affect the normal course of EHV1 infection. No effect was found on EHV1 replication when CHPZ, dynasore, cytochalasin D and IPA-3 were added to MDCC at 2 hpi. In contrast, the MP inhibitors amiloride and rottlerin did significantly reduce EHV1 replication (Figure 3a). To further confirm this effect, equine MDDC were treated with these MP inhibitors before inoculation with EHV1 and this resulted in an even greater reduction of EHV1 infection (Figure 3b). Therefore, we did not use these MP inhibitors for our actual experiments. Still, it is of interest to note that viruses, such as KSHV, HCMV, and vaccinia virus, have been shown to misuse MP to enter several cell types, including DC (Chakraborty et al., 2012; Haspot et al., 2012; Mercer and Helenius, 2012; Sandgren et al., 2010; Valiya Veettil et al., 2010). Therefore, it will be of interest to see whether EHV1 may (mis)use MP for its entry in MDDC.

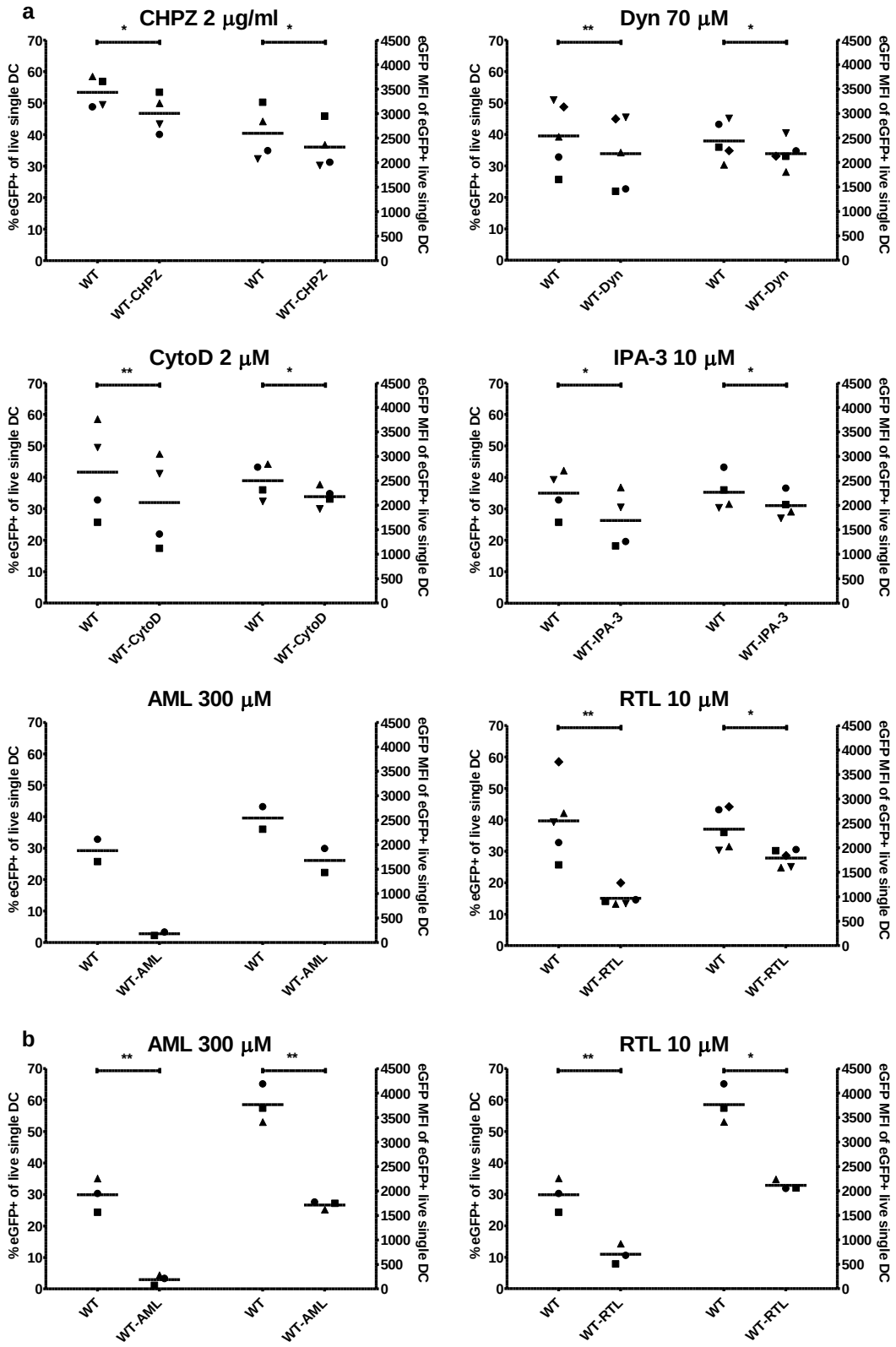


Figure 3: EHV1 infection of equine dendritic cells is drastically reduced after treatment with macropinocytosis inhibitors. Equine monocyte-derived dendritic cells (MDDC) were either 120 minutes after (a) or 30 minutes before (b) inoculation with EHV1, mock-treated or treated with the following inhibitors at the indicated concentrations: chlorpromazine (CHPZ, inhibits

clathrin coat formation), dynasore (Dyn, inhibits the dynamin GTPase domain), cytochalasin D (CytoD, inhibits actin polymerization), IPA-3 (inhibits group I PAK activation 3), amiloride (AML, inhibits Na+/H+ ATPase) or rottlerin (RTL, unknown mechanism). Equine MDDC were inoculated with the enhanced green fluorescent protein (eGFP) expressing EHV1 strain Ab4G at a MOI 10. At 16 hpi, the % and mean fluorescent intensity (MFI) of eGFP positive MDDC was analyzed by flow cytometry and expressed as the mean ± SEM of at least two horses. Two-sided Student's t test for paired observations was used (n > 2) to determine statistically significant differences (* = P < 0.05; ** = P < 0.01).

The drug CHPZ is widely used to inhibit clathrin-mediated endocytosis by misassembling clathrin triskelions and adaptor proteins on endosomes, which depletes the sub-membrane pool necessary to generate clathrin lattices on forming endosomes (Dutta and Donaldson, 2012; Mousavi et al., 2004). Here, we could demonstrate that treatment of EHV1-inoculated equine MDDC with CHPZ significantly inhibited MHCI downregulation (Figure 4a). The inhibitor dynasore, which inhibits the GTPase domain of dynamin (Dutta and Donaldson, 2012; Kirchhausen et al., 2008), was also able to significantly inhibit MHCI downregulation, to a similar extent as CHPZ (Figure 4b). Dynamin provides the membrane fission action that is necessary during several endocytosis pathways, including clathrin-, caveolae- and Arf6-dependent endocytosis (Dutta and Donaldson, 2012; Mercer and Greber, 2013). When looking at MP and phagocytosis, filamentous (F) actin dynamics have been shown to be important. F-actin content is controlled by the balance between addition of monomeric globular (G) actin at the plus end of F-actin versus dissociation of globular actin at the minus end of F-actin (Cooper, 1987; Dutta and Donaldson, 2012; Mercer and Greber, 2013; Sandgren et al., 2010). We found that the F-actin depolymerizing drug cytochalasin D (Cooper, 1987; Jacob et al., 2015), partially, albeit not statistically significant, inhibited MHCI downregulation (Figure 4c). Combining this finding with previous reports in which F-actin manipulation by different α-herpesviruses was demonstrated (Murata et al., 2000; Van Minnebruggen et al., 2003), prompted us to further explore the role of F-actin during EHV1-induced MHCI downregulation. To this end, we treated EHV1-infected MDDC with IPA-3,

known to inhibit group I p21-activated kinases (PAK-I) (Deacon et al., 2008) which, as fairly downstream signaling mediators controlling F-actin dynamics, have been shown to be involved in suid herpesvirus (SHV) 1-induced F-actin disruption (Van den Broeke et al., 2009). Somewhat surprisingly, we found that inhibition of PAK-I with IPA-3 could significantly inhibit MHCI downregulation on equine MDDC (Figure 4d). To find an explanation as to why cytochalasin D was not able to significantly inhibit MHCI downregulation, whereas IPA-3 was able to inhibit MHCI downregulation, it is important to note that the PAK-I kinases, targeted by IPA-3, are not only involved in F-actin dynamics but also in a multitude of other signaling processes such as gene expression, cell cycle regulation, apoptosis and mitogen activated protein kinase pathways (Pacheco and Chernoff, 2010). Interestingly, PAK1 activates the mitogen activated protein kinase pathway (Shrestha et al., 2012), which was shown to induce clathrin-mediated endocytosis (Cavalli et al., 2001; McLauchlan et al., 1998).

Our data point to an important role for clathrin-mediated endocytosis during EHV1-induced MHCI downregulation in MDDC. Involvement of clathrin during MHCI downregulation has been shown for KSHV-infected cells (e.g. human epithelial carcinoma cells) and depends on the ability of the viral proteins K3/K5 to exploit the function of the fission protein dynamin, the ubiquitination machinery and the clathrin adaptor protein epsin1 (which contains ubiquitin-interacting motifs) (Boname and Lehner, 2011; Cadwell and Coscoy, 2008; Coscoy et al., 2001; Coscoy and Ganem, 2000; Duncan et al., 2006).

The t-test/ANOVA statistical analyses performed during the current study have a low risk for type II statistical errors (not rejecting the null hypothesis while in fact it should be rejected, in other words missing significant differences between conditions). However, since the sample size does not allow testing for normality, t-test/ANOVA analyses may have a risk

for type I statistical errors (incorrectly assuming significant differences). Therefore, data were re-analyzed using more conservative non-parametric tests, which only resulted in statistically significant differences ($P < 0.05$) between "Mock" and "WT-10 16h", between "Mock" and "WT 4h" and between "Mock" and "Mock-CHX 4h" (Friedman test with Dunn's post test). Although other data were borderline significant in the non-parametric test (e.g. the difference between WT and WT treated with rottlerin post infection had a P-value of 0.06 with the Wilcoxon matched pair test), the other significant differences observed in the manuscript using t-test/ANOVA should be interpreted with caution and should be substantiated in further research, e.g. by performing additional independent replicates.

To further explore the endocytic mechanism(s) involved in the EHV1-mediated downregulation of MHCI in equine MDDC, following experiments could be proposed. First, a dilution series of the employed inhibitors could be performed with the appropriate controls to maximize specificity while minimizing off-target effects. Second, additional inhibitors targeting the same or other endocytosis components could be used. For example, not only the role of F-actin dynamics could be further ascertained using F-actin disruption (e.g. latrunculin A/B) or stabilization (e.g. jasplakinolide) agents, but also the role of clathrin (e.g. Pitstop2-100) or dynamin (e.g. Bis-T-23) could be strengthened by employing other inhibitors (Chaudhry et al., 2007; Dutta and Donaldson, 2012; Sandgren et al., 2010; Stahlschmidt et al., 2014). In addition, inhibitors (e.g. PYR-41) of the ubiquitin signaling pathway could be used to evaluate if ubiquitin tagging also plays a role in downregulating MHCI on EHV1-infected equine MDDC, as was demonstrated previously for MHCI internalization from the cell surface of KSHV-infected cells and EHV1-infected ED cells (Coscoy et al., 2001; Duncan et al., 2006; Huang et al., 2014). Third, overexpression of dysfunctional isoforms of central endocytosis components (e.g. caveolin 1, dynamin II, eps15) could significantly strengthen conclusions (Huang et al., 2014). Also, co-localization studies between internalized labeled

MHCI and labeled endocytosis components may further elucidate this process. In summary, our inhibitor studies thus far suggest that MHCI downregulation in EHV1-infected MDDC occurs via a dynamin-dependent (similar to ED cells) and clathrin-dependent (different from ED cells) endocytosis process (Huang et al., 2014).

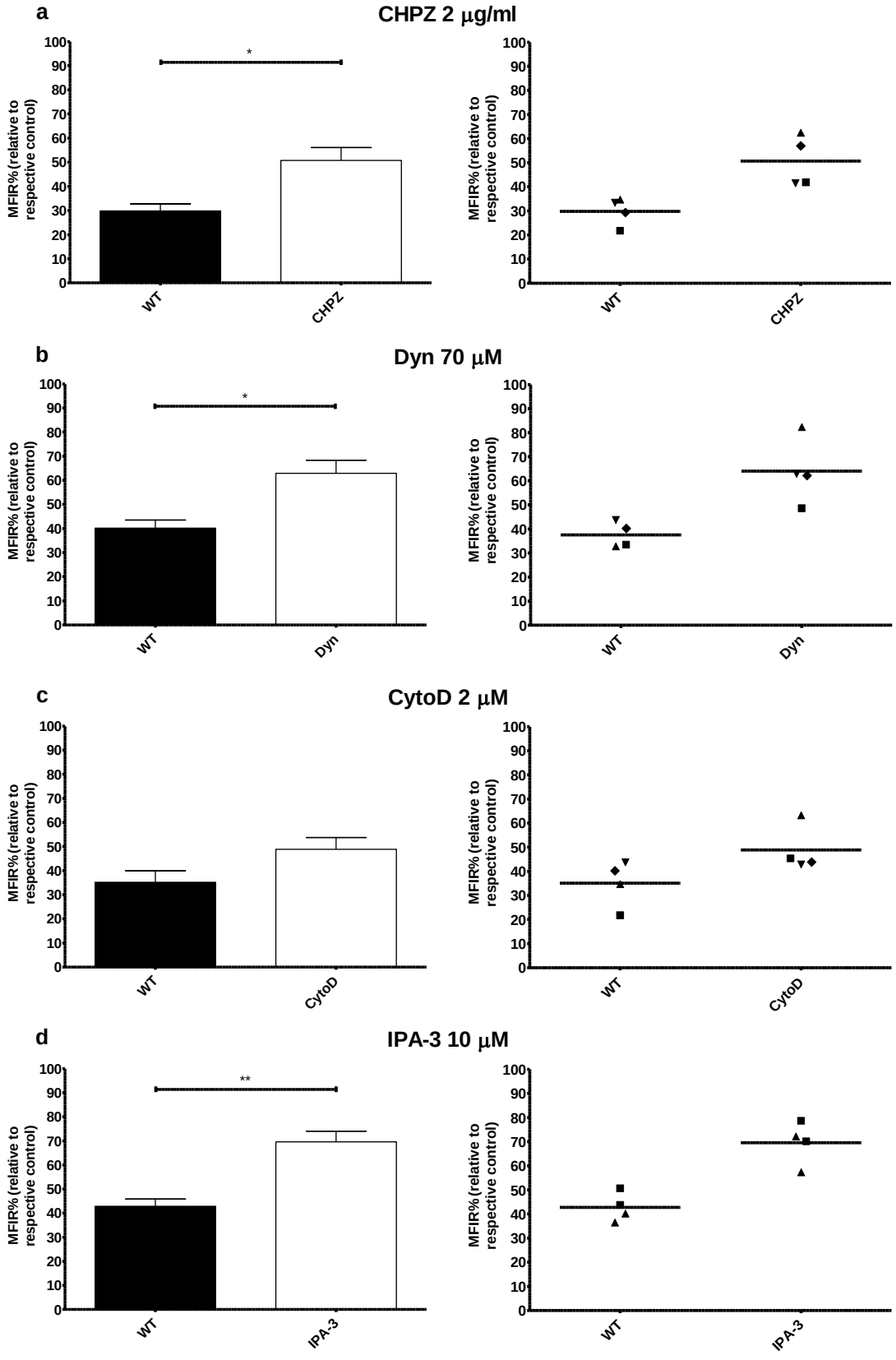


Figure 4: Effect of the inhibition of clathrin assembly, dynamin GTPase, actin cycling and group I PAK signaling on EHV1-mediated downregulation of MHCI on equine dendritic cells. Equine

monocyte-derived dendritic cells (MDDC) were mock-inoculated or inoculated with equine herpesvirus 1 strain Ab4G at a MOI 10. At 2 h post-inoculation (hpi), the following inhibitors were added at indicated concentrations: chlorpromazine (CHPZ, inhibits clathrin coat formation), dynasore (Dyn, inhibits the dynamin GTPase domain), cytochalasin D (CytoD, inhibits actin polymerization) or IPA-3 (inhibits group I PAK activation 3). At 16 hpi, the cell surface immunophenotype was analyzed by flow cytometry. The mean fluorescent intensity ± SEM of at least 4 independent repeats from at least 3 horses was normalized to the isotype control and expressed relative to mock-Ab4G inoculated MDDC which were either mock-treated or treated with the corresponding inhibitor (mean fluorescence intensity ratio, MFIR%). Two-sided Student's t test for paired observations was used to determine statistically significant differences (* = $P < 0.05$; ** = $P < 0.01$).

Chapter 5: EHV1 infection of equine mesenchymal stem cells induces a pUL56-dependent downregulation of select cell surface markers.

Adapted from:

Christophe Claessen, Herman Favoreel, Guanggang Ma, Nikolaus Osterrieder, Catharina De Schauwer, Sofie Piepers, Gerlinde R. Van de Walle

Veterinary Microbiology 176 (2015), 32-39

1. Abstract

Equid herpesvirus (EHV) 1 is a ubiquitous α-herpesvirus that can cause respiratory disease, abortion and central nervous disorders. EHV1 is known to infect a variety of different cell types *in vitro*, but its tropism for cultured primary equine mesenchymal stem cells (MSC) has never been explored. We report that equine MSC were highly permissive for EHV1 and supported lytic replication of the virus *in vitro*. Interestingly, we observed that an infection of MSC with EHV1 resulted in a consistent downregulation of cell surface molecules CD29 (β1-integrin), CD105 (endoglin), major histocompatibility complex (MHC) I and a variable downregulation of CD172a. In contrast, expression of CD44 and CD90 remained unchanged upon wild type infection. In addition, we found that this selective EHV1-mediated downregulation of cell surface proteins was dependent on the unique long protein (pUL) 56. So far, pUL56-dependent downregulation during EHV1 infection of equine cells has only been described for MHCI, but our present data indicate that pUL56 may have a broader function in downregulating cell surface proteins. Taken together, our results are the first to show that equine MSC are susceptible for EHV1 and that pUL56 induces downregulation of several cell surface molecules on infected cells. These findings provide a basis for future studies to evaluate the mechanisms underlying for this selective pUL56-induced downregulation and to evaluate the potential role of MSC during EHV1 pathogenesis.

2. Introduction

Equid herpesvirus type 1 (EHV1) is an ubiquitous α-herpesvirus that will infect most horses during their life time, sometimes resulting in serious clinical signs with a considerable negative impact on horse welfare and economics (Dunowska, 2014c). Clinical manifestations include respiratory disease, abortion, death of full term newborn foals and the emerging equine herpes myeloencephalopathy (EHM). During EHV1 pathogenesis, the virus is able to infect several cell types and tissues such as the respiratory epithelium and underlying connective tissues (primary replication). It can also cause a cell-associated viremia followed by infection of the endothelium of the pregnant uterus and the central nervous system (secondary replication) (Allen and Bryans, 1986; Edington et al., 1986; Kydd et al., 1994b). Although the exact identity of EHV1-infected cells during primary replication and viremia remains a matter of debate, a recent *in vivo* study demonstrated that most infected cells in the respiratory submucosa, draining lymph nodes and the blood are positive for the cell surface marker CD172a (Gryspeerdt et al., 2010). CD172a belongs to the family of signal regulatory protein (SIRP) and is typically found on the surface of neurons and myeloid cells (van Beek et al., 2005). In addition, CD172a has also been described to be expressed on the cell surface of human and rat MSC, but its expression on equine MSC has never been explored to date (Rooney et al., 2008; Vogel et al., 2003). MSC are multipotent adult stem cells which can differentiate into a variety of cell types of mesodermal origin (Stewart and Stewart, 2011) and are typically validated based on their specific immunophenotypic profile as well as their *in vitro* differentiation capacity towards the osteogenic, chondrogenic and adipogenic lineage (De Schauwer et al., 2012). Equine MSC have been shown to be present in a wide range of tissues and organs, including the blood (da Silva Meirelles et al., 2006; De Schauwer et al., 2012; Spaas et al., 2013).

Therefore, the aim of the present study was to evaluate whether blood-derived equine MSC are susceptible to EHV1 infection and whether EHV1 infection can alter the immunophenotypic profile of these cells, a frequently used technique to validate equine MSC (De Schauwer et al., 2012). Our salient findings were that EHV1 causes a productive infection in equine MSC *in vitro*. Moreover, we found that EHV1 infection of these cells resulted in a selective and consistent, pUL56-dependent, downregulation of the immunophenotypic cell surface markers CD29, CD105 and MHCI. In addition, there was a variable pUL56-mediated downregulation of CD172a. Based on our findings, it will be interesting to dissect the underlying mechanism of the apparently selective downregulation mediated by pUL56 and also, based on the presence of MSC in several tissues and blood, what the potential role of MSC could be during EHV1 pathogenesis.

3. Materials and Methods

3.1. Cells and viruses.

Rabbit kidney (RK13) cells and equine dermal fibroblasts (ED) (NBL6) were maintained in Dulbecco's Minimum Essential Medium (DMEM low glucose; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin (1% Pen/Strep; Sigma), at 5% CO_2 and 37° C. Equine MSC were isolated from the peripheral blood of healthy donor horses (EC2010/147) and cultured in low glucose (LG) Dulbecco's modified Eagle medium (DMEM) (Invitrogen), supplemented with 30% FBS (GIBCO), 10^{-11} M low dexamethasone, 50 µg/ml gentamicin, 10 µL/ml antibiotic–antimycotic solution, 250 ng/ml fungizone (all Sigma), and 2 mM ultraglutamine (Invitrogen) (MSC isolation medium), exactly as previously described (Spaas et al., 2013). MSC were further maintained in MSC isolation medium without dexamethasone (MSC maintenance medium). The parental EHV1 strain Ab4 (vAb4) and the enhanced green fluorescent protein (eGFP)-

expressing wild type Ab4G and mutant virus Ab4GΔ1, lacking the pUL56 encoding open reading frame (ORF) 1 gene, were propagated in RK13 cells and were described previously (Ma et al., 2012).

3.2. Antibodies.

MSC were labeled using the following panel of primary antibodies: CD29 (Chemicon, clone TDM29), CD44 (Abcam, clone IM7), CD45 (Serotec, clone F10-89-4), CD79a (Serotec, clone HM57), CD90 (VMRD, clone DH24A), CD105 (Serotec, clone SN6), CD172a (VMRD, clone DH59B), MHCI (VMRD, clone PT85A) MHCII (Novus, clone CVS20) and active caspase 3 (R&D, polyclonal rabbit). Rabbit anti-EHV1 pUL56 polyclonal antibody has been described previously (Ma et al., 2012). Secondary antibodies were Alexa647- or Cy5-conjugated (Invitrogen). Used isotype controls were commercial IgM (BD 557275) and in-house made IgG1 (anti-PRRSV) and IgG2a (anti-PCV2) antibodies.

3.3. *In vitro* growth kinetics.

To evaluate EHV1 replication in MSC, single-step growth kinetics were determined after infection of 2 x 10^5 MSC or ED (equine control cell line) with vAb4, Ab4G or its isogenic deletion mutant Ab4GΔ1 (lacking the pUL56 encoding ORF1 gene) at a multiplicity of infection (MOI) of 3, essentially as described previously (Van de Walle et al., 2008). Briefly, virus was allowed to attach and penetrate for 90 min at 37° C. Unbound virus was removed by washing the cells twice with phosphate buffered saline (PBS, pH = 7.6), followed by an incubation step for 3 minutes with ice-cold citrate buffered saline (CBS, pH = 3.0) after which the cells were washed again twice with PBS. Supernatant and cells were harvested separately at 0, 4, 8, 24 and 48 hpi, and cell-associated and extracellular viral titers were determined by plating onto RK13 cells, which were overlaid with carboxy-methylcellulose. At 2 days pi, cells were fixed with 90% aqueous acetone solution and stained with 0.1% crystal violet.

Plaques were counted and expressed as number of plaque forming units (PFU)/ml for three independent experiments.

3.4. Flow cytometry.

MSC were mock-infected or infected at an MOI of 10 with the various eGFP-expressing viruses for 16 h, unless indicated otherwise. In an additional experiment, MSC were incubated with supernatant of Ab4G-infected MSC. This supernatant was collected at 16 hpi and ultracentrifuged at 30,000xg to remove free virions. MSC were trypsinized, resuspended in MSC medium and washed once in DMEM LG with 1% BSA (DBSA) before labeling with primary antibodies for 15 min on ice. Cells to be incubated with pUL56 antibody were fixed first with 4% paraformaldehyde for 10 minutes and then permeabilized with 0.1% TritonX-100 (Sigma) for 2 min, both at room temperature. Cells were washed in PBS supplemented with 20% FCS (PFSC) and subsequently incubated with pUL56 antibody, diluted in PFSC, for 15 min on ice as well. Cells to be incubated with active caspase 3 antibody were first fixed with 3% paraformaldehyde for 10 minutes and then permeabilized with 0.1% saponin (Sigma) in PBS, both at RT. After 60 minutes incubation with caspase 3 antibody in 0.1% saponin at 37° C, cells were washed twice with 0.1% saponin in PBS. After incubation with the primary antibodies, cells were washed twice with DBSA. All cells, except for the active caspase 3 samples, were incubated for 15 min on ice with 10% goat serum (Invitrogen). Subsequently, cells were incubated with Alexa647- or Cy5-conjugated secondary antibodies, respectively, for 15 minutes on ice in the dark. For the active caspase 3 staining, cells were incubated for 60 minutes at 37° C with Alexa647-conjugated secondary antibody supplemented with 10% goat serum and 0.1% saponin. After two washing steps with DBSA, the viability of the MSC was assessed with 7-amino-actinomycin D (7AAD, Invitrogen). For each sample, at least 10,000 cells were analyzed using a FACSCanto flowcytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm

HeNe laser. Only 7AAD negative (live) cells were analyzed and in the case of virus-infected cells, only 7AAD negative/eGFP positive cells were evaluated. Apoptosis of Ab4G-infected cells was evaluated at indicated time points by gating against apoptosis levels of mock-infected cells. Isotype controls were applied to correct for unspecific binding and potential bleedthrough, due to spectral overlap between used fluorochromes, was compensated with an automatic calibration technique (FACSDiva software, Becton Dickinson). The expression levels of surface markers were presented as positive live cell percentage compared to the isotype control or as mean fluorescence intensities normalized to the isotype and expressed relatively to the appropriate control (mean fluorescence intensity ratio). All data were analyzed with the FACSDiva software (Becton Dickinson) and expressed as mean ± standard error of the mean (SEM).

3.5. Statistical analysis.

Statistical analysis was performed with Prism (GraphPad). A two-sided Student's t-test for paired observations was used to evaluate statistical differences between mock- and EHV1-infected MSC and between ED and MSC at $P < 0.05$. One-way repeated measures ANOVA analysis with the Tukey post-test was performed to evaluate statistical differences between mock-, Ab4G- and Ab4GΔ1-infected cells at $P < 0.05$. To determine the effect of EHV1-infection on the intracellular and extracellular PFU/ml in MSC, a linear mixed regression model with experiment as random effect (REPEATED statement) was fit using SAS 9.3 (PROC MIXED, SAS 9.3, SAS Institute Inc., NC, USA). A compound symmetry correlation structure was used to account for the clustering of repeated measurements within an experiment. A log10-transformation of PFU/ml was performed to obtain a normal distribution. The models with the log10-transformed intracellular PFU/ml and extracellular PFU/ml, respectively, as dependent variables included time point (5 levels – 0 hpi, 4 hpi, 8 hpi, 24 hpi and 48 hpi), cell type (2 levels – MSC and NBL-6) and the interaction term

between time point and cell type as categorical independent variable. Statistical significance was assessed at $P < 0.05$.

4. Results

4.1. Equine mesenchymal stem cells (MSC) are susceptible for EHV1 infection *in vitro*.

In general, MSC are characterized by three criteria: they (i) are plastic-adherent, (ii) display a specific cell surface marker phenotype, and (iii) are capable of differentiating into osteoblasts, chrondroblasts and adipocytes *in vitro* (Dominici et al., 2006). The equine peripheral blood-derived MSC used in this study showed a profile identical to what has been described previously (De Schauwer et al., 2012; Spaas et al., 2013). They were positive for CD29, CD44, CD90 and CD105; negative for CD45, CD79a and MHCII; and variably positive for MHCI (Figure 1). In addition, we also evaluated the expression of CD172a, since this cell surface marker has been used for immunophenotyping of human MSC (Vogel et al., 2003) and we found our equine MSC to be positive for CD172a also (Figure 1). Therefore, we propose CD172a as a novel addition to the panel used for immunophenotyping MSC of the horse.

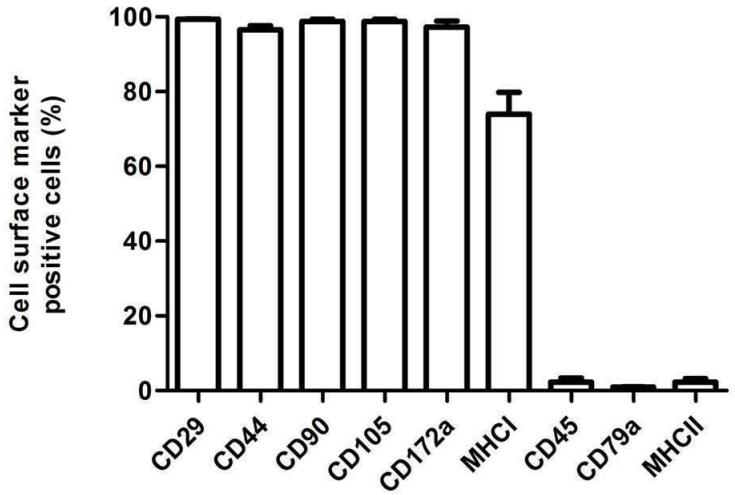
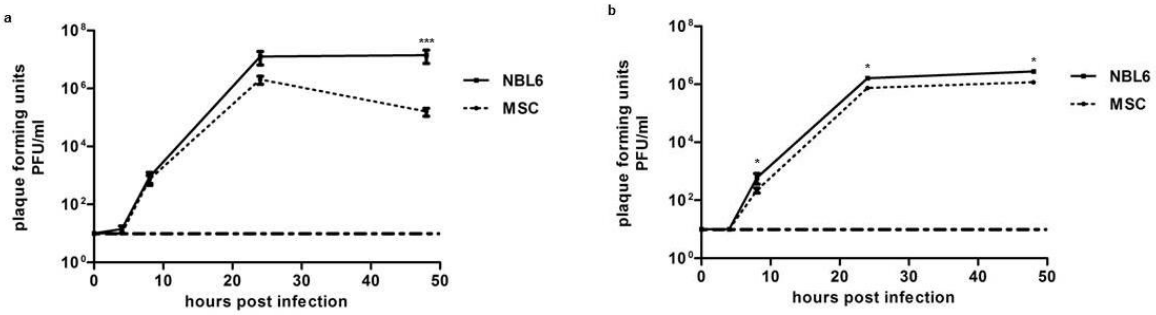


Figure 1: Immunophenotyping of equine MSC. Equine MSC were isolated from the peripheral blood of healthy donor horses and subjected to flow cytometry, as described in Methods, to

evaluate their immunophenotypic profile. The results are expressed as the mean ± SEM of at least three independent experiments.

To investigate the susceptibility of equine MSC towards EHV1 infection, single-step growth kinetics and plaque sizes were determined for MSC and compared to those for ED cells, an equine dermal fibroblast cell line routinely used in EHV1 research. We observed that EHV1 strain vAb4 exhibited similar intracellular and extracellular growth rates in MSC compared to ED cells although at 48 hours post infection (hpi), significantly lower intracellular virus titers were observed in MSC (1.5 x 10^5 PFU/ml) compared to ED (5.1 x 10^6 PFU/ml) (Figure 2a). Further statistical analysis also revealed small, but significantly different, extracellular virus titers between ED and MSC from 8 hpi on (Figure 2b). To address the possibility that the significant differences in intracellular virus titers at 48 hpi were due to differences in vitality or levels of apoptosis, flow cytometric analyses using vitality and apoptosis markers were performed. Briefly, no significant differences were observed in cell viability and apoptosis between EHV1-infected MSC and ED at 24 and 48 hpi, indicating that these do not account for the significant difference observed at these late time points post infection (Figure 3). The cell-to-cell spread capacity of EHV1 in MSC was comparable to that in ED, as indicated by virtual identical plaque sizes in both cell types (Figure 2c).



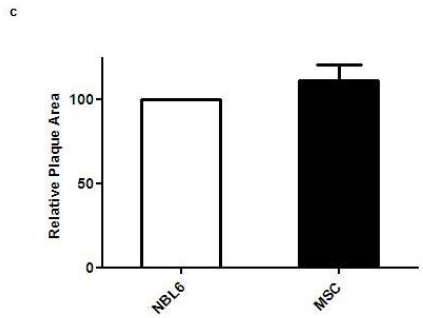


Figure 2: Equine MSC are susceptible to EHV1 *in vitro*. Single step growth kinetics of the EHV1 strain vAb4 in equine MSC and ED cells. Cellular (intracellular) (a) and supernatant (extracellular) (b) fractions were collected at the indicated time points and virus titers were determined by standard plaque assay. The results are expressed as the mean ± SEM of three independent experiments. The detection limit is displayed as the dashed-dotted line at 10 PFU/ml. A linear mixed regression model with experiment as random effect determined significant differences (* P < 0.001; * P < 0.05). (c) Plaque sizes ± SEM of EHV1-infected MSC and ED cells. Plaque sizes of 50 plaques were determined for equine MSC at 3 days pi with EHV1 Ab4G and compared to the plaque sizes of Ab4G infected ED cells which was set to a 100%. Two-sided Student's t-test for paired observations determined no significant differences (P > 0.05).**

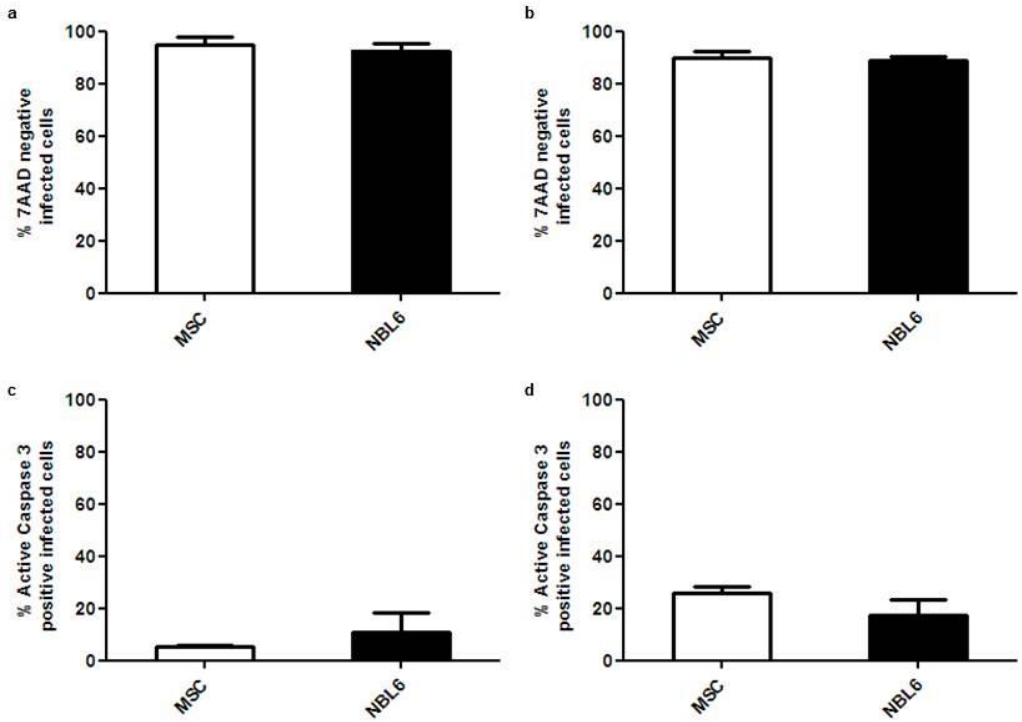


Figure 3: Vitality and apoptosis is not significantly different between EHV1-infected equine MSC and ED at 24 and 48 hpi. Equine MSC and ED were infected with Ab4G and analyzed by flow cytometry at 24 hpi (a, c) and 48 hpi (b, d) for vitality (7AAD negative) (a,b) or apoptosis (active caspase 3 positive) (c, d). All results are expressed relative to their respective negative control for three independent experiments. Two-sided Student's t tests for paired observations determined no significant differences (P > 0.05).

4.2. The immunophenotypic profile of equine MSC is altered following EHV1 infection.

Next, MSC were infected with recombinant Ab4G, which expresses eGFP, allowing flow cytometric identification of infected cells. At 16 hpi, the immunophenotypic profile of mock-infected or Ab4G-infected MSC was evaluated. A significant downregulation of MHCI, CD29 and CD105 was observed in eGFP+ EHV1-infected MSC when compared to mock-infected MSC (Figure 4). The cell surface of eGFP+ EHV1-infected MSC also showed a slight increase of CD44 and a minor reduction of CD90 and CD172a expression, albeit not significant, when compared to mock-infected MSC (Figure 4). The cell surface markers that are negative for MSC immunophenotyping, i.e. CD45, CD79a, and MHCII, remained negative on EHV1-infected MSC at 16 hpi (data not shown).

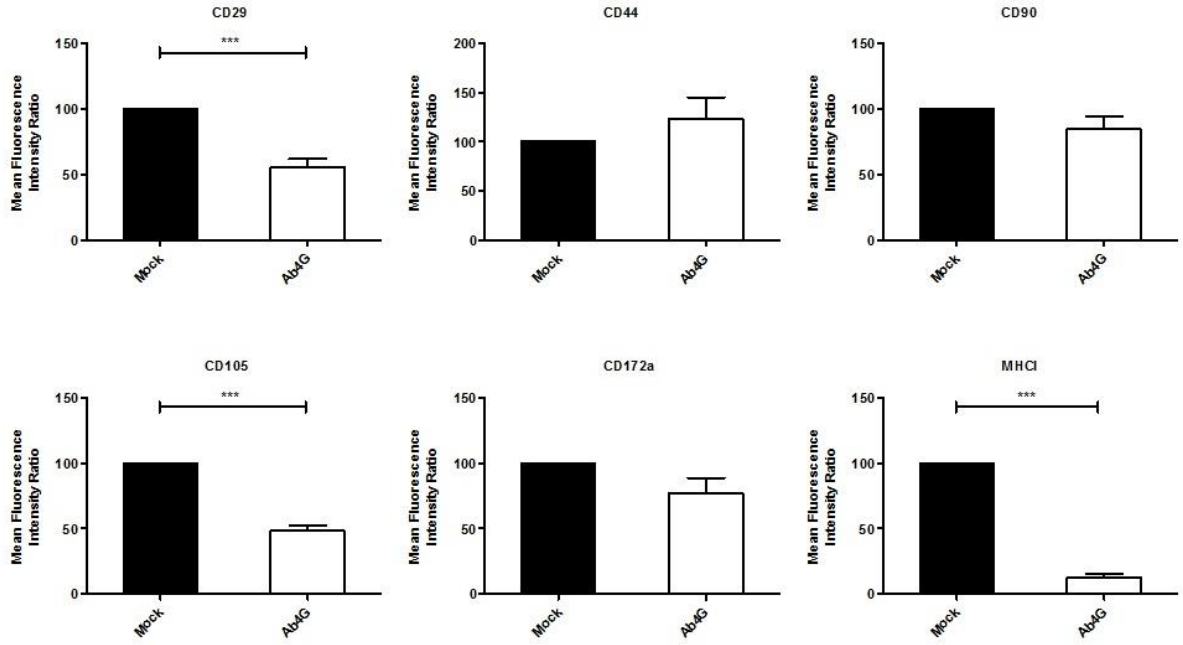


Figure 4: The immunophenotypic profile of equine MSC is altered upon EHV1 infection. MSC were mock-infected or infected with eGFP-expressing Ab4G and the expression of several cell surface markers was analyzed for mock and eGFP+ cells at 16 hpi using flow cytometry. The mean fluorescent intensity ± SEM of at least three independent experiments is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio). Two-sided Student's t-test for paired observations determined significant differences (* = P < 0.001).**

4.3. Selective downregulation of cell surface molecules in EHV1-infected cells is pUL56-dependent.

A significant downregulation of MHCI has been reported earlier in EHV1-infected ED cells and was observed at early times of infection (Ma et al., 2012). In order to investigate the kinetics of downregulation of MHCI, CD29 and CD105 from the cell surface of MSC, time course studies were performed with eGFP-expressing Ab4G. Downregulation of cell surface markers was initiated early in infection, i.e. from 4 hpi onwards, reached a plateau by 6 hpi and was maintained throughout the replication cycle (Figure 5). Interestingly, the initiation of downregulation of the different MSC markers coincided with the expression of the unique long protein (pUL) 56 (Figure 5).

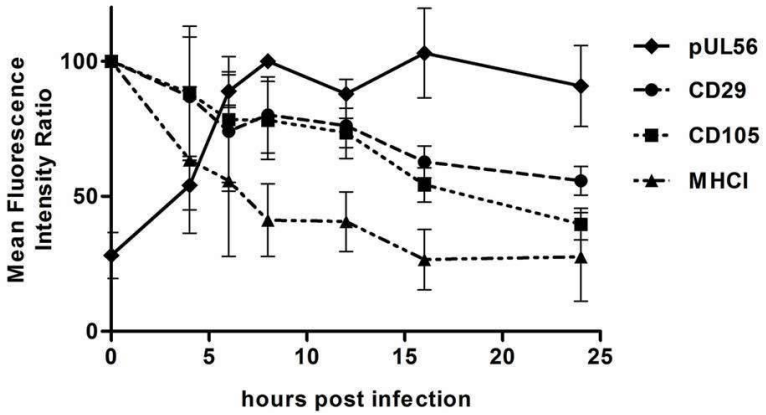


Figure 5: Time course kinetics of viral and cellular protein expression. MSC were mock-infected or infected with eGFP-expressing Ab4G and at the indicated time points, protein expression of MHCI, CD29, CD105 and the viral pUL56 protein was analyzed for mock or eGFP+ cells using flow cytometry. The mean fluorescent intensity is normalized to the isotype control and expressed as the mean ± SEM of three independent experiments. Cell surface markers and pUL56 MFIR were compared to time point 0 hpi and 8 hpi, respectively (mean fluorescence intensity ratio).

Since it has been previously demonstrated that downregulation of MHCI in ED depends on expression of this viral protein, we investigated whether pUL56 was involved in the downregulation of cell surface marker expression in EHV1-infected MSC also (Ma et al., 2012). To this end, MSC were infected with Ab4G or its isogenic mutant virus Ab4GΔ1, which lacks the ORF1 gene encoding pUL56, and the cell surface marker expression of

MHCI, CD29 and CD105 was evaluated by flow cytometry at 16 hpi. At this time point, approximately 35% of the cells were infected, as evaluated by their eGFP expression (data not shown). When MSC were infected with the mutant virus unable to express pUL56, the downregulation of MHCI, CD29 and CD105 was no longer observed in the eGFP+ cell population, representing the EHV1 infected MSC, indicating that the downregulation of these cell surface markers is indeed pUL56-dependent (Figure 6a).

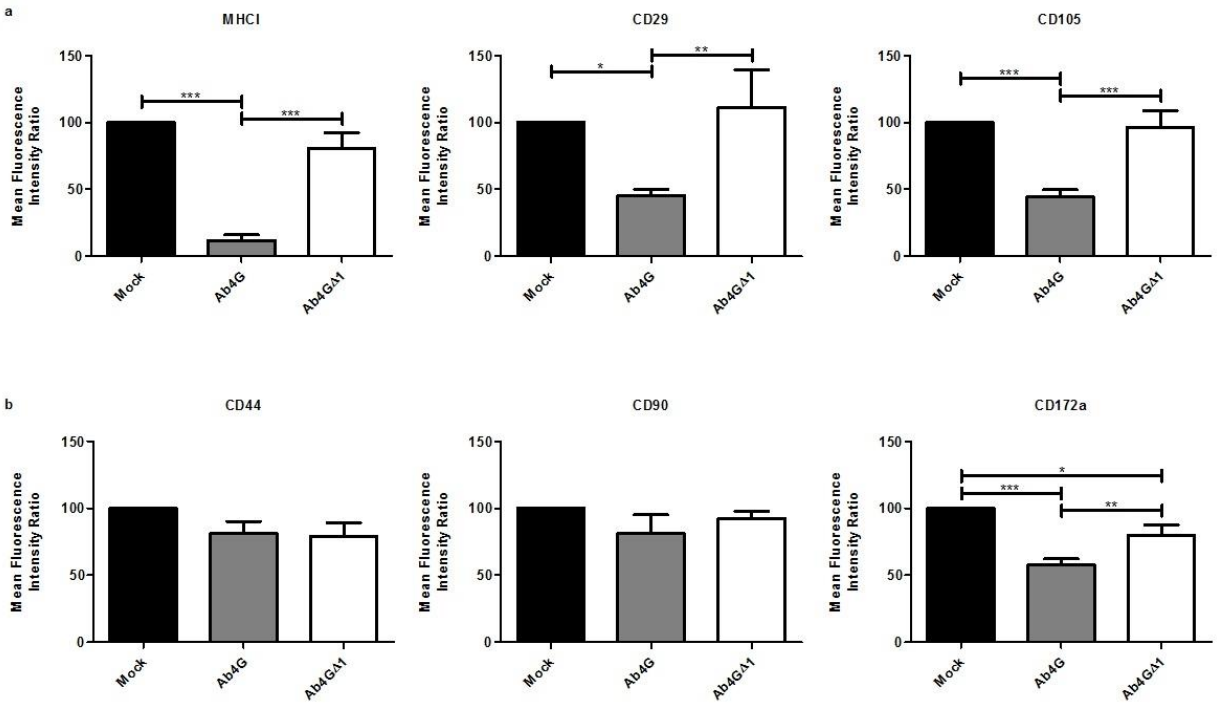


Figure 6: The selective downregulation of cell surface molecules in EHV1-infected cells is pUL56-dependent. MSC were mock-infected, infected with Ab4G or infected with the mutant virus Ab4GΔ1 which lacks the ORF1 gene encoding pUL56. At 16 hpi, eGFP+ cells were analyzed for cell surface expression of MHCI, CD29 and CD105 (a) or CD44, CD90, and CD172a (b). The mean fluorescent intensity is normalized to the isotype control and expressed as the mean ± SEM relative to the mock (mean fluorescence intensity ratio) for at least three independent experiments. One-way repeated measures ANOVA analysis determined statistical differences (* = P < 0.05; ** = P < 0.01; *** = P < 0.001).

In addition, Ab4G and Ab4GΔ1 demonstrated virtually identical growth kinetics in MSC (Figure 7), indicating that differences in replication efficiency between both viruses do not account for the observed difference in cell surface marker downregulation. Furthermore since MSC are known to secrete numerous factors, we also explored whether such soluble factors secreted by Ab4G-infected MSC could be responsible for the observed downregulation of

MHCI, CD29 and CD105 (Kyurkchiev, 2014). To this end we treated MSC for 16 h with either wild-type Ab4G virus or with virus-free supernatants. These virus-free supernatants were obtained after ultracentrifugation of MSC that had been infected with Ab4G for 16 h. Virus-free supernatant was unable to induce downregulation of cell surface markers, supporting the hypothesis that direct infection with EHV1 is necessary for the observed downregulation (Figure 8).

Additionally, we also examined the cell surface expression of CD44, CD90 and CD172a, the markers that were not significantly altered upon infection with wild type EHV1, when infected with the mutant virus Ab4GΔ1. Similar to what we found before, no significant alterations in CD44 and CD90 expression was seen in wild type, Ab4G-infected MSC, and infection with the mutant Ab4GΔ1 also did not result in significant alterations of these cell surface markers (Figure 6b). Somewhat surprisingly, while we observed a statistically non-significant trend of decreased CD172a levels in Ab4G-infected MSC in earlier experiments (Figure 4), this CD172a downregulation was statistically significant in these additional experiments (Figure 6b). Interestingly, and in contrast to CD29, CD105 and MHCI, this CD172a downregulation was only partially mediated by pUL56 expression as demonstrated by the absence of a full reversion to mock expression levels after Ab4GΔ1 incubation (Figure 6b).

Although elusive at this point, a potential explanation for this discrepancy in CD172a expression could be attributed to the fact that these additional experiments were done with equine MSC at a higher passage level and could indicate that the variable effects of EHV1 infection on CD172a expression in MSC are cell passage-dependent.

Taken together, we concluded from our results that the consistent EHV1-induced downregulation of CD29, CD105 and MHCI in equine MSC requires an active viral infection, starts at early times of infection, and depends on pUL56 expression.

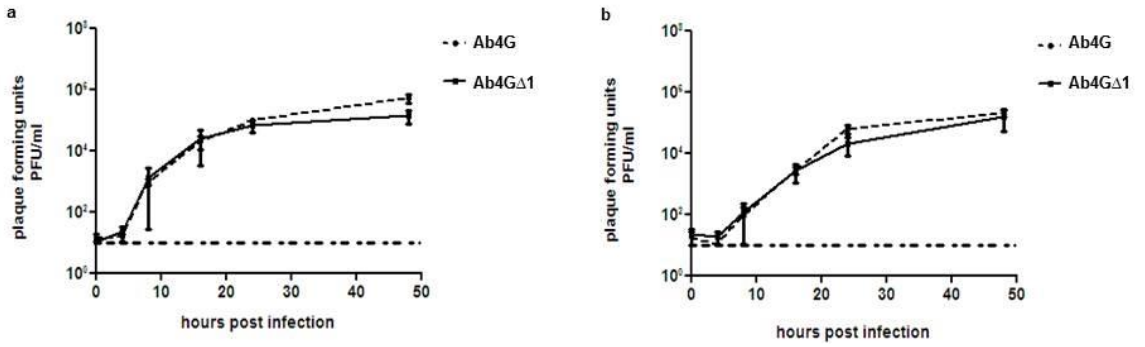


Figure 7: Similar growth rates in equine MSC for the Ab4G and Ab4GΔ1 EHV1 virus strains. Single step growth kinetics for EHV1 Ab4G and its isogenic deletion mutant Ab4GΔ1 in equine MSC. Cellular (a) (intracellular) and supernatant (extracellular) (b) fractions were collected at the indicated time points and virus titers were determined by standard plaque assay. The results are expressed as the mean ± SEM of three independent experiments. The detection limit is displayed as the dashed-dotted line at 10 PFU/ml.

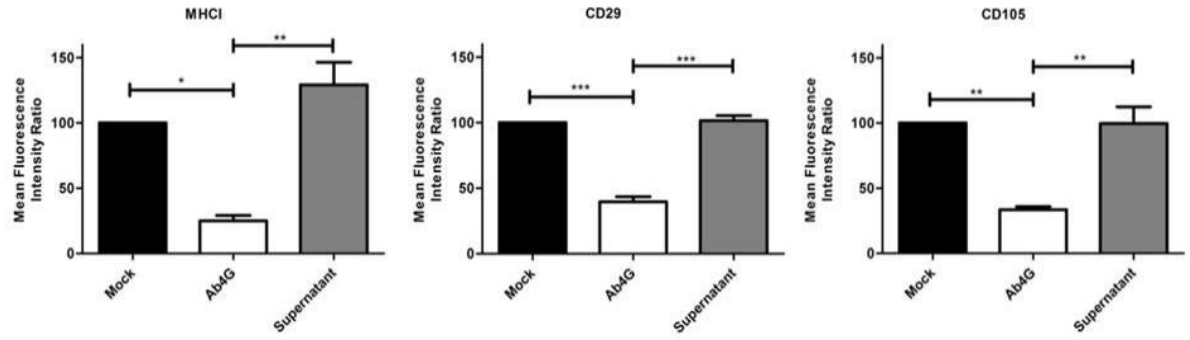


Figure 8: Virus-free supernatant could not induce downregulation of CD29, CD105 and MHCI in equine MSC. Equine MSC were mock-infected, infected with Ab4G for 16 h or treated with ultracentrifuged virus-free supernatant from 16 h Ab4G-infected equine MSC. At 16 hpi, eGFP+ cells were analyzed for cell surface expression of MHCI, CD29 and CD105. The mean fluorescent intensity ± SEM of three independent experiments is normalized to the isotype control and expressed relative to the mock (mean fluorescence intensity ratio). One-way repeated measures ANOVA analysis determined statistical differences (* = P < 0.05; ** = P < 0.01; *** = P < 0.001).

5. Discussion

The present study is the first to report on the susceptibility of equine MSC for the equine pathogen EHV1. To date, only a handful of studies are published that evaluate the susceptibility of MSC for herpesviruses. For example, human MSC were shown to be productively infected *in vitro* with the human α-herpesvirus herpes simplex virus (HSV) and the β-herpesvirus human cytomegalovirus (HMCV) (Choudhary et al., 2011; Smirnov et al.,

2007; Sundin et al., 2006). In addition, γ-herpesviruses such as human Kaposi's sarcoma-associated herpesvirus (KSHV) or bovine herpesvirus 4 are also capable of infecting MSC *in vitro* (Donofrio et al., 2005; Parsons et al., 2004).

Interestingly, we found that infection with EHV1 altered the expression of several, but not all, cell surface markers that are frequently used to immunophenotype MSC. A modulation of cell surface marker expression in infected MSC was described for HCMV, where downregulation of the cell surface markers MHCI, CD29, CD44, CD73, CD90 and CD105 was observed at 72 hpi (Smirnov et al., 2007). In line with this study, we also found a consistent downregulation of MHCI, CD29 and CD105 in the EHV1-MSC system, a less prominent downregulation of CD172a and slightly attenuated expression of CD44 and CD90, although the latter were not statistically significant. Hence, in contrast to HCMV-infected MSC, where infection-induced alteration was observed for all cell surface markers tested, we found a robust and consistent downregulation of three cell surface markers, i.e. MHCI, CD29 and CD105, in EHV1-infected MSC.

Downregulation of MHCI after EHV1 infection has been reported in a variety of cell types (Ambagala et al., 2004; Huang et al., 2014; Ma et al., 2012; Rappocciolo et al., 2003; Soboll Hussey et al., 2014) and we found in our present study that EHV1-induced MHCI downregulation also happens in adult stem cells. This MHCI downregulation is thought to be an immune evasion strategy used by EHV1 and other α-herpesviruses to avoid recognition and destruction by cytotoxic T lymphocytes (CTL) (Deruelle et al., 2009; Eisfeld et al., 2007; Koppers-Lalic et al., 2001; Rappocciolo et al., 2003). More recently, it was found, at least in human cells, that EHV1 also results in the downregulation of other cell surface markers and that this was mediated by the viral protein pUL56 (Huang et al., 2014; Ma et al., 2012; Soboll Hussey et al., 2014). Our study is the first to report that this occurs in equine cells as well. Indeed, we observed in EHV1-infected equine MSC a consistent, pUL56-dependent

downregulation of the cell surface markers CD29 and CD105, and a variable, partially pUL56-dependent downregulation of CD172a. It will be interesting to further investigate the underlying mechanisms of this selective, pUL56-dependent downregulation of different cell surface markers, but these studies may suggest that pUL56 promotes downregulation of particular clusters of proteins rather than single proteins, e.g., proteins concentrated in microdomains or lipid rafts. In this regard, it is interesting to note that pUL56 of the human α-herpesvirus HSV2 was found associated with lipid rafts (Koshizuka et al., 2002), as were the cellular surface molecules CD29, CD105, CD172a and MHCI (Kim et al., 2012).

Although speculative at this point, since we showed in our present study that equine MSC are susceptible for EHV1 *in vitro*, it is tempting to hypothesize that equine blood-derived MSC may also be infected *in vivo*. However, this proved extremely difficult to investigate since we cannot simply identify and sort out equine MSC in the blood of EHV1 infected horses due to the lack of a single specific MSC-marker. Still, we did find some indirect indications pointing towards *in vivo* susceptibility of equine MSC for EHV1. We were able to obtain peripheral blood mononuclear cell (PBMC) samples from horses (n=19) before and after infection with EHV1 (a kind gift from Dr. B. Wagner, College of Veterinary Medicine, Cornell University) and found that the efficiency to culture MSC from these samples dropped from 93% isolation efficiency before infection to 45% isolation efficiency after infection. Although speculative at this point, a potential explanation for this marked reduction in isolation efficiency could be that normally circulating, non-infected, MSC in the bloodstream home to extravascular EHV1 infected tissues, since it is well described that MSC migrate to injured organs and tissues (Sohni and Verfaillie, 2013). Another potential explanation could be that circulating MSC in the bloodstream do become infected with EHV1 and are either cleared from the blood circulation after lysis or leave the blood circulation due to increased migration and motility properties. For the latter, it is important to note that both CD29 and CD105 are involved in

cell adhesion and motility and these two proteins were found to be downregulated in EHV1 infected MSC. CD105 (endoglin) is involved in the organization of the actin cytoskeleton and reduced endoglin expression coincided with an enhanced migration capacity of human MSC (Jaganathan et al., 2007; Sanz-Rodriguez et al., 2004). CD29 (integrin β1-subunit), associates with α-integrins to form dimeric integrin complexes and expression of such integrin complex, for example α4β1, is required for transendothelial migration of human MSC to extravascular compartments (Rüster et al., 2006; Steingen et al., 2008). Hence, it will be interesting to investigate whether pUL56-mediated downregulation of CD29 and CD105 affects the adhesion and motility properties of EHV1 infected cells and hereby, may have functional consequences for enhanced viral spread and pathogenesis.

6. Conclusions

In summary, we have shown here that equine MSC are positive for the cell surface marker CD172a and are susceptible to infection with EHV1. Such infection did not only result in the downregulation of MHCI, but also in a consistent downregulation of the cell surface markers CD29 and CD105, and a variable downregulation of CD172a. This selective downregulation of certain cell surface markers was shown to be dependent on the expression of the viral protein pUL56.

Acknowledgements

This work was funded by a Concerted Research Action (GOA) of the Ghent University. Prof. Dr. Hans Nauwynck is greatly acknowledged for his support and we like to thank Prof. Dr. Wagner for generously sharing the samples of her EHV1 infection studies. We would also like to thank Silvia Janska and Matthew Pennington for the isolation of the MSC from these samples.

Chapter 6: General discussion

1. Outline

The respiratory epithelium, due to its continuous contact with the outer world, represents one of the primary entry sites of several pathogens, including viruses such as equine herpesvirus type (EHV) 1. Upon initial replication of EHV1 within the respiratory epithelium, the virus may spread through the tissues below the basement membrane. Earlier studies, both *in vitro* or *in vivo*, found that crossing the basement membrane by EHV1 occurs without overall disruption of this barrier and via infection of individual cells that are being used as Trojan horses (Gryspeerdt et al., 2010; Vandekerckhove et al., 2011, 2010). The majority of these single infected cells were found to be positive for the cell surface marker CD172a. Further on, it was demonstrated that the majority of EHV1-infected cells in the draining lymph nodes and blood were also positive for CD172a (Gryspeerdt et al., 2010). The research in the present PhD thesis was initiated to gain more knowledge on the interaction between EHV1 and CD172a-positive cell types. Specifically, dendritic cells (DC) and mesenchymal stem cells (MSC) were studied based on the fact that (i) DC, from a variety of species, and MSC, at least from humans and rats, are CD172a positive (Rooney et al., 2008; Vogel et al., 2003) and (ii) these two cell types are present in mucosal epithelium and blood (Jakob et al., 2010; Spaas et al., 2013; Van de Walle et al., 2009a).

The general aims of this thesis were to evaluate the effects of EHV1 infection on the typical cell surface markers (e.g. major histocompatibility complex (MHC)) of equine MDDC and identify the involved viral factor(s) (**Chapter 3**). Furthermore, we started to identify the cellular mechanism(s) misused by EHV1 to induce MHCI downregulation on equine MDDC (**Chapter 4**). In addition, if equine MSC proved to be susceptible to EHV1 infection, to evaluate the effects of EHV1 infection on select cell surface markers of equine MSC and to identify the viral factor(s) responsible for such modulation (**Chapter 5**).

2. EHV1 infection of equine MDDC results in downregulation of cell surface proteins important for proper DC functioning

In **Chapter 3**, we described that EHV1 infection of equine MDDC resulted in a significant downregulation of MHCI, CD83, CD86, CD206, CD29 and CD172a, but not of MHCII and CD11a/CD18. In contrast to previous reports on α-herpesvirus interactions with DC (Heilingloh et al., 2014; Kruse et al., 2000b; Kummer et al., 2007; Lampen et al., 2010; Samady et al., 2003), we demonstrated that downregulation of these cell surface markers on equine MDDC was not dependent on the viral expression of the virion host-shutoff (VHS) protein, the unique long protein (pUL) 49.5 or EHV1' infected cell protein (EICP) 0. In addition, virus-induced secretory factors were also not involved in the observed downregulation. However, we did found that downregulation of CD83 and CD86 on EHV1-infected equine MDDC was in part mediated by pUL56.

We found that EHV1 infection of equine MDDC did not induce a significant modulation of lymphocyte function associated antigen (LFA) 1 (CD11a/CD18; αL/β2) cell surface levels. This β2 integrin is, together with β1 integrins such as the very late antigen 4 protein (α4β1), involved in DC anchoring to the extracellular matrix (ECM) (Förster et al., 2012; Lämmermann et al., 2008; Sixt et al., 2006). Besides via increased or suppressed cell surface LFA-1 levels, cell anchoring may also be modulated by switching LFA-1 proteins from their inhibited to activated status and vice versa. Interestingly, infection of human MDDC with herpes simplex virus (HSV) 1 hampers the switch to a more migratory phenotype by inducing strong adhesion to fibronectin through persistent LFA-1 activation by strong degradation of the cytohesin interacting protein (Bedoui and Greyer, 2014; Theodoridis et al., 2011). Therefore, it would be interesting to evaluate if EHV1 may also enhance anchoring to the ECM via activation of LFA-1. This could

be interesting from an EHV1 pathogenic standpoint as this would prevent further spread of EHV1 via migrating DC and prevent homing of Ag-presenting DC to the lymph nodes. However, due to the lack of an antibody that specifically recognizes activated LFA-1 only that (cross-)reacts with equine LFA-1, we were unable to follow up on this potentially interesting hypothesis.

We found that EHV1-infected MDDC showed a significant reduction of cell surface CD29, also known as β1 integrin, which together with β2 and β3 integrins, in association with α integrins, form the canonical cell surface fibronectin receptors (Evans et al., 2009). Although speculative at this point, this may indicate that EHV1 can possibly shift the migratory balance towards release of MDDC from the ECM at the infection site, which would be in line with the hypothesis that EHV1 can hijack these cells for virus dissemination (Baghi, 2015; Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). For example, the adhesion of human MDDC to fibronectin-coated surfaces was severely reduced after treatment with β1 integrin blocking antibodies (Jancic et al., 1998). Further along these lines, it could be investigated whether or not DC lose their ability to sense the CC chemokine ligand 21 gradient secreted by the terminal lymphatic endothelial cells upon EHV1 infection (Girard et al., 2012; Prechtel et al., 2005). In this regard it is of interest to note that HSV1-infected MDDC show impaired CC chemokine receptor 7-dependent chemotaxis (Prechtel et al., 2005). Hence, it would be interesting to assess whether EHV1-infected DC retain normal transcription levels of CCR7 expression, for example by reverse transcriptase polymerase chain reaction (Cavatorta et al., 2009) or, even better, CCR7 protein levels using a cross-reacting anti-CCR7 antibody (which is currently not available).

Upon EHV1 infection of equine MDDC, we found that the cell surface expression of MHCI was drastically reduced, corroborating the findings of previous studies with HSV1-, HSV2- and

varicella zoster virus (VZV)-infected MDDC (Elboim et al., 2013; Morrow et al., 2003; Salio et al., 1999), although one study did report a lack of MHCI downregulation on HSV1-infected human MDDC (Mikloska et al., 2001). We found no downregulation of MHCII on equine MDDC, which is in agreement with previous reports on HSV1- and VZV-infected MDDC (Kruse et al., 2000b; Kummer et al., 2007; Mikloska et al., 2001; Morrow et al., 2003).

Next to the downregulation of the antigen (Ag) presentation molecule MHCI, we also found that EHV1 infection of equine MDDC significantly reduced the expression of the co-stimulatory molecule CD86 on the cell surface. This is in line with other reports where α-herpesviruses such as HSV1 and VZV induced downregulation of this B7.2 (CD86) co-stimulatory molecule on human MDDC (Mikloska et al., 2001; Morrow et al., 2003; Salio et al., 1999). Suppression of CD86 may substantially affect the generation of virus-specific cytotoxic T-lymphocytes (CTL). Indeed, by preventing the interaction of CD80/86 on antigen presenting cells (APC) with CD28 on T-lymphocytes using a soluble CD80/CD86 ligand, it was shown in mice that the number of HSV1-specific CD8+ CTL was reduced and such treatment also induced anergy (insensitivity to a specific Ag) of CD4+ T-lymphocytes. Next to CD80/86, CD40 is another important co-stimulatory molecule on DC, and its interaction with CD40L on T-lymphocytes has been shown to augment the interferon (IFN)-γ response of CD4+ T-lymphocytes during HSV-1 infection of mice (Edelmann and Wilson, 2001). Therefore, future experiments may aim at investigating whether CD40 and CD80 are also downregulated on equine MDDC upon EHV1 infection, as was demonstrated after HSV1-infection of human MDDC (Mikloska et al., 2001), when cross-reacting antibodies become available.

In addition to CD86, we also found that CD83 is downregulated on EHV1-infected equine MDDC, which is in line with what was observed for HSV1- and VZV-infected MDDC

(Heilingloh et al., 2014; Kruse et al., 2000b; Kummer et al., 2007; Morrow et al., 2003; Salio et al., 1999). In man, downregulation of this B7-like co-stimulatory cell surface molecule by siRNA resulted in reduced DC-mediated T-priming (Aerts-Toegaert et al., 2007; Prechtel et al., 2007). More specifically, *in vitro* selection, expansion, survival and cytotoxicity of low-frequency precursor human CD8+ CTL could only be accomplished with artificial APC (transduced K562 cells) if these APC also expressed CD83 (Hirano et al., 2006). Based on this, one could hypothesize that downregulation of CD83 on EHV1-infected equine MDDC may reduce the generation and longevity of EHV1-specific CD8+ CTL responses.

Due to the unavailability of a cross-reacting intercellular adhesion molecule (ICAM) 1 (CD54) antibody at the time of these experiments, we could not evaluate the cell surface expression of ICAM-1 on equine MDDC. However, when such antibodies would become available, it will be of interest to determine whether EHV1-infection can modulate ICAM-1 expression on equine MDDC. For HSV1-infected MDDC there have been conflicting data whether HSV1 infection leads to no modulation (Salio et al., 1999) or downregulation (Mikloska et al., 2001) of ICAM-1 on HSV1-infected human MDDC. Conversely, for HCMV, upregulation of ICAM-1 has been described in infected human cells including fibroblasts and MSC (Grundy and Downes, 1993; Smirnov et al., 2007). If EHV1 would induce downregulation of ICAM-1, then this could further contribute to one of the many immune evasion strategies used by EHV1. It was previously shown that blocking interaction between murine DC-expressed ICAM-1 and its ligands, such as LFA-1 and Mac (macrophage antigen) 1 (CD11b/CD18; αM/β2), on T-lymphocytes, significantly impairs the generation of long-surviving CD8+ T-memory cells (Scholer et al., 2008; Zhang et al., 2003) and so this could potentially contribute to the short-lived immunity against EHV1 after an EHV1 outbreak (Kydd et al., 2006a). If, on the other hand, EHV1 would

induce an upregulation of ICAM-1 on equine MDDC, this potentially could lead to longer lasting immunity by CD4+ and CD8+ T-cells, although this could also be subverted towards T-anergy when combined with our findings that MHCI, CD83 and CD86 are downregulated on EHV1-infected MDDC (Banchereau et al., 2000; Edelmann and Wilson, 2001).

Other interesting cell surface proteins associated with Ag-presentation to investigate on equine MDDC upon EHV1 infection are proteins belonging to the CD1 family. These molecules are also known as the non-classical MHCI, which resemble MHCI, but act like MHCII. More specifically, the lipid Ag presented by CD1 on APC are recognized by a specific T-subset, the NK (natural killer) T cells, which can assist B-lymphocytes or DC, lyse infected cells and enhance the NK-mediated immune response against the virus (Gelin et al., 2009). Two studies, using low virus inoculation titers, showed either upregulation or downregulation of CD1a on HSV1-infected human MDDC (Mikloska et al., 2001; Salio et al., 1999). Another study using HSV1-infected human MDDC showed only an upregulation of CD1b and CD1d, but no modulation of CD1a or CD1c at low virus inoculation titers, and, upon employing high titers of live HSV1 found all cell surface CD1 (a/b/c/d) to be reduced (Martin J Raftery et al., 2006). Viral clearance of HSV1 in CD1d knock-out or CD1d-restricted NKT knock-out mice was significantly reduced compared to WT mice (Grubor-Bauk et al., 2003). Further identification of (cross-) reacting antibodies, besides CD1c that was shown to be expressed on equine MDDC (Baghi et al., 2014), will aid to investigate the impact of EHV1 infection on CD1 molecules on equine MDDC.

Similar to the human herpesviruses HSV1, HSV2 and VZV, it was previously demonstrated that EHV1 infection of equine DC could impair their capacity to support T-lymphocyte proliferation (Kruse et al., 2000b; Morrow et al., 2003; Peretti et al., 2005; Salio et al., 1999; Siedek et al.,

1997). It will be interesting in future experiments to try to establish a direct link between this impaired capacity to support T-lymphocyte proliferation and our current observation of the downregulation of several cell surface markers on EHV1-infected MDDC.

One alternative factor that potentially could suppress CD8+ CTL-proliferation is the virus-induced release of secretory factors (e.g. cytokines). Antigen-specific T-proliferation can be significantly suppressed by prostaglandin E_2, nitric oxide, indoleamine 2,3-dioxygenase, transforming growth factor (TGF) β, IL-6 or IL-10 (Kushwah and Hu, 2011; Sela et al., 2011). This is particularly interesting since EHV1 infection of another cell type, more specifically equine respiratory epithelial cells (EREC) resulted in increased expression of IL-6, IL-10 and TGF-β, which, in the case of IL-10, was pUL56-dependent (Soboll Hussey et al., 2014). However, our data suggest that secreted factors are not involved in the EHV1-induced modulation of the evaluated cell surface markers on EHV1-infected equine MDDC. To directly investigate whether secreted factors are involved in EHV1-mediated suppression of T-proliferation, experiments could be designed to evaluate whether direct contact between EHV1-infected MDDC and T cells is required. This can be examined, for example, by adding virus-free supernatants from EHV1-infected MDDC to mock-infected MDDC mixed lymphocyte reactions or by physical separation of MDDC from T cells via a semi-permeable membrane in the presence of virus-neutralizing antibodies.

Another alternative explanation for suppressed T cell proliferation could be direct EHV1 infection of T cells via infected MDDC. To test this, EHV1-infected MDDC could be irradiated or fixed before testing their ability to suppress T lymphocyte proliferation. The role of specific downregulated MHCI, CD83 and CD86 on EHV1-mediated suppressed DC-T priming can be evaluated for example by mimicking the effects of EHV1-induced downregulation of cell surface

markers with function blocking antibodies or siRNA. Also, EHV1 mutants that show a reduced ability to downregulate DC cell surface proteins could be tested for their ability to suppress T cell proliferation. Unfortunately, thus far, only a UL56null EHV1 showed a partial reversion of cell surface levels of two proteins, CD83 and CD86 . The involvement of other viral proteins in downregulation of these and other cell surface proteins, using additional EHV1 mutants, should be evaluated. Because we could not find any indications for VHS, pUL49.5 or EICP0 involvement in the downregulation of MHCI, CD29, CD83, CD86, CD172a and CD206, it can be interesting to initially screen for the kinetic class of viral protein(s) (e.g. immediate early/late) that are involved in downregulation of these cell surface molecules, by applying inhibitors such as phosphonoacetic acid, actinomycin D (ActD) and cycloheximide (CHX). Subsequently, single deletion mutants of potential candidate genes can then be constructed and tested for their ability to downregulate these cell surface markers. Further on, if such single deletion mutants result in (partial) reversion of downregulation, as we observed for the UL56null EHV1 virus for CD83 and CD86, functional redundancy can be evaluated by comparing single deletion mutants with multiple deletion mutants (Aubert et al., 2006). For example, single or combined deletion of pUL56 and pUL43 did yield the same reversion of MHCI downregulation in EHV1-infected equine dermal fibroblasts (ED) (NBL6) cells, indicating no functional redundancy between these proteins in downregulation of MHCI (Huang et al., 2015).

3. The underlying mechanism of MHCI downregulation on equine MDDC involves enhanced internalization of cell surface MHCI

In **Chapter 4**, we found that EHV1-induced MHCI downregulation on equine MDDC is not directly associated with entry, is mediated by internalization of cell surface MHCI and, as shown

by inhibitor experiments, is partly dependent on clathrin, dynamin and group I p21-activated kinases (PAK-I).

Although MHCI was shown to be an entry receptor for EHV1 in ED cells and equine brain microvascular endothelial cells (Kurtz et al., 2010; Sasaki et al., 2011a), we found that MHCI on equine MDDC is not downregulated during entry of EHV1. This is in agreement with the inability of UV-inactivated EHV1 to downregulate MHCI on equine fibroblasts (Rappocciolo et al., 2003). However, other entry receptors such as herpes virus entry mediator and nectin1 are downregulated by other α-herpesviruses (e.g. HSV1) upon viral entry (Stiles et al., 2010; Stiles and Krummenacher, 2010), although nothing has been published thus far on the potential involvement of these entry receptors during EHV1 entry.

We found that EHV1 induces MHCI downregulation on equine MDDC by enhancing internalization, and this in agreement with the mechanism of MHCI downregulation in EHV1-infected equine fibroblasts and several Kaposi's sarcoma herpes virus (KSHV)-infected cell types (Coscoy et al., 2001; Coscoy and Ganem, 2000; Huang et al., 2014). To evaluate the possible internalization mechanism(s) involved in this MHCI downregulation on equine MDDC, we performed a screening with several inhibitors. Interestingly, when evaluating the effects of the inhibitors on EHV1 infection during the optimization stage, we found that EHV1 entry in equine MDDC is probably dependent on macropinocytosis (MP) since MP inhibitors substantially suppressed EHV1 infection. This is especially interesting since DC have a high constitutive activity of the MP-mediated uptake pathway (Mercer and Greber, 2013) and several viruses, such as vaccinia virus and HCMV, can enter human MDDC by MP (Haspot et al., 2012; Sandgren et al., 2010). Future entry studies of EHV1 in equine MDDC can include the assessment of other (non)-MP inhibitors on EHV1 infection, co-localization of EHV1 within

large fluid phase marker-filled macropinosomes, and/or counting of inside/outside virus particles after treatment with (non)-MP inhibitors. Furthermore, our inhibitor experiments point towards the involvement of clathrin-mediated endocytosis during MHCI downregulation, as we found a partial reversion of MHCI cell surface expression after applying inhibitors for clathrin, dynamin and PAK-I, but not after treating with an filamentous (F)-actin disrupting agent. These inhibitor experiments also demonstrated, in line with findings for NBL6 cells, that MHCI downregulation is dependent on dynamin (Huang et al., 2014). Clathrin-dependent EHV1-induced MHCI downregulation in MDDC, as we observed in our studies, is in contrast to EHV1-induced MHCI downregulation in NBL6 cells, indicating that this downregulation process is cell type-dependent (Huang et al., 2014). Along the same lines, MHCI downregulation in NBL6 is pUL56-dependent (Ma et al., 2012), unlike MHCI downregulation in MDDC. Remarkably, the inhibitor studies indicated that MHCI downregulation also depend on group I PAK, but not on F-actin dynamics. Since clathrin-mediated endocytosis is not dependent on F-actin (Mercer and Greber, 2013) and PAK-I is able to activate mitogen activated protein kinase signaling (Shrestha et al., 2012), which induces clathrin vesicle formation (Cavalli et al., 2001; McLauchlan et al., 1998), it could be interesting to further explore the involvement of signaling pathways upstream and downstream of PAK-I during EHV1-mediated MHCI downregulation in MDDC.

4. EHV1 can infect equine MSC and infection results in alterations of cell surface protein expression

In **Chapter 5**, we demonstrated that, similar to their rat and human counterparts (Rooney et al., 2008; Vogel et al., 2003), equine MSC are also positive for the cell surface marker CD172a. In addition, equine MSC proved to be susceptible to EHV1 infection and this infection resulted in a

consistent downregulation of cell surface MHCI, CD29, CD105 and a variable downregulation of CD172a. As shown for equine MDDC in **Chapter 3**, no role of EHV1-induced secreted factors could be found in the EHV1-induced downregulation of these cell surface markers on equine MSC. In line with the role of the viral protein UL56 in the downregulation of MHCI in other EHV1-infected cell types (Huang et al., 2014; Ma et al., 2012; Soboll Hussey et al., 2014), we also found that the MHCI downregulation on EHV1-infected equine MSC depends on the expression of pUL56. Recently, it was found that pUL56 can also be involved in the downregulation of cell surface markers other than MHCI, as was demonstrated for CD46 and CD63 on EHV1-infected human cells (Huang et al., 2014). We found that this can also be the case in equine cells, as we demonstrated that in equine MSC, pUL56 was involved in the downregulation of the cell surface markers CD29 and CD105 and was partially involved in the downregulation of cell surface CD172a. Future experiments can be planned to study whether pUL56 can exert these effects by itself, in the absence of other viral proteins, e.g. via transfection assays with a plasmid expressing pUL56. If no downregulation of these markers is observed in pUL56-transfected cells, then this could point towards the need for other viral (or cellular) interaction partner(s) to successfully downregulate the expression of cell surface markers. In this regard, it is interesting to mention that the early pUL43 protein was recently identified to be important for EHV1-mediated MHCI downregulation on equine dermal cells (Huang et al., 2015).

We found that equine MSC are susceptible to EHV1 infection *in vitro*, and that such infection results in downregulation of MHCI. Although speculative at this moment, these findings could have a pathogenic importance. For example, MHCI downregulation may disturb the acquisition of an APC phenotype by EHV1-infected MSC, and may further lower the ‘visibility’ of these

cells towards EHV1-specific CTLs, especially since basal levels of MHCI on MSC are typically already intermediate to low because of their hypo-immune status (Ankrum et al., 2014). Furthermore, since we detected an incomplete MHCI downregulation with our MHCI antibody (clone PT85A), it remains plausible that not all MHCI alleles are (equally well) downregulated. It would be of particular interest to assess whether particular NK cell-inhibiting MHCI alleles on the cell surface, comparable to human leukocyte antigen G in the human system, may be differentially modulated or not affected by EHV1 (Loch and Tampé, 2005; Mandelboim et al., 1997; Rappocciolo et al., 2003; Sparks-Thissen and Enquist, 1999). In man, the non-classical human leukocyte antigen G, either membrane-associated or secreted, belongs to the immune suppressive program of MSC which inhibits NK activity, monocyte to DC differentiation, DC maturation and T-proliferation (Fainardi et al., 2011; Naji et al., 2013).

Similar to our observations in equine MDDC, we observed an EHV1-induced downregulation of CD29 (β1 integrin) on equine MSC. In analogy with DC, integrins that are expressed on MSC can be viewed as anchoring elements. As part of the canonical fibronectin receptor (very late antigen 4), also known as α4β1 integrin, EHV1-induced downregulation of CD29 may affect the release of MSC in tissue (Evans et al., 2009). Although speculative, this could be the case in respiratory connective tissue ECM, where fibronectin forms the major constituent (Pankov, 2002). This could be further addressed *in vitro* by comparing adhesion of mock and EHV1-infected equine MSC on fibronectin coated surfaces (Ip et al., 2007). Furthermore, we also found a substantial downregulation of CD105 (endoglin) on EHV1-infected equine MSC. Since CD105 downregulation has been shown to result in disruption of F-actin stress fibers which coincided with enhanced migration capacity of human MSC towards plasma, EHV1-induced downregulation of CD105 on equine MSC could possibly enhance their migration towards blood

vessels (Jaganathan et al., 2007; Sanz-Rodriguez et al., 2004). CD105 can be considered as a modulator of TGF-β1/3 receptor signaling and, interestingly, murine MSC negative for CD105 suppressed the proliferation of CD4+ splenocytes more strongly than "normal" CD105-positive MSC and this more prominent suppression of proliferation coincided with enhanced IL-6 and inducible nitric oxide synthase mRNA expression in CD105-negative MSC. However, murine and human CD105 can be expressed in a short and long isoform with contrary effects on TGF-β1 receptor signaling and, therefore, it should be evaluated if equine MSC similarly express two CD105 isoforms and, if so, whether EHV1-induced downregulation predominantly entails the long amplifying isoform of CD105 which was the predominant CD105 isoform in murine MSC (Anderson et al., 2013; Blanco et al., 2008).

Furthermore, it should prove interesting to evaluate if, like HCMV infection of human bone marrow MSC, the expression of ICAM-1 is upregulated on equine EHV1-infected MSC and as such, could possibly allow these cells to interact with equine DC (Ren et al., 2010; Smirnov et al., 2007). Moreover, such ICAM-1 upregulation could not only result in contact and subsequent modulation of DC function, but also in enhanced binding and inhibition of T-lymphocytes as was demonstrated by ICAM-1, but also vascular cell adhesion molecule-1 (CD106) function blocking antibodies, which prevented direct contact-mediated inhibition of CD4+ T-blast proliferation by bone marrow MSC (Aldinucci et al., 2010; Ren et al., 2010).

Although human studies confirmed the inhibitory effects of MSC on mitogen- or allogen-driven T cell proliferation (Balan et al., 2014; Karlsson et al., 2008; Malcherek et al., 2014; Rasmusson et al., 2007; Sundin et al., 2006), they also showed that such inhibitory effects of MSC towards viral-Ag driven T cell proliferation were less pronounced (Karlsson et al., 2008; Malcherek et al., 2014; Sundin et al., 2006). However, the effects of direct viral infection (e.g. EHV1) on the

immune modulatory properties of (equine) MSC on T cell proliferation remains to be determined (Karlsson et al., 2008; Rasmusson et al., 2007; Sundin et al., 2006).

An important question that remains is whether MSC are also a target cell type for EHV1 *in vivo*. In a first attempt to try to answer this question, MSC were isolated from the peripheral blood of horses that were experimentally infected with EHV1 and uninfected control horses. Due to practical limitations of the long-term culture based isolation protocol and the absence of a single specific MSC marker, we were unable to confirm the presence of EHV-1 in these isolated MSC. We did find, however, that the isolation efficiency of MSC from peripheral blood of EHV1-infected horses was about 50% compared to 93% from peripheral blood of non-infected horses. This observation could potentially provide an indirect indication towards the *in vivo* susceptibility of equine peripheral blood MSC for EHV1 infection. Indeed, possible explanations for this reduced isolation efficiency compared to uninfected horses could be the lysis of EHV1-infected MSC or enhanced migration and motility out of the blood stream upon EHV1 infection of equine MSC. On the other hand, uninfected peripheral blood MSC could also simply migrate into EHV1-infected tissues and there possibly become infected by EHV1.

5. Concluding remarks and future prospects

5.1. Conclusion

Based on the results obtained in this thesis, following conclusions can be drawn:

1. EHV1 infection of equine MDDC induces a drastic downregulation of MHCI, CD83, a substantial downregulation of CD29, CD86 and CD206 and a moderate suppression of CD172a

2. EHV1 induced downregulation of cell surface markers on equine MDDC does not depend on pUL49.5, VHS or ECIP0
3. EHV1 induced downregulation of CD83 and CD86 on equine MDDC is in part mediated by pUL56
4. MHCI downregulation on EHV1-infected equine MDDC does not occur during viral entry, but is mediated by enhanced internalization of cell surface MHCI and potentially involves clathrin, dynamin and PAK-I
5. EHV1 possibly uses macropinocytosis to enter equine MDDC
6. Equine MSC are CD172a positive and susceptible to EHV1 infection
7. EHV1 infection of equine MSC induces a consistent drastic downregulation of MHCI, a substantial downregulation of CD29 and CD105 and a variable suppression of CD172a
8. MHCI, CD29 and CD105 downregulation depends on pUL56 expression

5.2. Future prospects

Most of the EHV1-infected cells below the basement membrane are positive for the cell surface marker CD172a. In this work we evaluated the interaction of EHV1 with two CD172a positive cell types, namely dendritic cells (DC) and mesenchymal stem cells (MSC). We found that EHV1 infection of equine DC induced downregulation of MHCI, CD83 and CD86 but not of MHCII which is especially interesting during further evaluation of how EHV1 subverts the capacity of equine DC to support T-proliferation. In addition, we found downregulation of CD29, but not of LFA-1 and these are interesting findings for further evaluation if EHV1-infected DC are able to reach the lymph nodes or are more prone to stay and die at the infection site. In future studies, the putative effect of EHV1 infection on the expression of other interesting

markers that have not been investigated in the current thesis, such as CD40 or CD80, may also be measured at the mRNA level or, even better, directly at the protein level if (cross-)reacting antibodies become available. Next to this important immune cell type, we found that also equine MSC are positive for CD172a and could sustain a productive EHV1 infection, just like equine DC. This creates the possibility that EHV1 may misuse MSC as a replication reservoir and even misuse this immune modulatory cell type as a virus dissemination vehicle by preventing detection through MHCI downregulation and, although speculative at this stage, possibly increase migration towards the blood or lymph nodes by CD29 and CD105 downregulation. In addition to lowering their own detection, MSC could also prevent destruction of other infected cells by modulating immune response within the lymph node or at the primary or secondary infection site. Future work should evaluate if EHV1 induces immune suppressive or stimulating properties of MSC. In addition, it should prove interesting to evaluate if EHV1 can infect MSC *in vivo*, not only for further understanding of EHV1 pathogenesis, but also towards prevention of possible EHV1 transmission through allogeneic MSC transfer.

In search for the viral factors involved in the downregulation of cell surface markers on equine MSC and DC, we found that VHS and pUL49.5 were not involved in the downregulation of cell surface markers on EHV1-infected equine DC. Deletion of the viral protein pUL56 partly prevented CD83 and CD86 downregulation on equine MDDC and ablated downregulation of MHCI, CD29 and CD105 on equine MSC. However, pUL56 was not involved in any of the other observed downregulations on EHV1-infected MDDC and this includes the downregulation of MHCI and CD29, which was unexpected. In addition, we found that MHCI downregulation on EHV1-infected DC probably occurs through clathrin-dependent MHCI endocytosis and these observations contrast the pUL56-dependent clathrin-independent endocytosis reported for MHCI

downregulation on EHV1-infected equine fibroblasts (Huang et al., 2014; Ma et al., 2012). These findings are not only interesting in elucidating EHV1 biology, but most importantly, may be of importance for future vaccine development work where a safe modified live vaccine with targeted deletion of immune evasion genes could be evaluated for their improved ability to generate an effective cytotoxic T-lymphocyte response. Although pUL56 is an important candidate in this respect, our work has demonstrated that EHV1 possesses other, still to be identified, viral proteins that trigger downregulation of biologically important cell surface proteins.

Chapter 7: Summary/Samenvatting

Summary

Equine herpesvirus (EHV) 1 is an α-herpesvirus which is endemic in horse populations worldwide and can induce respiratory disease, reproductive disorders (e.g. abortion) and central nervous disorders (e.g. equine herpesvirus myeloencephalopathy, EHM). After inhalation of the virus, EHV1 will replicate in the upper respiratory epithelium and can subsequently spread via a cell-associated viremia towards the other target organs. Recent studies demonstrated that the majority of the EHV1-infected cells in the respiratory tissue, the draining lymph nodes and the blood were positive for the cell surface marker CD172a and have led to the assumption that EHV1 'hijacks' CD172a+ cells as carrier cells to spread the virus in the host. The respiratory tract harbors several cell populations, including two highly migratory (potentially) CD172a+ cell types, namely the dendritic cell (DC) and the mesenchymal stem cell (MSC). Although MSC have been demonstrated to be CD172a+ in man and rodent, this had not yet been analyzed in horse. In addition, although DC have been shown before to be susceptible to EHV1 infection, this had not yet been investigated for MSC. Infection of DC/MSC with EHV1 may substantially affect the biological activities of these cell types. Indeed, it was previously shown that EHV1 infection of equine DC resulted in a reduced capacity of these cells to support T-proliferation. However, it was not known if, as is the case with other herpesviruses, EHV1 infection of equine DC results in a modulation of cell surface markers associated with antigen (Ag) presentation, co-stimulation and adhesion. Likewise, for equine MSC, it was unknown what the impact of a putative infection could be on the expression pattern of typical MSC cell surface markers. Therefore, the aims of this thesis were to (i) evaluate CD172a expression and EHV1 susceptibility of equine MSC, (ii) determine the effect of EHV1 infection on different cell surface markers on equine monocyte-derived dendritic cells (MDDC) and MSC, and (iii) investigate the potential

involvement of viral factors and cellular mechanisms that may underlie such cell surface marker modulation.

In **Chapter 1**, a general introduction is provided on the classification, virion structure, and viral replication of EHV-1 and the epidemiology, prevention, and treatment of EHV1 infection. Next, we discussed the immunological response against EHV1 infection and the viral mechanisms to avoid this response. Specifically, we focused on viral factors that are of particular importance for this thesis with regard to their putative role in the modulation of cell surface proteins. We finished by discussing the potential role of the two CD172a positive cell types that were investigated in this thesis, namely the DC and the MSC, in the pathogenesis of and immune response against herpesviruses.

In **Chapter 2**, a general outline of the thesis is given.

In **Chapter 3**, we found that EHV1-infected equine MDDC showed drastic downregulation of major histocompability complex (MHC) I and CD83, substantial reduction of CD29, CD86 and CD206, and moderate lowering of CD172a. However, MHCII and lymphocyte function associated antigen (LFA) 1 were not modulated on EHV1-infected equine MDDC. In addition, virus-free supernatant of EHV1-infected MDDC was unable to induce any of the downregulations observed with wild type EHV1 and only induced a very slight upregulation of MHCI on mock-infected MDDC. Next, we evaluated the role of the viral proteins virion host-shutoff (VHS), unique long protein (pUL) 49.5, EHV1' infected cell protein (EICP) 0 (only CD83 evaluated) and pUL56 in downregulation of cell surface proteins. We found no involvement of VHS in the downregulation of any of the evaluated cell surface markers on EHV1-infected equine MDDC, despite previous report showing that VHS is involved in downregulation of CD83 and CD86 in HSV1-infected MDDC. Also, no role for EICP0 was found in reducing CD83 on EHV1-infected equine MDDC, in contrast to the role of the

HSV1 ICP0 homolog in downregulating CD83 in human MDDC. Moreover, neither pUL49.5 nor pUL56 were involved in downregulation of equine MDDC cell surface proteins, with the exception of a partial elevation of CD83 and CD86 in a pUL56 deletion mutant virus compared to wild type EHV1. For CD29 we found a minor involvement of pUL56 in EHV1-mediated downregulation.

In **Chapter 4,** we started to explore the cellular mechanism (mis)used by EHV1 to downregulate MHCI on equine MDDC. Despite the fact that MHCI is an EHV1 entry receptor for certain equine cell types and that other entry receptors were previously shown to be downregulated upon entry of other α-herpesviruses, we found that MHCI downregulation on equine MDDC is not a direct entry-associated event. We did, however, demonstrate that EHV1 downregulates MHCI on equine MDDC by enhancing internalization of cell surface residing MHCI, which is in line with previous findings in EHV1-infected equine fibroblasts and various Kaposi's sarcoma herpesvirus (KSHV) infected cell types. MHCI internalization in EHV1-infected equine fibroblasts has been reported to depend on dynamin, but not clathrin and this contrasts MHCI internalization in KSHV-infected cells, which depends on both clathrin and dynamin. To evaluate the possible endocytosis mechanism(s) involved in MHCI downregulation in EHV1-infected equine MDDC, we performed a screening with several inhibitors of endocytic pathways. This led to the additional finding that EHV1 entry in equine MDDC probably depends on macropinocytosis. Furthermore, these inhibitor experiments pointed towards the involvement of clathrin-dependent endocytosis in downregulation of MHCI during EHV1 infection of equine MDDC, as we found partial reversion of MHCI downregulation upon applying inhibitors for clathrin and dynamin.

In **Chapter 5**, we found that equine MSC are CD172a positive, susceptible to EHV1 infection and that such infection resulted in the downregulation of select cell surface markers in a pUL56-dependent fashion. While EHV1 infection of equine MSC did drastically

downregulate cell surface MHCI and substantially reduced CD29 and CD105, we found no modulation of CD44 or CD90 upon EHV1 infection. EHV1-infected equine MSC also showed a variable and moderate downregulation of CD172a. We found that virus-free supernatant of EHV1-infected MSC was unable to induce any of the modulations found after infection of MSC with EHV1 and this in contrast to human cytomegalovirus (HCMV)-mediated modulation of MSC cell surface proteins. In line with previous findings in other EHV1-infected cell types, we also found that MHCI downregulation in EHV1-infected equine MSC depends on the expression of pUL56. In addition, we found for the first time in an equine cell type that expression of EHV1 pUL56 is required for downregulation of CD29 and CD105 and contributes to downregulation of CD172a.

In **Chapter 6**, a general discussion is given on the results shown in this thesis.

Conclusion

In conclusion, the results from this thesis show that EHV1 infects CD172a+ cell populations, including DC and MSC, and causes downregulation of several important cell surface proteins on these infected cells. Although the exact underlying mechanisms and viral proteins involved in this downregulation need to be further defined, the results from this thesis add to our understanding of how EHV1 highjacks and manipulates its target cells for its own benefits.

Samenvatting

Equine herpesvirus (EHV) 1 is een α-herpesvirus dat endemisch is in paardenpopulaties wereldwijd en ademhalingsstoornissen, reproductieve problemen (bijv. abortus) en zenuwstoornissen (bijv. equine herpesvirus myeloencephalopathy, EHM) kan veroorzaken. Na inhalatie zal EHV1 zich vermeerderen in het epitheel van de bovenste ademhalingswegen en hierna kan het virus zich verspreiden via een cel-geassocieerde viraemie naar de verschillende doelorganen. Recente studies hebben aangetoond dat het grootste deel van de EHV1-geïnfecteerde cellen in de ademhalingsweefsels, de drainerende lymfeknopen en het bloed positief zijn voor de cellulaire oppervlaktemerker CD172a en deze bevindingen hebben geleid tot de veronderstelling dat EHV1 deze CD172a+ cellen gebruikt als drager cellen om zich te verspreiden in de gastheer. Het ademhalingsstelsel is opgebouwd uit verschillende cel populaties waaronder ook twee sterk mobiele CD172a+ cel types, namelijk de dendritische cel (DC) en de mesenchymale stam cel (MSC). Alhoewel bij de mens en bij knaagdieren al werd aangetoond dat MSC positief zijn voor CD172a, diende dit voor het paard nog bevestigd te worden. Daarenboven diende ook nog te worden onderzocht of equine MSC, net als equine DC, ook gevoelig zijn voor EHV1 infectie. Een infectie met EHV1 kan de biologische functies van equine DC en MSC beïnvloeden. Voor DC zijn er reeds indicaties voor EHV1-gemedieerde modulatie van de biologische activiteit, aangezien EHV1 infectie leidt tot een gereduceerde capaciteit van de DC cellen om T-cel proliferatie te ondersteunen. Het was echter nog niet geweten of EHV1 infectie van DC, zoals reeds werd aangetoond voor andere herpesvirussen, ook leidt tot een gewijzigde expressie van biologisch belangrijke cellulaire oppervlaktemerkers die geassocieerd zijn met antigeen (Ag) presentatie, co-stimulatie en adhesie. Ook voor equine MSC, indien ze gevoelig zijn voor infectie met EHV1, is het niet bekend of EHV1 infectie een modulatie van cel oppervlakte merkers kan induceren. Daarom werden volgende doelstellingen voor deze thesis bepaald: (i) nagaan of equine MSC positief

zijn voor CD172a en gevoelig zijn voor EHV1, (ii) het effect van EHV1 infectie op verschillende cellulaire oppervlaktemerkers onderzoeken voor equine monocyte-derived dendritic cells (MDDC) en MSC, en (iii) onderzoeken welke virale factoren en cellulaire mechanismen betrokken zijn bij de modulatie van deze cellulaire oppervlaktemerker(s).

In **hoofdstuk 1** wordt een algemene inleiding gegeven over de classificatie, de structuur van het virion en de virale replicatie cyclus van EHV1 alsook een korte beschrijving van de epidemiologie, preventie en behandeling van EHV1 infectie. Vervolgens wordt de immunologische reactie tegen een EHV1 infectie besproken en de virale mechanismen die deze afweer proberen te ontwijken. De focus ligt voornamelijk op virale factoren die een rol kunnen spelen tijdens de mogelijke modulatie van cellulaire oppervlaktemerkers tijdens EHV1 infectie. De introductie eindigt met het bespreken van de mogelijke rol van de in deze thesis onderzochte CD172a+ celtypes (DC en MSC) tijdens de pathogenese en immuun respons van een EHV1 infectie.

In **hoofdstuk 2** wordt een algemeen overzicht gegeven van de doelstellingen van deze thesis.

In **hoofdstuk 3** wordt beschreven dat EHV1 infectie van equine MDDC leidt tot een sterke downregulatie van het major histocompatibility complex (MHC) I en CD83 op het celoppervlak, een aanzienlijke reductie van CD29, CD86 en CD206, en een matige vermindering van de CD172a oppervlakte expressie. Daarentegen vertoonden het MHCII complex en de lymphocyte function associated antigen (LFA) 1 merker geen gewijzigde expressie op EHV1-geïnfecteerde MDDC. Ook bleek blootstelling van equine MDDC aan virusvrij supernatant afkomstig van EHV1-geïnfecteerde equine MDDC geen downregulatie te veroorzaken van de onderzochte oppervlaktemerkers en enkel een lichte opregulatie van MHCI, wat suggereert dat gesecreteerde factoren niet betrokken zijn bij de waargenomen downregulaties. Voor verschillende virale eiwitten werd de betrokkenheid bij deze

downregulaties onderzocht, namelijk: virion host-shutoff (VHS), unique long protein (pUL) 49.5, pUL56 en EHV1 infected cell protein (EICP) 0 (voor deze laatste werd enkel de impact op CD83 geëvalueerd). Alhoewel VHS betrokken is bij de downregulatie van CD83 en CD86 op HSV1-geïnfecteerde MDDC, bleek dit viraal eiwit niet betrokken te zijn in de downregulatie van CD83, CD86 en alle andere geëvalueerde merkers op EHV1-geïnfecteerde equine MDDC. Ook EICP0 bleek niet betrokken te zijn bij de downregulatie van CD83 op EHV1-geïnfecteerde equine MDDC, alhoewel HSV1 ICP0 wel downregulatie van CD83 induceert op humane MDDC. De virale eiwitten pUL49.5 en pUL56 bleken ook niet betrokken bij de downregulatie van cellulaire oppervlakte-eiwitten op EHV1-geïnfecteerde equine MDDC met uitzondering van een partiële betrokkenheid van pUL56 bij de downregulatie van CD83 en CD86. Ook de downregulatie van CD29 was iets minder uitgesproken in equine MDDC die geïnfecteerd werden met een pUL56-gedeleteerd mutant virus ten opzichte van het overeenkomstige wildtype EHV1.

In **hoofdstuk 4** werd een aanzet gegeven naar het ontrafelen van het cellulaire mechanisme dat door EHV1 wordt misbruikt om MHCI oppervlakte expressie te onderdrukken op equine MDDC. Ondanks het feit dat MHCI een entry receptor is voor EHV1 voor bepaalde celtypes en dat bij andere α-herpesvirussen dergelijke entry receptoren worden gedownreguleerd tijdens virus binnenkomst, werden geen indicaties gevonden dat EHV1 MHCI direct downreguleert tijdens binnenkomst in equine MDDC. Wel werd vastgesteld dat EHV1 de MHCI downregulatie op equine MDDC bewerkstelligt door het bevorderen van de internalisatie van MHCI van het celoppervlak, wat in overeenstemming is met eerdere bevindingen in verband met EHV1-geïnduceerde downregulatie van MHCI in equine fibroblasten. MHCI internalisatie in EHV1-geïnfecteerde fibroblasten is afhankelijk van dynamin, maar niet van clathrin. Ter identificatie van de mogelijke endocytose mechanismen die door EHV1 worden misbruikt om MHCI te downreguleren op equine MDDC, werd een

screening met verschillende inhibitoren uitgevoerd. Deze initiële experimenten wezen in de richting van clathrin-afhankelijke endocytose die door EHV1 wordt misbruikt om MHCI te downreguleren vermits een partiële reversie van MHCI downregulatie werd vastgesteld bij behandeling met clathrin en dynamin inhibitoren. Deze inhibitor experimenten leidden ook tot de bijkomende bevinding dat EHV1 waarschijnlijk via macropinocytosis de equine MDDC binnendringt.

In **hoofdstuk 5** wordt aangetoond dat equine MSC positief zijn voor CD172a, gevoelig zijn voor EHV1 infectie en dat deze infectie een downregulatie van bepaalde cellulaire oppervlaktemerkers veroorzaakt. Er werd een sterke downregulatie van MHCI en een aanzienlijke reductie van CD29 en CD105 waargenomen op EHV1-geïnfecteerde equine MSC, maar geen modulatie van CD44 of CD90. EHV1-geïnfecteerde equine MSC vertoonden ook een variabele en matige downregulatie van CD172a. Virusvrij supernatant van EHV1-geïnfecteerde equine MSC veroorzaakte geen modulatie van de onderzochte cellulaire oppervlaktemerkers. Dit suggereert dat gesecreteerde factoren niet betrokken zijn bij de waargenomen downregulaties, in tegenstelling tot bijvoorbeeld humaan cytomegalovirus (HCMV) gemedieerde modulatie van cellulaire oppervlaktemerkers op humane MSC. In overeenstemming met diverse andere celtypes bleek de downregulatie van MHCI op EHV1-geïnfecteerde equine MSC afhankelijk van de expressie van pUL56. Dit viraal eiwit bleek ook betrokken te zijn bij de reductie van CD29 en CD105 en bij te dragen tot de vermindering van CD172a expressie op EHV1-geïnfecteerde equine MSC.

In **hoofdstuk 6** worden de resultaten van deze thesis besproken en in de context van de bestaande literatuur geplaatst.

Conclusie

De resultaten bekomen in deze thesis geven aan dat CD172a+ cel populaties, zoals de equine MSC en DC, door EHV1 kunnen geïnfecteerd worden en dat dergelijke infectie aanleiding geeft tot downregulatie van verschillende biologisch belangrijke cellulaire oppervlaktemerkers. Alhoewel de onderliggende mechanismen en virale factoren betrokken in deze downregulaties verder dienen onderzocht te worden, dragen de resultaten van deze thesis bij tot een beter begrip hoe EHV1 zijn doelcellen kaapt en manipuleert.

References

Ackerman, A.L., Cresswell, P., 2003. Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. J. Immunol. 170, 4178–88.

Aerts-Toegaert, C., Heirman, C., Tuyaerts, S., Corthals, J., Aerts, J.L., Bonehill, A., Thielemans, K., Breckpot, K., Breckpot, K., 2007. CD83 expression on dendritic cells and T cells: Correlation with effective immune responses. Eur. J. Immunol. 37, 686–695. doi:10.1002/eji.200636535

Ahn, K., Meyer, T.H., Uebel, S., Sempé, P., Djaballah, H., Yang, Y., Peterson, P.A., Früh, K., Tampé, R., 1996. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. EMBO J. 15, 3247–55.

Alblas, J., Honing, H., de Lavalette, C.R., Brown, M.H., Dijkstra, C.D., van den Berg, T.K., 2005. Signal regulatory protein alpha ligation induces macrophage nitric oxide production through JAK/STAT- and phosphatidylinositol 3-kinase/Rac1/NAPDH oxidase/H2O2-dependent pathways. Mol. Cell. Biol. 25, 7181–7192. doi:10.1128/MCB.25.16.7181-7192.2005

Aldinucci, A., Rizzetto, L., Pieri, L., Nosi, D., Romagnoli, P., Biagioli, T., Mazzanti, B., Saccardi, R., Beltrame, L., Massacesi, L., Cavalieri, D., Ballerini, C., 2010. Inhibition of immune synapse by altered dendritic cell actin distribution: a new pathway of mesenchymal stem cell immune regulation. J. Immunol. 185, 5102–5110. doi:10.4049/jimmunol.1001332

Allan, R.S., Waithman, J., Bedoui, S., Jones, C.M., Villadangos, J. a., Zhan, Y., Lew, A.M., Shortman, K., Heath, W.R., Carbone, F.R., 2006. Migratory Dendritic Cells Transfer Antigen to a Lymph Node-Resident Dendritic Cell Population for Efficient CTL Priming. Immunity 25, 153–162. doi:10.1016/j.immuni.2006.04.017

Allen, G., Yeargan, M., Costa, L.R., Cross, R., 1995. Major histocompatibility complex class I-restricted cytotoxic T-lymphocyte responses in horses infected with equine herpesvirus 1. J. Virol. 69, 606–612.

Allen, G.P., 2006. Antemortem detection of latent infection with neuropathogenic strains of equine herpesvirus-1 in horses. Am. J. Vet. Res. 67, 1401–1405. doi:10.2460/ajvr.67.8.1401

Allen, G.P., 2008. Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses. Am. J. Vet. Res. 69, 1595–1600. doi:10.2460/ajvr.69.12.1595

Allen, G.P., Bolin, D.C., Bryant, U., Carter, C.N., Giles, R.C., Harrison, L.R., Hong, C.B., Jackson, C.B., Poonacha, K., Wharton, R., Williams, N.M., 2008. Prevalence of latent, neuropathogenic equine herpesvirus-1 in the Thoroughbred broodmare population of central Kentucky. Equine Vet. J. 40, 105–110. doi:10.2746/042516408X253127

Allen, G.P., Bryans, J.T., 1986. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. Prog. Vet. Microbiol. Immunol. 2, 78–144.

Allen, G.P., Murray, M.J., 1962. Equid herpesvirus 2 and equid herpesvirus 5 infections, in: Coetzer JAW, Tustin RC (Eds). Infectious Diseases of Livestock. Volume 2. Pp 829–59. Oxford University Press, Oxford, UK.

Ambagala, A.P.N., Gopinath, R.S., Srikumaran, S., 2004. Peptide transport activity of the transporter associated with antigen processing (TAP) is inhibited by an early protein of equine herpesvirus-1. J. Gen. Virol. 85, 349–353. doi:10.1099/vir.0.19563-0

Anderson, P., Carrillo-GÃ¡lvez, A.B., GarcÃ-a-PÃ©rez, A., Cobo, M., MartÃ-n, F., 2013. CD105 (Endoglin)-Negative Murine Mesenchymal Stromal Cells Define a New Multipotent Subpopulation with Distinct Differentiation and Immunomodulatory Capacities. PLoS One 8, e76979. doi:10.1371/journal.pone.0076979

Ankrum, J.A., Ong, J.F., Karp, J.M., 2014. Mesenchymal stem cells: immune evasive, not immune privileged. Nat. Biotechnol. 32, 252–60. doi:10.1038/nbt.2816

References

Arrode, G., Boccaccio, C., Abastado, J.P., Davrinche, C., 2002. Cross-presentation of human cytomegalovirus pp65 (UL83) to CD8+ T cells is regulated by virus-induced, soluble-mediator-dependent maturation of dendritic cells. J Virol 76, 142–150.

Aubert, M., Krantz, E.M., Jerome, K.R., 2006. Lymphocyte-Induced Cytotoxicity 19, 391–408.

Azab, W., Lehmann, M.J., Osterrieder, N., 2013. Glycoprotein H and α4β1 integrins determine the entry pathway of alphaherpesviruses. J. Virol. 87, 5937–48. doi:10.1128/JVI.03522-12

Azab, W., Osterrieder, N., 2012. Glycoproteins D of equine herpesvirus type 1 (EHV-1) and EHV-4 determine cellular tropism independently of integrins. J. Virol. 86, 2031–44. doi:10.1128/JVI.06555-11

Azab, W., Zajic, L., Osterrieder, N., 2012. The role of glycoprotein H of equine herpesviruses 1 and 4 (EHV-1 and EHV-4) in cellular host range and integrin binding. Vet. Res. 43, 61. doi:10.1186/1297-9716-43-61

Baghi, H.B., 2015. Effects of EHV-1 infection on the migration behavior of monocytic cells and on components of the basement membrane in the respiratory mucosa.

Baghi, H.B., Laval, K., Favoreel, H., Nauwynck, H.J., 2014. Isolation and characterization of equine nasal mucosal CD172a+ cells. Vet. Immunol. Immunopathol. 157, 155–163.

Baghi, H.B., Nauwynck, H.J., 2014. Impact of equine herpesvirus type 1 (EHV-1) infection on the migration of monocytic cells through equine nasal mucosa. Comp. Immunol. Microbiol. Infect. Dis. 37, 321–329.

Balan, A., Lucchini, G., Schmidt, S., Schneider, A., Tramsen, L., Kuçi, S., Meisel, R., Bader, P., Lehrnbecher, T., 2014. Mesenchymal stromal cells in the antimicrobial host response of hematopoietic stem cell recipients with graft-versus-host disease--friends or foes? Leukemia 28, 1941–8. doi:10.1038/leu.2014.127

Balkow, S., Heinz, S., Schmidbauer, P., Kolanus, W., Holzmann, B., Grabbe, S., Laschinger, M., 2010. LFA-1 activity state on dendritic cells regulates contact duration with T cells and promotes T-cell priming. Blood 116, 1885–1894. doi:10.1182/blood-2009-05-224428

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., Palucka, K., 2000. Immunobiology of dendritic cells. Annu. Rev. Immunol. 18, 767–811. doi:10.1146/annurev.immunol.18.1.767

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245–252. doi:10.1038/32588

Bannazadeh Baghi, H., Nauwynck, H.J., 2016. Effect of equine herpesvirus type 1 (EHV-1) infection of nasal mucosa epithelial cells on integrin alpha 6 and on different components of the basement membrane. Arch. Virol. 161, 103–110. doi:10.1007/s00705-015-2643-4

Baxi, M.K., Efstathiou, S., Lawrence, G., Whalley, J.M., Slater, J.D., Field, H.J., 1995. The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. J. Gen. Virol. 76 (Pt 12, 3113–8.

Bedoui, S., Greyer, M., 2014. The role of dendritic cells in immunity against primary herpes simplex virus infections. Front. Microbiol. 5, 1–9. doi:10.3389/fmicb.2014.00533

Bentz, B.G., Maxwell, L.K., Erkert, R.S., Royer, C.M., Davis, M.S., MacAllister, C.G., Clarke, C.R., 2006. Pharmacokinetics of acyclovir after single intravenous and oral administration to adult horses. J. Vet. Intern. Med. 20, 589–94.

Bhatia, S., Edidin, M., Almo, S.C., Nathenson, S.G., 2006. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. Immunol. Lett. 104, 70–5. doi:10.1016/j.imlet.2005.11.019

Birbrair, A., Zhang, T., Wang, Z.-M., Messi, M.L., Mintz, A., Delbono, O., 2015. Pericytes at the intersection between tissue regeneration and pathology. Clin. Sci. (Lond). 128, 81–93. doi:10.1042/CS20140278

Blanco, F.J., Grande, M.T., Langa, C., Oujo, B., Velasco, S., Rodriguez-Barbero, A., Perez-Gomez, E., Quintanilla, M., López-Novoa, J.M., Bernabeu, C., 2008. S-endoglin expression is induced in senescent endothelial cells and contributes to vascular pathology. Circ. Res. 103, 1383–92.

doi:10.1161/CIRCRESAHA.108.176552

Bleijs, D. a., Geijtenbeek, T.B.H., Figdor, C.G., Van Kooyk, Y., 2001. DC-SIGN and LFA-1: A battle for ligand. Trends Immunol. 22, 457–463.

Boname, J.M., Lehner, P.J., 2011. What has the study of the K3 and K5 viral ubiquitin E3 ligases taught us about ubiquitin-mediated receptor regulation? Viruses 3, 118–31. doi:10.3390/v3020118

Borchers, K., Thein, R., Sterner-Kock, A., 2006. Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation. Equine Vet. J. 38, 283–7.

Borgman, K.J.E., Van Zanten, T.S., Manzo, C., Cabezón, R., Cambi, A., Benítez-Ribas, D., Garcia-Parajo, M.F., 2014. Priming by chemokines restricts lateral mobility of the adhesion receptor LFA-1 and restores adhesion to ICAM-1 nano-aggregates on human mature dendritic cells. PLoS One 9, 1–11. doi:10.1371/journal.pone.0099589

Borowski, A.B., Boesteanu, A.C., Mueller, Y.M., Carafides, C., Topham, D.J., Altman, J.D., Jennings, S.R., Katsikis, P.D., 2007. Memory CD8+ T cells require CD28 costimulation. J. Immunol. 179, 6494–6503. doi:179/10/6494 [pii]

Boutell, C., Everett, R.D., 2013. Regulation of alphaherpesvirus infections by the ICP0 family of proteins. J. Gen. Virol. 94, 465–481. doi:10.1099/vir.0.048900-0

Braun, J., Hack, A., Weis-Klemm, M., Conrad, S., Treml, S., Kohler, K., Walliser, U., Skutella, T., Aicher, W.K., 2010. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. Am. J. Vet. Res. 71, 1228–36. doi:10.2460/ajvr.71.10.1228

Breathnach, C.C., Yeargan, M.R., Sheoran, A.S., Allen, G.P., 2001. The mucosal humoral immune response of the horse to infective challenge and vaccination with equine herpesvirus-1 antigens. Equine Vet. J. 33, 651–657.

Breathnach, C.C., Yeargan, M.R., Timoney, J.F., Allen, G.P., 2006. Detection of equine herpesvirus-specific effector and memory cytotoxic immunity in the equine upper respiratory tract. Vet. Immunol. Immunopathol. 111, 117–25. doi:10.1016/j.vetimm.2006.01.014

Bresgen, C., Lämmer, M., Wagner, B., Osterrieder, N., Damiani, A.M., 2012. Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines. Vet. Microbiol. 160, 9–16. doi:10.1016/j.vetmic.2012.04.042

Bridges, C.G., Edington, N., 1986. Innate immunity during Equid herpesvirus 1 (EHV-1) infection. Clin. Exp. Immunol. 65, 172–81.

Bridges, C.G., Edington, N., 1987. The proteins of equid herpesvirus 1 (EHV 1) recognised by equine antisera and their ability to promote antibody-dependent cell-mediated cytotoxicity. Tierarztl. Prax. Suppl. 2, 47–9.

Brosnahan, M.M., Damiani, A., van de Walle, G., Erb, H., Perkins, G.A., Osterrieder, N., 2010. The effect of siRNA treatment on experimental equine herpesvirus type 1 (EHV-1) infection in horses. Virus Res 147, 176–181. doi:10.1016/j.virusres.2009.10.017

Brown, K. a, Bedford, P., Macey, M., McCarthy, D. a, Leroy, F., Vora, a J., Stagg, a J., Dumonde, D.C., Knight, S.C., 1997. Human blood dendritic cells: binding to vascular endothelium and expression of adhesion molecules. Clin. Exp. Immunol. 107, 601–607. doi:10.1046/j.1365-2249.1997.d01-951.x

Bryant, N.A., 2003. Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. EMBO J. 22, 833–846. doi:10.1093/emboj/cdg092

Burgess, B.A., Tokateloff, N., Manning, S., Lohmann, K., Lunn, D.P., Hussey, S.B., Morley, P.S., 2012. Nasal shedding of equine herpesvirus-1 from horses in an outbreak of equine herpes myeloencephalopathy in Western Canada. J. Vet. Intern. Med. 26, 384–92. doi:10.1111/j.1939-1676.2012.00885.x

Bürki, F., Rossmanith, W., Nowotny, N., Pallan, C., Möstl, K., Lussy, H., 1990. Viraemia and abortions are not prevented by two commercial equine herpesvirus-1 vaccines after experimental challenge of horses. Vet. Q. 12, 80–6. doi:10.1080/01652176.1990.9694249

References

Burrows, R., Goodridge, D., Denyer, M.S., 1984. Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. Vet. Rec. 114, 369–74.

Cadwell, K., Coscoy, L., 2008. The specificities of Kaposi's sarcoma-associated herpesvirus-encoded E3 ubiquitin ligases are determined by the positions of lysine or cysteine residues within the intracytoplasmic domains of their targets. J. Virol. 82, 4184–9. doi:10.1128/JVI.02264-07

Cameron, C.M., Barrett, J.W., Mann, M., Lucas, A., McFadden, G., 2005. Myxoma virus M128L is expressed as a cell surface CD47-like virulence factor that contributes to the downregulation of macrophage activation in vivo. Virology 337, 55–67. doi:10.1016/j.virol.2005.03.037

Campioni, D., Rizzo, R., Stignani, M., Melchiorri, L., Ferrari, L., Moretti, S., Russo, A., Bagnara, G.P., Bonsi, L., Alviano, F., Lanzoni, G., Cuneo, A., Baricordi, O.R., Lanza, F., 2009. A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. Cytometry B. Clin. Cytom. 76, 225–30. doi:10.1002/cyto.b.20461

Caplan, A.I., 2008. All MSCs are pericytes? Cell Stem Cell 3, 229–30. doi:10.1016/j.stem.2008.08.008

Carrade, D.D., Lame, M.W., Kent, M.S., Clark, K.C., Walker, N.J., Borjesson, D.L., 2012. Comparative Analysis of the Immunomodulatory Properties of Equine Adult-Derived Mesenchymal Stem Cells(). Cell Med. 4, 1–11. doi:10.3727/215517912X647217

Carrigan, M., Cosgrove, P., Kirkland, P., Sabine, M., 1991. An outbreak of Equid herpesvirus abortion in New South Wales. Equine Vet. J. 23, 108–10.

Carvalho, R., Oliveira, A.M., Souza, A.M., Passos, L.M., Martins, A.S., 2000. Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction. Arch. Virol. 145, 1773–87.

Cavalli, V., Vilbois, F., Corti, M., Marcote, M.J., Tamura, K., Karin, M., Arkinstall, S., Gruenberg, J., 2001. The Stress-Induced MAP Kinase p38 Regulates Endocytic Trafficking via the GDI:Rab5 Complex. Mol. Cell 7, 421–432. doi:10.1016/S1097-2765(01)00189-7

Cavatorta, D.J., Erb, H.N., Felippe, M.J., 2012. Activation-induced FoxP3 expression regulates cytokine production in conventional T cells stimulated with autologous dendritic cells. Clin. Vaccine Immunol. 19, 1583–1592. doi:10.1128/CVI.00308-12

Cavatorta, D.J., Erb, H.N., Flaminio, M.J.B.F., 2009. Ex vivo generation of mature equine monocyte-derived dendritic cells. Vet. Immunol. Immunopathol. 131, 259–267. doi:10.1016/j.vetimm.2009.04.019

Chakraborty, S., Veettil, M.V., Chandran, B., 2012. Kaposi's Sarcoma Associated Herpesvirus Entry into Target Cells. Front. Microbiol. 3, 6. doi:10.3389/fmicb.2012.00006

Chang, W.L.W., Baumgarth, N., Yu, D., Barry, P. a, 2004. Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. J. Virol. 78, 8720–8731. doi:10.1128/JVI.78.16.8720-8731.2004

Charan, S., Palmer, K., Chester, P., Mire-Sluis, A.R., Meager, A., Edington, N., 1997. Transforming growth factor-beta induced by live or ultraviolet-inactivated equid herpes virus type-1 mediates immunosuppression in the horse. Immunology 90, 586–91.

Chaudhry, A., Das, S.R., Jameel, S., George, A., Bal, V., Mayor, S., Rath, S., 2007. A two-pronged mechanism for HIV-1 Nef-mediated endocytosis of immune costimulatory molecules CD80 and CD86. Cell Host Microbe 1, 37–49. doi:10.1016/j.chom.2007.01.001

Chesters, P.M., Allsop, R., Purewal, A., Edington, N., 1997. Detection of latency-associated transcripts of equid herpesvirus 1 in equine leukocytes but not in trigeminal ganglia. J. Virol. 71, 3437–43.

Chong, Y.C., Duffus, W.P.H., Hannant, D., 1992. Natural killer cells in normal horses and specific-pathogen-free foals infected with equine herpesvirus. Vet. Immunol. Immunopathol. 33, 103–113. doi:10.1016/0165-2427(92)90038-R

Choudhary, S., Marquez, M., Alencastro, F., Spors, F., Zhao, Y., Tiwari, V., 2011. Herpes simplex virus type-1 (HSV-1) entry into human mesenchymal stem cells is heavily dependent on heparan sulfate. J. Biomed.

Biotechnol. 2011, 264350. doi:10.1155/2011/264350

Claessen, C., Favoreel, H., Ma, G., Osterrieder, N., De Schauwer, C., Piepers, S., Van de Walle, G.R., 2015. Equid herpesvirus 1 (EHV1) infection of equine mesenchymal stem cells induces a pUL56-dependent downregulation of select cell surface markers. Vet. Microbiol. 176, 32–9.

Coombs, D.K., Patton, T., Kohler, A.K., Soboll, G., Breathnach, C., Townsend, H.G.G., Lunn, D.P., 2006. Cytokine responses to EHV-1 infection in immune and non-immune ponies. Vet. Immunol. Immunopathol. 111, 109–16. doi:10.1016/j.vetimm.2006.01.013

Cooper, J.A., 1987. Effects of cytochalasin and phalloidin on actin. J. Cell Biol. 105, 1473–8.

Coscoy, L., Ganem, D., 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc. Natl. Acad. Sci. U. S. A. 97, 8051–6. doi:10.1073/pnas.140129797

Coscoy, L., Sanchez, D.J., Ganem, D., 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. J Cell Biol 155, 1265–1273. doi:10.1083/jcb.200111010

Crabb, B.S., Allen, G.P., Studdert, M.J., 1991. Characterization of the major glycoproteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3 using monoclonal antibodies. J. Gen. Virol. 72 (Pt 9), 2075–82.

Crisan, M., Yap, S., Casteilla, L., Chen, C.-W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P.-N., Traas, J., Schugar, R., Deasy, B.M., Badylak, S., Buhring, H.-J., Giacobino, J.-P., Lazzari, L., Huard, J., Péault, B., 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3, 301–13. doi:10.1016/j.stem.2008.07.003

Crispin, S.M., Matthews, A.G., Parker, J., 1990. The equine fundus. I: Examination, embryology, structure and function. Equine Vet. J. Suppl. 42–9.

Crowhurst, F.A., Dickinson, G., Burrows, R., 1981. An outbreak of paresis in mares and geldings associated with equid herpesvirus 1. Vet. Rec. 109, 527–8.

Cuxson, J.L., Hartley, C. a., Ficorilli, N.P., Symes, S.J., Devlin, J.M., Gilkerson, J.R., 2014. Comparing the genetic diversity of ORF30 of Australian isolates of 3 equid alphaherpesviruses. Vet. Microbiol. 169, 50–57. doi:10.1016/j.vetmic.2013.12.007

da Silva Meirelles, L., Chagastelles, P.C., Nardi, N.B., 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J. Cell Sci. 119, 2204–13. doi:10.1242/jcs.02932

Dauber, B., Saffran, H. a, Smiley, J.R., 2014. The HSV-1 virion host shutoff protein enhances translation of viral late mRNAs by preventing mRNA overload. J. Virol. 88, 9624–9632. doi:10.1128/JVI.01350-14

David-Watine, B., Israël, A., Kourilsky, P., 1990. The regulation and expression of MHC class I genes. Immunol. Today 11, 286–292. doi:10.1016/0167-5699(90)90114-O

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order Herpesvirales. Arch. Virol. 154, 171–7. doi:10.1007/s00705-008-0278-4

de Andrés, C., Teijeiro, R., Alonso, B., Sánchez-Madrid, F., Martínez, M.L., de Villoria, J.G., Fernández-Cruz, E., Sánchez-Ramón, S., 2012. Long-term decrease in VLA-4 expression and functional impairment of dendritic cells during natalizumab therapy in patients with multiple sclerosis. Public Libr. Sci. ONE 7, e34103. doi:10.1371/journal.pone.0034103

Deacon, S.W., Beeser, A., Fukui, J.A., Rennefahrt, U.E.E., Myers, C., Chernoff, J., Peterson, J.R., 2008. An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. Chem. Biol. 15, 322–31. doi:10.1016/j.chembiol.2008.03.005

De Schauwer, C., Goossens, K., Piepers, S., Hoogewijs, M.K., Govaere, J.L.J., Smits, K., Meyer, E., Van Soom, A., Van de Walle, G.R., 2014. Characterization and profiling of immunomodulatory genes of equine

mesenchymal stromal cells from non-invasive sources. Stem Cell Res. Ther. 5, 6. doi:10.1186/scrt395

De Schauwer, C., Meyer, E., Cornillie, P., De Vliegher, S., van de Walle, G.R., Hoogewijs, M., Declercq, H., Govaere, J., Demeyere, K., Cornelissen, M., Van Soom, A., 2011. Optimization of the isolation, culture, and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng. Part C. Methods 17, 1061–70. doi:10.1089/ten.tec.2011.0052

De Schauwer, C., Piepers, S., Van de Walle, G.R., Demeyere, K., Hoogewijs, M.K., Govaere, J.L.J., Braeckmans, K., Van Soom, A., Meyer, E., 2012. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry. A 81, 312–23. doi:10.1002/cyto.a.22026

Del Piero, F., Wilkins, P.A., 2001. Pulmonary vasculotropic EHV-1 infection in equids. Vet. Pathol. 38, 474.

Del Piero, F., Wilkins, P.A., Timoney, P.J., Kadushin, J., Vogelbacker, H., Lee, J.W., Berkowitz, S.J., La Perle, K.M., 2000. Fatal nonneurological EHV-1 infection in a yearling filly. Vet. Pathol. 37, 672–6.

Deruelle, M.J., Van den Broeke, C., Nauwynck, H.J., Mettenleiter, T.C., Favoreel, H.W., 2009. Pseudorabies virus US3- and UL49.5-dependent and -independent downregulation of MHC I cell surface expression in different cell types. Virology 395, 172–181. doi:10.1016/j.virol.2009.09.019

Dietze, B., Cierpka, E., Schäfer, M., Schill, W., Lutz, M.B., 2008. An improved method to generate equine dendritic cells from peripheral blood mononuclear cells: Divergent maturation programs by IL-4 and LPS. Immunobiology 213, 751–758. doi:10.1016/j.imbio.2008.07.024

Doll, E.R., Bryans, J.T., 1963. Immunization of young horses against viral rhinopneumonitis. Cornell Vet. 53, 24–41.

Doll, E.R., Crowe, M.E.W., Bryans, J.T., McCollum, W.H., 1955. Infection immunity in equine virus abortion. Cornell Vet. 45, 387–410.

Doll, E.R., McCollum, W.H., Bryans, J.T., Crowe, M.E., 1959. Effect of physical and chemical environment on the viability of equine rhinopneumonitis virus propagated in hamsters. Cornell Vet. 49, 75–81.

Doll, E.R., McCollum, W.H., Wallace, M.E., Bryans, J.T., Richards, M.G., 1953. Complement-fixation reactions in equine virus abortion. Am. J. Vet. Res. 14, 40–5.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–7. doi:10.1080/14653240600855905

Donofrio, G., Colleoni, S., Galli, C., Lazzari, G., Cavirani, S., Flammini, C.F., 2005. Susceptibility of bovine mesenchymal stem cells to bovine herpesvirus 4. J. Virol. Methods 127, 168–70. doi:10.1016/j.jviromet.2005.02.019

Dossa, R.G., Alperin, D.C., Hines, M.T., Hines, S. a., 2014. The equine CD1 gene family is the largest and most diverse yet identified. Immunogenetics 66, 33–42. doi:10.1007/s00251-013-0741-6

Drummer, H.E., Studdert, M.J., Crabb, B.S., 1998. Equine herpesvirus-4 glycoprotein G is secreted as a disulphide-linked homodimer and is present as two homodimeric species in the virion. J. Gen. Virol. 79 (Pt 5), 1205–13. doi:10.1099/0022-1317-79-5-1205

Duncan, L.M., Piper, S., Dodd, R.B., Saville, M.K., Sanderson, C.M., Luzio, J.P., Lehner, P.J., 2006. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. EMBO J. 25, 1635–45. doi:10.1038/sj.emboj.7601056

Dunowska, M., 2014a. A review of equid herpesvirus 1 for the veterinary practitioner. Part A: clinical presentation, diagnosis and treatment. N. Z. Vet. J. 62, 171–178. doi:10.1080/00480169.2014.899945

Dunowska, M., 2014b. A review of equid herpesvirus 1 for the veterinary practitioner. Part B: Pathogenesis and epidemiology. N. Z. Vet. J. 37–41. doi:10.1080/00480169.2014.899946

Dunowska, M., 2014c. A review of equid herpesvirus 1 for the veterinary practitioner. Part B: pathogenesis and epidemiology. N. Z. Vet. J. 62, 37–41. doi:10.1080/00480169.2014.899946

Dunowska, M., 2016. How common is equine herpesvirus type 1 infection? Vet. Rec. 178, 67–9. doi:10.1136/vr.i190

Durán, M.C., Willenbrock, S., Carlson, R., Feige, K., Nolte, I., Escobar, H.M., 2013. Enhanced protocol for CD14+ cell enrichment from equine peripheral blood via anti-human CD14 mAb and automated magnetic activated cell sorting. Equine Vet. J. 45, 249–253. doi:10.1111/j.2042-3306.2012.00616.x

Dutta, D., Donaldson, J.G., 2012. Search for inhibitors of endocytosis: Intended specificity and unintended consequences. Cell. Logist. 2, 203–208. doi:10.4161/cl.23967

Duttagupta, P. a, Boesteanu, A.C., Katsikis, P.D., 2009. Costimulation signals for memory CD8+ T cells during viral infections. Crit. Rev. Immunol. 29, 469–486. doi:10.1615/CritRevImmunol.v29.i6.20

Edelmann, K.H., Wilson, C.B., 2001. Role of CD28/CD80-86 and CD40/CD154 costimulatory interactions in host defense to primary herpes simplex virus infection. J. Virol. 75, 612–621.

Edington, N., Bridges, C.G., Griffiths, L., 1989. Equine interferons following exposure to equid herpesvirus-1 or -4. J. Interferon Res. 9, 389–92.

Edington, N., Bridges, C.G., Huckle, A., 1985. Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. Equine Vet. J. 17, 369–72.

Edington, N., Bridges, C.G., Patel, J.R., 1986. Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: Equine stroke. Arch. Virol. 90, 111–124. doi:10.1007/BF01314149

Edington, N., Smyth, B., Griffiths, L., 1991. The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions. J. Comp. Pathol. 104, 379–87.

Edington, N., Welch, H.M., Griffiths, L., 1994. The prevalence of latent Equid herpesviruses in the tissues of 40 abattoir horses. Equine Vet. J. 26, 140–2.

Eggenhofer, E., Luk, F., Dahlke, M.H., Hoogduijn, M.J., 2014. The life and fate of mesenchymal stem cells. Front. Immunol. 5, 148. doi:10.3389/fimmu.2014.00148

Ehlers, B., Borchers, K., Grund, C., Frölich, K., Ludwig, H., Buhk, H.J., 1999. Detection of new DNA polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and deoxyinosine-substituted primers. Virus Genes 18, 211–20.

Eisenberg, R.J., Atanasiu, D., Cairns, T.M., Gallagher, J.R., Krummenacher, C., Cohen, G.H., 2012. Herpes virus fusion and entry: a story with many characters. Viruses 4, 800–32. doi:10.3390/v4050800

Eisfeld, A.J., Yee, M.B., Erazo, A., Abendroth, A., Kinchington, P.R., 2007. Downregulation of class I major histocompatibility complex surface expression by varicella-zoster virus involves open reading frame 66 protein kinase-dependent and -independent mechanisms. J Virol 81, 9034–9049. doi:10.1128/JVI.00711-07

Elboim, M., Grodzovski, I., Djian, E., Wolf, D.G., Mandelboim, O., 2013. HSV-2 specifically down regulates HLA-C expression to render HSV-2-infected DCs susceptible to NK cell killing. PLoS Pathog 9, e1003226. doi:10.1371/journal.ppat.1003226

Elion, G.B., 1982. Mechanism of action and selectivity of acyclovir. Am. J. Med. 73, 7–13.

Evans, R., Patzak, I., Svensson, L., De Filippo, K., Jones, K., McDowall, A., Hogg, N., 2009. Integrins in immunity. J. Cell Sci. 122, 215–225. doi:10.1242/jcs.019117

Everett, R.D., Boutell, C., McNair, C., Grant, L., Orr, A., 2010. Comparison of the biological and biochemical activities of several members of the alphaherpesvirus ICP0 family of proteins. J. Virol. 84, 3476–3487. doi:10.1128/JVI.02544-09

Fainardi, E., Castellazzi, M., Stignani, M., Morandi, F., Sana, G., Gonzalez, R., Pistoia, V., Baricordi, O.R.,

Sokal, E., Peña, J., 2011. Emerging topics and new perspectives on HLA-G. Cell. Mol. Life Sci. 68, 433–51. doi:10.1007/s00018-010-0584-3

Farini, A., Sitzia, C., Erratico, S., Meregalli, M., Torrente, Y., 2014. Clinical applications of mesenchymal stem cells in chronic diseases. Stem Cells Int. 2014, 306573. doi:10.1155/2014/306573

Feng, X., Thompson, Y.G., Lewis, J.B., Caughman, G.B., 1996. Expression and function of the equine herpesvirus 1 virion-associated host shutoff homolog. J. Virol. 70, 8710–8718.

Flaminio, M.J.B.F., Borges, A.S., Nydam, D. V, Horohov, D.W., Hecker, R., Matychak, M.B., 2007. The effect of CpG-ODN on antigen presenting cells of the foal. J. Immune Based Ther. Vaccines 5, 1. doi:10.1186/1476-8518-5-1

Foote, C.E., Love, D.N., Gilkerson, J.R., Rota, J., Trevor-Jones, P., Ruitenberg, K.M., Wellington, J.E., Whalley, J.M., 2005. Serum antibody responses to equine herpesvirus 1 glycoprotein D in horses, pregnant mares and young foals. Vet. Immunol. Immunopathol. 105, 47–57. doi:10.1016/j.vetimm.2004.12.012

Foote, C.E., Love, D.N., Gilkerson, J.R., Wellington, J.E., Whalley, J.M., 2006a. EHV-1 and EHV-4 infection in vaccinated mares and their foals. Vet. Immunol. Immunopathol. 111, 41–6. doi:10.1016/j.vetimm.2006.01.007

Foote, C.E., Love, D.N., Gilkerson, J.R., Whalley, J.M., 2002. Serological responses of mares and weanlings following vaccination with an inactivated whole virus equine herpesvirus 1 and equine herpesvirus 4 vaccine. Vet. Microbiol. 88, 13–25.

Foote, C.E., Raidal, S.L., Pecenpetelovska, G., Wellington, J.E., Whalley, J.M., 2006b. Inoculation of mares and very young foals with EHV-1 glycoproteins D and B reduces virus shedding following respiratory challenge with EHV-1. Vet. Immunol. Immunopathol. 111, 97–108. doi:10.1016/j.vetimm.2006.01.012

Förster, R., Braun, A., Worbs, T., 2012. Lymph node homing of T cells and dendritic cells via afferent lymphatics. Trends Immunol. 33, 271–80. doi:10.1016/j.it.2012.02.007

Frampton, A.R., Stolz, D.B., Uchida, H., Goins, W.F., Cohen, J.B., Glorioso, J.C., 2007. Equine herpesvirus 1 enters cells by two different pathways, and infection requires the activation of the cellular kinase ROCK1. J. Virol. 81, 10879–10889. doi:10.1128/JVI.00504-07

Frampton, A.R., Uchida, H., von Einem, J., Goins, W.F., Grandi, P., Cohen, J.B., Osterrieder, N., Glorioso, J.C., 2010. Equine herpesvirus type 1 (EHV-1) utilizes microtubules, dynein, and ROCK1 to productively infect cells. Vet. Microbiol. 141, 12–21. doi:10.1016/j.vetmic.2009.07.035

Friday, P.A., Scarratt, W.K., Elvinger, F., Timoney, P.J., Bonda, A., 2000. Ataxia and paresis with equine herpesvirus type 1 infection in a herd of riding school horses. J. Vet. Intern. Med. 14, 197–201.

Fulton, A., Peters, S.T., Perkins, G.A., Jarosinski, K.W., Damiani, A., Brosnahan, M., Buckles, E.L., Osterrieder, N., Van de Walle, G.R., 2009. Effective treatment of respiratory alphaherpesvirus infection using RNA interference. PLoS One 4, e4118. doi:10.1371/journal.pone.0004118

Gardiner, D.W., Lunn, D.P., Goehring, L.S., Chiang, Y.-W., Cook, C., Osterrieder, N., McCue, P., Del Piero, F., Hussey, S.B., Hussey, G.S., 2012. Strain impact on equine herpesvirus type 1 (EHV-1) abortion models: viral loads in fetal and placental tissues and foals. Vaccine 30, 6564–72. doi:10.1016/j.vaccine.2012.08.046

Garré, B., Baert, K., Nauwynck, H., Deprez, P., De Backer, P., Croubels, S., 2009a. Multiple oral dosing of valacyclovir in horses and ponies. J. Vet. Pharmacol. Ther. 32, 207–12. doi:10.1111/j.1365-2885.2008.01025.x

Garré, B., Gryspeerdt, A., Croubels, S., De Backer, P., Nauwynck, H., 2009b. Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies. Vet. Microbiol. 135, 214–21. doi:10.1016/j.vetmic.2008.09.062

Garré, B., van der Meulen, K., Nugent, J., Neyts, J., Croubels, S., De Backer, P., Nauwynck, H., 2007. In vitro susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir,

PMEDAP and foscarnet. Vet. Microbiol. 122, 43–51. doi:10.1016/j.vetmic.2007.01.004

Gelin, C., Sloma, I., Charron, D., Mooney, N., 2009. Regulation of MHC II and CD1 antigen presentation: from ubiquity to security. J. Leukoc. Biol. 85, 215–24. doi:10.1189/jlb.0308206

Gibson, J.S., Slater, J.D., Awan, A.R., Field, H.J., 1992. Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. Arch. Virol. 123, 351–66.

Gilkerson, J.R., Bailey, K.E., Diaz-Méndez, A., Hartley, C. a., 2015. Update on Viral Diseases of the Equine Respiratory Tract. Vet. Clin. North Am. Equine Pract. 31, 91–104. doi:10.1016/j.cveq.2014.11.007

Gilkerson, J.R., Love, D.N., Whalley, J.M., 2000. Incidence of equine herpesvirus 1 infection in thoroughbred weanlings on two stud farms. Aust. Vet. J. 78, 277–8.

Gilkerson, J.R., Whalley, J.M., Drummer, H.E., Studdert, M.J., Love, D.N., 1999. Epidemiology of EHV-1 and EHV-4 in the mare and foal populations on a Hunter Valley stud farm: are mares the source of EHV-1 for unweaned foals. Vet. Microbiol. 68, 27–34. doi:10.1016/S0378-1135(99)00058-9

Girard, J.-P., Moussion, C., Förster, R., 2012. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nat. Rev. Immunol. 12, 762–73. doi:10.1038/nri3298

Gleeson, L.J., Coggins, L., 1980. Response of pregnant mares to equine herpesvirus 1 (EHV1). Cornell Vet. 70, 391–400.

Glorieux, S., Favoreel, H.W., Steukers, L., Vandekerckhove, A.P., Nauwynck, H.J., 2011. A trypsin-like serine protease is involved in pseudorabies virus invasion through the basement membrane barrier of porcine nasal respiratory mucosa. Vet. Res. 42, 58. doi:10.1186/1297-9716-42-58

Goehring, L.S., Hussey, G.S., Ashton, L. V, Schenkel, A.R., Lunn, D.P., 2011. Infection of central nervous system endothelial cells by cell-associated EHV-1. Vet. Microbiol. 148, 389–95. doi:10.1016/j.vetmic.2010.08.030

Goehring, L.S., Landolt, G.A., Morley, P.S., 2010. Detection and management of an outbreak of equine herpesvirus type 1 infection and associated neurological disease in a veterinary teaching hospital. J. Vet. Intern. Med. 24, 1176–83. doi:10.1111/j.1939-1676.2010.0558.x

Goehring, L.S., van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., 2005. Neurological syndromes among horses in The Netherlands. A 5 year retrospective survey (1999-2004). Vet. Q. 27, 11–20. doi:10.1080/01652176.2005.9695182

Goehring, L.S., van Winden, S.C., van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., 2006. Equine herpesvirus type 1-associated myeloencephalopathy in The Netherlands: a four-year retrospective study (1999-2003). J. Vet. Intern. Med. 20, 601–7.

Goehring, L.S., Wagner, B., Bigbie, R., Hussey, S.B., Rao, S., Morley, P.S., Lunn, D.P., 2010. Control of EHV-1 viremia and nasal shedding by commercial vaccines. Vaccine 28, 5203–5211. doi:10.1016/j.vaccine.2010.05.065

Goodman, L.B., Loregian, A., Perkins, G. a., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palù, G., Smith, K.C., Osterrieder, N., Davis-Poynter, N., 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog. 3, 1583–1592. doi:10.1371/journal.ppat.0030160

Goodman, L.B., Wagner, B., Flaminio, M.J.B.F., Sussman, K.H., Metzger, S.M., Holland, R., Osterrieder, N., 2006. Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1). Vaccine 24, 3636–45. doi:10.1016/j.vaccine.2006.01.062

Goodman, L.B., Wimer, C., Dubovi, E.J., Gold, C., Wagner, B., 2012. Immunological correlates of vaccination and infection for equine herpesvirus 1. Clin. Vaccine Immunol. 19, 235–41. doi:10.1128/CVI.05522-11

Greenwood, R.E., Simson, A.R., 1980. Clinical report of a paralytic syndrome affecting stallions, mares and foals on a thoroughbred studfarm. Equine Vet. J. 12, 113–7.

Grubor-Bauk, B., Simmons, A., Mayrhofer, G., Speck, P.G., 2003. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. J. Immunol. 170, 1430–4.

Grundy, J.E., Downes, K.L., 1993. Up-regulation of LFA-3 and ICAM-1 on the surface of fibroblasts infected with cytomegalovirus. Immunology 78, 405–12.

Gryspeerdt, A.C., Vandekerckhove, A.P., Garre, B., Barbe, F., Van de Walle, G.R., Nauwynck, H.J., 2010. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Vet Microbiol 142, 242–253. doi:10.1016/j.vetmic.2009.10.015

Hammond, S. a., Horohov, D., Montelaro, R.C., 1999. Functional characterization of equine dendritic cells propagated ex vivo using recombinant human GM-CSF and recombinant equine IL-4. Vet. Immunol. Immunopathol. 71, 197–214.

Hannant, D., Jessett, D.M., O'Neill, T., Dolby, C.A., Cook, R.F., Mumford, J.A., 1993. Responses of ponies to equid herpesvirus-1 ISCOM vaccination and challenge with virus of the homologous strain. Res. Vet. Sci. 54, 299–305.

Harty, R.N., Colle, C.F., Grundy, F.J., O'Callaghan, D.J., 1989. Mapping the termini and intron of the spliced immediate-early transcript of equine herpesvirus 1. J. Virol. 63, 5101–10.

Haspot, F., Lavault, A., Sinzger, C., Laib Sampaio, K., Stierhof, Y.-D., Pilet, P., Bressolette-Bodin, C., Halary, F., 2012. Human cytomegalovirus entry into dendritic cells occurs via a macropinocytosis-like pathway in a pH-independent and cholesterol-dependent manner. PLoS One 7, e34795. doi:10.1371/journal.pone.0034795

Heilingloh, C.S., Muhl-Zurbes, P., Steinkasserer, A., Kummer, M., 2014. Herpes simplex virus type 1 ICP0 induces CD83 degradation in mature dendritic cells independent of its E3 ubiquitin ligase function. J Gen Virol 95, 1366–1375. doi:10.1099/vir.0.062810-0

Heldens, J.G., Hannant, D., Cullinane, A.A., Prendergast, M.J., Mumford, J.A., Nelly, M., Kydd, J.H., Weststrate, M.W., van den Hoven, R., 2001a. Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn EHV1,4). Vaccination/challenge experiments in foals and pregnant mares. Vaccine 19, 4307–17.

Heldens, J.G., Kersten, A.J., Weststrate, M.W., van den Hoven, R., 2001b. Duration of immunity induced by an adjuvanted and inactivated equine influenza, tetanus and equine herpesvirus 1 and 4 combination vaccine. Vet. Q. 23, 210–7. doi:10.1080/01652176.2001.9695116

Henninger, R.W., Reed, S.M., Saville, W.J., Allen, G.P., Hass, G.F., Kohn, C.W., Sofaly, C., 2007. Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. J. Vet. Intern. Med. 21, 157–165. doi:10.1892/0891-6640(2007)21[157:OONDCB]2.0.CO;2

Hennrick, K.T., Keeton, A.G., Nanua, S., Kijek, T.G., Goldsmith, A.M., Sajjan, U.S., Bentley, J.K., Lama, V.N., Moore, B.B., Schumacher, R.E., Thannickal, V.J., Hershenson, M.B., 2007. Lung cells from neonates show a mesenchymal stem cell phenotype. Am. J. Respir. Crit. Care Med. 175, 1158–64. doi:10.1164/rccm.200607-941OC

Herrera, M.B., Bussolati, B., Bruno, S., Morando, L., Mauriello-Romanazzi, G., Sanavio, F., Stamenkovic, I., Biancone, L., Camussi, G., 2007. Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. Kidney Int. 72, 430–41. doi:10.1038/sj.ki.5002334

Hewitt, E.W., 2003. The MHC class I antigen presentation pathway: Strategies for viral immune evasion. Immunology 110, 163–169. doi:10.1046/j.1365-2567.2003.01738.x

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., Johnson, D., 1995. Herpes simplex virus turns off the TAP to evade host immunity. Nature 375, 411–415. doi:10.1038/375411a0

Hirano, N., Butler, M.O., Xia, Z., Ansén, S., Von Bergwelt-Baildon, M.S., Neuberg, D., Freeman, G.J., Nadler, L.M., 2006. Engagement of CD83 ligand induces prolonged expansion of CD8+ T cells and preferential

enrichment for antigen specificity. Blood 107, 1528–1536.

Holmes, M.A., Townsend, H.G.G., Kohler, A.K., Hussey, S., Breathnach, C., Barnett, C., Holland, R., Lunn, D.P., 2006. Immune responses to commercial equine vaccines against equine herpesvirus-1, equine influenza virus, eastern equine encephalomyelitis, and tetanus. Vet. Immunol. Immunopathol. 111, 67–80. doi:10.1016/j.vetimm.2006.01.010

Holt, P.G., Stumbles, P. a, 2000. Characterization of dendritic cell populations in the respiratory tract. J. Aerosol Med. 13, 361–367.

Hong, C.B., Donahue, J.M., Giles, R.C., Petrites-Murphy, M.B., Poonacha, K.B., Roberts, a W., Smith, B.J., Tramontin, R.R., Tuttle, P. a, Swerczek, T.W., 1993. Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. J. Vet. Diagn. Invest. 5, 560–566. doi:10.1177/104063879300500410

Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S., Keating, A., 2005. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 7, 393–5. doi:10.1080/14653240500319234

Huang, T., Lehmann, M.J., Said, A., Ma, G., Osterrieder, N., 2014. Major histocompatibility complex class I downregulation induced by equine herpesvirus type 1 pUL56 is through dynamin-dependent endocytosis. J. Virol. 88, 12802–12815.

Huang, T., Ma, G., Osterrieder, N., 2015. Equine Herpesvirus 1 Multiply Inserted Transmembrane Protein pUL43 Cooperates with pUL56 in Downregulation of Cell Surface Major Histocompatibility Complex Class I. J. Virol. 89, 6251–6263.

Huemer, H.P., Nowotny, N., Crabb, B.S., Meyer, H., Hübert, P.H., 1995. gp13 (EHV-gC): a complement receptor induced by equine herpesviruses. Virus Res. 37, 113–126. doi:10.1016/0168-1702(95)00027-N

Hussey, G.S., Goehring, L.S., Lunn, D.P., Hussey, S.B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., Slater, J., 2013. Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Vet. Res. 44, 118. doi:10.1186/1297-9716-44-118

Imai, T., Koyanagi, N., Ogawa, R., Shindo, K., Suenaga, T., Sato, A., Arii, J., Kato, A., Kiyono, H., Arase, H., Kawaguchi, Y., 2013. Us3 kinase encoded by herpes simplex virus 1 mediates downregulation of cell surface major histocompatibility complex class I and evasion of CD8+ T cells. PLoS One 8, e72050. doi:10.1371/journal.pone.0072050

Ip, J.E., Wu, Y., Huang, J., Zhang, L., Pratt, R.E., Dzau, V.J., 2007. Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. Mol. Biol. Cell 18, 2873–82. doi:10.1091/mbc.E07-02-0166

Iversen, M.B., Paludan, S.R., 2010. Mechanisms of type III interferon expression. J. Interferon Cytokine Res. 30, 573–8. doi:10.1089/jir.2010.0063

Jackson, T.A., Osburn, B.I., Cordy, D.R., Kendrick, J.W., 1977. Equine herpesvirus 1 infection of horses: studies on the experimentally induced neurologic disease. Am. J. Vet. Res. 38, 709–19.

Jacob, T., Van den Broeke, C., Grauwet, K., Baert, K., Claessen, C., De Pelsmaeker, S., Van Waesberghe, C., Favoreel, H.W., 2015. Pseudorabies virus US3 leads to filamentous actin disassembly and contributes to viral genome delivery to the nucleus. Vet. Microbiol. 177, 379–385. doi:10.1016/j.vetmic.2015.03.023

Jaganathan, B.G., Ruester, B., Dressel, L., Stein, S., Grez, M., Seifried, E., Henschler, R., 2007. Rho inhibition induces migration of mesenchymal stromal cells. Stem Cells 25, 1966–74. doi:10.1634/stemcells.2007-0167

Jakob, M., Hemeda, H., Janeschik, S., Bootz, F., Rotter, N., Lang, S., Brandau, S., 2010. Human nasal mucosa contains tissue-resident immunologically responsive mesenchymal stromal cells. Stem Cells Dev. 19, 635–44. doi:10.1089/scd.2009.0245

Jancic, C., Chuluyan, H.E., Morelli, A., Larregina, A., Kolkowski, E., Saracco, M., Barboza, M., Leiva, W.S.,

Fainboim, L., 1998. Interactions of dendritic cells with fibronectin and endothelial cells. Immunology 95, 283–90. doi:10.1046/j.1365-2567.1998.00586.x

Karlsson, H., Samarasinghe, S., Ball, L.M., Sundberg, B., Lankester, A.C., Dazzi, F., Uzunel, M., Rao, K., Veys, P., Le Blanc, K., Ringdén, O., Amrolia, P.J., 2008. Mesenchymal stem cells exert differential effects on alloantigen and virus-specific T-cell responses. Blood 112, 532–41. doi:10.1182/blood-2007-10-119370

Kim, Y.M., Kim, J., Heo, S.C., Shin, S.H., Do, E.K., Suh, D.-S., Kim, K.-H., Yoon, M.-S., Lee, T.G., Kim, J.H., 2012. Proteomic identification of ADAM12 as a regulator for TGF-β1-induced differentiation of human mesenchymal stem cells to smooth muscle cells. PLoS One 7, e40820. doi:10.1371/journal.pone.0040820

Kirchhausen, T., Macia, E., Pelish, H.E., 2008. Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis. Methods Enzymol. 438, 77–93. doi:10.1016/S0076-6879(07)38006-3

Kleiboeker, S.B., Schommer, S.K., Johnson, P.J., Ehlers, B., Turnquist, S.E., Boucher, M., Kreeger, J.M., 2002. Association of two newly recognized herpesviruses with interstitial pneumonia in donkeys (Equus asinus). J. Vet. Diagn. Invest. 14, 273–80.

Kleiboeker, S.B., Turnquist, S.E., Johnson, P.J., Kreeger, J.M., 2004. Detection and Nucleotide Sequencing of a DNA-Packaging Protein Gene of Equine Gammaherpesviruses. J. Vet. Diagnostic Investig. 16, 67–74. doi:10.1177/104063870401600112

Kobelt, D., Lechmann, M., Steinkasserer, A., 2003. The interaction between dendritic cells and herpes simplex virus-1. Curr. Top. Microbiol. Immunol. 276, 145–161.

Koppers-Lalic, D., Rijsewijk, F.A., Verschuren, S.B., van Gaans-Van den Brink, J.A., Neisig, A., Ressing, M.E., Neefjes, J., Wiertz, E.J., 2001. The UL41-encoded virion host shutoff (vhs) protein and vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1. J Gen Virol 82, 2071–2081.

Koppers-Lalic, D., Verweij, M.C., Lipińska, A.D., Wang, Y., Quinten, E., Reits, E. a., Koch, J., Loch, S., Rezende, M.M., Daus, F., Bieńkowska-Szewczyk, K., Osterrieder, N., Mettenleiter, T.C., Heemskerk, M.H.M., Tampé, R., Neefjes, J.J., Chowdhury, S.I., Ressing, M.E., Rijsewijk, F. a M., Wiertz, E.J.H.J., 2008. Varicellovirus UL49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP. Public Libr. Sci. Pathog. 4, e1000080. doi:10.1371/journal.ppat.1000080

Koshizuka, T., Goshima, F., Takakuwa, H., Nozawa, N., Daikoku, T., Koiwai, O., Nishiyama, Y., 2002. Identification and characterization of the UL56 gene product of herpes simplex virus type 2. J Virol 76, 6718–6728.

Krampera, M., 2011. Mesenchymal stromal cell "licensing": a multistep process. Leukemia 25, 1408–14. doi:10.1038/leu.2011.108

Kruse, M., Rosorius, O., Krätzer, F., Bevec, D., Kuhnt, C., Steinkasserer, a, Schuler, G., Hauber, J., 2000a. Inhibition of CD83 cell surface expression during dendritic cell maturation by interference with nuclear export of CD83 mRNA. J. Exp. Med. 191, 1581–1590. doi:10.1084/jem.191.9.1581

Kruse, M., Rosorius, O., Krätzer, F., Stelz, G., Kuhnt, C., Schuler, G., Hauber, J., Steinkasserer, A., 2000b. Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. J. Virol. 74, 7127–7136.

Kukreja, A., Love, D.N., Whalley, J.M., Field, H.J., 1998a. Study of the protective immunity of co-expressed glycoprotein H and L of equine herpesvirus-1 in a murine intranasal infection model. Vet. Microbiol. 60, 1–11.

Kukreja, A., Walker, C., Fitzmaurice, T., Awan, A., Love, D.N., Whalley, J.M., Field, H.J., 1998b. Protective effects of equine herpesvirus-1 (EHV-1) glycoprotein B in a murine model of EHV-1-induced abortion. Vet. Microbiol. 62, 303–11.

Kummer, M., Turza, N.M., Muhl-Zurbes, P., Lechmann, M., Boutell, C., Coffin, R.S., Everett, R.D., Steinkasserer, A., Prechtel, A.T., 2007. Herpes simplex virus type 1 induces CD83 degradation in mature dendritic cells with immediate-early kinetics via the cellular proteasome. J. Virol. 81, 6326–6338.

Kurtz, B.M., Singletary, L.B., Kelly, S.D., Frampton, A.R., 2010. Equus caballus major histocompatibility complex class I is an entry receptor for equine herpesvirus type 1. J. Virol. 84, 9027–34. doi:10.1128/JVI.00287-10

Kushwah, R., Hu, J., 2011. Role of dendritic cells in the induction of regulatory T cells. Cell Biosci. 1, 20. doi:10.1186/2045-3701-1-20

Kydd, J.H., Case, R., Minke, J., Audonnet, J.-C., Wagner, B., Antczak, D.F., 2014. Immediate-early protein of equid herpesvirus type 1 as a target for cytotoxic T-lymphocytes in the Thoroughbred horse. J. Gen. Virol. 95, 1783–9. doi:10.1099/vir.0.065888-0

Kydd, J.H., Davis-Poynter, N.J., Birch, J., Hannant, D., Minke, J., Audonnet, J.-C., Antczak, D.F., Ellis, S.A., 2006a. A molecular approach to the identification of cytotoxic T-lymphocyte epitopes within equine herpesvirus 1. J. Gen. Virol. 87, 2507–15. doi:10.1099/vir.0.82070-0

Kydd, J.H., Hannant, D., Mumford, J.A., 1996. Residence and recruitment of leucocytes to the equine lung after EHV-1 infection. Vet. Immunol. Immunopathol. 52, 15–26.

Kydd, J.H., Slater, J., Osterrieder, N., Lunn, D.P., Antczak, D.F., Azab, W., Balasuriya, U., Barnett, C., Brosnahan, M., Cook, C., Damiani, A., Elton, D., Frampton, A., Gilkerson, J., Goehring, L., Horohov, D., Maxwell, L., Minke, J., Morley, P., Nauwynck, H., Newton, R., Perkins, G., Pusterla, N., Soboll-Hussey, G., Traub-Dargatz, J., Townsend, H., Van de Walle, G.R., Wagner, B., 2012. Third International Havemeyer Workshop on Equine Herpesvirus type 1. Equine Vet. J. 44, 513–7. doi:10.1111/j.2042-3306.2012.00604.x

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994a. Distribution of equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications for cellular immunity. Equine Vet. J. 26, 470–3.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A., 1994b. Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine Vet. J. 26, 466–9.

Kydd, J.H., Townsend, H.G.G., Hannant, D., 2006b. The equine immune response to equine herpesvirus-1: the virus and its vaccines. Vet. Immunol. Immunopathol. 111, 15–30. doi:10.1016/j.vetimm.2006.01.005

Kydd, J.H., Wattrang, E., Hannant, D., 2003. Pre-infection frequencies of equine herpesvirus-1 specific, cytotoxic T lymphocytes correlate with protection against abortion following experimental infection of pregnant mares. Vet. Immunol. Immunopathol. 96, 207–217. doi:10.1016/j.vetimm.2003.08.004

Kyurkchiev, D., 2014. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J. Stem Cells 6, 552. doi:10.4252/wjsc.v6.i5.552

Lakshmipathy, U., Verfaillie, C., 2005. Stem cell plasticity. Blood Rev. 19, 29–38. doi:10.1016/j.blre.2004.03.001

Lama, V.N., Smith, L., Badri, L., Flint, A., Andrei, A.-C., Murray, S., Wang, Z., Liao, H., Toews, G.B., Krebsbach, P.H., Peters-Golden, M., Pinsky, D.J., Martinez, F.J., Thannickal, V.J., 2007. Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts. J. Clin. Invest. 117, 989–96. doi:10.1172/JCI29713

Lambrecht, B.N., Hammad, H., 2012. Lung Dendritic Cells in Respiratory Viral Infection and Asthma: From Protection to Immunopathology. Annu. Rev. Immunol. 30, 243–270. doi:10.1146/annurev-immunol-020711-075021

Lämmermann, T., Bader, B.L., Monkley, S.J., Worbs, T., Wedlich-Söldner, R., Hirsch, K., Keller, M., Förster, R., Critchley, D.R., Fässler, R., Sixt, M., 2008. Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature 453, 51–5. doi:10.1038/nature06887

Lampen, M.H., Verweij, M.C., Querido, B., van der Burg, S.H., Wiertz, E.J.H.J., van Hall, T., 2010. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. J. Immunol. 185, 6508–6517. doi:10.4049/jimmunol.1001774

Laval, K., Favoreel, H.W., Nauwynck, H.J., 2014. Equine herpesvirus type 1 (EHV-1) replication is delayed in CD172a+ monocytic cells and controlled by histone deacetylases. J. Gen. Virol. doi:10.1099/vir.0.067363-0

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. doi:10.1128/JVI.01589-15

Lee, H.K., Iwasaki, A., 2007. Innate control of adaptive immunity: dendritic cells and beyond. Semin Immunol 19, 48–55. doi:10.1016/j.smim.2006.12.001

Lee, H.K., Zamora, M., Linehan, M.M., Iijima, N., Gonzalez, D., Haberman, A., Iwasaki, A., 2009. Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. J. Exp. Med. 206, 359–70. doi:10.1084/jem.20080601

Lehman, I.R., Boehmer, P.E., 1999. Replication of Herpes Simplex Virus DNA. J. Biol. Chem. 274, 28059–28062. doi:10.1074/jbc.274.40.28059

Lim, J.-H., Kim, J.-S., Yoon, I.-H., Shin, J.-S., Nam, H.-Y., Yang, S.-H., Kim, S.-J., Park, C.-G., 2010. Immunomodulation of delayed-type hypersensitivity responses by mesenchymal stem cells is associated with bystander T cell apoptosis in the draining lymph node. J. Immunol. 185, 4022–9. doi:10.4049/jimmunol.0902723

Loch, S., Tampé, R., 2005. Viral evasion of the MHC class I antigen-processing machinery. Pflugers Arch. Eur. J. Physiol. 451, 409–417. doi:10.1007/s00424-005-1420-8

Lovati, A.B., Corradetti, B., Lange Consiglio, A., Recordati, C., Bonacina, E., Bizzaro, D., Cremonesi, F., 2011. Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells. Vet. Res. Commun. 35, 103–21. doi:10.1007/s11259-010-9457-3

Luce, R., Shepherd, M., Paillot, R., Blacklawst, B., Wood, J.L.N., Kydd, J.H., 2007. Equine herpesvirus-1-specific interferon gamma (IFNgamma) synthesis by peripheral blood mononuclear cells in thoroughbred horses. Equine Vet. J. 39, 202–9.

Ludewig, B., Ehl, S., Karrer, U., Odermatt, B., Hengartner, H., Zinkernagel, R.M., 1998. Dendritic cells efficiently induce protective antiviral immunity. J. Virol. 72, 3812–3818.

Ludewig, B., Oehen, S., Barchiesi, F., Schwendener, R. a, Hengartner, H., Zinkernagel, R.M., 1999. Protective antiviral cytotoxic T cell memory is most efficiently maintained by restimulation via dendritic cells. J. Immunol. 163, 1839–1844.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J., Horohov, D.W., Osterrieder, K., Pusterla, N., Townsend, H.G., 2009a. Equine herpesvirus-1 consensus statement. J Vet Intern Med 23, 450–461. doi:10.1111/j.1939-1676.2009.0304.x

Lunn, D.P., Horohov, D.W., Osterrieder, K., Pusterla, N., 2009b. EHV-1 Consensus Statement 450–461.

Ma, G., Azab, W., Osterrieder, N., 2013. Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol 167, 123–134. doi:10.1016/j.vetmic.2013.06.018

Ma, G., Feineis, S., Osterrieder, N., Van de Walle, G.R., 2012. Identification and characterization of equine herpesvirus type 1 pUL56 and its role in virus-induced downregulation of major histocompatibility complex class I. J. Virol. 86, 3554–3563.

Malcherek, G., Jin, N., Hückelhoven, A.G., Mani, J., Wang, L., Gern, U., Diehlmann, A., Wuchter, P., Schmitt, A., Chen, B., Ho, A.D., Schmitt, M., 2014. Mesenchymal stromal cells inhibit proliferation of virus-specific CD8(+) T cells. Leukemia 28, 2388–94. doi:10.1038/leu.2014.273

Mandelboim, O., Pazmany, L., Davis, D.M., Valés-Gómez, M., Reyburn, H.T., Rybalov, B., Strominger, J.L., 1997. Multiple receptors for HLA-G on human natural killer cells. Proc. Natl. Acad. Sci. U. S. A. 94, 14666–70.

References

Martinez-Pomares, L., 2012. The mannose receptor. J. Leukoc. Biol. 92, 1177–1186. doi:10.1189/jlb.0512231

Matsumura, T., Kondo, T., Sugita, S., Damiani, A.M., O'Callaghan, D.J., Imagawa, H., 1998. An equine herpesvirus type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses. Virology 242, 68–79. doi:10.1006/viro.1997.8984

Mauel, S., Steinbach, F., Ludwig, H., 2006. Monocyte-derived dendritic cells from horses differ from dendritic cells of humans and mice. Immunology 117, 463–473. doi:10.1111/j.1365-2567.2005.02319.x

Maxwell, L.K., Bentz, B.G., Bourne, D.W.A., Erkert, R.S., 2008. Pharmacokinetics of valacyclovir in the adult horse. J. Vet. Pharmacol. Ther. 31, 312–20. doi:10.1111/j.1365-2885.2008.00957.x

McCartan, C.G., Russell, M.M., Wood, J.L., Mumford, J.A., 1995. Clinical, serological and virological characteristics of an outbreak of paresis and neonatal foal disease due to equine herpesvirus-1 on a stud farm. Vet. Rec. 136, 7–12.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., Smythe, E., 1998. A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. Curr. Biol. 8, 34–45.

Mensing, N., Gasse, H., Hambruch, N., Haeger, J.-D., Pfarrer, C., Staszyk, C., 2011. Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse. BMC Vet. Res. 7, 42. doi:10.1186/1746-6148-7-42

Mercer, J., Greber, U.F., 2013. Virus interactions with endocytic pathways in macrophages and dendritic cells. Trends Microbiol. 21, 380–388. doi:10.1016/j.tim.2013.06.001

Mercer, J., Helenius, A., 2012. Gulping rather than sipping: Macropinocytosis as a way of virus entry. Curr. Opin. Microbiol. 15, 490–499. doi:10.1016/j.mib.2012.05.016

Mettenleiter, T.C., 1999. Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis--state of the art, June 1999. Vet. Res. 31, 99–115. doi:10.1051/vetres:2000110

Mettenleiter, T.C., Klupp, B.G., Granzow, H., 2009. Herpesvirus assembly: An update. Virus Res. 143, 222–234. doi:10.1016/j.virusres.2009.03.018

Mikloska, Z., Bosnjak, L., Cunningham, A.L., 2001. Immature monocyte-derived dendritic cells are productively infected with herpes simplex virus type 1. J. Virol. 75, 5958–5964.

Minke, J.M., Audonnet, J.-C., Fischer, L., 2004. Equine viral vaccines: the past, present and future. Vet. Res. 35, 425–43. doi:10.1051/vetres:2004019

Minke, J.M., Fischer, L., Baudu, P., Guigal, P.M., Sindle, T., Mumford, J. a., Audonnet, J.C., 2006. Use of DNA and recombinant canarypox viral (ALVAC) vectors for equine herpes virus vaccination. Vet. Immunol. Immunopathol. 111, 47–57. doi:10.1016/j.vetimm.2006.01.008

Mittelbrunn, M., Molina, A., Escribese, M.M., Yáñez-Mó, M., Escudero, E., Ursa, A., Tejedor, R., Mampaso, F., Sánchez-Madrid, F., 2004. VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses. Proc. Natl. Acad. Sci. U. S. A. 101, 11058–11063. doi:10.1073/pnas.0307927101

Miyamoto, S., Teramoto, H., Coso, O.A., Gutkind, J.S., Burbelo, P.D., Akiyama, S.K., Yamada, K.M., 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131, 791–805.

Morrow, G., Slobedman, B., Cunningham, A.L., Abendroth, A., 2003. Varicella-zoster virus productively infects mature dendritic cells and alters their immune function. J. Virol. 77, 4950–4959.

Mousavi, S.A., Malerød, L., Berg, T., Kjeken, R., 2004. Clathrin-dependent endocytosis. Biochem. J. 377, 1–16. doi:10.1042/BJ20031000

Moyo, N. a., Marchi, E., Steinbach, F., 2013. Differentiation and activation of equine monocyte-derived dendritic cells are not correlated with CD206 or CD83 expression. Immunology 139, 472–483. doi:10.1111/imm.12094

Mumford, J.A., Rossdale, P.D., Jessett, D.M., Gann, S.J., Ousey, J., Cook, R.F., 1987. Serological and virological investigations of an equid herpesvirus 1 (EHV-1) abortion storm on a stud farm in 1985. J. Reprod. Fertil. Suppl. 35, 509–18.

Murata, T., Goshima, F., Daikoku, T., Takakuwa, H., Nishiyama, Y., 2000. Expression of herpes simplex virus type 2 US3 affects the Cdc42/Rac pathway and attenuates c-Jun N-terminal kinase activation. Genes to Cells 5, 1017–1027. doi:10.1046/j.1365-2443.2000.00383.x

Murray, I.R., Péault, B., 2015. Q&A: Mesenchymal stem cells — where do they come from and is it important? BMC Biol. 13, 99. doi:10.1186/s12915-015-0212-7

Murray, M.J., del Piero, F., Jeffrey, S.C., Davis, M.S., Furr, M.O., Dubovi, E.J., Mayo, J.A., 1998. Neonatal equine herpesvirus type 1 infection on a thoroughbred breeding farm. J. Vet. Intern. Med. 12, 36–41.

Naji, A., Rouas-Freiss, N., Durrbach, A., Carosella, E.D., Sensébé, L., Deschaseaux, F., 2013. Concise review: combining human leukocyte antigen G and mesenchymal stem cells for immunosuppressant biotherapy. Stem Cells 31, 2296–303. doi:10.1002/stem.1494

Neubauer, A., Braun, B., Brandmuller, C., Kaaden, O.R., Osterrieder, N., 1997. Analysis of the contributions of the equine herpesvirus 1 glycoprotein gB homolog to virus entry and direct cell-to-cell spread. Virology 227, 281–94. doi:10.1006/viro.1996.8336

Neubauer, A., Osterrieder, N., 2004. Equine herpesvirus type 1 (EHV-1) glycoprotein K is required for efficient cell-to-cell spread and virus egress. Virology 329, 18–32. doi:10.1016/j.virol.2004.07.034

Novak, N., Peng, W.M., 2005. Dancing with the enemy: the interplay of herpes simplex virus with dendritic cells. Clin. Exp. Immunol. 142, 405–10. doi:10.1111/j.1365-2249.2005.02927.x

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J. a, Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. J. Virol. 80, 4047–4060. doi:10.1128/JVI.80.8.4047-4060.2006

O'Callaghan, D.J., Colle 3rd, C.F., Flowers, C.C., Smith, R.H., Benoit, J.N., Bigger, C.A., 1994. Identification and initial characterization of the IR6 protein of equine herpesvirus 1. J Virol 68, 5351–5364.

O'Neill, T., Kydd, J.H., Allen, G.P., Wattrang, E., Mumford, J.A., Hannant, D., 1999. Determination of equid herpesvirus 1-specific, CD8+, cytotoxic T lymphocyte precursor frequencies in ponies. Vet. Immunol. Immunopathol. 70, 43–54.

Orabona, C., Grohmann, U., Belladonna, M.L., Fallarino, F., Vacca, C., Bianchi, R., Bozza, S., Volpi, C., Salomon, B.L., Fioretti, M.C., Romani, L., Puccetti, P., 2004. CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. Nat. Immunol. 5, 1134–42. doi:10.1038/ni1124

Osterrieder, N., 1999. Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. Virus Res. 59, 165–77.

Osterrieder, N., 1999. Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. Virus Res. 59, 165–177. doi:10.1016/S0168-1702(98)00134-8

Osterrieder, N., Neubauer, A., Brandmuller, C., Braun, B., Kaaden, O.R., Baines, J.D., 1996. The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. J. Virol. 70, 4110–5.

Osterrieder, N., Neubauer, a, Fakler, B., Brandmüller, C., Seyboldt, C., Kaaden, O.R., Baines, J.D., 1997. Synthesis and processing of the equine herpesvirus 1 glycoprotein M. Virology 232, 230–239.

Osterrieder, N., Wagner, R., Brandmüller, C., Schmidt, P., Wolf, H., Kaaden, O.R., 1995. Protection against EHV-1 challenge infection in the murine model after vaccination with various formulations of recombinant glycoprotein gp14 (gB). Virology 208, 500–10. doi:10.1006/viro.1995.1181

Ostlund, E.N., 1993. The equine herpesviruses. Vet. Clin. North Am. Equine Pract. 9, 283–94.

Pacheco, A., Chernoff, J., 2010. Group I p21-activated kinases: emerging roles in immune function and viral pathogenesis. Int. J. Biochem. Cell Biol. 42, 13–6. doi:10.1016/j.biocel.2009.09.006

Packiarajah, P., Walker, C., Gilkerson, J., Whalley, J.M., Love, D.N., 1998. Immune responses and protective efficacy of recombinant baculovirus-expressed glycoproteins of equine herpesvirus 1 (EHV-1) gB, gC and gD alone or in combinations in BALB/c mice. Vet. Microbiol. 61, 261–78.

Paillot, R., Case, R., Ross, J., Newton, R., Nugent, J., 2008. Equine Herpes Virus-1: Virus, Immunity and Vaccines. Open Vet. Sci. J. 2, 68–91. doi:10.2174/1874318800802010068

Paillot, R., Daly, J.M., Juillard, V., Minke, J.M., Hannant, D., Kydd, J.H., 2005. Equine interferon gamma synthesis in lymphocytes after in vivo infection and in vitro stimulation with EHV-1. Vaccine 23, 4541–51. doi:10.1016/j.vaccine.2005.03.048

Paillot, R., Daly, J.M., Luce, R., Montesso, F., Davis-Poynter, N., Hannant, D., Kydd, J.H., 2007. Frequency and phenotype of EHV-1 specific, IFN-gamma synthesising lymphocytes in ponies: the effects of age, pregnancy and infection. Dev. Comp. Immunol. 31, 202–14. doi:10.1016/j.dci.2006.05.010

Paillot, R., Ellis, S.A., Daly, J.M., Audonnet, J.C., Minke, J.M., Davis-Poynter, N., Hannant, D., Kydd, J.H., 2006. Characterisation of CTL and IFN-gamma synthesis in ponies following vaccination with a NYVAC-based construct coding for EHV-1 immediate early gene, followed by challenge infection. Vaccine 24, 1490–500. doi:10.1016/j.vaccine.2005.10.019

Pankov, R., 2002. Fibronectin at a glance. J. Cell Sci. 115, 3861–3863. doi:10.1242/jcs.00059

Pardoe, I., Dargan, D., 2002. PREPS and L-particles: a new approach to virus-like particle vaccines. Expert Rev. Vaccines 1, 427–32. doi:10.1586/14760584.1.4.427

Parsons, C.H., Szomju, B., Kedes, D.H., 2004. Susceptibility of human fetal mesenchymal stem cells to Kaposi sarcoma-associated herpesvirus. Blood 104, 2736–8. doi:10.1182/blood-2004-02-0693

Patel, J.R., Edington, N., Mumford, J.A., 1982. Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. Arch. Virol. 74, 41–51.

Patel, J.R., Heldens, J., 2005. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)--epidemiology, disease and immunoprophylaxis: a brief review. Vet. J. 170, 14–23. doi:10.1016/j.tvjl.2004.04.018

Peretti, S., Shaw, A., Blanchard, J., Bohm, R., Morrow, G., Lifson, J.D., Gettie, A., Pope, M., 2005. Immunomodulatory effects of HSV-2 infection on immature macaque dendritic cells modify innate and adaptive responses. Blood 106, 1305–1313. doi:10.1182/blood-2004-12-4899

Perkins, G.A., Goodman, L.B., Tsujimura, K., Van de Walle, G.R., Kim, S.G., Dubovi, E.J., Osterrieder, N., 2009. Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984-2007). Vet. Microbiol. 139, 375–8. doi:10.1016/j.vetmic.2009.06.033

Perkins, G.A., Van de Walle, G.R., Pusterla, N., Erb, H.N., Osterrieder, N., 2013. Evaluation of metaphylactic RNA interference to prevent equine herpesvirus type 1 infection in experimental herpesvirus myeloencephalopathy in horses. Am. J. Vet. Res. 74, 248–56. doi:10.2460/ajvr.74.2.248

Perkins, G., Ainsworth, D.M., Erb, H.N., Del Piero, F., Miller, M., Wilkins, P.A., Palmer, J., Frazer, M., 1999. Clinical, haematological and biochemical findings in foals with neonatal Equine herpesvirus-1 infection compared with septic and premature foals. Equine Vet. J. 31, 422–6.

Pollara, G., Kwan, A., Newton, P.J., Handley, M.E., Chain, B.M., Katz, D.R., 2005. Dendritic cells in viral pathogenesis: protective or defective? Int. J. Exp. Pathol. 86, 187–204. doi:10.1111/j.0959-9673.2005.00440.x

Pomeranz, L.E., Reynolds, A.E., Hengartner, C.J., 2005. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiol. Mol. Biol. Rev. 69, 462–500. doi:10.1128/MMBR.69.3.462-500.2005

Ponta, H., Sherman, L., Herrlich, P.A., 2003. CD44: from adhesion molecules to signalling regulators. Nat. Rev. Mol. Cell Biol. 4, 33–45. doi:10.1038/nrm1004

Popova, A.P., Bozyk, P.D., Goldsmith, A.M., Linn, M.J., Lei, J., Bentley, J.K., Hershenson, M.B., 2010. Autocrine production of TGF-beta1 promotes myofibroblastic differentiation of neonatal lung mesenchymal stem cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 298, L735-43. doi:10.1152/ajplung.00347.2009

Poulsom, R., 2007. CD44 and hyaluronan help mesenchymal stem cells move to a neighborhood in need of regeneration. Kidney Int. 72, 389–90. doi:10.1038/sj.ki.5002398

Prazma, C.M., Tedder, T.F., 2008. Dendritic cell CD83: a therapeutic target or innocent bystander? Immunol. Lett. 115, 1–8. doi:10.1016/j.imlet.2007.10.001

Prechtel, A.T., Turza, N.M., Kobelt, D.J., Eisemann, J.I., Coffin, R.S., McGrath, Y., Hacker, C., Ju, X., Zenke, M., Steinkasserer, A., 2005. Infection of mature dendritic cells with herpes simplex virus type 1 dramatically reduces lymphoid chemokine-mediated migration. J. Gen. Virol. 86, 1645–57. doi:10.1099/vir.0.80852-0

Prechtel, A.T., Turza, N.M., Theodoridis, A. a, Steinkasserer, A., 2007. CD83 knockdown in monocyte-derived dendritic cells by small interfering RNA leads to a diminished T cell stimulation. J. Immunol. 178, 5454–5464. doi:10.4049/jimmunol.178.9.5454

Pusterla, N., 2014. Equine Herpesvirus 1 Myeloencephalopathy. Vet. Clin. NA Equine Pract. 30, 489–506. doi:10.1016/j.cveq.2014.08.006

Qi, J., Chen, A., You, H., Li, K., Zhang, D., Guo, F., 2011. Proliferation and chondrogenic differentiation of CD105-positive enriched rat synovium-derived mesenchymal stem cells in three-dimensional porous scaffolds. Biomed. Mater. 6, 15006. doi:10.1088/1748-6041/6/1/015006

Raftery, M.J., Wieland, D., Gronewald, S., Kraus, A. a, Giese, T., Schönrich, G., 2004. Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. J. Immunol. 173, 3383–3391. doi:173/5/3383 [pii]

Raftery, M.J., Winau, F., Kaufmann, S.H., Schaible, U.E., Schonrich, G., 2006. CD1 antigen presentation by human dendritic cells as a target for herpes simplex virus immune evasion. J Immunol 177, 6207–6214.

Raftery, M.J., Winau, F., Kaufmann, S.H.E., Schaible, U.E., Schonrich, G., Schönrich, G., 2006. CD1 antigen presentation by human dendritic cells as a target for herpes simplex virus immune evasion. J Immunol 177, 6207–6214. doi:10.4049/jimmunol.177.9.6207

Rappocciolo, G., Birch, J., Ellis, S. a., 2003. Down-regulation of MHC class I expression by equine herpesvirus-1. J. Gen. Virol. 84, 293–300. doi:10.1099/vir.0.18612-0

Rasmusson, I., Le Blanc, K., Sundberg, B., Ringdén, O., 2007. Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand. J. Immunol. 65, 336–43. doi:10.1111/j.1365-3083.2007.01905.x

Rege, T.A., Hagood, J.S., 2006. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. FASEB J. 20, 1045–54. doi:10.1096/fj.05-5460rev

Ren, G., Zhao, X., Zhang, L., Zhang, J., L'Huillier, A., Ling, W., Roberts, A.I., Le, A.D., Shi, S., Shao, C., Shi, Y., 2010. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J. Immunol. 184, 2321–8. doi:10.4049/jimmunol.0902023

Ricciardi, M., Malpeli, G., Bifari, F., Bassi, G., Pacelli, L., Nwabo Kamdje, A.H., Chilosi, M., Krampera, M., 2012. Comparison of epithelial differentiation and immune regulatory properties of mesenchymal stromal cells derived from human lung and bone marrow. PLoS One 7, e35639. doi:10.1371/journal.pone.0035639

Roizman & Pellet, (eds), 2001. The Family Herpesviridae: A Brief Introduction. Lipincott Williams & Wilkins, Philadelphia.

Rolandsson, S., Andersson Sjöland, A., Brune, J.C., Li, H., Kassem, M., Mertens, F., Westergren, A., Eriksson, L., Hansson, L., Skog, I., Bjermer, L., Scheding, S., Westergren-Thorsson, G., 2014. Primary

mesenchymal stem cells in human transplanted lungs are CD90/CD105 perivascularly located tissue-resident cells. BMJ open Respir. Res. 1, e000027. doi:10.1136/bmjresp-2014-000027

Romieu-Mourez, R., Francois, M., Boivin, M.-N., Stagg, J., Galipeau, J., 2007. Regulation of MHC Class II Expression and Antigen Processing in Murine and Human Mesenchymal Stromal Cells by IFN- , TGF- , and Cell Density. J. Immunol. 179, 1549–1558. doi:10.4049/jimmunol.179.3.1549

Rooney, G.E., Moran, C., McMahon, S.S., Ritter, T., Maenz, M., Flügel, A., Dockery, P., O'Brien, T., Howard, L., Windebank, A.J., Barry, F.P., 2008. Gene-Modified Mesenchymal Stem Cells Express Functionally Active Nerve Growth Factor on an Engineered Poly Lactic Glycolic Acid (PLGA) Substrate. Tissue Eng. Part A 14, 681–690. doi:10.1089/tea.2007.0260

Rouzaut, A., Garasa, S., Teijeira, Á., González, I., Martinez-Forero, I., Suarez, N., Larrea, E., Alfaro, C., Palazón, A., Dubrot, J., Hervás-Stubbs, S., Melero, I., 2010. Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN-α. Eur. J. Immunol. 40, 3054–3063. doi:10.1002/eji.201040523

Rudolph, J., Seyboldt, C., Granzow, H., Osterrieder, N., 2002. The gene 10 (UL49.5) product of equine herpesvirus 1 is necessary and sufficient for functional processing of glycoprotein M. J. Virol. 76, 2952–2963. doi:10.1128/JVI.76.6.2952-2963.2002

Ruitenberg, K.M., Love, D.N., Gilkerson, J.R., Wellington, J.E., Whalley, J.M., 2000. Equine herpesvirus 1 (EHV-1) glycoprotein D DNA inoculation in horses with pre-existing EHV-1/EHV-4 antibody. Vet. Microbiol. 76, 117–127. doi:10.1016/S0378-1135(00)00237-6

Rüster, B., Göttig, S., Ludwig, R.J., Bistrian, R., Müller, S., Seifried, E., Gille, J., Henschler, R., 2006. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood 108, 3938–44. doi:10.1182/blood-2006-05-025098

Sabatini, F., Petecchia, L., Tavian, M., Jodon de Villeroché, V., Rossi, G.A., Brouty-Boyé, D., 2005. Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab. Invest. 85, 962–71. doi:10.1038/labinvest.3700300

Said, A., Azab, W., Damiani, A., Osterrieder, N., 2012. Equine herpesvirus type 4 UL56 and UL49.5 proteins downregulate cell surface major histocompatibility complex class I expression independently of each other. J Virol 86, 8059–8071. doi:10.1128/JVI.00891-12

Salio, M., Cella, M., Suter, M., Lanzavecchia, a, 1999. Inhibition of dendritic cell maturation by herpes simplex virus. Eur. J. Immunol. 29, 3245–3253. doi:10.1002/(SICI)1521-4141(199910)29:10<3245::AID-IMMU3245>3.0.CO;2-X

Samady, L., Costigliola, E., MacCormac, L., McGrath, Y., Cleverley, S., Lilley, C.E., Smith, J., Latchman, D.S., Chain, B., Coffin, R.S., 2003. Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs- HSV vectors for dendritic cell-mediated immunotherapy. J. Virol. 77, 3768–3776.

Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14, 778–809, table of contents. doi:10.1128/CMR.14.4.778-809.2001

Sandgren, K.J., Wilkinson, J., Miranda-Saksena, M., McInerney, G.M., Byth-Wilson, K., Robinson, P.J., Cunningham, A.L., 2010. A differential role for macropinocytosis in mediating entry of the two forms of vaccinia virus into dendritic cells. PLoS Pathog. 6, e1000866. doi:10.1371/journal.ppat.1000866

Sansom, D.M., Manzotti, C.N., Zheng, Y., 2003. What's the difference between CD80 and CD86? Trends Immunol. 24, 313–318. doi:10.1016/S1471-4906(03)00111-X

Sanz-Rodriguez, F., Guerrero-Esteo, M., Botella, L.-M., Banville, D., Vary, C.P.H., Bernabéu, C., 2004. Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. J. Biol. Chem. 279, 32858–68. doi:10.1074/jbc.M400843200

Sasaki, M., Hasebe, R., Makino, Y., Suzuki, T., Fukushi, H., Okamoto, M., Matsuda, K., Taniyama, H., Sawa, H., Kimura, T., 2011a. Equine major histocompatibility complex class I molecules act as entry receptors

that bind to equine herpesvirus-1 glycoprotein D. Genes to cells 16, 343–57. doi:10.1111/j.1365-2443.2011.01491.x

Sasaki, M., Kim, E., Igarashi, M., Ito, K., Hasebe, R., Fukushi, H., Sawa, H., Kimura, T., 2011b. Single amino acid residue in the A2 domain of major histocompatibility complex class I is involved in the efficiency of equine herpesvirus-1 entry. J. Biol. Chem. 286, 39370–8. doi:10.1074/jbc.M111.251751

Scholer, A., Hugues, S., Boissonnas, A., Fetler, L., Amigorena, S., 2008. Intercellular Adhesion Molecule-1-Dependent Stable Interactions between T Cells and Dendritic Cells Determine CD8+ T Cell Memory. Immunity 28, 258–270. doi:10.1016/j.immuni.2007.12.016

Scholler, N., Hayden-Ledbetter, M., Hellström, K.E., Hellström, I., Ledbetter, J. a, 2001. CD83 is an I-type lectin adhesion receptor that binds monocytes and a subset of activated CD8+ T cells [corrected]. J. Immunol. 166, 3865–3872. doi:10.4049/jimmunol.166.6.3865

Scott, J.C., Dutta, S.K., Myrup, A.C., 1983. In vivo harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. Am. J. Vet. Res. 44, 1344–8.

Sela, U., Olds, P., Park, A., Schlesinger, S.J., Steinman, R.M., 2011. Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice. J. Exp. Med. 208, 2489–96. doi:10.1084/jem.20110466

Sentsui, H., Wu, D., Murakami, K., Kondo, T., Matsumura, T., 2010. Antiviral effect of recombinant equine interferon-gamma on several equine viruses. Vet. Immunol. Immunopathol. 135, 93–9. doi:10.1016/j.vetimm.2009.11.006

Shrestha, Y., Schafer, E.J., Boehm, J.S., Thomas, S.R., He, F., Du, J., Wang, S., Barretina, J., Weir, B.A., Zhao, J.J., Polyak, K., Golub, T.R., Beroukhim, R., Hahn, W.C., 2012. PAK1 is a breast cancer oncogene that coordinately activates MAPK and MET signaling. Oncogene 31, 3397–408. doi:10.1038/onc.2011.515

Siedek, E.M., Edington, N., Hamblin, A., 1997. Equine dendritic cell infection with equid herpesvirus type 1 reduces their ability to support mitogenic T cell proliferation. Biochem. Soc. Trans. 25, 283S.

Siedek, E.M., Whelan, M., Edington, N., Hamblin, A., 1999. Equine herpesvirus type 1 infects dendritic cells in vitro: stimulation of T lymphocyte proliferation and cytotoxicity by infected dendritic cells. Vet Immunol Immunopathol 67, 17–32.

Sinclair, K., Yerkovich, S.T., Chambers, D.C., 2013. Mesenchymal stem cells and the lung. Respirology 18, 397–411. doi:10.1111/resp.12050

Sixt, M., Bauer, M., Lämmermann, T., Fässler, R., 2006. Beta1 integrins: zip codes and signaling relay for blood cells. Curr. Opin. Cell Biol. 18, 482–90. doi:10.1016/j.ceb.2006.08.007

Slater, J., 2007. Equine Herpesviruses, Second Edi. ed, Equine Infectious Diseases. Elsevier Inc. doi:10.1016/B978-1-4160-2406-4.50018-1

Slater, J.D., Borchers, K., Thackray, A.M., Field, H.J., 1994. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. J. Gen. Virol. 75 (Pt 8), 2007–16.

Slater, J.D., Gibson, J.S., Barnett, K.C., Field, H.J., 1992. Chorioretinopathy associated with neuropathology following infection with equine herpesvirus-1. Vet. Rec. 131, 237–9.

Smirnov, S. V, Harbacheuski, R., Lewis-Antes, A., Zhu, H., Rameshwar, P., Kotenko, S. V, 2007. Bone-marrow-derived mesenchymal stem cells as a target for cytomegalovirus infection: implications for hematopoiesis, self-renewal and differentiation potential. Virology 360, 6–16. doi:10.1016/j.virol.2006.09.017

Smith, D., Hamblin, A., Edington, N., 2002. Equid herpesvirus 1 infection of endothelial cells requires activation of putative adhesion molecules: an in vitro model. Clin. Exp. Immunol. 129, 281–7.

Smith, D.J., Hamblin, A.S., Edington, N., 2001. Infection of endothelial cells with equine herpesvirus-1 (EHV-1) occurs where there is activation of putative adhesion molecules: a mechanism for transfer of virus. Equine

Vet. J. 33, 138–42.

Smith, D.J., Iqbal, J., Purewal, A., Hamblin, A.S., Edington, N., 1998. In vitro reactivation of latent equid herpesvirus-1 from CD5+/CD8+ leukocytes indirectly by IL-2 or chorionic gonadotrophin. J. Gen. Virol. 79 (Pt 12, 2997–3004.

Smith, K.C., Borchers, K., 2001. A study of the pathogenesis of equid herpesvirus-1 (EHV-1) abortion by DNA in-situ hybridization. J. Comp. Pathol. 125, 304–10. doi:10.1053/jcpa.2001.0513

Smith, K.C., Mumford, J.A., Lakhani, K., 1996. A comparison of equid herpesvirus-1 (EHV-1) vascular lesions in the early versus late pregnant equine uterus. J. Comp. Pathol. 114, 231–47.

Smith, K.C., Whitwell, K.E., Mumford, J.A., Hannant, D., Blunden, A.S., Tearle, J.P., 2000. Virulence of the V592 isolate of equid herpesvirus-1 in ponies. J. Comp. Pathol. 122, 288–97. doi:10.1053/jcpa.1999.0373

Smith, K.L., Allen, G.P., Branscum, A.J., Frank Cook, R., Vickers, M.L., Timoney, P.J., Balasuriya, U.B.R., 2010. The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions. Vet. Microbiol. 141, 5–11. doi:10.1016/j.vetmic.2009.07.030

Smith, P.M., Kahan, S.M., Rorex, C.B., von Einem, J., Osterrieder, N., O'Callaghan, D.J., 2005. Expression of the full-length form of gp2 of equine herpesvirus 1 (EHV-1) completely restores respiratory virulence to the attenuated EHV-1 strain KyA in CBA mice. J. Virol. 79, 5105–15. doi:10.1128/JVI.79.8.5105-5115.2005

Smith, R.H., Caughman, G.B., O'Callaghan, D.J., 1992. Characterization of the regulatory functions of the equine herpesvirus 1 immediate-early gene product. J. Virol. 66, 936–45.

Smith, R.H., Holden, V.R., O'Callaghan, D.J., 1995. Nuclear localization and transcriptional activation activities of truncated versions of the immediate-early gene product of equine herpesvirus 1. J. Virol. 69, 3857–62.

Soboll, G., Breathnach, C.C., Kydd, J.H., Hussey, S.B., Mealey, R.M., Lunn, D.P., 2010. Vaccination of ponies with the IE gene of EHV-1 in a recombinant modified live vaccinia vector protects against clinical and virological disease. Vet. Immunol. Immunopathol. 135, 108–17. doi:10.1016/j.vetimm.2009.11.009

Soboll, G., Hussey, S.B., Whalley, J.M., Allen, G.P., Koen, M.T., Santucci, N., Fraser, D.G., Macklin, M.D., Swain, W.F., Lunn, D.P., 2006. Antibody and cellular immune responses following DNA vaccination and EHV-1 infection of ponies. Vet. Immunol. Immunopathol. 111, 81–95. doi:10.1016/j.vetimm.2006.01.011

Soboll, G., Whalley, J.M., Koen, M.T., Allen, G.P., Fraser, D.G., Macklin, M.D., Swain, W.F., Lunn, D.P., 2003. Identification of equine herpesvirus-1 antigens recognized by cytotoxic T lymphocytes. J. Gen. Virol. 84, 2625–34.

Soboll Hussey, G., Ashton, L. V, Quintana, A.M., Van de Walle, G.R., Osterrieder, N., Lunn, D.P., 2014. Equine herpesvirus type 1 pUL56 modulates innate responses of airway epithelial cells. Virology 464–465, 76–86. doi:10.1016/j.virol.2014.05.023

Sohni, A., Verfaillie, C.M., 2013. Mesenchymal stem cells migration homing and tracking. Stem Cells Int. 2013, 130763. doi:10.1155/2013/130763

Spaas, J.H., De Schauwer, C., Cornillie, P., Meyer, E., Van Soom, A., Van de Walle, G.R., 2013. Culture and characterisation of equine peripheral blood mesenchymal stromal cells. Vet. J. 195, 107–13. doi:10.1016/j.tvjl.2012.05.006

Sparks-Thissen, R.L., Enquist, L.W., 1999. Differential regulation of Dk and Kk major histocompatibility complex class I proteins on the cell surface after infection of murine cells by pseudorabies virus. J. Virol. 73, 5748–56.

Spear, P.G., 2004. Herpes simplex virus: receptors and ligands for cell entry. Cell. Microbiol. 6, 401–10. doi:10.1111/j.1462-5822.2004.00389.x

Spear, P.G., Longnecker, R., 2003. Herpesvirus Entry: an Update. J. Virol. 77, 10179–10185. doi:10.1128/JVI.77.19.10179-10185.2003

Spiesschaert, B., Goldenbogen, B., Taferner, S., Schade, M., Mahmoud, M., Klipp, E., Osterrieder, N., Azab, W., 2015a. Role of gB and pUS3 in Equine Herpesvirus 1 Transfer between Peripheral Blood Mononuclear Cells and Endothelial Cells: a Dynamic In Vitro Model. J. Virol. 89, 11899–908. doi:10.1128/JVI.01809-15

Spiesschaert, B., Osterrieder, N., Azab, W., 2015b. Comparative analysis of glycoprotein B (gB) of equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) in cellular tropism and cell-to-cell transmission. Viruses 7, 522–542. doi:10.3390/v7020522

Sprecher, E., Becker, Y., 1986. Skin Langerhans cells play an essential role in the defense against HSV-1 infection. Arch. Virol. 91, 341–9.

Sprecher, E., Becker, Y., 1989. Langerhans cell density and activity in mouse skin and lymph nodes affect herpes simplex type 1 (HSV-1) pathogenicity. Arch. Virol. 107, 191–205.

Stahlschmidt, W., Robertson, M.J., Robinson, P.J., McCluskey, A., Haucke, V., 2014. Clathrin terminal domain-ligand interactions regulate sorting of mannose 6-phosphate receptors mediated by AP-1 and GGA adaptors. J. Biol. Chem. 289, 4906–18. doi:10.1074/jbc.M113.535211

Steinbach, F., Bischoff, S., Freund, H., Metzner-Flemisch, S., Ibrahim, S., Walter, J., Wilke, I., Mauel, S., 2009. Clinical application of dendritic cells and interleukin-2 and tools to study activated T cells in horses-First results and implications for quality control. Vet. Immunol. Immunopathol. 128, 16–23. doi:10.1016/j.vetimm.2008.10.317

Steingen, C., Brenig, F., Baumgartner, L., Schmidt, J., Schmidt, A., Bloch, W., 2008. Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells. J. Mol. Cell. Cardiol. 44, 1072–84. doi:10.1016/j.yjmcc.2008.03.010

Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449, 419–426. doi:10.1038/nature06175

Stewart, M.C., Stewart, A.A., 2011. Mesenchymal stem cells: characteristics, sources, and mechanisms of action. Vet. Clin. North Am. Equine Pract. 27, 243–61. doi:10.1016/j.cveq.2011.06.004

Stiles, K.M., Krummenacher, C., 2010. Glycoprotein D actively induces rapid internalization of two nectin-1 isoforms during herpes simplex virus entry. Virology 399, 109–19. doi:10.1016/j.virol.2009.12.034

Stiles, K.M., Whitbeck, J.C., Lou, H., Cohen, G.H., Eisenberg, R.J., Krummenacher, C., 2010. Herpes simplex virus glycoprotein D interferes with binding of herpesvirus entry mediator to its ligands through downregulation and direct competition. J. Virol. 84, 11646–60. doi:10.1128/JVI.01550-10

Stokes, A., Alber, D.G., Cameron, R.S., Marshall, R.N., Allen, G.P., Killington, R.A., 1996. The production of a truncated form of baculovirus expressed EHV-1 glycoprotein C and its role in protection of C3H (H-2Kk) mice against virus challenge. Virus Res. 44, 97–109.

Stokes, A., Wardley, R.C., 1988. ADCC and complement-dependent lysis as immune mechanisms against EHV-1 infection in the horse. Res. Vet. Sci. 44, 295–302.

Sugahara, Y., Matsumura, T., Kono, Y., Honda, E., Kida, H., Okazaki, K., 1997. Adaptation of equine herpesvirus 1 to unnatural host led to mutation of the gC resulting in increased susceptibility of the virus to heparin. Arch. Virol. 142, 1849–56.

Sun, Y., MacLean, A.R., Aitken, J.D., Brown, S.M., 1996. The role of the gene 71 product in the life cycle of equine herpesvirus 1. J. Gen. Virol. 77 (Pt 3), 493–500.

Sundin, M., Örvell, C., Rasmusson, I., Sundberg, B., Ringdén, O., Le Blanc, K., 2006. Mesenchymal stem cells are susceptible to human herpesviruses, but viral DNA cannot be detected in the healthy seropositive individual. Bone Marrow Transplant. 37, 1051–1059. doi:10.1038/sj.bmt.1705368

Tallmadge, R.L., Campbell, J.A., Miller, D.C., Antczak, D.F., 2010. Analysis of MHC class I genes across horse MHC haplotypes. Immunogenetics 62, 159–72. doi:10.1007/s00251-009-0420-9

Tearle, J.P., Smith, K.C., Boyle, M.S., Binns, M.M., Livesay, G.J., Mumford, J. a, 1996. Replication of equid

herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus. J. Comp. Pathol. 115, 385–397. doi:10.1016/S0021-9975(96)80073-9

Tearle, J.P., Smith, K.C., Platt, A.J., Hannant, D., Davis-Poynter, N.J., Mumford, J.A., 2003. In vitro characterisation of high and low virulence isolates of equine herpesvirus-1 and -4. Res. Vet. Sci. 75, 83–86. doi:10.1016/S0034-5288(03)00031-6

Telford, E. a R., Watson, M.S., McBride, K., Davison, a. J., 1992. The DNA sequence of equine herpesvirus-1. Virology 189, 304–316. doi:10.1016/0042-6822(92)90706-U

Tewari, D., Del Piero, F., Cieply, S., Feria, W., Acland, H., 2013. Equine herpesvirus 1 (EHV-1) nucleotide polymorphism determination using formalin fixed tissues in EHV-1 induced abortions and myelopathies with real-time PCR and pyrosequencing. J. Virol. Methods 193, 371–3. doi:10.1016/j.jviromet.2013.06.039

Tewari, D., Nair, S. V, De Ungria, M.C., Lawrence, G.L., Hayden, M., Love, D.N., Field, H.J., Whalley, J.M., 1995. Immunization with glycoprotein C of equine herpesvirus-1 is associated with accelerated virus clearance in a murine model. Arch. Virol. 140, 789–97.

Tewari, D., Whalley, J.M., Love, D.N., Field, H.J., 1994. Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model. J. Gen. Virol. 75 (Pt 7), 1735–41.

Theodoridis, a. a., Eich, C., Figdor, C.G., Steinkasserer, A., 2011. Infection of dendritic cells with herpes simplex virus type 1 induces rapid degradation of CYTIP, thereby modulating adhesion and migration. Blood 118, 107–115.

Thomson, G.R., Mumford, J.A., Campbell, J., Griffiths, L., Clapham, P., 1976. Serological detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet. J. 8, 58–65.

Thormann, N., Van de Walle, G.R., Azab, W., Osterrieder, N., 2012. The role of secreted glycoprotein G of equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) in immune modulation and virulence. Virus Res. 169, 203–11. doi:10.1016/j.virusres.2012.07.029

Tsujimura, K., Murase, H., Bannai, H., Nemoto, M., Yamanaka, T., Kondo, T., 2015. Efficacy of five commercial disinfectants and one anionic surfactant against equine herpesvirus type 1. J. Vet. Med. Sci. doi:10.1292/jvms.15-0030

Tsujimura, K., Shiose, T., Yamanaka, T., Nemoto, M., Kondo, T., MATSUMURA, T., 2009. Equine Herpesvirus Type 1 Mutant Defective in Glycoprotein E Gene as Candidate Vaccine Strain. J. Vet. Med. Sci. 71, 1439–1448. doi:10.1292/jvms.001439

Turtinen, L.W., Allen, G.P., 1982. Identification of the envelope surface glycoproteins of equine herpesvirus type 1. J. Gen. Virol. 63, 481–5.

Valiya Veettil, M., Sadagopan, S., Kerur, N., Chakraborty, S., Chandran, B., 2010. Interaction of c-Cbl with myosin IIA regulates Bleb associated macropinocytosis of Kaposi's sarcoma-associated herpesvirus. PLoS Pathog. 6, e1001238. doi:10.1371/journal.ppat.1001238

van Beek, E.M., Cochrane, F., Barclay, a N., van den Berg, T.K., 2005. Signal regulatory proteins in the immune system. J. Immunol. 175, 7781–7787. doi:10.4049/jimmunol.175.12.7781

Van de Walle, G.R., Cox, E., Nauwynck, H., Favoreel, H.W., 2009a. The role of dendritic cells in alphaherpesvirus infections: archetypes and paradigms. Rev. Med. Virol. 19, 57–64. doi:10.1002/rmv.628

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G.A., Osterrieder, N., 2009b. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. J. Infect. Dis. 200, 20–5. doi:10.1086/599316

Van de Walle, G.R., Jarosinski, K.W., Osterrieder, N., 2008a. Alphaherpesviruses and chemokines: pas de deux not yet brought to perfection. J. Virol. 82, 6090–7. doi:10.1128/JVI.00098-08

Van de Walle, G.R., Kaufer, B.B., Chbab, N., Osterrieder, N., 2009c. Analysis of the herpesvirus chemokine-

binding glycoprotein G residues essential for chemokine binding and biological activity. J. Biol. Chem. 284, 5968–76. doi:10.1074/jbc.M808127200

Van de Walle, G.R., May, M.A., Peters, S.T., Metzger, S.M., Rosas, C.T., Osterrieder, N., 2010. A vectored equine herpesvirus type 1 (EHV-1) vaccine elicits protective immune responses against EHV-1 and H3N8 equine influenza virus. Vaccine 28, 1048–55. doi:10.1016/j.vaccine.2009.10.123

Van de Walle, G.R., May, M.L., Sukhumavasi, W., von Einem, J., Osterrieder, N., 2007. Herpesvirus Chemokine-Binding Glycoprotein G (gG) Efficiently Inhibits Neutrophil Chemotaxis In Vitro and In Vivo. J. Immunol. 179, 4161–4169. doi:10.4049/jimmunol.179.6.4161

Van de Walle, G.R., Peters, S.T., VanderVen, B.C., O'Callaghan, D.J., Osterrieder, N., 2008b. Equine herpesvirus 1 entry via endocytosis is facilitated by alphaV integrins and an RSD motif in glycoprotein D. J. Virol. 82, 11859–68. doi:10.1128/JVI.00868-08

Van de Walle, G.R., Sakamoto, K., Osterrieder, N., 2008c. CCL3 and viral chemokine-binding protein gg modulate pulmonary inflammation and virus replication during equine herpesvirus 1 infection. J. Virol. 82, 1714–22. doi:10.1128/JVI.02137-07

Van den Broeke, C., Radu, M., Deruelle, M., Nauwynck, H., Hofmann, C., Jaffer, Z.M., Chernoff, J., Favoreel, H.W., 2009. Alphaherpesvirus US3-mediated reorganization of the actin cytoskeleton is mediated by group A p21-activated kinases. Proc. Natl. Acad. Sci. U. S. A. 106, 8707–8712. doi:10.1073/pnas.0900436106

van der Meulen, K., Caij, B., Pensaert, M., Nauwynck, H., 2006. Absence of viral envelope proteins in equine herpesvirus 1-infected blood mononuclear cells during cell-associated viremia. Vet. Microbiol. 113, 265–73. doi:10.1016/j.vetmic.2005.11.048

van der Meulen, K.M., Nauwynck, H.J., Buddaert, W., Pensaert, M.B., 2000. Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. J. Gen. Virol. 81, 21–5.

van der Meulen, K.M., Nauwynck, H.J., Pensaert, M.B., 2001. Mitogen stimulation favours replication of equine herpesvirus-1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts. J. Gen. Virol. 82, 1951–7.

van der Meulen, K.M., Nauwynck, H.J., Pensaert, M.B., 2003. Absence of viral antigens on the surface of equine herpesvirus-1-infected peripheral blood mononuclear cells: a strategy to avoid complement-mediated lysis. J. Gen. Virol. 84, 93–7.

van Maanen, C., 2002. Equine herpesvirus 1 and 4 infections: an update. Vet. Q. 24, 58–78.

van Maanen, C., Sloet van Oldruitenborgh-Oosterbaan, M.M., Damen, E.A., Derksen, A.G., 2001. Neurological disease associated with EHV-1-infection in a riding school: clinical and virological characteristics. Equine Vet. J. 33, 191–6.

Van Minnebruggen, G., Favoreel, H.W., Jacobs, L., Nauwynck, H.J., 2003. Pseudorabies virus US3 protein kinase mediates actin stress fiber breakdown. J. Virol. 77, 9074–80.

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J., 2010. Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J Gen Virol 91, 2019–2028. doi:10.1099/vir.0.019257-0

Vandekerckhove, A.P., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Van Doorsselaere, J., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J., 2011. Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect mononuclear cells during early steps of infection in nasal mucosal explants. Vet. Microbiol. 152, 21–8. doi:10.1016/j.vetmic.2011.03.038

Vengust, M., Wen, X., Bienzle, D., 2008. Herpesvirus-Associated Neurological Disease in a Donkey. J. Vet. Diagnostic Investig. 20, 820–823. doi:10.1177/104063870802000620

References

Vogel, W., Grunebach, F., Messam, C., Kanz, L., Brugger, W., Buhring, H., 2003. Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. Haematologica 88, 126–133.

von Einem, J., Schumacher, D., O'Callaghan, D.J., Osterrieder, N., 2006. The alpha-TIF (VP16) homologue (ETIF) of equine herpesvirus 1 is essential for secondary envelopment and virus egress. J. Virol. 80, 2609–2620. doi:10.1128/JVI.80.6.2609-2620.2006

Vossen, M.T.M., Westerhout, E.M., Söderberg-Nauclér, C., Wiertz, E.J.H.J., 2002. Viral immune evasion: A masterpiece of evolution. Immunogenetics 54, 527–542.

Wagner, B., Wimer, C., Freer, H., Osterrieder, N., Erb, H.N., 2011. Infection of peripheral blood mononuclear cells with neuropathogenic equine herpesvirus type-1 strain Ab4 reveals intact interferon-alpha induction and induces suppression of anti-inflammatory interleukin-10 responses in comparison to other viral strains. Vet Immunol Immunopathol 143, 116–124. doi:10.1016/j.vetimm.2011.06.032

Walter, J., Balzer, H.-J., Seeh, C., Fey, K., Bleul, U., Osterrieder, N., 2012. Venereal shedding of equid herpesvirus-1 (EHV-1) in naturally infected stallions. J. Vet. Intern. Med. 26, 1500–4. doi:10.1111/j.1939-1676.2012.00997.x

Wei, X., Yang, X., Han, Z., Qu, F., Shao, L., Shi, Y., 2013. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol. Sin. 34, 747–54. doi:10.1038/aps.2013.50

Welch, H.M., Bridges, C.G., Lyon, A.M., Griffiths, L., Edington, N., 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. J. Gen. Virol. 73 (Pt 2), 261–8.

Whalley, J.M., Love, D.N., Tewari, D., Field, H.J., 1995. Characteristics of equine herpesvirus 1 glycoproteins expressed in insect cells. Vet. Microbiol. 46, 193–201.

Whitwell, K.E., Blunden, A.S., 1992. Pathological findings in horses dying during an outbreak of the paralytic form of Equid herpesvirus type 1 (EHV-1) infection. Equine Vet. J. 24, 13–9.

Wildy, P., Watson, D.H., 1962. Electron microscopic studies on the architecture of animal viruses. Cold Spring Harb. Symp. Quant. Biol. 27, 25–47.

Wilsterman, S., Soboll-Hussey, G., Lunn, D.P., Ashton, L. V, Callan, R.J., Hussey, S.B., Rao, S., Goehring, L.S., 2011. Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia. Vet. Microbiol. 149, 40–7. doi:10.1016/j.vetmic.2010.10.004

Wong, S.-P., Rowley, J.E., Redpath, A.N., Tilman, J.D., Fellous, T.G., Johnson, J.R., 2015. Pericytes, mesenchymal stem cells and their contributions to tissue repair. Pharmacol. Ther. 151, 107–120. doi:10.1016/j.pharmthera.2015.03.006

Zaichick, S. V, Bohannon, K.P., Smith, G.A., 2011. Alphaherpesviruses and the cytoskeleton in neuronal infections. Viruses 3, 941–81. doi:10.3390/v3070941

Zehn, D., Cohen, C.J., Reiter, Y., Walden, P., 2004. Extended presentation of specific MHC-peptide complexes by mature dendritic cells compared to other types of antigen-presenting cells. Eur. J. Immunol. 34, 1551–60. doi:10.1002/eji.200324355

Zhang, Q.-W., Kish, D.D., Fairchild, R.L., 2003. Absence of Allograft ICAM-1 Attenuates Alloantigen-Specific T Cell Priming, But Not Primed T Cell Trafficking into the Graft, to Mediate Acute Rejection. J. Immunol. 170, 5530–5537. doi:10.4049/jimmunol.170.11.5530

Zhang, Y., Charvat, R.A., Kim, S.K., O'Callaghan, D.J., 2014. The EHV-1 UL4 protein that tempers viral gene expression interacts with cellular transcription factors. Virology 449, 25–34. doi:10.1016/j.virol.2013.11.005

Zuo, J., Rowe, M., 2012. Herpesviruses placating the unwilling host: manipulation of the MHC class II antigen presentation pathway. Viruses 4, 1335–1353. doi:10.3390/v4081335

Curriculum vitae

Personalia

Christophe Claessen werd geboren op 16 juni 1986 te Lier. Hij behaalde in 2011 het diploma van Master in de Diergeneeskunde (optie onderzoek) aan de UGent met onderscheiding. Vanaf september 2011 tot oktober 2015 beschikte hij over een doctoraatsbeurs van het Bijzonder Onderzoeksfonds van de UGent die kaderde in een Geconcerteerde Onderzoeksacties (GOA) aan de Vakgroep Virologie, Parasitologie en Immunologie aan de Faculteit Diergeneeskunde van de UGent. Dit onderzoek handelde over de interactie van EHV1 met dendritische cellen en mesenchymale stamcellen. Christophe Claessen is auteur en medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften.

Publicaties in peer-reviewed internationale wetenschappelijke tijdschriften

Claessen, C., De Lange V., Huang T., Ma, G., Osterrieder, N., Van de Walle, G.R., Favoreel, H.W. Equine herpesvirus type 1 (EHV1) induces alterations in the immunophenotypic profile of equine monocyte-derived dendritic cells. Vet. J. 210, 85–88. doi:10.1016/j.tvjl.2015.12.008

Claessen, C., Favoreel, H., Ma, G., Osterrieder, N., De Schauwer, C., Piepers, S., Van de Walle, G.R., 2015. Equid herpesvirus 1 (EHV1) infection of equine mesenchymal stem cells induces a pUL56-dependent downregulation of select cell surface markers. Vet. Microbiol. 176, 32–9.

Jacob, T., Van den Broeke, C., Grauwet, K., Baert, K., **Claessen, C.**, De Pelsmaeker, S., Van Waesberghe, C., Favoreel, H.W., 2015. Pseudorabies virus US3 leads to filamentous actin disassembly and contributes to viral genome delivery to the nucleus. Vet. Microbiol. 177, 379–385. doi:10.1016/j.vetmic.2015.03.023

Beurzen

Mei 2013	FCWO reisbeurs

Bijgewoonde conferenties met posterpresentaties (p) en mondelinge presentaties (pr)

International Herpesvirus Workshop (IHW) 2013	Grand Rapids	20-24 juli 2013	p + pr
Belgian Society for Microbiology (BSM) 2013	Brussel	27 november 2013	p
Belgian Society of Virology	Brussel	8 november 2013	p

Doctoral schools

Vaardigheidstraining

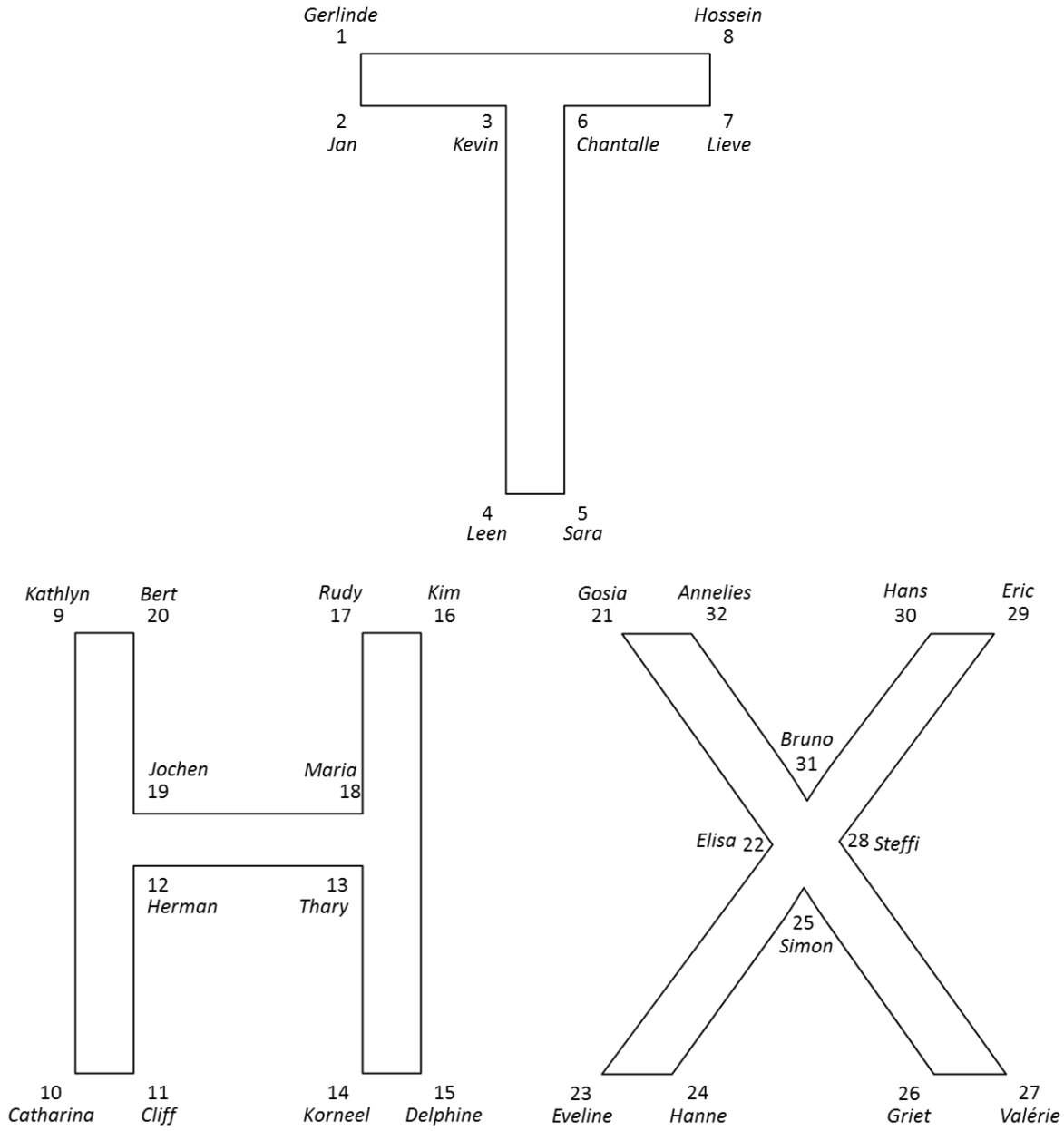
Advanced academic English: academic posters – doctoral schools – UGent	2014
Effective graphical displays – doctoral schools – UGent	2014

Verdiepende studies

Applied flow cytometry – (pre)clinical hematological analysis – doctoral schools – UGent	2013

Dankwoord

To following persons,



for helping me connect the dots along the road of my PhD.

More specifically I would like to thank my promotors Professor Dr. Herman Favoreel and Professor Dr. Gerlinde Van de Walle for letting me conduct this research in the laboratories of Comparative Physiology and, afterwards, Immunology.

I would also like to especially thank the members of the reading- and exam committee, namely Professor Dr. Hans Nauwynck, Professor Dr. Bruno Verhasselt, Dr. Catharina De Schauwer and Dr. Annelies Vandekerckhove for their effort and critical suggestions.