Aus dem Institut für Virologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Development and molecular studies on Equine Herpesvirus type 1 (EHV-1) vaccine against foot and mouth disease virus

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Mohamed Salah Kamal Kamel
Tierarzt aus Giza, Ägypten

Berlin 2018
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For the soul of my Father

For my Mother, my brothers and my sisters

For my wife and my lovely daughter

For my supervisors

Mohamed Kamel

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3 Abbreviations

Abbreviation	Refers to
3D	3 dimensions
AHV	Asinine herpesvirus
AHV-4	Asinine herpesvirus 4
AHV-5	Asinine herpesvirus 5
AHV-6	Asinine herpesvirus 6
ANOVA	One-way analysis of variance
APS	Ammonium persulfate
BAC	Bacterial artificial chromosome
Bac-EgB	Recombinant Baculovirus with EHV gB
B-cell	Bone marrow cells
BEI	Binary ethyleneimine
BoHV-4	Bovine herpesvirus
BoHV-1	Bovine herpesvirus 1
BoHV-2	Bovine herpesvirus 2
bp	Base pairs
BSA	Bovine serum albumin
BVD	Bovine viral diarrhea
C	Complement
C3	Complement component 3
Cam	Chloramphenicol
CCD	Charge-coupled device
CD	Cluster of differentiation
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CDV	Canine distemper virus
CHO-K1	Chinese hamster ovarian epithelial cell line
CIP	Calf Intestinal
CIV	Canine influenza virus
CMV	Cytomegalovirus
ConA	Concanavalin A
CTLs	Cytotoxic T lymphocytes
CXCL8	CXC chemokine ligand 8
DAPI	4′,6-diamidino-2-phenylindole

DC	Dendritic cells
ddH2O	Double distilled water
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
dpi	Days post infection
dpv	Days post-vaccination
ds	Double strand
DTT	Dithiothreitol
E	Early
ER	Endoplasmic reticulum
E. coli	Escherichia Coli
EC	Endothelial cells
ECM	Extracellular matrix
ED	Equine dermal
EDTA	Ethylenediaminetetraacetate
EHM	Equine herpes myeloencephalopathy
EHV- 7	Equine herpesvirus 7
EHV-1	Equine herpesvirus 1
EHV-2	Equine herpesvirus 2
EHV-3	Equine herpesvirus 3
EHV-4	Equine herpesvirus 4
EHV-5	Equine gamma herpesvirus 5
EHV-6	Equine herpesvirus 6
EHV-8	Equine herpesvirus 8
EHV-9	Equine herpesvirus 9
ELISA	Enzyme-linked immunosorbent serologic assay
ERECs	Equine respiratory epithelial cell
FACS	Fluorescence-activated cell sorting
FBS or FCS	Fetal calf serum or Fetal Bovine Serum (FBS)
FHV-1	Feline herpesvirus 1

FITC	Fluorescence isothiocyanate
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
F-plasmids	Fertility plasmid
GAGs	Glycosaminoglycans
gB	Glycoprotein B
gB1	Glycoprotein B of EHV-1
gB4	Glycoprotein B of EHV-4
gC	Glycoprotein C
gD	Glycoprotein D
gD1	Glycoprotein D of EHV-1
gD4	Glycoprotein D of EHV-4
gE	Glycoprotein E
gE-	Deleted glycoprotein E
GFP	Green Fluorescent Proteins
gG	Glycoprotein G
gH	Glycoprotein H
GHV	Gazelle herpesvirus
gI	Glycoprotein I
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gp2	Glycoprotein P2
h or hr	Hour
HCl	Hydrochloric
HCMV	Human cytomegalovirus
hep	Heparin
HIV	Human immunodeficiency virus type I
HRP	Horseradish peroxidase
HRs	Homologous recombinations
hrs	Hours
HSV-1	Herpes simplex type 1
HSV-2	Herpes simplex type 2
HTLV-1	Human T-cell leukemia virus-1
HVB	Herpesvirus B

HveA	Herpes virus entry A
HveB	Herpes virus entry B
HveC	Herpes virus entry C
HVS-1	Herpesvirus saimiri 1
HVT	Herpesvirus of turkeys
ICAM-1	Intercellular adhesion molecule-1
ICAM-3	Intercellular adhesion molecule-3
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-8	Interleukin 8
ILTV	Infectious laryngotracheitis virus
IMDM	Iscove's modified Dulbecco's medium
Inf	Infected
IR	Internal repeats
IRL	Internal repeat long
IRS	Internal repeat short
ISCOMs	Immune stimulating complexes
Kana	Kanamycin
KanR	Kanamycin resistance gene
kb	Kilobases
kbp	Kilobase pairs
KCL	Potassium Chloride
kDa	Kilodalton
L	Late
LAT	Latency Associated Transcript
LB	Luria-Bertani or lysogeny broth
LFA-1	Leucocyte function-associated antigen
LLV	Leaderless virus
Luc	Luciferase
M	Marker
MDDC	Dendritic cells
MDV-1	Marek's disease virus 1

MDV-2	Marek's disease virus 2
MEM	Minimum Essential Media Eagle
MeOH	Methanol
MgCl2	Magnesium chloride
MHC	Major histocompatibility complex
min	Minutes
miRNA	Micro-ribonucleic acid
MOI	Multiplicity of infection
mRFP	Monomeric red fluorescent protein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
n	Number
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NP	Non-structural proteins
o/n	Overnight
OD 600	Optical density, 600 nm wavelength
ORF	Open reading frame
ORF1.1	ORF1 of EHV-1
ORF1.4	ORF1 of EHV-4
ORFs	Open reading frames
p.i	Post infection
P/S	Penicillin/streptomycin
PAGE	Polyacrylamide gel electrophoresis
PAGE	Polyacrylamide gel electrophoretic
PBMC	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBS-T	PBS- Tween
PCR	Polymerase chain reaction
PD50	50% protective dose
PEI	Polyethyleneimine
PFA	Paraformaldehyde
Pfu	Plaque-forming units
Pic	Pichia pastoris
pip	Pipetting

PRV	Pseudorabies virus
PVDF	Polyvinyl diisopropyl fluoride
RBCs	Red blood cells
Revert	Revertant
RFLP	Restriction endonuclease fragment length polymorphism
RFP	Red fluorescent protein
rH	RacH
rH_P1	Recombinant RacH_P1 FMD
RIPA	Radioimmunoprecipitation assay
RK13	Rabbit kidney cells
RLU	Luciferase luminescence Unit
RNA	Ribonucleic acids
ROCK	Rho-associated protein kinase
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
RSD	Arginine, serine, and aspartic acid
RT	Room temperature
RVFV	Rift valley fever virus
S	Segment
SA8	Simian agent 8
SAT 1,2,3	South African Territories 1,2,3
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second
SOB	Super Optimal broth
SOC	Super Optimal broth with Catabolite Repression
SOI	Sequence of interest
SPSS	Statistical Package for the Social Sciences
Sup	Supernatant
TAE	Tris acetate-EDTA
TAP	Transporter associated with Antigenic Processing
T-cell	Thymus cells
TEM	Transmission electron microscope

Th1	T-helper 1
TK-	Thymidine kinase deleted
Tm	Melting temperature
TM	Transmembrane
TNF	Tumor necrosis factor
TNTs	Tunneling nanotubes
TR	Terminal repeat
Trans	Transfected
TRS	Terminal repeats
UL	Unique long
UL56.1	UL56 of EHV-1
UL56.4	UL56 of EHV-4
URT	Upper respiratory tract
US	Unique short
UV	Ultra Violet
VCAM	Vascular cell adhesion molecules
vCKBP	Viral chemokine-binding protein
VLA-4	Very late antigen-4
VLPs	Viral-like particles
VP	Viral protein
VS	Virological synapse
VZV	Varicella-zoster virus
w/v	Weight by volume
WAHV	Wild ass herpesvirus
WGA	Wheat Germ Agglutinin
WT or wt	Wild-type
ZHV	Zebra herpesvirus
µm	Micormeter

4 Introduction

4.1 Virus taxonomy and the Phylogenetic background.

4.1.1 *Herpesvirales order; taxonomy and classification.*

The International Committee on Taxonomy of Viruses (ICTV), in 2009, had assigned a new order, *Herpesvirales* which is classified into three families: *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae*. This order has more than 100 large DNA virus members with a broad spectrum of hosts, including humans, mammals, birds, amphibians, reptiles, bony fish and invertebrates. The *Herpesviridae* comprises herpesviruses of mammals, birds, and reptiles viruses; the *Alloherpesviridae* includes fish and frog viruses, and the *Malacoherpesviridae* contains only a virus of oysters (bivalve mollusk virus) [1-3]. The *Herpesviridae* family has been categorized into three subfamilies *alpha*, *beta*, and *gammaherpesvirinae* based on viral reproductive cycle. These sub-families have different host range, tissue tropism, clinical symptoms, disease severity and latent infection`s characteristic [4]. *Alphaherpesvirinae* and *Betaherpesvirinae* can be deliberated as 'lytic' as they produce and induce a lytic replication in a wide range of cells. However, they may remain lifelong in a latent stage in specific types of cells (i.e., neurons) [2, 5]. In contrast, *gammaherpesvirinae* has narrow permissive cells range and is often latent and persistent in cells. The subfamilies are further subdivided into distinct genera, based on similarities in DNA sequence homology, genome sequence arrangement, genome size and structure, and relatedness of viral proteins [6].

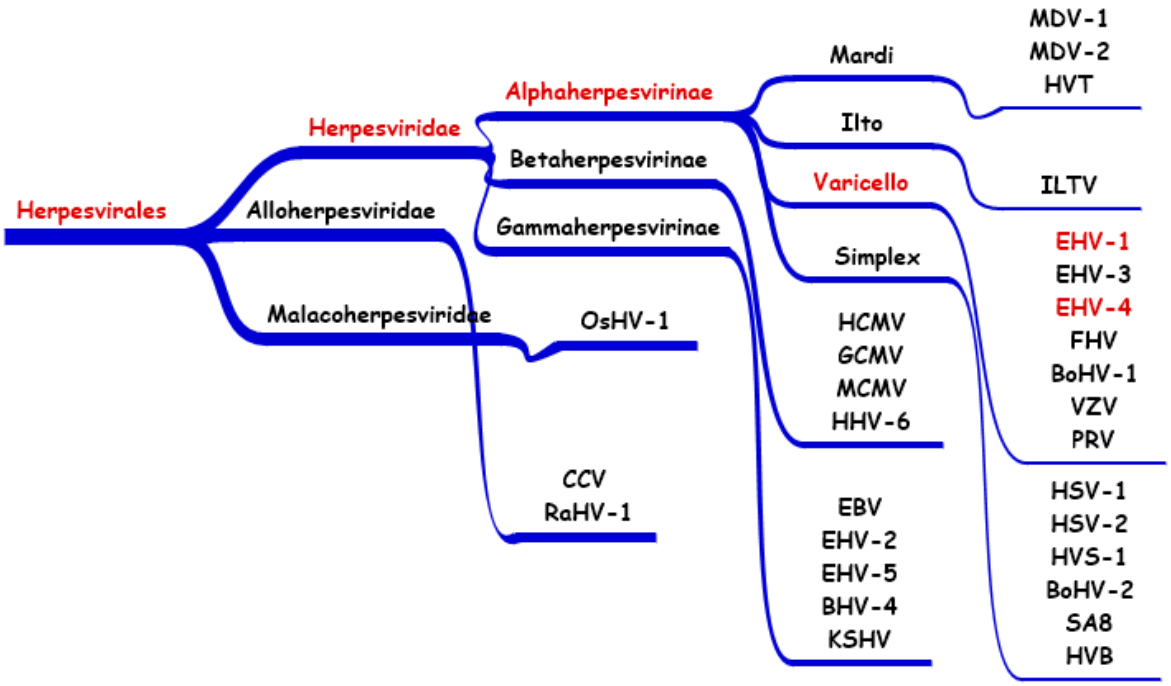


Figure 1: **Schematic illustration showing the phylogenetic tree of the Herpesvirales** (modified from [7])

4.1.2 *Alphaherpesvirinae subfamily; characteristics and classification.*

Alphaherpesviruses infect wide host range with relative short replication cycle. Mostly, they have a rapid lytic replication and capable of establishing latency in sensory ganglia and immune cells. On the other hand, Betaherpesviruses are mostly restricted host range with a long replication cycle that often leads to cytomegaly (enlargement of infected cells). Betaherpesviruses can establish latency in secretory glands, kidneys, lymphoreticular cells, and other several tissues. Gammaherpesviruses have a limited host range and the virus establish latency in lymphoid tissues [2, 5].

Based on molecular biological criteria and genomic sequence analysis, the *Alphaherpesvirinae* is divided into five genera; *Simplexvirus*, *Varicellovirus*, *Scutavirus*, *Iltovirus,* and *Mardivirus* [6] **(Figure 1)**. The *Simplexvirus* genus includes herpes simplex type 1 and type 2 (HSV-1, HSV-2), herpesvirus saimiri 1 (HVS-1), herpesvirus B (HVB), bovine herpesvirus 2 (BoHV-2), and cercopithecine herpesvirus 2 (CeHV2) namely simian agent 8 (SA8). Equine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4) have been grouped within the *Varicellovirus* genus together with pseudorabies virus (PRV), feline herpesvirus 1 (FHV-1), equine herpesvirus 3 (EHV-3), bovine herpesvirus 1 (BoHV-1) and varicella zoster virus (VZV). Marek's disease virus type 1 (MDV-1), Marek's disease virus type 2 (MDV-2) and herpesvirus of turkeys (HVT) belong to the *Mardivirus* genus and *Iltovirus* genus includes infectious laryngotracheitis virus (ILTV) [4, 8].

4.1.3 *Taxonomy of the known herpesviruses affecting equids.*

Nine herpesviruses have been ascertained so far that infect the *Equid* populations. These are included into two subfamilies; *alphaherpesvirinae* subfamily includes equine herpesvirus 1 (EHV-1), equine herpesvirus 3 (EHV-3), equine herpesvirus 4 (EHV-4), equine herpesvirus 6 (EHV-6), equine herpesvirus 8 (EHV-8) and equine herpesvirus 9 (EHV-9). The *gammaherpesvirinae* subfamily includes equine herpesvirus 2 (EHV-2), equine herpesvirus 5 (EHV-5), and equine herpesvirus 7 (EHV- 7). Equine herpesvirus-1 to EHV-5 infect horses, while EHV-6 to EHV-8 infect donkeys and are denominated as asinine herpesvirus (AHV, AHV-1 to 3), and gazelle herpesvirus (GHV) or EHV- 9 infects several species as zebras, onagers, polar bears and Thomson's gazelles [2, 3, 9-12]. In addition, the donkeys can also be infected by another gamma herpesviruses as asinine herpesvirus 4 (AHV-4), asinine herpesvirus 5 (AHV-5) and asinine herpesvirus 6 (AHV-6). The

gammaherpesvirinae includes zebra herpesvirus (ZHV) and wild ass herpesvirus (WAHV) infecting zebra and wild ass subsequently [13].

Viruses and their common diseases name	**Host affected**	**Clinical signs**
Equine herpesvirus 1 (EHV-1) (**Equine abortion virus)**	Domestic horse	Respiratory disease, abortion, nervous system disorders, chorioretinopathy
Equine herpesvirus 2 (EHV-2) (**Equine herpesvirus 2)**		Respiratory disease, conjunctivitis, malaise
Equine herpesvirus 3 (EHV-3) (**Equine coital exanthema virus)**		Coital exanthema
Equine herpesvirus 4 (EHV-4) (**Equine rhinopneumonitis virus)**		Respiratory disease
Equine herpesvirus 5 (EHV-5) (**Equine herpesvirus 5)**		Respiratory disease, Association with EMPF (multinodular pulmonary fibrosis)
Equine herpesvirus 6 (EHV-6) (**Asinine herpesvirus 1)**	Donkey	Lesions on external genitalia and udder
Equine herpesvirus 7 (EHV-7) (**Asinine herpesvirus 2)**		Unknown
Equine herpesvirus 8 (EHV-8) (**Asinine herpesvirus 3**		Respiratory disease, other?
Asinine herpesvirus 4 (AHV-4) (**Asinine herpesvirus 4)**		Pneumonia
Asinine herpesvirus 5 (AHV-5) (**Asinine herpesvirus 5)**		Pneumonia
Asinine herpesvirus 6 (AHV-6) (**Asinine herpesvirus 6)**		Pneumonia
Zebra herpesvirus (ZHV) (**Zebra herpesvirus)**	Zebra	Pneumonia

Equine herpesvirus 9 (EHV-9) (**Gazelle herpesvirus)**	Gazelle	Respiratory disease and encephalitis
Wild ass herpesvirus (WAHV) (**Wild ass herpesvirus)**	Wild ass	Pneumonia

Table 1: **The known herpesviruses in equids showing the host affected and their characteristic clinical signs.**

4.2 Virus structure

Equine herpesvirus type 1 (EHV-1) virion comprises of four morphologically distinct main structural components: viral genome, capsid, tegument and the envelope. The complete viral genome, a linear double-stranded DNA of 150 kbp, contains at least 76 open reading frames (ORFs) in contrast to EHV-4 genome which is 146 kbp in length. They encode 76 genes, with four duplicated genes in EHV-1 and three duplicated genes in EHV-4 within the repeat regions. The genome is arranged into two major regions (the unique long and short regions), which are flanked by inverted repeat sequences; the internal repeat (IR) regions and the terminal repeat (TR) [6, 14-16]. EHV-1 and EHV-4 have 55-84% nucleotide sequence homology and share cross-reactivity to antibodies [14, 16]. Although they are considered close relative to each other, they exhibit distinct clinical outcomes. The virion core is enclosed in an icosahedral capsid consisting of 162 capsomeres (150 hexameric and 12 pentameric) with an approximate diameter of 125 - 130 nm [4, 17]. The core and capsid form what is called nucleocapsid that is surrounded by unstructured tegument protein. It fills the space between the capsid and the envelope consisting of thousands of densely-packaged protein molecules that have functions in virion assembly and termination of host protein synthesis [18-20]. The viral envelope, enclosing nucleocapsid and tegument, makes up the outer layer of the virus deriving from the cellular trans-Golgi network. It is made up of bilayer of phospholipids with irregular shape in which 12 different glycoproteins are embedded. Eleven out of them have been found to be homologous to other alphaherpesvirus glycoproteins (Herpes simplex virus (HSV), PRV or BoHV) and therefore, they are named in accordance with the nomenclature established for HSV [21]. EHV-1 encodes an additional unique glycoprotein, gp2. These 12 envelope glycoproteins are key players in virus attachment, penetration, egress, pathogenicity, virulence and cell-to-cell spread [22, 23]. In addition, enveloped glycoproteins are also important in vaccine development as they are the targets of the host's immune response. The five glycoproteins gB, gD, gH, gK, and gL are shown to be exhaustively essential and necessary for virus replication in tissue culture while others not [15, 22].

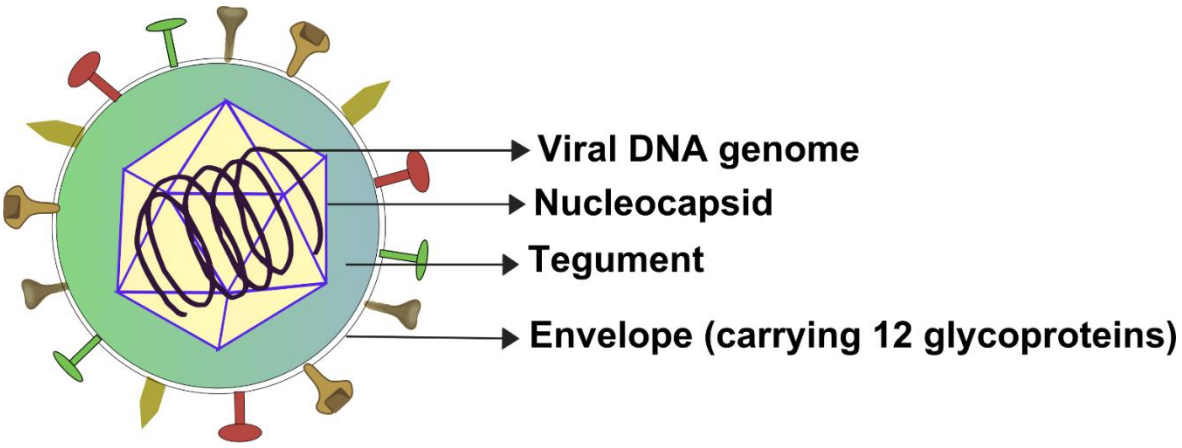


Figure 2: Schematic representation of equine herpesvirus morphology.

4.2.1 *An overview of EHV-1 glycoproteins; general characteristics with their main functions.*

4.2.1.1 Glycoprotein B (gB)

Glycoprotein B is highly conserved enveloped glycoprotein throughout the *Herpesviridae* family. Glycoprotein B together with the major nucleocapsid proteins constitute the most antigenic proteins in EHV-1 [24]. The cleavage of EHV-1 gB is mastered by furin. The ^{544}RLHK^{547} motif is shown to be the principal cleavage site of gB because the virus growth is greatly affected by altering this motif [25]. EHV-1 gB plays a crucial role in attachment, membrane fusion, penetration and in virion direct cell-to-cell spreading [26-29]. This cell-to-cell fusion, mediated by gB, is suggested to be facilitated by perturbations of its C-terminal domains [28].

4.2.1.2 Glycoprotein D (gD)

The EHV-1 envelope gD consists of 385-amino acid with 20% homology with HSV-1 gD and is encoded by the unique short (US) segment of EHV-1 genome [30-33]. Several functions of glycoprotein D had been previously recorded as cell penetration and entry, host cell specificity and cellular tropism and virus cell-to-cell spread [32].

The role of gD in viral entry via endocytosis and its cellular counterpart/s.

gD has a crucial role in EHV-1 entry into cells. Major histocompatibility complex type I (MHC-I) is playing a key role as a cellular counterpart of glycoprotein D (a functional gD receptor). MHC-I molecules are used by EHV-1 and EHV-4 as entry receptors into cells [34]. The endocytic entry pathway of EHV-1 has been described to infect several cell populations. The interaction between cellular integrins and their counterpart arginine, serine, and aspartic acid (RSD) motif at amino acid positions 152-154 of EHV-1 envelope gD was demonstrated to play

a crucial role for virus penetration and entry [35] but in contrast results showed that mutation at position 152 of gD in EHV-4 (EHV-4_gD^{152D} mutant) failed to infect or bind CHO-K1 but it has no effect in infection and productive infection in Vero cells or PBMC [36]. Another third counterpart that is involved in virus entry via endocytic way of some alphaherpesviruses for particular cell types such as CHO-K1 and HeLa is the integrin alpha [37, 38]. Generally, the cellular gD receptors include integrins, members of the family of tumor necrosis factor (TNF) receptor (HveA), the poliovirus receptor family (HveB) and immunoglobulin superfamily (HveC) and the 3-O-sulfated heparan sulfate which is the modified form of heparan sulfate. Therefore, the EHV-1 could infect any cells express heparan sulfate, alpha V, HveA, HveB, and HveC, making it as a wide range of cellular tropism [36, 39]. Neutralization of EHV-1 infection via hampering their penetration into the cells was accomplished through monoclonal antibodies against gD [35, 40].

Its role in the fusion entry pathway to cells

Regarding its significance in viral entry through fusion routes, its mechanism is shared among alphaherpesviruses [41] as the C-terminus segment involving residues 260 to 310 of HSV-1 gD is indispensable for triggering membrane fusion [42, 43].

4.2.1.3 Glycoprotein G (gG)

Glycoprotein G exists in three isoforms: membrane-anchored forms and a secreted form. membrane-anchored forms are either a full-length form or a small form that lacks most of the extracellular domain. The secreted soluble form comprises the released extracellular domain resulting from the membrane anchor cleavage [22, 44].

gG is a viral chemokine-binding protein (vCKBP) [45, 46]. It prevents the chemokines binding to their specific receptors or glycosaminoglycans [47]. The highly variable region is responsible for this chemokine binding ability [48]. The leucocyte migration to the organs is markedly affected by gG [22]. It could hinder transmigration of leucocytes in response to recombinant equine interleukin 8 (IL8, also recognized as CXCL8) [46, 47]. The chemokines binding capability results from the secreted and membrane-anchored forms of the EHV-1 gG [22, 35].

4.2.1.4 Glycoprotein E (gE)

Generally, glycoprotein E along with glycoprotein I (gE/gI complex) form a non-covalently bound heterodimer that has a critical role in viral cell-to-cell spread and spreading of infection transsynaptically throughout the host nervous system [49]. gE is a “nonessential” glycoprotein, except for two herpesviruses (varicella-zoster virus (VZV) and Marek's disease virus serotype 1) [50].

Despite the relevance of the gE/gI and their role in cell-to-cell spread of EHV-1, it is not needed for virus attachment and penetration or virus maturation and release processes [51].

The gE/gI negative mutants resulted in small plaques compared to parental viruses due to the severe effect of cell-to-cell spread [52]. The attenuated gE deleted mutant, virulence factor determinant, was partially protecting the intramuscularly injected foals against the EHV-1 respiratory disease [53].

4.2.1.5 Glycoprotein H (gH)

Glycoprotein H comprises a heterodimer with glycoprotein L forming the (complex gH/gL). This complex serves as a regulator for the fusion process of many herpesviruses [27, 54]. Three distinct domains in the N-terminal region (domain H1) were analyzed structurally of the gH and shown to bind to gL [55]. The resulting gH polypeptide following its transfection into cells doesn`t undergo either folding or correct processing in the absence of gL highlighting the impact of gL in gH folding and processing inside the cells. EHV-1 gH also with the cellular α4β1 integrins has been entangled as a pivotal determinant in the selection of virus cellular entry pathways [54].

4.2.1.6 Glycoprotein I (gI)

Glycoprotein I binds non-covalently with gE forming the complex heterodimer gI/gE. This complex facilitates the cell-to-cell spread of virions and is one of the important EHV-1 virulence factors which is speculated to occur through selective sorting mechanism of nascent virions to cell junctions enhancing cell-to-cell spread [51]. Regarding EHV-1 gI structure and function, some aspects of them remains ambiguous and needs more and deep investigation.

4.2.1.7 Glycoprotein K (gK)

gK was proven to be an essential protein in EHV-4 replication in vitro [56]. This late protein is affected by immediate-early protein (IEP) as it binds to their transcription initiation site of their promoter sequence resulting in repressing their transcription [57, 58]. This envelope protein exists in virion and in the cellular plasma membrane. The glycoprotein gK is implicated in EHV-1 cell-to-cell spread, virus entry and egress [59].

4.2.1.8 Glycoprotein L (gL)

Glycoprotein L is associated with gH, but the specific role of gH / gL complex in herpesviridae family is the least understood. Although gL is a key glycoprotein in some herpesviruses, its role the EHV-1 replication cycle is still largely unknown [60, 61]. A heterozygous gH / gL is required for virus binding, entry and in virus cell-cell fusion transmission occurrence [27]. Even though alphaherpesviruses lacking the gH / gL complex cannot invade and enter cells, they are able to attach to their cell surface. Therefore, the role of gH / gL in viral entry is important

during the fusion of the plasma membrane of the viral envelope proteins but not necessary to the virion-receptor binding [27]

4.2.1.9 Glycoprotein M (gM)

gM is a multiply hydrophobic type III integral membrane protein encoding eight putative transmembrane domains. It is a non-essential and conserved glycoprotein. As the glycoprotein N (pUL49.5) forming a complex with glycoprotein M, its expression also is very important for gM processing in infected cells [62]. Its deletion in EHV-1 strain RacL11 resulted in 50% plaque size reduction in comparison to the wild-type [63]. Its role not only in virus egress and affecting the production of extracellular viruses but it also has an influence on virus cell-to-cell spread [63, 64].

4.2.1.10 Glycoprotein N (gN)

The function of gN needs more investigations, characterization and addressing. Only some reports addressed their function in functional gM processing [65]. Its importance in alphaherpesviruses immunoevasion is to evade the immune response by evading its elimination by T cytotoxic lymphocytes and inhibiting TAP and peptide transport to ER which subsequently affect MHC-I antigen presentation function, but actually, it has no noticeable effect in downregulation of MHC-I [66].

4.2.1.11 Glycoprotein P2 (gp2)

The gp2 (also known as gp300) is a glycoprotein of approximately 250-kDa [67-69]. The glycoprotein 2 (gp2) is a unique glycoprotein in equine herpesviruses (EHV-1, EHV-4 and asinine herpesvirus). It has been reported to have several functions as follows 1- immunomodulatory protein [65, 67] 2- virus egress and is cogitated to be a major viral determinant of virulence, however, it is not included in virus cell-to-cell spread or secondary envelopment [15, 62]. It is considered as a non-essential protein as a mutation in their encoding gene 71 didn`t inhibit virus replication in murine lung [39, 67] 4- gp2 acts as a signaling molecule [65, 67].

4.2.1.12 Glycoprotein C (gC)

EHV-1 gC has a substantial role in virus entry, egress and inhibition of complement cascades. EHV-1 gC is binding to the heparan sulfate that initiates virus attachment and entry to the cell. The hydrophilic part of gC is more likely responsible for this binding to heparan sulfate [28, 70, 71]. gC plays not only a role in virus entry, but it has also a great function in virion release and egress [72]. It has a significant role in protecting the cell-free viruses from complement-mediated inactivation or protecting infected cells from complement-mediated lysis of infected

cells through binding the complement component 3 (C3) [70, 73]. EHV-1 gC is responsible for haemagglutination activity of equine RBCs which is not inhibited by heparin [74].

4.2.2 *Tegument proteins (*UL56 as one of the important tegument proteins)

pUL56 is an early type II membrane protein that has a function with other proteins (one or more cellular partners induced by virus or viral partners) for efficient MHC class I interference [75]. This immunomodulatory protein has various forms with predominant localization in Golgi membranes. Their transmembrane (TM) domain is indispensable for their proper function of localization. The expression of MHC class I started to be downregulated after 2hr of virus infection with completion at 8 hr infection [75, 76]. UL56 has an extra, functional in-frame start codon. It is located in 17 codons downstream of the first start codon. The translated protein originated from the second in-frame AUG of UL56 is short but still functional. The early UL56 gene is relevant and required for cell-surface MHC-I downregulation for both EHV-1 and EHV-4 infection at early time. Although UL56 is phosphorylated protein, its phosphorylation and even the N-terminal 17 aa were not necessary for UL56 to downregulate MHC-I [75]. The US3 or UL13 has no role in phosphorylation of UL56 [75]. pUL56 alone on the plasmid level is insufficient to provoke MHC-I downregulation. EHV-4 UL56 could also downregulate cell α4β1 integrin cell surface molecule in infected NBL-6 cells [76]. Claessen et al showed that pUL56 induced downregulation of various cell surface molecules in infected mesenchymal stem cells (MSC) during EHV-1 pathogenesis [77].

In vivo studies demonstrated the role of EHV-1 pUL56 in modulation of immunity in the respiratory epithelium rather than PBMC (cytokine expression, chemotaxis and antigen presentation) [78]. As EHV-1 pUL56 leads to *in vitro* reduction in cell surface MHC-1 expression [75], together with ORF2 could show impacts in the clinical disease outcome, nasal virus shedding, moreover TH-1-specific T box transcription factor (T-bet) and IL-8 responses in experimentally-infected ponies [79]. Although EHV-1 UL56 suppressed the interferon α and IL-10 induction in equine respiratory epithelial cells (ERECs), no response or effect had been observed on PBMC [78].

In infected equine monocyte-derived dendritic cells (MDDC), pUL56 was demonstrated to have no role or effect on MHCI down-regulation, marginal role in CD206 and CD172a downregulation, but had a significant contribution in CD29 down-regulation and substantial role in CD83 and CD86 [80]. In Human HeLa cells (CD63 and CD46 down-regulation) and in equine mesenchymal stem cells (MSC)(CD29, CD172a, CD105) were shown to be affected and downregulated by UL56 [81, 82]. pUL43 and pUL56 cooperate together to mitigate MHC-I expression on the surface of transfected cells and it was suggested that PPxY motifs in pUL56 have a role in cooperated function [83].

4.3 Virus genome, replication cycle and latency of EHV

4.3.1 *Genomic organization*

The entire genome sequence of EHV-1 containing 80 open reading frames (ORFs) with only 76 distinct ORFs of them has been sequenced and elucidated by Telford et al. in 1992 [14]. The viral genome is a linear double-stranded type D DNA of approximately 150kbp with a base composition of 56 to 57 % G + C [14]. Four of these ORFs are duplicated, and the genome contains at least 76 unique genes with a potential coding for 77 proteins due to ORF64 splicing occurrence [14, 84]. The EHV-1 genome is divided into 2 covalently linked components: the short component (S) that consists of a unique (US) sequence and the long region (L) that comprises a unique (UL) sequence. The UL and US are flanked by two inverted repeat regions, namely the internal repeat (IR) and terminal repeat (TR), [14, 85]. (Fig 3).

Figure 3: EHV-1 genomic organization.; US, unique short region; UL, unique long region; IR, internal repeat; TR, terminal repeat

4.3.2 *EHV-1 replication cycle*

Entrance of EHV-1 free viruses occurs either through membrane fusion or the endocytic route [35, 39, 54, 86]. The attachment of EHV-1 viruses to cellular heparan sulfate occurs via glycoprotein C [87, 88], followed by intimate attachment through glycoprotein D [89, 90]. Glycoproteins gC, gD, gB, gH/L had been demonstrated to play a role in virus entry not only in EHV but almost in alphaherpesviruses [91]. Following fusion membrane, entrance the nucleic acid which passes through nuclear membrane [92, 93] making cell host protein shut off is achieved [91]. Three types of genes are recognized in the reproductive cycle, immediate early (IE), early and late genes. Each gene is translated into its respective protein with important specific function. Immediate early (IE) genes lead to activation of both early and late genes [94]. The early genes encode for proteins playing a role in viral replication. On the other hand, late genes encode viral structural proteins and proteins that have a role in virus assembly [94].

Masterminding the immediate-early (IE) gene transcription is done via a transactivator protein, VP16 [95]. This transactivator protein brought as a tegument protein and transactivates the only EHV-1 immediate early protein encoded by ORF64 (HSV-1 ICP4 homologue). The transcription is controlled by cellular RNA polymerase II [96]. The early (E) genes encode for products of UL5, UL8, UL9, UL29, UL30, UL42, and UL52 gene. IE gene

products (proteins and enzymes) are primarily involved in virus replication (nucleotide metabolism and DNA synthesis) [97]. The transcription continues to membrane proteins, structural proteins, enzymes responsible for assembly [98]. By the aid of these proteins together with US3 protein kinase, the viral nucleocapsids bud via the inner nuclear membrane gaining tegument proteins and a preliminary envelope.

Assembly of EHV nucleocapsids occurs near scaffolding proteins in the nucleus before encapsidation of the DNA. Envelopment of the tegument proteins surrounding the nucleocapsids and containing glycoproteins occurs in the inner nuclear membrane but will be lost in the outer nuclear membrane. After leaving the nucleus and reaching endoplasmic reticulum (ER) or exocytotic vesicles, a second envelopment containing all viral glycoproteins will be added. Mature viruses will migrate via the excretory pathway (Golgi apparatus) [98, 99]. The viruses will be then excreted extracellularly or fused to other cells as virus cell-to-cell spread. Generally, most of the EHV-1 replication cycle occurs in the nucleus including viral DNA replication, viral genome transcription, and assembly of new capsids assembly but all viral proteins are synthesized in the cytoplasm.

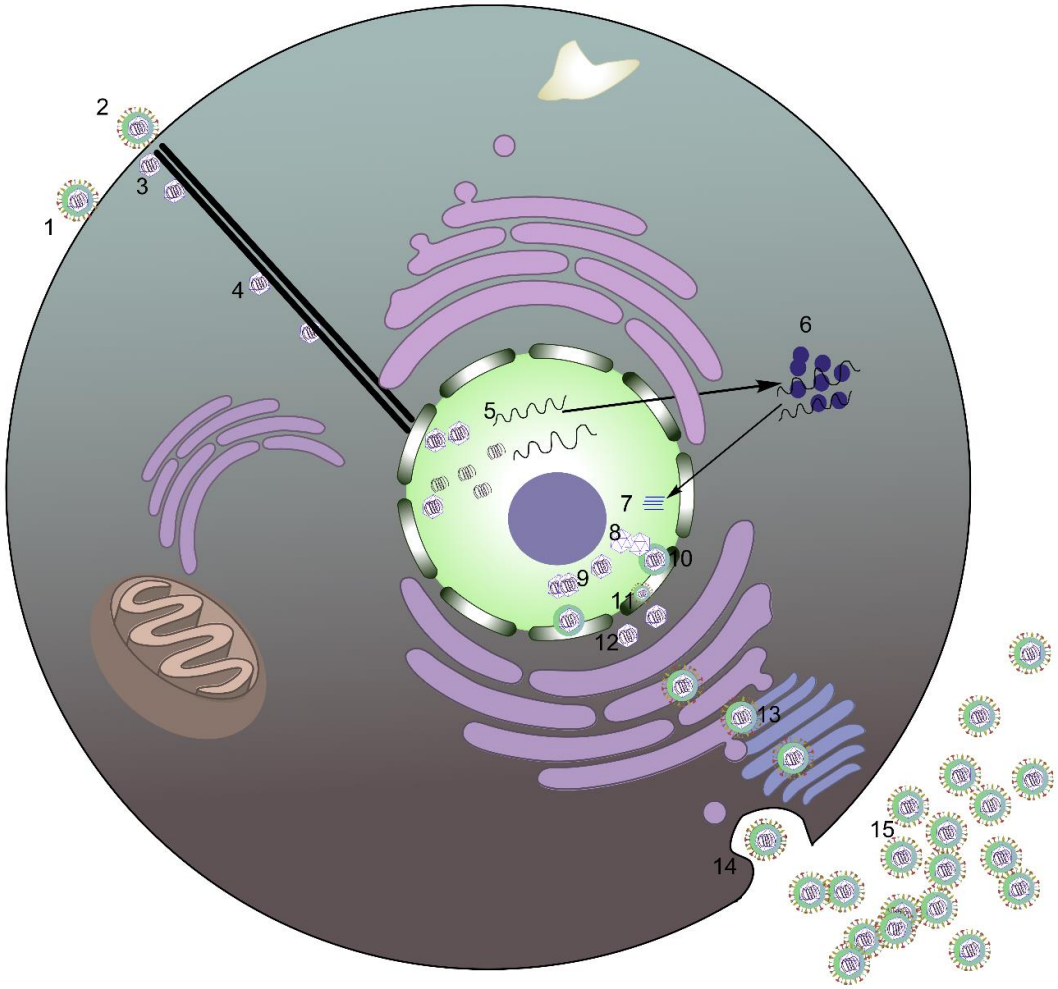


Figure 4: The EHV-1 replication cycle. EHV-1 replication cycle starts with the free virus attachment to the target cell surface through the binding receptors (unstable attachment and includes viral envelope glycoproteins gB and gC interaction with heparan sulfate) (1) virus attachment is followed by virus cell entry via endocytosis or membrane fusion (2) according to the infected cell type as shown in figure (3). Releasing of the nucleocapsids into the cytoplasm (3) is followed by its transport to the nucleus via microtubules (4) for viral DNA replication and transcription that happens in the nucleus and RNA molecules transportation to the cytoplasm to be translated there. The genome is transcribed in a cascade-like manner with first the immediate-early (IE) genes, then the early (E) genes and finally the late (L) genes (5). The translated capsid proteins in the cytoplasm (6) are then redirected again to the nucleus (7) for their assembly (8) and pulling the DNA into this newly formed capsid and consequently, nucleocapsids are formed and assembled (9). The nucleocapsids leave the nucleus via budding of the nucleocapsids through the inner leaflet of the nuclear membrane

acquiring their primary envelope (10 and 11). Their fusion with the outer leaflet of the nuclear membrane results in the entrance into the cytoplasm as a naked nucleocapsid (12) which after that goes to the Golgi apparatus where the second envelopment occurs (13) being transported within secretory vesicles to the cell surface leaving it via exocytosis (15) as cell-free viruses (15).

Virus entry pathways into different cell types.

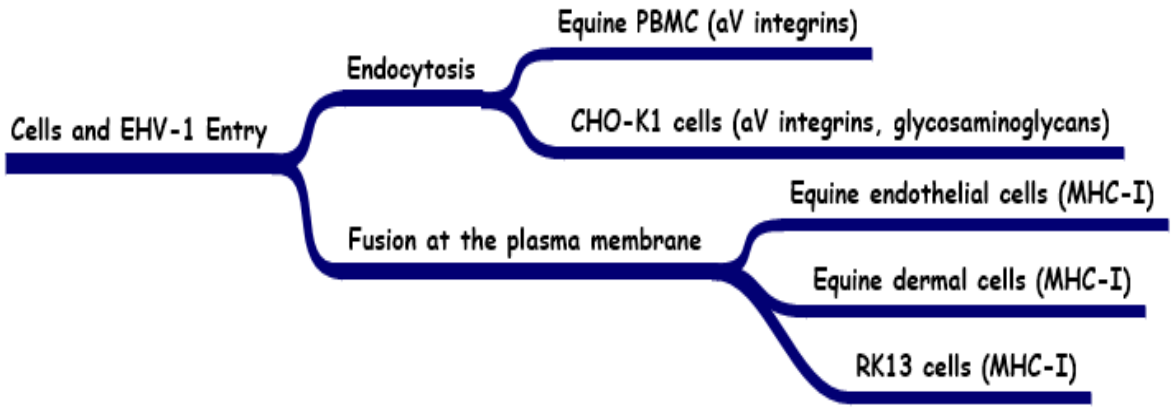


Figure 5: Pathways of EHV-1 entry. The pathway of virus entry either through endocytosis or membrane fusion had been studied for several cells as PBMC [35, 36], equine endothelial cells [34, 87], equine dermal cells [88, 100], RK13 cells [88, 100] and CHO-K1 cells [35, 39]. The figure presented different types in several different cells with their cellular counterparts.

4.3.3 *Latency*

EHV-1 enters in latent state in sensory nerve bodies of the trigeminal ganglia, lymphoid tissues and leucocytes [101-103]. In this case, no transcription and expression of viral proteins were detected except transcription and expression of immediate early (IE) gene, latency associated transcript (LAT) [96]. Mostly 40-60% of equine populations establish EHV-1 latency. This latently infected horse exhibits no cell-associated viremia and clinical signs which make their diagnosis and control more difficult. Following immunosuppression or long term-corticosteroid administration [104], reactivation of latent infection occurs and the infected animals exhibit viremia, clinical signs and spreading the EHV-1 to the others which is very crucial in the

epidemiology of EHV virus [105-107]. The latency had also been stated for EHV-4 in the majority of adult equine population in neural and lymphoid tissues [108, 109].

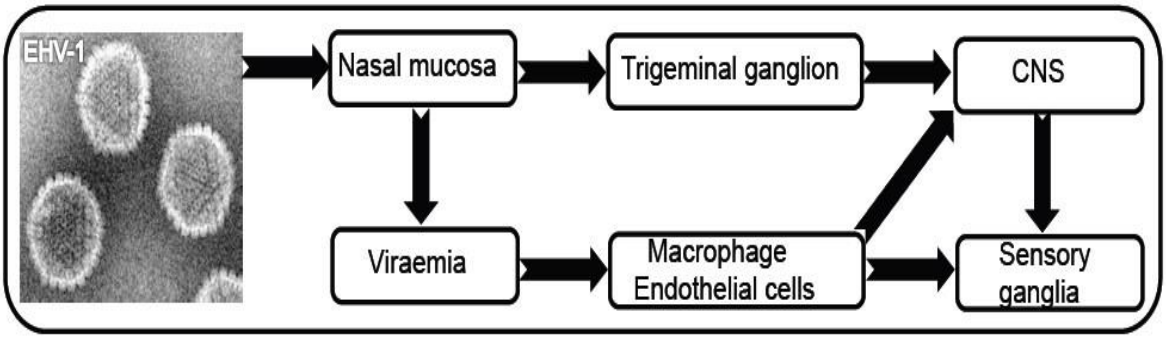


Figure 6: Schematic representation of EHV-1 latency. EHV-1 is able to remain latent state in the trigeminal ganglion via nasal mucosa, modified from [110, 111].

4.4 Pathogenesis of EHV-1

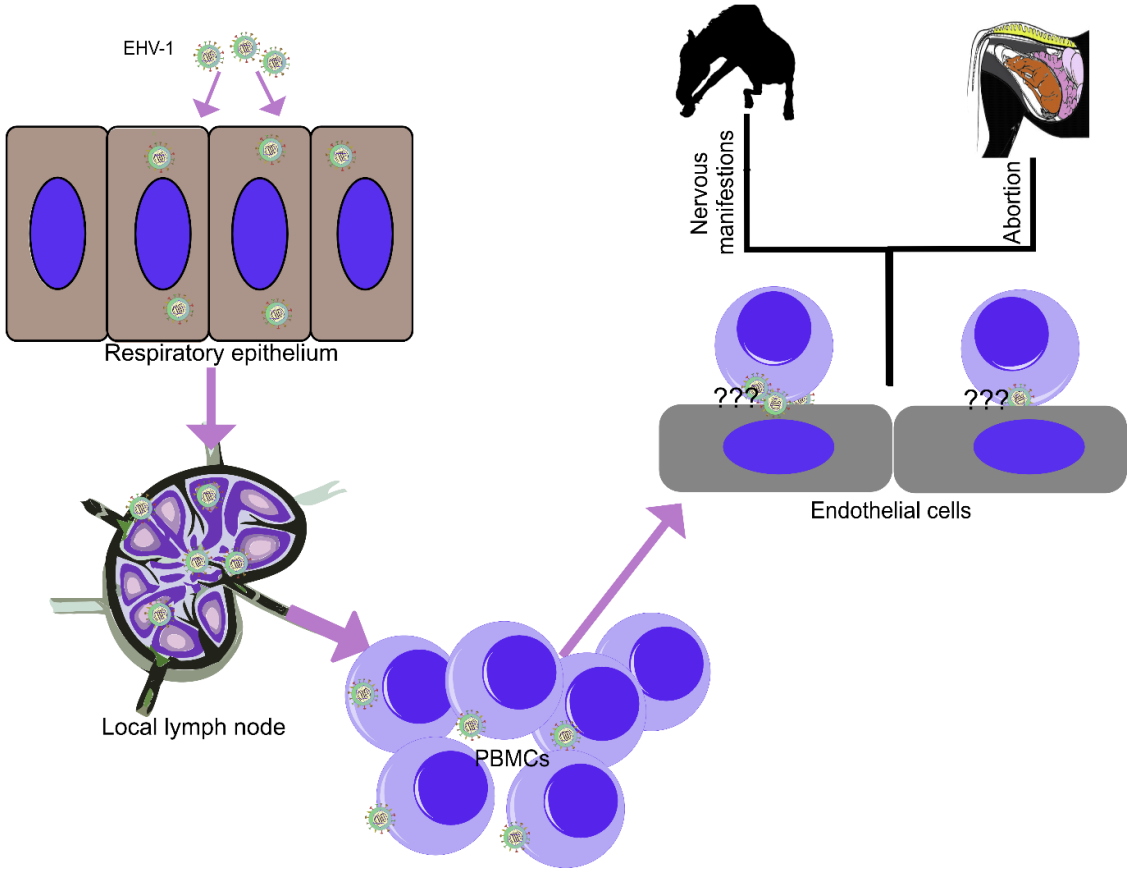


Figure 7: Schematic illustration of the pathogenesis of EHV-1. After inhalation, EHV-1 replicates in the epithelial cells lining the upper respiratory tract (URT). Then, by infecting monocytic cells and T lymphocytes, it reaches lymph nodes and blood vessels. Second replication of EHV-1 in the endothelial cells of the blood vessels of the

pregnant uterus or the CNS is established via cell-associated viremia in the mononuclear leukocytes inducing neurological disease or abortion. Viral latency could be established after primary infection and can be reactivated periodically that may or may not cause clinical signs to appear.

4.4.1 *Infection of the respiratory epithelium*

Following inhalation, EHV-1 replicates in the horses upper respiratory resulting in distinct herpetic lesions and virus shedding [112]. Generally, virus shedding begins at 1-day post infection (dpi) and remains for 7 to 14 dpi or even longer as in equine herpes myeloencephalopathy (EHM) infection [101, 113, 114]. Shedding of large quantities of viruses occurs from the respiratory mucosa of upper respiratory tract (nasal septum, soft palate turbinates, nasopharynx and trachea) into the environment [113, 115, 116]. 2-13 dpi, the infection of lower respiratory tract including the epithelium, endothelium and leucocyte of the lung is established with a peak at 9 dpi. The infection may be subclinical or severe infection following vasculitis, thrombosis and edema manifested as severe respiratory distress namely rhinopneumonitis which involves rhinopharyngitis and tracheobronchitis [112]. Although plaque formation in the infected upper respiratory tract mucosal epithelium is established, these plaques don`t break the basement membrane barrier. The virus hijacks the leucocytes (primarily monocytic cells and T lymphocytes) which then cross the basement membrane barriers reaching to the lamina propria of blood vessels and the local drainage lymph nodes [113, 115-117].

4.4.2 *Cell-associated viremia*

The resulted cell-associated viremia is lasting up from 8 to 18 dpi [105, 106, 113, 118]. The peripheral blood mononuclear cells (PBMC) were displayed to be susceptible to EHV-1 infection in vitro and in vivo [48, 119-121]. These cells are monocytes, T lymphocytes and B lymphocytes [113, 121].

When the infected PBMC reach the endothelial lining of central nervous and reproductive systems, nervous manifestations and abortions occur respectively. The abortion doesn`t result from the virus itself, but it occurs due to severe infection of endothelial lining of reproductive system as EHV-1 is an endotheliotropic virus [11, 122]. The pathological lesions include vasculitis, thrombosis, edema and vascular necrosis [123-126]. The abortion happens mainly at last trimester of pregnancy with one or few reports of 4 months abortion [127, 128]. It has been documented that endometrium endothelial cells adhesion molecules as intracellular adhesion molecules (ICAM) and selectin are increased and upregulated, which can thereby facilitate the infected PBMC to transfer the virus and subsequently cause the pathological effect [125, 126]. It has been thought that the late pregnancy stage hormonal changes of

progesterone, estrogen and cortisone modulate these adhesion molecules, and subsequently, events happen. The late pregnancy stages are also considered a stress factor triggering the reactivation of the latent state of infection [125, 129]. The aborted fetus may be negative-virus or positive-virus depending on the severity of the endothelial lining of endometrium so the recovery of the virus from aborted fetus depends on the severity of infection [130]. If the lesions are less severe, the virus can pass to the umbilical vessels endothelial cells. The aborted fetus shows hepatic necrosis, pulmonary edema, pleural edema, spleen enlargement and any other postmortem lesions [131, 132]. Detachment of the fetal membranes and fetus occurs due to endometrial vascular damage. The fetus may be born alive exhibiting jaundice, weakness and respiratory distress but it will die within 7 days [101, 133].

Although the virus is neurotropic, the resulting clinical outcomes are mainly due to endothelial cells lesions not due to virus replication in neurons [122, 134]. It has been thought that strains that cause long and high viremia tend to cause nervous manifestations than others. The neuropathogenic strains are differentiated than the non-neuropathogenic one via a point mutation in their DNA polymerase [119, 135]. The clinical signs appear as paresis [122], forelimb paralysis, then complete paralysis, head tilting, eye lesion, faecal and/or urinary incontinence, distal limb edema, edema of testes, penis prolapse and even blindness [110, 136, 137]. The lack of nutrients and oxygen causes neurons degeneration and eventually leads to equine herpes myeloencephalopathy (EHM). Infection of the ocular endothelium causes chorioretinopathy, which results in permanent lesions. These lesions can be focal, multifocal, or rarely diffuse that affect the entire eye [138, 139].

Infection of EHV-4, in contrast to EHV-1, is mainly restricted to the upper respiratory tract. These differences in their pathogenesis are potentially related to the fact that EHV-4 gG couldn`t modulate chemokines which are pivotal for regulating leukocyte migration, the diversity in cellular tropism that is primarily determined by gD, the rarity or short time of a leukocyte-associated viremia of EHV-4 [22, 140].

4.5 PBMC-EC interface

4.5.1 *PBMC-EC interaction.*

The mechanisms of PBMC rolling, and transmigration through endothelial cells had been previously investigated to understand this complicated process. The process of initial capture, rolling, firm adhesion and transmigration is controlled through a set of adhesion molecules [141-145]. Each adhesion molecule has a crucial role in this complex process. Expression of selectins as endothelial cell adhesion molecules has a key role in the initial capture of leukocytes over them. These selectins have many types according to the cells they originate as for examples endothelial cell, E-selectin and Platelets, P-selectin [146-148]. Selectins are

a family like transmembrane glycoproteins. The binding of E-selectin together with their counter ligand activates integrin family through signaling cascade. The integrin has two subunits α and a β subunit. These integrins are very late antigen-4 (VLA-4; α4β1-integrin) and their counterpart on endothelial cells vascular cell adhesion molecules (VCAM-1) and leucocyte function-associated antigen (LFA-1) and their counterpart on EC is Intracellular cell adhesion molecules (ICAM) [149]. These integrins cause slow rolling of leucocyte and their accumulation in PBMC-EC interface are responsible for intimate adhesion to endothelial cells [150-152]. The intimate adhesion, marked by accumulation of integrins in the interface of the two cells, is followed by transmigration of PBMC [153].

Static condition between PBMC and EC is the most extensively used in vitro systems for studying PBMC-EC interaction. For simulating what`s happens in vivo, flow chamber assays setups represent a model simulating the same conditions happening in vivo including the shear forces and all dynamic fluid flow in their physiological environment [154, 155]. In the flow conditions, the role of adhesion molecules has been assessed and elucidated in endothelial cells. The upregulation of endothelial cells adhesion molecules during late pregnancy is responsible for preference of EHV to endothelial cells at this time [156]. Also, in the nervous system preference, astrocyte medium on the endothelium make attraction to the T lymphocyte [157]. On the other hand, the up/down-regulation had been investigated in equine dermal cells (EC) for both EHV-1/4 [76]. On the surface of PBMC, down-regulation of adhesion molecules was observed in EHV-4 infection but it wasn`t affected either by down-regulation or upregulation upon EHV-1 infection [158].

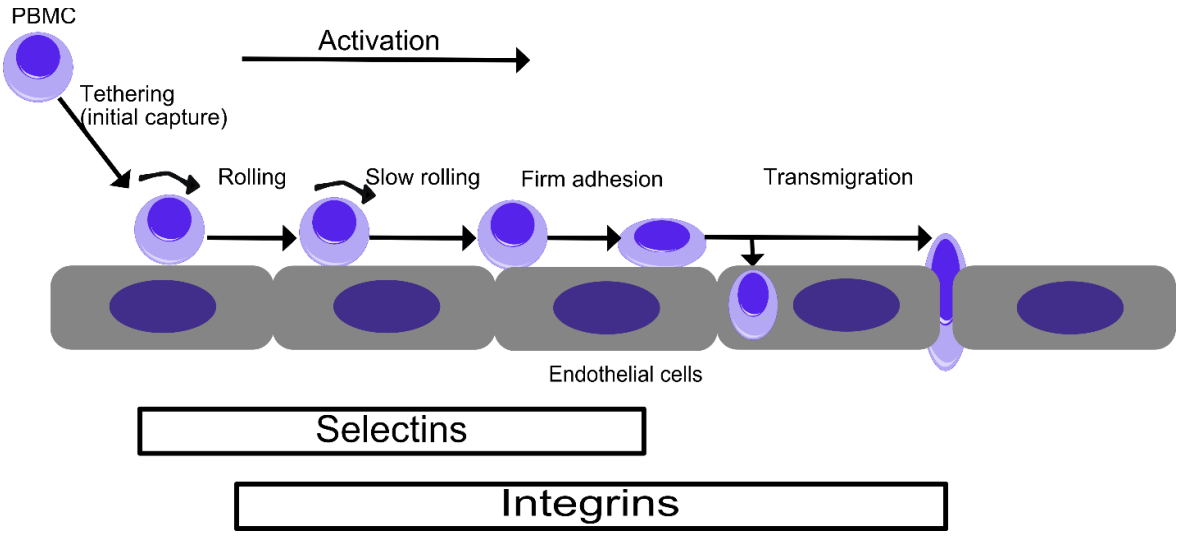


Figure 8: Schematic illustration of PBMC-endothelial cells interaction showing the initial capture of PBMC to the endothelial cells followed by their rolling over endothelial cells and their slowing to be finally firmly adhered to endothelial cell followed by transmigration. Two mechanisms of transmigration were shown transcellular and paracellular transmigration. Initiation of leukocyte rolling is referred to selectin-mediated

adhesion, and leukocyte adhesion stabilization is attributable to integrin-mediated adhesion.

Infection of PBMC plays a key role in disease outcomes since these cells have access to all body tissues. Although non-adherent, PBMC can adhere to EC after stimulation, as is presumably the case after certain virus infections, where viruses can attach to, enter and replicate in EC [159-164]. PBMC-EC interface represents a crucial spot that can influence virus pathogenesis and disease outcomes. Infection of EC through infected-PBMC has been documented for members of several virus families, including Bunya-, Herpes-, Filo-, Flavi-, Toga-, and Arena-viruses [159-169]. However, the mechanism of how these viruses spread from infected PBMC to EC needs to be elucidated. The most fatal disease outcomes of these viral infections are to humans and animals are due to hemorrhagic fevers, severe respiratory disease, neurological and/or congenital disorders. Raveling and unveiling the molecular mechanisms of PBMC-EC virus transfer is very important for understanding virus pathogenesis and subsequently controlling it.

4.5.2 *Transcellular and paracellular PBMC migration through endothelial cells; Brief mechanisms and their fate.*

Transendothelial migration needs an orchestrated series of events to facilitate movement of the leukocyte paracellularly in an ameboid fashion between the endothelial borders and occasionally transcellularly via the endothelial cell body itself [144]. Several mechanisms as, ICAM-1 and VCAM-1 clustering, loosening or disruption of adherens junctions, and influx of membrane from the lateral border recycling compartment are critical for transmigration to occur, and any interference with one of them is enough to block transmigration [144, 145, 170]. Although the majority of transmigration tends to be paracellular, there are in vivo situations and certainly in vitro occurrence of transcellular migration. The mechanisms of transcellular and paracellular migration may be in a similar way than in a different way. The occurrence of transcellular migration tends to happen when the endothelial cell junctions are remarkably tight (such as in the blood-brain barrier), when the leukocytes have difficulty getting the cell junctions, or because of the strong activation of the leukocytes before reaching the endothelial cell border [144, 171]. One of the PBMC activations is the activation following viral infection [172, 173] which might trigger the transendothelial migration to happen. The fate of the transendothelial migratory cells are returning back to circulation as another phenotypic cells or arrested in the endothelial cell or cross the bloodstream to the tissue as for an example for that, the macrophage, if they enter the tissue, a fraction of these become tissue

macrophages. The part that reversely transmigrating is called phenotypically dendritic cells (DC) or the last part stays in the endothelial cells and killed (dead) [174].

4.6 Cell-to-cell transmission of herpesviruses

Spreading of viruses occurs through two distinct modes either via cell-free viruses (diffusion) or through cell-associated viruses (cell-to-cell contact spread). Each route has its own advantages and disadvantages. Although the viruses can spread via cell-free transmission throughout the host and to other hosts more faster and to long distances within the host, they have to be released in large numbers, resistant to be cleared by immune cells and virus neutralizing antibodies, stable, and have the ability to bind and infect the target cells. Viruses cell-to-cell contact spread is more efficient and could bypass phagocytic cells, neutralizing antibodies, complement cascades and most obstacles of cell-free viruses' transmission. Prevention of direct cell-to-cell contact was performed by coculturing the infected and target cells utilizing porous transwell that permit only virus diffusion to the target cells [175, 176]. Herpesviruses represent large DNA viruses infecting a wide range of hosts including humans and animals. Based on the target cells or tissues and the affecting virus species, cell-to-cell spread of herpesviruses varies greatly. Several mechanisms including cell-cell fusion, filopodial bridges, nanotubes, synapse formation were publicized for varicella zoster virus, herpes simplex virus 1, cytomegaloviruses, bovine herpesvirus 1 (BoHV-1), and pseudorabies virus [177-187].

4.6.1 Tight junction

The virus directs their assembly toward the intercellular tight junction. Movement of the viruses from the infected cells to the target non-infected cells occurs through basolateral exit from the infected one and their trapping in the intercellular tight junction between their cell membranes. This trapping in tight junction is followed by their entry through the targets cells receptors which is mainly by fusion pathway.

The sealing of the two cell membranes particularly the adherent cells by the adhesion proteins especially claudin making them impermeable to fluids and small particles. This tight junction could also prevent the cell-free viruses from apical surfaces [188]. Several glycoproteins as gB, gD, gH, gE and gI were implicated in direct cell-to-cell spread. The most studied glycoproteins are gE/gI and their role in virus trafficking of the polarized cells through trans Golgi network. The gE/gI negative-virus mutants lead to reduced plaque size and cell-to-cell spread with controlling of cell-free viruses [189, 190]. The tight junction has another role in epithelial polarization by restricting molecules mobility and targeting them between basolateral and the apical domains [191-193].

4.6.2 Tunneling nanotubes (membrane nanotubes) (intercellular nanotubes)

Tunneling nanotubes (TNTs) are extension and protrusion of cell membrane that sometimes reach more than 100um to reach other cells. This nanotube either short-type less than 0.7um diameter, includes only actin and portion from plasma membrane or the other one is larger than 0.7 um diameter, includes actin, microtubules and cytoplasmic content (as lipid, proteins, calcium and other components) to the other cells. This thin cell plasma membrane protrusion provides a direct physical connection to the remote cells plasma membranes allowing the in-between intercellular exchange of cellular organelles and even signaling molecules [194-196]. Several microorganisms have been demonstrated to exploit these structures for spreading and transmission. It is not only the transfer of viral proteins that occurs via tunneling nanotubes but also transfer the viral genome to the naïve cells as HIV-1 and influenza virus [197-201]. The molecular basis for TNTs induction and formation and their role in viral genome delivery has been reviewed [202, 203]. TNTs are very important and a novel way for intercommunication among cells and herpesvirus transmission in between. The entry of transmitted herpesvirus to target uninfected cells occurs in a fusion-and receptor-dependent manner [187].

4.6.3 Cell-to-cell fusion

Change of cell morphology and its fusion to other cells to form new cells called cell-cell fusion namely syncytia (if the fusion is complete between the cells). Once the virus has the fusion protein machinery, the cell-cell fusion mechanism will start. It is notably that cell-to-cell fusion is proceeded by cell-cell adhesion and in some cases cell migration [204].
Herpesviruses gB, gD, gH and gL play a key role in cell-to-cell fusion especially with the gD receptors on the target cell [205]. Although syncytia are formed during cell-to-cell fusion, only microfusion foci localized events were recorded between CMV and PRV infected leucocytes and endothelial cells. The infectious viral material is transmitted between the two cells [185, 206, 207].

4.6.4 Neural synapses

Paramyxoviruses, rhabdoviruses, and herpesviruses can be transmitted through neural synapses into the uninfected synaptic cells [208, 209]. The viruses either mature or naked virion are assembled in pre or postsynaptic then bud via the synaptic membrane or released through the synaptic space into the synaptic cleft. The virus can then enter the other opposing synaptic cell via fusion or endocytosis. The entrance of herpesviruses through the sensory nerves of skin and mucosa to reach the synaptic cell bodies to become latent occurs via retrograde movement. Virus reactivation occurs during immunosuppression and its movement

anterograde and synaptic transmission occurs. Several enveloped glycoproteins as gE, gI, gD, and gB have been implicated in this neural synapse transmission [210].

4.6.5 Actin containing protrusion (viral synapses)

The virological synapse (VS) is a specialized complex supramolecular structure found in the interface between infected and uninfected cells and expedites the transmission of certain viruses in between. The VS is highly enriched in the adhesion molecule LFA-1 and its counterpart ligands ICAM-1 and ICAM-3 [211, 212]. Herpesviruses could be transmitted via actin containing protrusion from infected cells to neighboring cells [213]. Trafficking of HSV and PRV via these protrusions happens in antibody resistant way [214]. The conserved US3 protein kinase or the inhibition of the Rho-associated kinase (ROCK) of PRV is important for this transmission. The US3 kinase induces cytoskeletal rearrangement and extensions through which an alphaherpesviruses are enhanced to move and spread [182].

4.6.6 Herpesviruses transmission from PBMC to EC

Infection of PBMC with PRV, CMV and VZV and subsequently their transfer to endothelial cells represents the critical step for herpesviruses' pathogenesis as PRV, CMV and VZV are infecting PBMC and subsequently spread and transfer to endothelial cells.Two of them (CMV and PRV) were documented to spread via cell-cell microfusion between PBMC and EC [185, 207, 215]. EHV-1 is a herpesvirus that can infect PBMC and establish cell-associated viremia until reach the EC and transmitted resulting in abortion, neurological disorders, and death [115, 216]. However, EHV-1 spread from PBMC to EC is a yet unknown mechanism. PBMC-EC virus spread was previously shown to be affected by certain adhesion molecules [126, 158, 217, 218]. Tethering and rolling of EHV-1 infected PBMC over EC were controlled and maintained by EHV-1, which resulted in its transfer from PBMC to EC [169]. Interestingly, there was no or highly limited or restricted EHV-1-replication infection in PBMC. The productive virus replication is very low and highly restricted, but it is possible to be triggered by the virus through activating signals inductions [169, 218, 219].

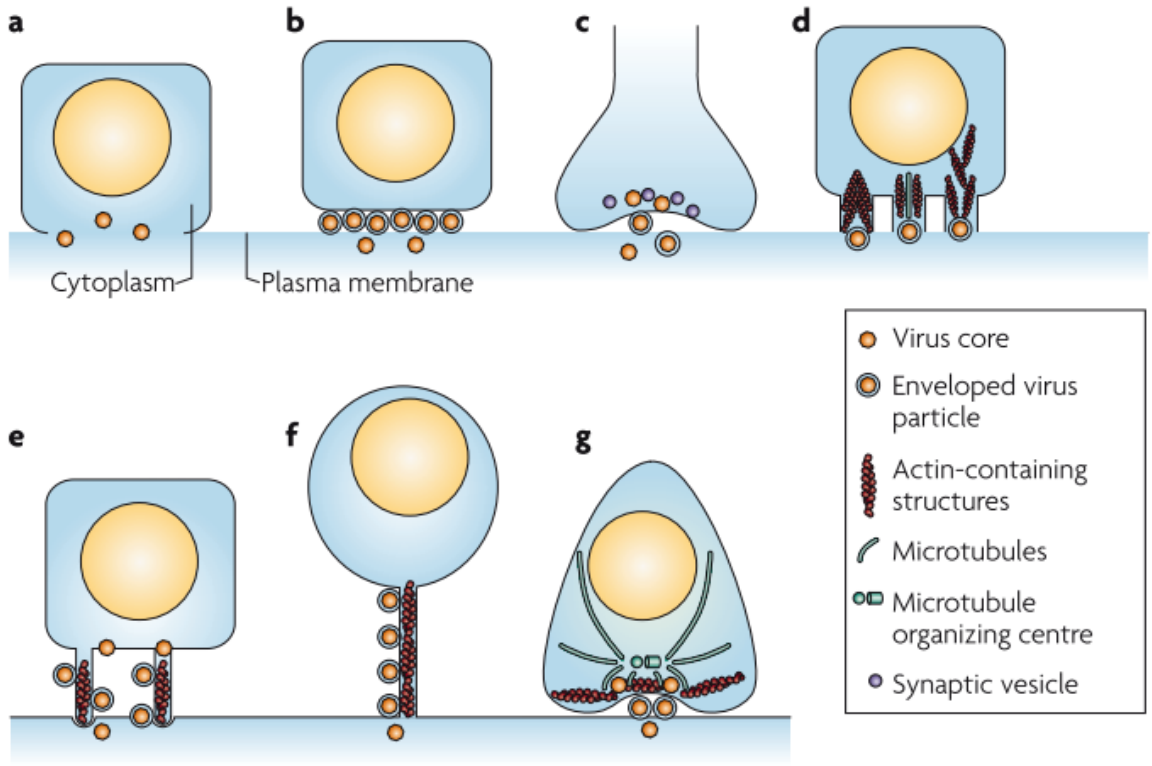


Figure 9: Schematic illustration modified from [214] showing several ways of virus-cell-to-cell transmission: a) Cell-to-cell-membrane fusion b) Tight junction c) Neural synapse d) Viral induction of actin- or tubulin-containing structures e) Viral subversion of actin-containing structures f) Membrane nanotube g) Virological synapses.

4.7 Extracellular matrix and viruses.

The extracellular matrix (ECM) presents in all tissues. ECM is a well-organized networks of macromolecules (including, polysaccharides and proteins) assembling to a three-dimensional structure with distinct biomechanical and biochemical properties [220]. It is not only supplying a physical support for integrity and elasticity of tissues but is also regulating cell growth, communication, survival, motility, differentiation and intercellular adhesion [220, 221]. ECM is constantly rebuilt, remodeled in response to different physiological and pathological cell conditions such as diseases and infections [221, 222]. Several viruses have evolved various mechanisms to overcome the powerful and strong barrier of ECM and use it for their entry and spread [220, 223-227]. One of these viruses is HTLV-that has been shown budding into the ECM of infected lymphocyte using the virus-induced extracellular matrix components (namely biofilm-like structure) allowing them to spread and infect other T lymphocytes [228].

4.8 Immunoevasion

There are numerous examples and methods for immunoevasion that EHV-1 does for escaping both humoral and cellular host immunity. These methods are as follows: downregulating MHC class I expression in the infected cells [81, 229], interfering viral peptide transport to MHC class I in the ER by repressing TAP protein [75, 76, 230], inhibiting antibody cell-mediated cell lysis by inhibition or downregulation of viral enveloped proteins or viral antigens expression on the surface of circulating infected cells [231] which are speculated to be expressed on the surface upon adhesion between PBMC and EC .

4.9. Vaccination

4.9.1 *General types of vaccines*

Vaccination is one of the methods aimed to prevent and control life-threatening infectious diseases infecting humans and animals. It keeps welfare and health of both humans and animals. Vaccination prevents economic losses and social consequences resulting from microbes' infection and outbreaks. Many strategies have been conducted for vaccine development. These strategies are based on rudimentary information about the infectious agent. Vaccines protect humans and animals from the infectious agent via the induction of protective immune responses. In addition, vaccination has helped a lot in the infectious disease epidemiology of limiting or precluding disease transmission in reservoir hosts and from reservoirs to animals and humans. The current vaccines can be categorized into first- and second-generation vaccines. The first-generation vaccines include inactivated and live attenuated vaccines. The second-generation vaccines comprise gene-deleted, subunit, DNA and recombinant vector vaccines.

4.9.1.1 Live attenuated vaccines

Live attenuated vaccines are created from attenuated organisms with a reduced virulence but still replicating and alive. Attenuation can be achieved by several strategies; by (1) several *in vitro* passages either in embryonated eggs or in cell culture (2) selection of temperature-sensitive mutants either the spontaneous or induced ones (3) co-infection two different viruses with segmented genomes at the same cell and using the resulting reassortants virus and (4) generating mutation of the genes encoding virulence factors. These vaccines are capable of triggering cellular and humoral immune response and its immunity can be long-lasting after only one or two vaccinations. Each type of vaccine has advantages and disadvantages. Live attenuated virus vaccine can potentially revert to its virulence state in vaccinated animals or lack its safety in immunocompromised and pregnant animals [232, 233]. Another drawback is

that these vaccines usually require to be kept in refrigerator to keep and sustain their biological activity.

4.9.1.2 Killed or inactivated vaccines

The virus grown on tissue culture can be inactivated physically by heat, radiation or chemically by formaldehyde or any other chemical agent to produce inactivated or killed vaccine. Although the resulting vaccine is able to induce good humoral immunity, it has several disadvantages. Their disadvantages are as follows 1- it needs strong long-lasting adjuvant 2- multiple administrations are required due to the short immunity course. 3- infection spreading is possible especially if there is incomplete inactivation. 4- the difficulty of differentiating vaccinated and non-vaccinated animals which is very important in infection epidemiology. 5- vaccinated animals can become asymptomatic carriers after their exposure to natural infection 6- the weak immune response which is mainly humoral immune response.

4.9.1.3 Subunit vaccines

Subunit vaccine is like the inactivated whole virus vaccine but contains only the antigenic part of the virus (mainly protein). It may include one or several antigens responsible for stimulating the immune response. This antigen could be taken directly from the virus or they are inserted into DNA viruses then culturing them, purifying them which called recombinant subunit vaccine. They need multiple doses to provoke the long-lasting immune response, but they are safe as they are only part from the virus and also not stressful for the animal immune systems.

4.9.1.4 DNA vaccines

DNA vaccine is mainly a plasmid carrying DNA targeting the immune systems. The main advantages are as follows. 1- not exhausting the host immune system 2- triggering both B and T cells 3- safer as it doesn`t contain infectious agent 4- easily to be manufactured 5- stable and doesn`t need cold chain and therefore cost effective. 6- co-expression of multiple antigenic sites, could incorporate marker gene and rapid incorporation of field strain sequences. [234, 235]. The main problems and obstacles are that they have mainly targeted to protein antigen, not to lipopolysaccharide, the possibility of antibody against host DNA, tolerance to the antigen produced, multiple doses of inoculation with large amount are needed to achieve their function. Many DNA vaccines have been generated for viruses as rabies, hepatitis B, west Nile fever, malaria and influenza viruses [236-240].

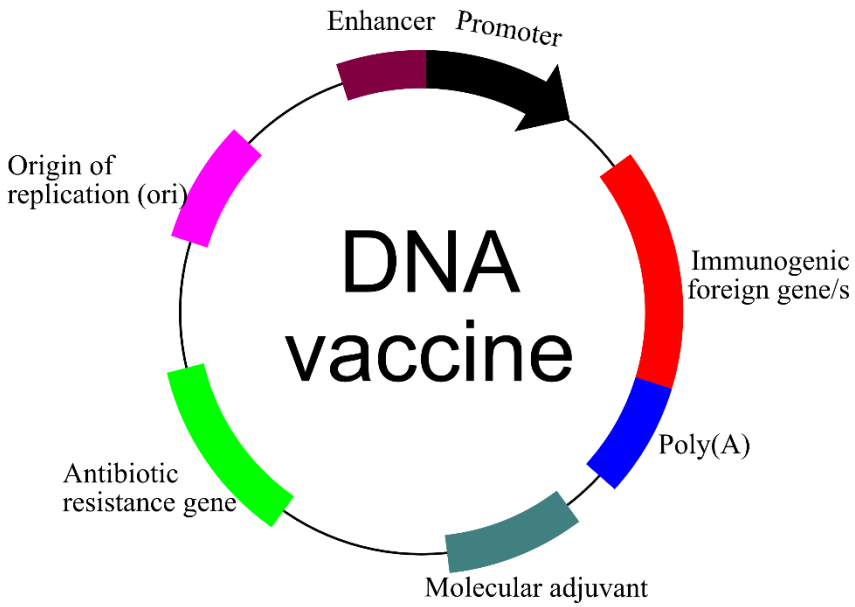


Figure 10: Schematic illustration of DNA vaccines structure.

4.9.1.5 Recombinant vector vaccines

Live attenuated vector DNA vaccine is used to carry the gene of interest to the host instead of DNA plasmid. It can provoke both cell-mediated and humoral immunity. The mostly used viral vectors are the large DNA viruses as poxvirus, vaccinia virus, herpesviruses and others [241-244]. This type of vaccine has several advantages, it could harbor and carry heterologous gene of interests, could provoke cell-mediated and humoral immunity, could differentiate between vaccinated and non-vaccinated animals, could stimulate strong and long-lasting immunity, easily and mass production in tissue culture facility than the inactivated one. Herpesviruses are of these viruses that had been used as vector vaccine. Deletion of the non-essential virus genes for replication, virulence genes or genes responsible for immune evasion and replacing them with the gene of interest of specific pathogen to protect against it.

4.10 Vaccines used for protection against EHV-1

The ideal vaccine needs to fulfill the criteria of preventing clinical symptoms, preventing virus spread, preventing virus replication in the respiratory tract, preventing cell-associated viremia and subsequently preventing the virus spreading to internal organs as uterus and nervous system [217, 245]. The ideal vaccine should provoke cell-mediated immunity, humoral immunity and local mucosal immunity. Most of the available commercial vaccines affect only virus spread and reduce the clinical signs but unfortunately, they have little or no effect on cell-

associated viremia or nervous manifestations or abortion [217]. The ideal vaccine has to provide protective immunity after 1-day postvaccination with only one dose, prevent carrier and primary infection and provoke long-lasting immunity.

4.10.1 *Attenuated, commercial and experimental vaccines.*

Virus attenuation can be done through their passage in heterologous cells or tissue or hosts or at different temperatures. The attenuation could also be obtained by removal of virulence genes from the wild-type viruses. The disadvantage of utilizing this kind of vaccine is the likelihood of reversion and mutation to its original virulent state and causing the disease. In contrast to its disadvantages, the major advantage of these vaccines is triggering the CTLs.

Table 2: A complete list of the developed commercial and experimental attenuated vaccines and their efficacy in EHV prevention.

Vaccine	Efficacy	Reference
Prevaccinol and Rhinomune	• Viremia could not be prevented • The abortion cases were declined after the Prevaccinol vaccination [246-248] • The Rhinomune vaccine surprisingly protected against EHM in a later experiment [249]. It could also lower nasal shedding and clinical symptoms in infected ponies [250]	[249, 251]
Kentucky (KyA) strain, adapted to mouse fibroblast cells	Could reduce the duration of viremia	[252]
C147 strain	Restricted for growth at temperatures above normal body temperature, fewer horses became viremic after infection and abortion	[253, 254]

	percentage had been declined	
the TK^- strain or with the gE/gI deficient (gE^-/gI^-) strain	Could not guarantee a decline in percentage of viremic horses, nor abbreviate the duration of viremia	[51, 255]
gE^- strain	Reduction in the infected PBMC upon infection	[53]
the attenuated vaccine strain rNY03ΔIR6/1gp2S (NY03ΔIR6/1gp2S_H3 strain)	• Vaccinated animals did not develop viremia upon infection compared to the mock-vaccinated horses • It elicited antibodies against both EHV-1 and equine influenza virus	[256]

4.10.2 *Inactivated vaccines generated for protection against EHV.*

Virus inactivation includes the whole virus or viral envelope glycoprotein combined with adjuvant. The biggest disadvantage is that inability to stimulate CTLs which are crucial for killing infected cells [257].

Table 3: List of the developed inactivated vaccines and their efficacy in EHV prevention.

Vaccine	Efficacy	Reference
Pneumabort K an inactivated oil adjuvant whole virus vaccine including EHV-1, given to pregnant mares, yearlings and two-year-old ponies	No effect on viremia	[251, 258]

Duvaxyn EHV1,4 tested in pregnant mares and naïve foals inactivated carbomer-adjuvanted a whole virus vaccine containing both EHV-1 and EHV-4	In pregnant mares, no reduction in viremic horses, nor in the duration of viremia was observed. In contrast, 30% of the vaccinated foals compared to 80% of the control foals showed viremia upon challenge,	[114]
Some field studies showed a decline of the abortion percentage upon vaccination with Pneumabort K or Duvaxyn EHV1,4 [247]		
Flu-Vac Innovator 6	No reduction of the number of viremic horses, nor diminishing duration of viremia	[249]
ISCOMs (immune stimulating complexes) vaccine containing all the pivotal EHV-1 glycoproteins	Declined the duration of viremia upon infection	[259]
gD/gB, gB/gC/gD and IE	Can`t reduce the level, nor the duration of viremia	[96, 260-262]

4.10.3 *DNA vaccines*

DNA vaccines consist of plasmids that contain genes encoding glycoproteins flanked by promoter for provoking the immune response [263]. The vaccine is administered intramuscular or intradermal. Once host cells take up the plasmid, viral proteins are expressed, transported into the ER and cleaved into peptides by cellular proteases. These peptides are subsequently bound to MHC I molecules in the ER. Through MHC I molecules, the viral peptides are expressed at the cellular surface and immunity is then elicited. Their advantages are that, next to a humoral response, they can induce cellular immunity by activating CTLs. Despite this promising fact, vaccination with the experimental gB/gC/gD could not significantly reduce viremia upon challenge.

Table 4: List of some developed DNA vaccines to protect against EHV

Vaccine	Efficacy	Reference
gB/gC/gD	Could not significantly reduce viremia upon challenge	[261]
IE/Unique Long (UL)-DNA vaccine		[264]

4.10.4 *Vector vaccines constructed for protecting equines from EHV infection.*

The main recombinant vaccines expressing the major immunogenic glycoproteins eliciting a protective immune response for EHV-1 prevention are listed in the table (5).

Table 5: List of the recombinant vaccine and their evaluated efficacy in EHV-1 prevention.

Vaccine	Efficacy	Reference
Herpes simplex virus strain VC2–EHV-1–gD	After three vaccination doses, IgG1 and IgG2a antibodies, as well as strong cellular immune responses were induced.	[265]
Baculovirus-expressing EHV-1 glycoproteins gB, gC and gD alone or in combination	Vaccinated mice were protected against a challenge with EHV-1 and antibody was evoked. No clinical signs in EHV-1 challenged mice, rapid elimination of EHV-1 from the lungs and a decrease in lungs herpesviruses characteristic lesions was observed following gB or gD vaccination. The challenged mice that were previously vaccinated with gB, gD or gB+gD showed evoked CMI responses manifested by T cells aggregations around blood vessels and bronchioles in the lungs .	[266]

Baculovirus expressing EHV-1 gD	• It was used as a boost in a prime-boost strategy to the plasmid EHV-1 gD DNA construct in murine animal model. • The prime-boost immunization increased neutralizing and ELISA antibody titers to EHV-1 and accelerated its elimination from lungs. It induced also the T-helper 2 (Th 2) type together with Th 1 immune response.	[267]
Pichia pastoris expressing EHV-1 gD (Pic-gD)	The DNA prime/protein boost strategy provoked high neutralizing and ELISA antibody titers into the protected challenged BALB/c mice.	[268]
a pathogenic vaccinia virus MVA expressing EHV-1 gC	Recombinant MVA followed by plasmid DNA vaccination induced humoral and cell-mediated immunity in hamster	[269]
Bac-EgB	Bac-EgB infected insect cells could induce complement dependent virus neutralizing antibody at a low level. It induced protective immune responses in murine models.	[270]

4.11 EHV as a viral vector vaccine for protection of animals against several diseases

4.11.1 *Recombinant herpesviruses viral vector and techniques*

4.11.1.1 Bacterial artificial chromosome (BAC)

A bacterial artificial chromosome (BAC) is an artificial DNA construct used to clone large sizable DNA in *E. coli* [271]. BACs are based on functional F-plasmids which play a vital role in their construction by containing machinery genes enhancing the even and equal distribution of the plasmids during cell division. Due to the large herpesvirus genomes, ligating them into the BAC plasmid vector (BAC cloning) is not a simple issue. The BAC plasmid cassette is then easily to be shuttled into the herpesvirus genome via the homologous recombination in infected cells. During replication of herpesviruses, circularization of their linear double-

stranded DNA genome befalls [272]. Isolation of the circular replication intermediate of the BAC mutant can be then achieved and introduced into the competent *E. coli* cells by DNA transformation. The herpesvirus BAC can then be propagated as well as mutated in *E. coli*. The viral genome is kept in E.coli by the BAC technique which can be then transfected into permissive eukaryotic cells for reconstitution and the viral progeny production resulting in infectious viral BAC [273]. Thus, the construction of recombinant viral genomes, for instance, the insertion of foreign genes is expeditious, fast and feasible.

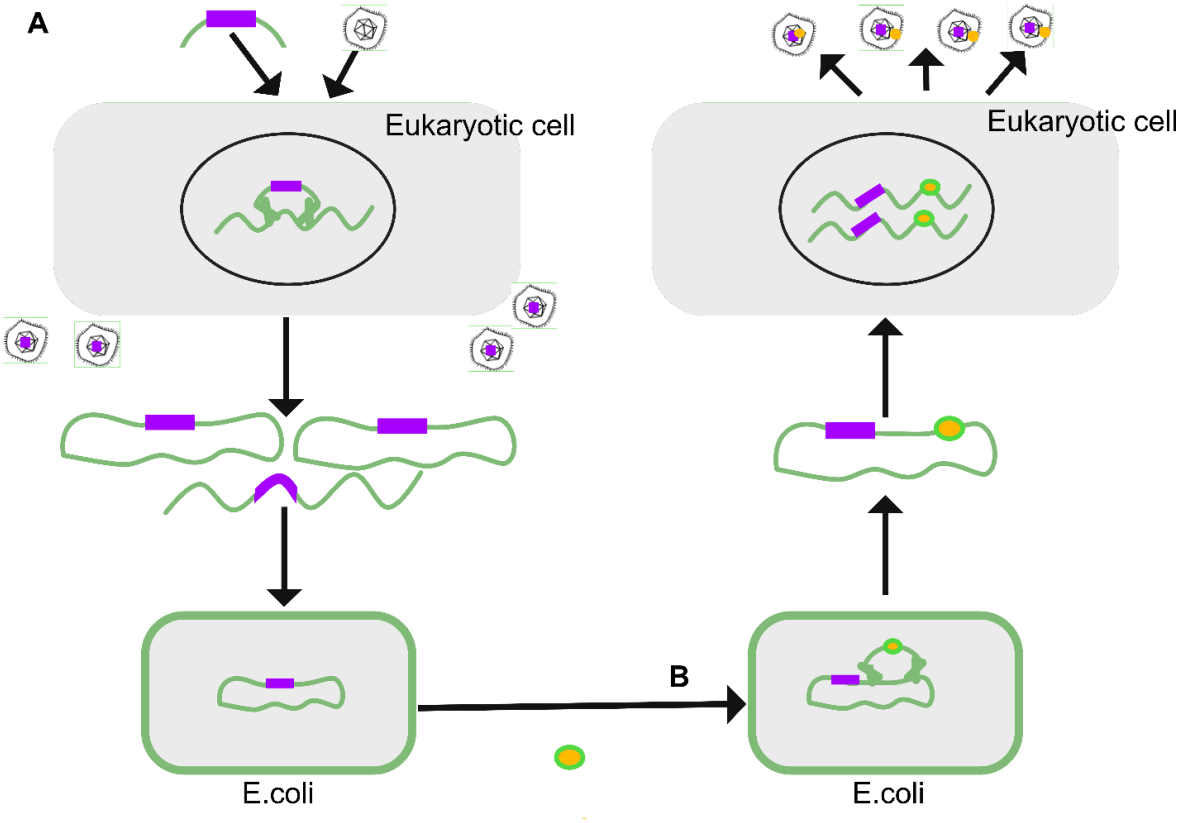


Figure 11: **Cloning and mutagenesis of herpesvirus genome as bacterial artificial chromosomes in *E. coli*.** (a) The herpesvirus incorporating the BAC cassette (violet) has been generated by infected eukaryotic cells through homologous recombination achieved between BAC plasmid cassette and the herpesvirus genome. Circular replication intermediates of the BAC mutant are then isolated and shuttled into the competent *E. coli* cells by DNA transformation to obtain viral BAC. (b) Via genetic engineering, the produced BAC-cloned viral genome in *E. coli is* used for introducing a mutation (yellow). The resulting BAC harboring the target viral genome with the introduced mutation results in producing mutant viral progeny after their transfection in susceptible eukaryotic permissive cells.

4.11.1.2 BAC technology application in herpesviruses

Experimental manipulation of herpesviruses faces huge difficulties [274] due to the largest

and complicated genome [275]. Several herpesviruses BACs have been generated, for instance, murine cytomegalovirus, pseudorabies virus, human simplex virus, equine herpesvirus and bovine herpesvirus. A substantial progress in the field of molecular biology and recombinant vaccines generation is achieved as one of the applications of BAC technology. The availability of BACs-cloned herpesvirus genomes makes it easy to do any genetic modification on viral genomes including deletion and insertion of sequences. The BAC system is a powerful and effective tool for delivering several genes from many pathogens in the recombinant vaccine fields. The BACs technology has a plethora of applications including gene therapy. These viruses could effectively serve as vectors in both vaccine development and gene therapy.

4.11.2. Equine herpesvirus as a viral vector vaccine against several pathogens.

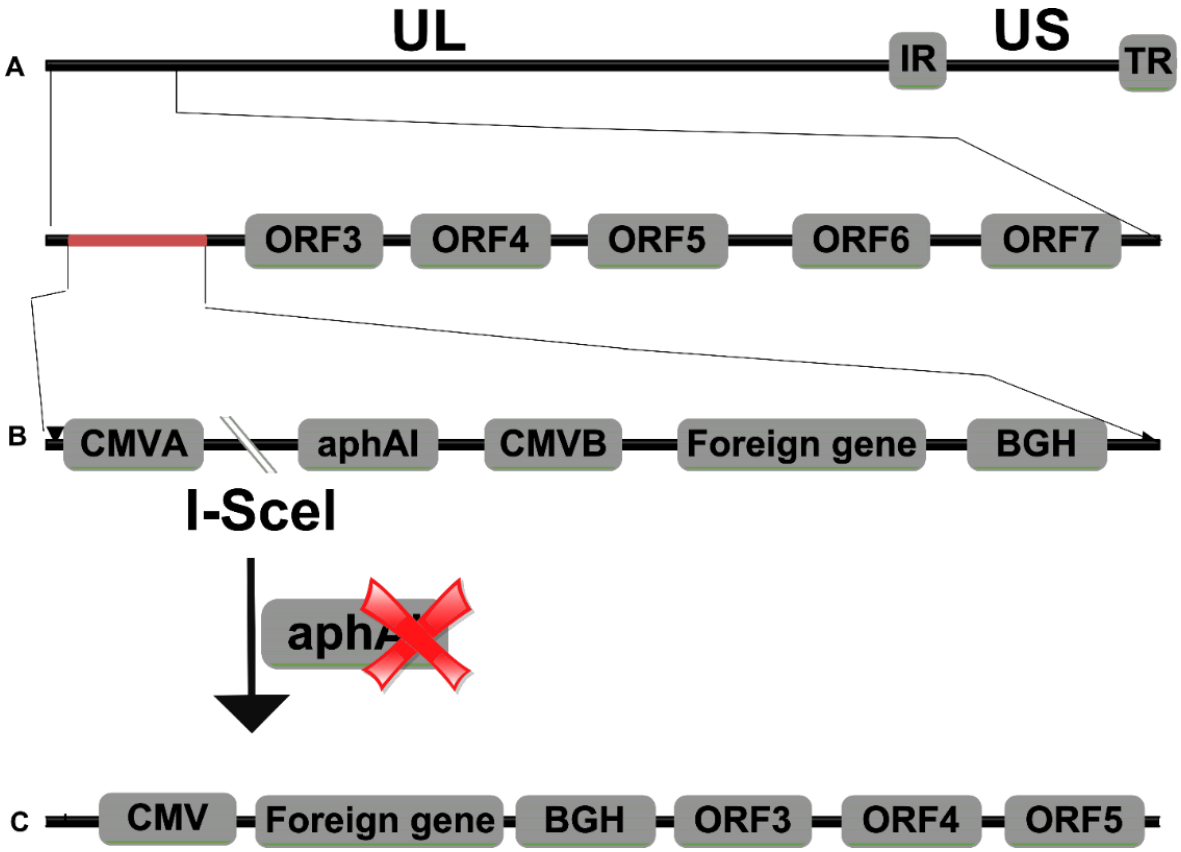


Figure 12: Schematic presentation of strategies for the construction of recombinant RacH strain of EHV-1 expressing any pathogen foreign gene (Two-step red en-passant mutagenesis). (A) Depiction of the left terminus of the UL segment of RacH BAC genome. (B) Introduction of foreign gene, kanamycin resistance gene and HCMV promoter in the ORF1/2 locus of the RacH genome occurs during first recombination step. (C) The second recombination event leads to the excision of kanamycin resistance gene (KanR) and the

final construct recombinant RacH expressing foreign gene with its correct pattern is obtained.

The EHV-1 strain RacH had been established as a BAC [276] and introduced as a universal live vector. The most remarkable characteristic of RacH is its capability to enter and infect numerous cell types of different origins [277]. RacH has been demonstrated to be safe and sound in equine and several other species. Its attenuation comes up from the deletion of both copies of the gene 67 and further genomic modulations and modifications [278-280] Previous studies [281-287] reported that the EHV-1 strain RacH can express several immunogenic proteins in an effective and stable way. It can also induce the cellular and humoral immunity that provide protection to the vaccinated animals after heterologous virus challenging. Furthermore, its efficacy has been demonstrated in non-equine animals, primarily because of its capability to convey several foreign genes of interest in cells of several species including mice, cattle and dogs [281-284, 287] due to the lack or absence of pre-existing anti-EHV-1 immunity in these species. One of its advantages in diagnostic epidemiology field is that there is no cross-reactivity between the induced neutralizing antibody during immunization with recombinant EHV-1 expressing bovine viral diarrhea (BVD) and bovine herpesvirus type 1(BHV-1) [281]. The lack of pre-existing anti-EHV-1 viral vector immunity in animals other than equine will avoid interference with the vector itself. Table (6)

Table 6: List of experimental studies used for developing recombinant vaccines for protection against several pathogens using EHV as a viral vector vaccine

	Disease to protect against	**Foreign gene of interest**	**Challenged animal**	**Produced Immunity**	**Reference**
Equine herpes virus	Swine influenza virus (H1N1)	Hemagglutinin H1	Pigs	Abs and Partial protection	[286]
	Blue tongue	VP2 and VP5	Mice	Abs and complete protection	[287]
	Bovine viral diarrhea virus (BVDV)	Tructural proteins (C, E^{rns}, E1, E2)	Cattle	Neutralizing-antibody response reduced viremia levels and decreased nasal	[281]

				virus shedding	
	West Nile virus	prM and E proteins	Horses	E-protein-specific IgG(T), IgGb, and neutralizing antibodies	[284]
	Venezuelan equine encephalitis virus	Structural proteins	Mice	Complete protection	[283]
	Canine influenza virus (CIV)	H3 of EIV	Mice and dogs	In Mice and Dogs, Abs produced. In Dogs, there was a protection against clinical signs.	[288]
	Canine distemper virus (CDV)	Hemagglutinin protein and nucleoprotein	Pups	Protective neutralizing antibodies levels were induced in these constructs (EHV-H and EHV-H&EHV-N) that protected challenged pups that showed no clinical symptoms. On the other hand, vaccination with EHV-N didn`t protect pups and showed moderate symptoms.	[289]
	Rift valley fever virus	RVFV Gn and Gc	Sheep	Protective titers obtained post-	[290]

	(RVFV)			immunization	

4.12 Foot and mouth disease: disease history and recent vaccines.

The wide species tropism, high infectious capabilities, wide genetic diversity, fast replication rate and remarkable transmissibility make foot and mouth disease virus (FMDV) harder to be controlled. The high and rapid re-emergency of FMDV is mainly due to increase in international trade, animal and human movement, rapid change of environment and the population growth. The control measures include either eradication or vaccination campaigns and strict hygienic biosecurity and control. The severity of clinical signs and outcomes is mainly dependent on several factors including species susceptibility, previous exposure to infection or immunization, genetic makeup and the inherent viral properties of the serotype [291, 292]. Infection of adult animals is mainly resolved within 2 weeks, but it is highly severe in young animals with high mortality rate due to infection-related myocarditis [291, 293].

FMD is highly contagious transboundary diseases affecting domestic cloven-hoofed animals as cattle, goats, sheep, and swine as well as wild cloven-hoofed animals [291]. The infection is mainly starting in cattle as nasopharyngeal and in swine as oropharyngeal followed by systematic spreading and typical vesicular lesion of mouth, coronary band, interdigital cleft, udder, teat and claws [294, 295]. Establishment of carrier ruminant in their oropharyngeal tissue had been recorded but epidemiologically, their role isn't elucidated even though FMD virus or viral DNA is isolated [296]. Infection of susceptible animals principally occurs via direct or indirect transmission as fomites and aerosols [297, 298]. Strict control measures combined with effective vaccination campaigns had eradicated and controlled the virus in Europa, some African and South American countries [299]. The problems face FMDV controls are mainly epidemiological complexity between both domestic and wild animals in virus maintenance. Virus introduction into naïve animals makes severe economic losses and high financial losses to eradicate and control as happens in England [300]. FMDV is a positive sense RNA genome encapsulated in an icosahedral capsid without envelopment. The RNA genome is composed of large ORF encoding for 4 proteins, VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A) and several non-structural proteins as, Lpro, 2A, 2B, 2C, and 3A, 3B, 3Cpro, 3Dpol **Figure (13)** [301] . The structural polypeptides are encoded by FMD P1 region. The FMD P2 and P3 regions encode the nonstructural proteins associated with replication. The 5' and 3' untranslated non-coding regions have important roles in virus replication and polyprotein translation [302]. FMD is antigenically distinct and separated into seven immunologically and serologically distinct serotypes (A, O, C, SAT 1, SAT 2, SAT 3 and Asia-1) [303]. They have unequal and varied distribution in endemic regions as in Asia (A, O, C and Asia 1), South America (A, O and C) Africa (A, O, C and SAT1-3) [303].

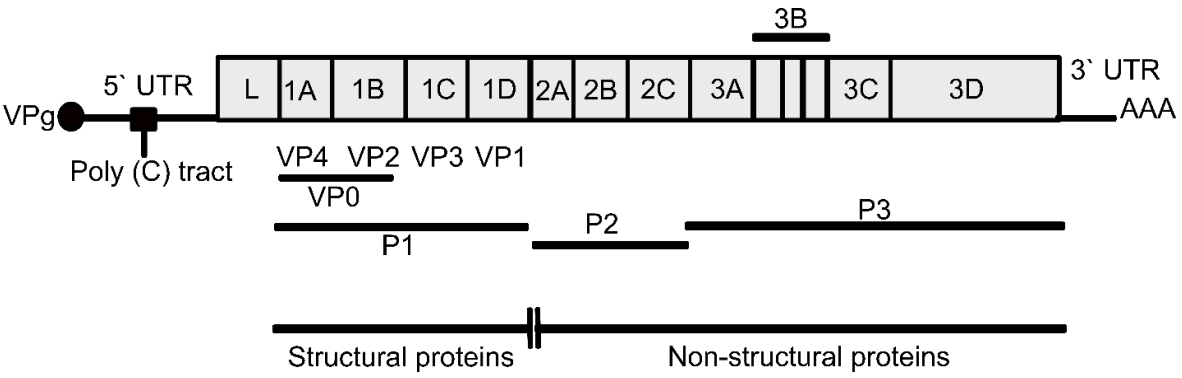


Figure 13: Schematic illustration of FMD genome organization and structure.

Vaccination programs are mainly based on virus strains in each pool. International trade, globalization and people movement make threat to endemic and non-endemic regions for inter viral pool transmission as the vaccination programs are only directed to a specific pool. Most of the commercially available vaccines of FMD are **inactivated (killed vaccines)** with ethyleneimine with depletion of non-structural proteins (NP) that may be monovalent, bivalent or multivalent [304, 305]. They are either aqueous based or oil emulsion based or aluminium based inactivated vaccines. After antigen concentration, they can be stored in liquid nitrogen for long time preservation. The inactivated vaccine may be highly concentrated equal to six times PD50 or as the normal 3 times PD50. This antigen load and concentration depend on manufacturer, antigen and purpose [306, 307]. The highly concentrated vaccine provides protection against challenge within 7 days. Most recommendations are mainly aimed for a booster dose 30 days after the initial vaccination and repeated every 6 months until two years old then repeated annually [305]. The main disadvantages of the currently used inactivated vaccines are as follow: the need for high containment facility as lab biosafety III (prevention of virus escape during manufacturing), multiple serotypes are needed, highly stressful on animal immune system and the need of cold chain as the virus is heat sensitive [308]. Most commonly used vaccines only protect against disease generalization but not prevent from primary infection as more than 50% from vaccinated animals become carriers with only DIVA to differentiate between diseased and vaccinated one [308, 309]. The new marked inactivated vaccines are binary ethyleneimine (BEI) inactivated a virulent FMD with various adjuvants having an intrinsic marker in the different non-structural proteins (NP) (Lpro and 3AB) [310]

The viral vectors are utilized for delivery of structural proteins for their expression inside or through vector infected cells to provoke both cellular and humoral immune response. Different vectors have been used for this purpose as fowl pox, vaccinia pseudorabies, semliki forest virus, alphaviruses and replication-defective human adenovirus virus. All of them provoke partial protection in swine or cattle or not tested in natural host [311-314] except replication-

defective human adenovirus virus which induces protective immune response during delivering FMD capsid proteins [315, 316]. It is the only licensed vaccine until now to be used during emergency situation. It doesn`t need high containment facilities, DIVA capabilities and is genetically stable. The main disadvantage of future utilization is their high cost in their production especially for veterinary utilizing in mass vaccination for a large number of animals in developing countries. Recent advances to deliver of adenovirus viral vector for FMD (Ads-FMD) together with interferon on the same vector or separated are achieved [317].

DNA vaccines

DNA vaccination with modified full-length FMD or empty capsid even with small FMD regions alone or sometimes with immunoregulatory genes has been tested in animal models, cattle and swine [318, 319]. The major obstacles are the need for large amount of DNA with multiple doses. DNA plasmid to deliver both FMD B and T cell epitopes and some modification for targeting antigen presenting cells in them provoked a total protective immunity against the homologous FMD [320]. The improvement of the T cell response is achieved by co-expression of Bcl-Xl antiapoptotic proteins with FMDV T and B cell epitopes [321]. DNA nanoparticles vaccine showed enhancement in the protection and the immunological parameters [322].

The peptide vaccine is mainly stable and relatively has a low-cost production. The peptide vaccines either contain single linear peptide [323] targeting B cell and T cells [324] or FMD capsid proteins. On the other hand, using the conformational 3D peptides leads to full protection. Addition of Poly(I:C) is also very essential in provoking T cytotoxic cytokines and Interferon Gamma [325]. Utilization of dendrimers displayed full protection in swine or cattle (one copy of an FMDV T-cell epitope branching into several copies of a B-cell epitope wedge or radial branched macromolecules) [326, 327].

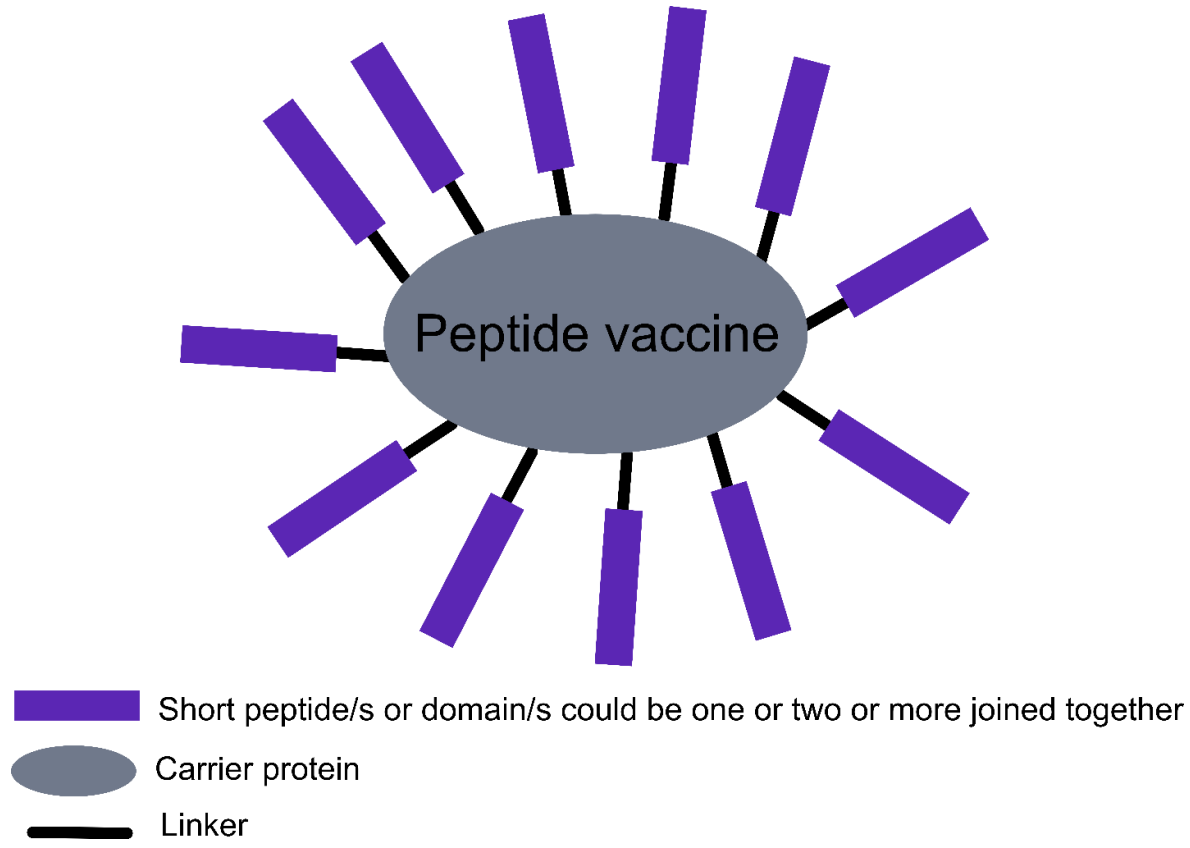


Figure 14: Schematic illustration of peptide vaccine structure.

Live attenuated vaccine

The FMDV attenuation could be achieved through 4 different methods either through removal of full Lpro (leaderless virus (LLV) (induce a non-sufficient antibody response for protection in cattle or swine) [328, 329] or removal of the SAP from Lpro (protected swine against homologous challenge as early as 2 days postvaccination (dpv)) [330] or using chimeric FMDV with the closely related Lpro of bovine rhinitis B virus (BRVB) [331], which is attenuated in cattle and induced remarkable protective immunity against FMDV homologous challenge, or using codon pair bias deoptimization to attenuate the FMDV. All of these approaches confer virus attenuation with triggering high titers of neutralizing antibodies in swine and mice [332]

Viral-like particles (VLP) is expressed in eukaryotic or prokaryotic. Several expression systems to deliver the viral capsid proteins without the infectious genome were reported. Those include some edible plants which are easy to be introduced to animals. Baculovirus/insect cells and larvae, bacteria and plants had been used as an expression system for expressing Viral-like particles (VLP) [333-335].

Vaccine	Efficacy	References
SUMO fusion protein system E. coli	Three capsid proteins of FMDV (VP0, VP1, and VP3) were used	[333]

	in vaccine construction and provided a complete protection against FMD challenge in guinea pigs, swine and cattle.	
Alfalfa, tobacco or tomato fruit have been designated as FMD VLPs production platforms	Immunized mice were protected against experimental challenge but not tested in natural host challenges.	[334, 335]

Table 7 Viral-like particles for vaccine development against FMD

4.13 Project outline

One important feature in EHV-1 pathogenesis, after the virus establishes infection through the respiratory route and replicates in epithelial tissues of the upper respiratory tract (URT), is the infection of mononuclear cells in local lymphatic tissues associated with URT that ultimately enter the bloodstream and lead to cell-associated viremia [336]. EHV-1 can be carried by the infected peripheral blood mononuclear cells (PBMC) and disseminated to the vasculature of target tissues mainly the pregnant uterus or central nervous system, where the virus can subsequently infect and replicate in the lining endothelial cells [337]. The resulting clinical outcomes and economic losses from abortion and nervous manifestations are resulting from spread of virus from infected PBMC to endothelial cells causing the endothelial cells pathological lesions (vasculitis, thrombosis and other pathological lesions). This PBMC-EC interaction represents a crucial interface for the transmission that occurs in vaccinated animals and in an antibody resistant way to the endothelial cells. Until now, this how-to-transmit mechanism(s) is not fully addressed. This area of study is not only important for EHV but for another herpesvirus or any viruses spread from PBMC to EC. Most of these viruses cause severe human and animal threats. Although seeing is believing, combining mutagenesis, functional assays (contact assays, transwell assays and flow chamber assays) together with immunofluorescence data is very important.

In the first part of my study, we documented for the first evidence of unveiling the mechanisms undelaying virus transmission between PBMC to endothelial cell. Here, we document a novel pathway of virus transmission that includes embedding of virus particles in the extracellular matrix. These ECM-associated viruses are protected from the immune system and efficiently spread from infected PBMC to another or to endothelial cells. Furthermore, we show an event of leukocyte transmigration through the endothelial cells where ECM-associated viruses can be delivered directly inside EC. Finally, we also documented the classical cell-cell fusion events between infected PBMC and EC. Two events had also been documented for the first time for

equine herpesvirus, but they need further and deep investigation in the infected EC, microvesicle containing EHV viral particle and tight junction between the infected and uninfected EC harboring EHV particle as a likelihood cell-to-cell transmission for escaping from neutralizing antibodies.

In the second part of my study, I investigated the role of viral protein UL56 in EHV-1 transfer from PBMC to EC. UL56 tegument protein has a role in MHC-I and some cell surface molecules expression effect in different types of cells in EHV-1 and EHV-4. Their role in virus transfer is until now unknown. To address their role, multiple EHV-1 and EHV-4 mutants were constructed by two-step en passant mutageneses. Their in vitro growth properties (plaque size and growth kinetics) were assessed. We here document that UL56 is dispensable for EHV-1 or EHV-4 transmission from infected PBMC to EC in both static and dynamic conditions.

The two previous sections “molecular events associated with EHV-1 pathogenesis and cell to cell transmission” are very relevant for better understanding of vaccine development and improvement. In the last part of my study, I tried to generate FMDV-recombinant vaccine using EHV-1 as a vaccine vector. FMDV is the causative agent of a highly contagious and economically important disease affecting cloven-hoofed animals. FMD outbreaks have devastating economic and social consequences peculiarly in FMDV-free areas with serologically naïve susceptible livestock as it results in culling of the infected and healthy contact animals [338]. The currently used chemically inactivated virion vaccines have a number of difficulties in their production and applications such as the risk of incomplete virus inactivation and the need to use the circulating serotype or even the subtype to produce the vaccine as there is no cross-immunity between FMDV serotypes and subtypes [339]. Other disadvantages of the currently used vaccines include the need for high–biosecurity production facilities, inability to induce immediate protection, short-lived immunity and the inability to differentiate between infected and vaccinated animals [339].

Most of the approaches towards the development of alternative vaccines that do not require live virus material, such as DNA vaccines, subunit vaccines, synthetic peptides, and recombinant virus vaccines, have been investigated extensively [340-342]. Unlike DNA vaccines limitations such as low relative efficacy and multiple boosts with high doses requirements, recombinant virus vaccines are more stable and versatile for manufacturing and storage. Several virus vectors such as fowl pox virus, pseudo rabies virus (PRV), bovine herpesvirus type 1 (BHV-1) and adenovirus were used [343-345].

Here, I used EHV-1 (RacH strain) as a vaccine vector. EHV-1 is a double-stranded DNA (150 Kbp) virus and belongs to the genus Varicellovirus within *Alphaherpesvirinae* subfamily. EHV-1 is capable of infecting a wide range of cell types of different origins including those of bovine origin [346]. Furthermore, we expect that the efficacy of delivery of FMDV antigens through EHV-1 vector will be maximized as there is no pre-existing anti-vector immunity in the target

animals. Here, we constructed equine herpesvirus harboring FMD P1 gene using two en passant mutagenesis and confirmation was done by PCR, RFLP, and sequencing. The in vitro growth properties (plaque size and growth kinetics) were compared to wild-type RaCH. The P1 expression was confirmed by immunofluorescence and western blot. The efficacy of the recombinant vaccine is ongoing under investigation by means of challenged animal experimen

5 Materials and methods

5.1 Materials

The chemicals, enzymes, antibodies, media, and instruments used in this study were used according to the manufacturer's recommendations.

5.1.1 *Chemicals, Consumables and Equipments*

Chemicals

Chemical	Features/Cat. Nu.	Supplier
100 mM Hepes buffer containing 0.1 % (w/v) tannic acid		Science Services, Germany
Acetic acid (CH_3COOH)	Cat. No. A3686, 2500	AppliChem, Darmstadt
Acrylamid		Carl-Roth, Karlsruhe
Agar (agar bacteriological)	Cat. No. 2266.2	Carl-Roth, Karlsruhe
Agarose-Standard Roti® grade	Cat. No.3810.4	Carl-Roth, Karlsruhe
Albumin Bovine Fraction V (BSA)	Cat. No. A6588.0100	AppliChem, Darmstadt
Ammonium persulfate (APS)	Cat. No. K38297601	Merck, Darmstadt
Ampicillin Na-salt	Cat. No.K029.2	Carl-Roth, Karlsruhe
Bromophenol blue	Cat. No. B1793	Alfa Aesar, Karlsruhe
Chloramphenicol	Cat. No. 3886.1	Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Cat. No. 1.02952.2500	Merck, Darmstadt
Dithiotheritol (DTT)	Cat. No. 3483-12-3	Sigma-Aldrich, St. Louis
dNTP Mix (10mM)	Cat. No. BIO-39053	Bioline, Luckenwalde
Dulbecco's MEM (DMEM)	Cat. No. MP0239-TS	Biochrom AG, Berlin
EDTA	Cat. No. A2937, 1000	AppliChem, Darmstadt
Ethanol	Cat. No. A1613	AppliChem, Darmstadt
Ethidium bromide (1%)	Cat. No. 2218.2	Carl-Roth, Karlsruhe
FACS Clean		Bechman Coulter, Germany
FACS Rinse		Bechman Coulter, Germany
Fetal Bovine Serum (FBS)	Cat. No. 10270106	Biochrom AG, Berlin
Glutaraldehyde	Grade I	Sigma, Germany,

Glycerol	Cat. No. A2926, 2500	AppliChem, Darmstadt
Heparin		Sigma Aldrich, St Louis
Hoechst	Cat. No 33342	Thermo Fischer Scientific, Germany
hydrochloric acid (HCl)	Cat. No. 4625.2	Roth, Karlsruhe
Isopropyl alcohol (2-propanol)	Cat. No. A0892	AppliChem, Darmstadt
KCL	HN02-3	Roth, Karlsruh
L-(+)-Arabinose	Cat. No. A11921	Alfa Aesar, Karlsruhe
Lipofectamine 2000	Ref 11668-027	Invitrogen, USA
Methyl cellulose M0262-500gG		Sigma Aldrich, St Louis
$MgCl_2$	Cat. No.5883.025	Merck, Darmstadt
Opti-mem I	Cat. No. 31985062	Life Tech., Carlsbad
Osmium tetroxide	Used as 0.5 % (v/v)	Science Services GmbH, Germany
Paraformaldehyde	Cat. No. P6148	Sigma-Aldrich, St Louis
PEI (polyethyleneimine linear	Cat. No. 23966-2	Polysciences
Reynolds lead citrate		Sigma-Aldrich, Merck, Germany
Sodium chloride (NaCl)	Cat. No. A3597, 5000	AppliChem, Darmstadt
sodium dodecyl sulfate (SDS)	Cat. No. 75746	Sigma-Aldrich, St Louis
Sodium hydroxide (NaOH)	Cat. No. 1.06462	Merck, Darmstadt
SPURR (100 %)	Low Viscosity Spurr Kit	Ted Pella, CA, USA
ß-mercaptoethanol	Cat. No.28625	Serva, Heidelberg
Temed	Cat. No. 2367.3	Roth, Karlsruhe
Tris	Cat. No. A1086, 5000	AppliChem, Darmstadt
Triton X-100	Cat. No. 8603	Merck, Darmstadt
Trypsin/EDTA 0, 05%	Cat. No. 25300054	LifeTechnologies, Grand Island
Tween-20	Cat. No. 9127.2	Roth, Karlsruhe
Uranyl acetate	Used as 2 % (w/v)	Sigma-Aldrich, Merck, Germany

Water Molecular biology grade	Cat. No. A7398	AppliChem, Darmstadt

Consumables

Name	Features/Cat. Nu.	Manufacturer
Collagen type IV cellware 24-well plate	24-well collagen-coated transwell tissue culture inserts. Cat.Nu 354430	Corning, NY, USA
Transwell tissue culture inserts	0.4 µm pore size	Sarstedt, Nümbrecht,
15 u slide 8 well	8-well multi-chamber ibidi slides (Cat.Nu: 80826	ibidi GmbH, Martinsried,
Cell culture dishes	6-well, 24-well, 96 well	Sarstedt, Nümbrecht, Germany
Cell culture flasks	25 ml, 75 ml	Sarstedt, Nümbrecht,
Collagen type IV cellware 24-well plate 60 mm dish	Cat.Nu: 354416	Corning, NY, USA
Conical test tubes	17x120 15 ml	Sarstedt, Nümbrecht,
Conical test tubes 30x115	50 ml, with and without feet	Sarstedt, Nümbrecht,
Cryotubes	1.8 ml	Nunc, Roskilde
Electroporation cuvettes	1mm	Biodeal, Markkleeberg
Eppendorf tubes 1.5 ml	1.5	Sarstedt, Nümbrecht
Falcon bacteria	13ml	Sartsedt, Nümbrecht
Falcon tubes	15 ml, 50 ml	BDFalcon, Heidelberg,GER
Formvar Carbon coated TEM copper slot grids		Plano GmbH, Germany
Ibidi flow chamber slide (µIV-0.4)	Cat.No: 80606	Ibidi GmbH, Martinsried, Germany
Latex gloves Size	L	Unigloves, Troisdorf
LCMC34A	Electron microscope coverslips (FIN-45717, D-35578)	Plano GmbH, Germany
Parafilm® M		Bems, Neenah
PCR tube	0.2 ml	Applied Biosystems, UK
Petri dish for bacteria		Sartsedt, Nümbrecht
Petri dish for cell culture	60mm, 100mm, 150mm	Sartsedt, Nümbrecht
Pipette tips	(1000, 200, 100 and 10)	VWR International, West
PVDF membrane	Cat. No. T830	Roth, Karlsruhe
Single use syringe (different diameter)		several companies

Sterile Pipettes for culture	5, 10, 25 ml	Sartsedt, Nümbrecht
Transfection polypropylene tubes	round, short 17.1 x 105	TPP, Trasadingen
Whatman blotting paper	3MM	GE Healthcare, Freiburg

Equipments

Name	**Feature / Cat. Nu.**	**Company**
1. General Equipment:		
Aclar Embedding Film		Science Services, Germany
Bacterial incubator	07-26860	Binder, Turtlingen
Bacterial incubator shaker	Innova 44	New Brunswick Scientific, New Jersev
CASY counter		Roche Innovatis, Bielefeld, Germany
Cell incubator	Excella ECO-1	New Brunswick Scientific, New Jersey
Cell scraper (different sizes)		Sarstedt, Germany
Centrifuge 5424	Rotor FA-45-24-11	Eppendorf, Hamburg
Centrifuge 5804R	Rotors A-4-44	Eppendorf, Hamburg
Centrifuge Beckman Coulter Avanti J-25 J-25		Beckman Instruments, Palo Alto, CA, USA
Centrifuge Sorvall RC 6+		Thermo Scientific, Dreieich
Electrophoresis power supply	F45-30-11	VWR International, West Chester
Electroporator	Genepulser Xcell	Bio-Rad, München
Freezer	-20°C	Liebherr, Bulle
Freezer	-80°C	GFL,Burgwedel
Gel chambers Mini Protean	2D	Bio-Rad, München
Gel electrophoresis chamber	SUB-Cell GT	Bio-Rad, München
Heating, mixing and cooling thermomixer		Eppendorf, Hamburg
Ice machine	AF100 AF100	Scotsman, Vernon Hills
Leica UC7 ultramicrotome equipped with a 3 mm diamond knife		Diatome, Biel, Switzerland
Magnetic stirrer	RH basic KT/C	IKA, Staufen
Mini centrifuge	Galaxy	VWR International, West Chester
Nanodrop 1000		Peqlab, Erlangen
8 Channel Syringe Pump	NE-1800	New Era Pump Systems Inc.
2 Channel Syringe Pump	NE-4000	New Era Pump Systems Inc

Name	Feature / Cat. Nu.	Company
Nucleofector™ II		Lonza, Basel
Orbital shaker	0S-10	Peqlab, Erlangen
Oven		Memmert, Germany
pH-meter	RHBKT/C WTW pH level 1	Inolab, Weilheim
Pipetboy	INTEGRA	Integrated Biosciences
Pipets	P1000, P200, P100, P10	Eppendorf, Hamburg
Sterile laminar flow	ScanLaf, Mars, Safetv Class 2	Bleymehl, Inden
Thermocyclar Flexcyclar		Analytik Jena, Jena
Thermocycler T-Gradient		Biometra, Göttingen
UV Transiluminator	Bio-Vision-3026	Peqlab, Erlangen
Vortex Genie 2™		Bender&Hobein AG, Zurich
Water bath shaker	C76	Brunswick Scientific, New Jersev
Water baths	TW2 and TW12	Julabo, Seelbach
2. Microscopes		
a Tecnai Spirit transmission electron microscope (FEI)	Operated at 120 kV, which was equipped with a 4kx4k F416 CMOS	FEI
CellDiscoverer 7	equipped with Axiocam 506	Carl Zeiss, Germany
Fluorescence microscope	Axiovert S 100	Carl Zeiss MicroImagi, Jena
Fluorescence microscope	Axio Imager M1	Carl Zeiss MicroImagi, Jena
Inverted microscope	AE20	Motic, Wetzlar
VisiScope scanning disk confocal laser microscope		Visitron Systems GmbH, Germany

Softwares

Software	Version	Supplier or Reference
AxioVision Microscopy	4.8	Carl Zeiss MicroImaging, Jena
BLAST		NCBI, Bethesda, USA.
Chemi-Capt		Vilber-Lourmat, Eberhardzell
Endnote	X5	Thomson Reuters

Software	Version	Supplier or Reference
FlowJo 7.6.5,		FlowJo, LLC. Photos courtesy Graham Lewis.
Graphpad Prism 7	7	Graphpad Software Inc.
Image J 1.41	1.41	NIH, Bethesda
Metamorph		Metamorph software (Universal Imaging Corporation, West Chester, Pa.).
ND-1000	3.0.7	PeqLab, Erlangen
Vector NTI 9	9	Invitrogen Life Technologies, Grand Island
Vision-Capt		Vilber-Lourmat, Eberhardzel

5.1.2 Enzymes and Markers

Enzyme	Catalog Number	Supplier
Antarctic Phosphatase	M0289L	New England Biolabs, Ipswich
BamHI	R0136	New England Biolabs, Ipswich
BamHI-HF®	R3136	New England Biolabs, Ipswich
CIP	M0290S	New England Biolabs, Ipswich
DpnI	ER1701	New England Biolabs, Ipswich
EcoRI	R0101S	New England Biolabs, Ipswich
EcoRV	R0195	New England Biolabs, Ipswich
EcoRV-HF®	R3195	New England Biolabs, Ipswich
Generuler TM 1kb Plus DNA Ladder	SM0311	Darmstadt Fermentas, Mannheim
1 kb DNA Ladder		Solis BioDyne, Estonia
HindIII	R0104	New England Biolabs, Ipswich
KpnI	R0142	New England Biolabs, Ipswich
Long Amp	M0323	New England Biolabs, Ipswich
NotI	R0189	New England Biolabs, Ipswich
Phusion-High Fidelity DNA Polymerase	M0530S	Thermo Scientific, Rochester
Protein Prestained plus	26619	Thermo Scientific
Proteinase K	7528.2	Roth, Karlsruhe
RNase A	2326466	AppliChem, Darmstadt
RQ1-RNas-free DNase	M6101	Promega, USA
SacI	R0156	New England Biolabs, Ipswich

SmaI	R0141	New England Biolabs, Ipswich
SphI	R0182	New England Biolabs, Ipswich
T4 DNA Ligase	01-1020	Peqlab, Erlangen
Taq DNA-Polymerase	01-1020	Peqlab, Erlangen
XbaI	R0145	New England Biolabs, Ipswich
XhoI	R0146	New England Biolabs, Ipswich
Heparinase III	P0737	New England Biolabs, Ipswich and also from sigma

5.1.3 *Antibodies*

Antibody	**Catalog Number**	**Company or Reference**
Agrin Antibody (D-2)	sc-374117	Santa Cruz Biotechnology, Heidelberg, Germany
Ezrin Antibody (3C12)	sc-58758	Santa Cruz Biotechnology, Heidelberg, Germany
Fluorescein isothiocyanate (FITC) labeled lectin from Triticum vulgaris	WGA, lectin from Triticum vulgaris	Sigma-Aldrich, Germany
Fluorescein isothiocyanate (FITC) labeled lectins ConA	ConA-FITC	Sigma-Aldrich, Germany
Goat anti-mouse Alexa-Fluor-647, 568, 488		Thermo Fisher Scientific, Darmstadt, Germany
Goat anti-rabbit IgG conjugated with Alexa Fluor 568		Thermo Fisher Scientific, Darmstadt, Germany
Goat anti-mouse IgG conjugated with Alexa Fluor 568		Thermo Fisher Scientific, Darmstadt, Germany
Neutralizing antibody with a titer 1:2.028 with a dilution 1:100		[347]
Polyclonal antibody anti-FMD (Rabbit)	A-MKS-O	FLI institute
Collagen I/II/III/IV/V antibody	Kaninch 146S Rabbit anti-Human	Bio-Rad Laboratories, Hercules, CA
Secondary antibody was goat anti-rabbit IgG conjugated with Alexa Fluor 568		Invitrogen anti-Rabbit IgG, HRP
Anti-CD11+CD18 antibody LFA	Ab23917	Abcam, Cambridge, UK

Antibody	Catalog Number	Company or Reference
Anti-gp2 (MAbs)		[62]
Anti integrin alpha4+Beta 1 antibody (P4C2)	Ab24695	Abcam, Cambridge, UK

5.1.4 *Kits*

Name	Catalog Number	Company
Amaxa® Cell Line Nucleofector® Kit V	VCA-1003	Lonza
ECL Prime Western Blotting Detection Reagent	RPN2236	Amersham Biosciences
GF-1 AmbiClean PCR/Gel Purification kit	GF-GC-200	Vivantis, Malaysia
Hi Yield Gel/PCR DNA kit	30 HYDF100-1	SLG, Gauting
Luciferase Assay System kit (E1500)	E1500	Promega, USA
Monarch DNA Gel Extraction Kit	T1020S	New England Biolabs, Ipswich
PeqGold Plasmid Mini Kit	12-6942-02	Peqlab, Erlangen
Qiagen Plasmid Midi Kit	12145	Qiagen, Hilden
QIAGEN® Large-Construct Kit	12462	Qiagen, Hilden
QIAGEN® DNeasy Blood & Tissue Kit	69506	Qiagen, Hilden

5.1.5 *Antibiotics*

Name and Cat. No.	Working Sol. Conc.	Manufacturer
Ampicillin Cat. No. K0292	100 µg/ml in ddH2O	Roth, Karlsruhe
Chloramphenicol Cat. No. 3886.3	30 µg/ml in 96 % EtOH	Roth, Karlsruhe
Kanamycin sulphate Cat. No. T832.3	50 µg/ml in ddH2O	Roth, Karlsruhe
Penicillin (P) Cat. N. A1837	100 U/ml diluted in MEM	Applichem, Darmstadt
Streptomycin (S) Cat. N. A1852	100 U/ml diluted in MEM	Applichem, Darmstadt

5.1.6 *Bacteria, cells, viruses and plasmids*

Name	Genotype	Reference
Bacteria		
GS1783	DH10B λcI857 Δ(cro-bioA)<>araC-PBAD, I-SceI	[348]
Top10	F-mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ (ara-leu) 7697 galE15 galK16 rpsL(Str R) endA1	Invitrogen, Carlsbad

Cells

293T	Human epithelial kidney cell line, SV-40 T-antigen	ATCC CRL-11268
EC	Primary equine carotid artery endothelial cells	Primary cells
ED	Equine dermal cells	Primary cells
PBMC	Mononuclear cells	Primary cells
RK13	Rabbit epithelial kidney cell line	ATCC CCL-37

Viruses

EHV-1	EHV-1 strain Ab4	[119]
EHV-4	EHV-4 strain TH20p	[349]
EHV-1	EHV-1 vaccine strain RacH	[62, 350]

Name	Genotype	Reference
Plasmids		
pCAGT7	Plasmid expressing T7 RNA polymerase plasmids	Gift from Richard Longnecker [351, 352]
pT7EMCLuc	Plasmid expressing luciferase gene downstream to T7 promoter	Gift from Richard Longnecker [351, 352]
pBluescript II KS		Invitrogen
p71H expressing the full-length glycoprotein gp2		[44, 62]
pcDNA3.1	Mammalian expression vector; Catalog #V790-20	Invitrogen
pEP Kan-S	Mammalian expression vector; T7prom, f1 ori, SV40 ori, SV40 pr, KanR, I-Sce-I restriction site, AmpR, ColE1 ori, NeoR	[353]
pEPgP2	pEP vector with gp2 (pEP-MCS-in-Belo-gp2)	
pEPmRFP1		[353]
pEPmRFP1-in		[353]

5.1.7 *Buffers, Gels and Media*

Buffers and Gels:

Media and supplements for propagation of bacteria (E.coli)

Media	Composition
LB medium (for 1L)	10 g Bacto™ Tryptone 5 g Bacto™ Yeast Extract 10 g NaCl 15 g Bacto™ Agar
SOB medium (1L pH to 7.0)	20 g Bacto™ Tryptone 5 g Bacto™ Yeast Extract 0.584 g NaCl 0.186 g KCl
SOC medium	SOB medium 20 mM Glucose

Plasmid and BAC DNA preparation buffers

Buffer	Composition
Buffer (P1)	50 mM Tris HCL pH 8.0 10 mM EDTA 100 µg/ml RNase
Lysis buffer (P2)	200 mM NaOH 1 % SDS
Genomic DNA Lysis buffer	mM Tris-Cl pH 8.0 0.1 M EDTA pH 8.0 0.5% (w/v) SDS 20 ug/ml RNase A
Neutralization buffer (P3)	3M K-acetate pH 5.5
Buffer TE	10 mM Tris HCl pH 7.4 1 mM Na_2EDTA

Media and supplements for cultivation of mammalian cells

Name	Catalog No.	Company
DMEM	Cat. No. F 0435	Biochrom AG, Berlin
Fetal bovine serum (FBS)	Cat. No. S 0415	Biochrom AG, Berlin
glutamine	Cat.No. K 0302	Biochrom AG, Berlin
Iscove's modified Dulbecco's medium (IMDM)	Cat.No. P04-20350	Invitrogen, Darmstadt, Germany
L-alanyl-L-Glutamine	Cat.No. K 0302	Biochrom AG, Berlin
Minimum essential Medium Eagle (MEM)	Cat.No. F 0315	Biochrom AG, Berlin
non-essential amino acids	Cat.No. K 0293	Biochrom AG, Berlin

RPMI(1640)	Cat.No. P04-18500	Biochrom AG, Berlin
Sodium pyruvate	Cat.No. L 0473	Biochrom AG, Berlin
Trypsin	Cat.No. L 2103-20G	Biochrom AG, Berlin
DMEM	Cat.No. P04-05545	Biochrom AG, Berlin

Buffer or gel	**Composition**
1x PBS	2 Mm KH2PO4 10 Mm Na2HPO4 137 Mm NaCl 2.7 mM KCl pH 7.3
1x TAE	40 mM Tris 1mM Na2EDTAx2H2O 20 mM Acetic acid 99%, PH 8.0
10x SDS-page running buffer	250mM Tris 1.9M Glycine 1% SDS
Western Blot transfer buffer	25 mM Tris 192 mM Gycine 20 % (v/v) MeOH
10x Lämmli buffer	1.25 M Tris-HCl pH 6.8 10 % SDS 0.2% Bromophenol blue
RIPA I buffer	20 mM Tris-HCl 150 mM NaCl 1 % (v/v) Nonidet P-40 0.5 % (w/v) Sodium deoxycholate 0.1 % (w/v) SDS Complete® Mini protease/phosphatase inhibitor cocktail
RIPA II	RIPAI 1mM EDTA
0.8 % Agarose Gel	80 mM Agarose 1x TAE buffer 4 uL Ethidium bromide 10 mg/ml
6x SDS sample Loading buffer	0.35 M Tris-HCl (pH6.8) 10% SDS 30% glycerol 10% β-mercaptoethanol 0.6% bromophenol blue

Cell culture media and buffers.

Live cell imaging medium	**Trypsin**
Dulbecco's modified Eagle's medium (DMEM) (P04-05545) 20% fetal bovine serum (FBS) 1% nonessential amino acids 1% penicillin-streptomycin.	1.5 M NaCl 0.054 M KCl 0.055 M C6H12O6 0.042 M NaHCO3 106 U Penicillin (P) 1457.4 Streptomycin (S) 0.0084 M Versene (EDTA) Ethylene diamine tetra cetate Trypsin 1:250
RK13 and 293T Medium	**PBMC medium**
Dulbecco's modified Eagle's medium (DMEM) 10% fetal bovine serum(FBS) 1% penicillin-streptomycin	RPMI 1640 10% FBS 0.3 mg/ml glutamine 1% non-essential amino acids 1% penicillin-streptomycin.
ED Medium	**Endothelial cells medium**
Iscove's modified Dulbecco's medium (IMDM) 20% FBS 1% non-essential amino acids 1% 100 mM sodium pyruvate 1% penicillin-streptomycin.	Dulbecco's modified Eagle's medium (DMEM) 20% fetal bovine serum (FBS) 1% non-essential amino acids 1% penicillin-streptomycin.

5.1.8 *Primers*

Primers used in Virus cell-to-cell transmission Project, EHV-1 VP26 mRFP.

Table 8: The used primers were referenced in [54, 353]

Primer/product	Sequence
Primers used for two-step Red-mediated mutagenesis	
mRFPKan-1 For	TGATAACTATCCTAAACCAGAACATCGATGAACTGGATTACA C CAAATACATGGCCTCCTCCGAGGACGTCATC
mRFPKan-1Rev	GCAGTTCCCATAAACAGCTGCTTTAACCCTTCATTAATTTCAT C GTCGCTCAAGGCGCCGGTGGAGTGG
Primers used for PCR and sequencing confirmation	
VP26_For	TAGTGTATCTGTTTTTCAAT
VP26_Rev	GACTACTCGAAACTGCGCTA

Table 9: Primers used in studying the Role of UL56 in virus transfer between PBMC and endothelial cell (UL56 ,ORF1; UL56.1,orf1.1; UL56.4,orf1.4)

Nu	Primer	Sequence
P1	ORF1.1	ATTGGATCCATGAGACCCGAGGGAGTTTC
P2	ORf1.1	CACGAATTCTTATTTCTCCTTCTTGCCGT
P3	ORF1.4	ATTGAATTCATGAGGCCCACGGAAGTTTC
P4	ORF1.4	CACGGATCCTTAATTTTTGGATTTTGTGA
P5	KanR 1	GGCCGCGGGCGTGGACGGCGAGCGTGATAGGATGACGACGA TAAGTAGGG
P6	KanR 1	GCCCGCGGCCGCTCTGCCCCGCGTTCCACAACCAATTAACCA ATTCTGATTAG
P7	KanR 4	GCGGCATGCTGTGCACCTCCGCGTTTGGAAGGATGACGACGA TAAGTAGGG
P8	KanR 4	ACAGCATGCCGCGTGGCTGTGTTGTGGAGCAACCAATTAACC AATTCTGATTAG
P9	ORF1.1 del	TCCACCTGCACCTTTTCCATCTCCTCTCCAACTCGCCGCCAAC GACTGTAGTACCGCAAAAGGATGACGACGATAAGTAGGG
P10	ORF1.1 del	AAAAATAAATGCGATTAACCTTTGCGGTACTACAGTCGTTGGC GGCGAGTTGGAGAGGAGCAACCAATTAACCAATTCTGATTAG

P11	ORF1.4 del	CCGCTCCTGTCTGCTTACACTTTACACTTTTCTGCTCGTCAACA AGTGGTTCAACACAGTAGGATGACGACGATAAGTAGGG
P12	ORF1.4 del	GTGAAAATAAACATAATACAACTGTGTTGAACCACTTGTTGACG AGCAGAAAAGTGTAAACAACCAATTAACCAATTCTGATTAG
P13	ORF1.1 KanR	ATTTAGCCTTCCGCTCCTGTCTGCTTACACTTTACACTTTTCTG CTCGTCATGAGACCCGAGGGAGTTTC
P14	ORF1.1 KanR	AGGGGTGTTTGTGAAAATAAACATAATACAACTGTGTTGAACC ACTTGTTTTATTTCTCCTTCTTGCCGT
P15	ORF1.4 KanR	TTCCACTTTCTCCACCTGCACCTTTTCCATCTCCTCTCCAACTC GCCGCCATGAGGCCCACGGAAGTTTC
P16	ORF1.4 KanR	GAGTGCATGTAAAAATAAATGCGATTAACCTTTGCGGTACTAC AGTCGTTTTAATTTTTGGATTTTGTGA
P17	ORF1.1 revert	TTCCACTTTCTCCACCTGCACCTTTTCCATCTCCTCTCCAACTC GCCGCCATGAGACCCGAGGGAGTTTC
P18	ORF1.1 revert	GAGTGCATGTAAAAATAAATGCGATTAACCTTTGCGGTACTAC AGTCGTTTTATTTCTCCTTCTTGCCGT
P19	ORF1.4 revert	ATTTAGCCTTCCGCTCCTGTCTGCTTACACTTTACACTTTTCTG CTCGTCATGAGGCCCACGGAAGTTTC
P20	ORF1.4 revert	AGGGGTGTTTGTGAAAATAAACATAATACAACTGTGTTGAACC ACTTGTTTTAATTTTTGGATTTTGTGA
P21	ORF1.1Sequencing (Pre ORF1.1)	GGCTCCTCCCTTTTGGCTCTGG
P22	ORF1.1 Sequencing (Post ORF1.1)	TCTGGTGCTGATCGGAATAGTGTA
P23	ORF1.4 Sequencing (Pre ORF1.1)	CGTGTGCTTATCACGATGGCGATTA
P24	ORF1.4 Sequencing (Post ORF1.1)	CCGTGGATTTGGAAAGGGGT

Table 10 Primers used for sequencing and PCR confirmation for rH-P1 of FMD BAC and viral genomic DNA

Primer	**Sequence**
FMD-P1 For	GGATCCGCCACCATGGGAGC
FMD-P1 Rev	TCTAGATTACAGGGTCTGCT
FMD-P1 Pre For	CAGCCCACTAGATTTCCCAACTCG
FMD-P1 Post Rev	CGCACCTCATCTCCACCAG
FMD-P1 Seq For	CAGATCAAGGTGTACGCCAATA

5.2 Methods

5.2.1 Viruses, cells and plasmids

5.2.1.1 Viruses.

All the viruses included and used in this study were produced and recovered from infectious bacterial artificial chromosome clones of EHV-1 strain Ab4 [119], EHV-4 strain TH20p [349], modified BACs EHV-1_gB4 and EHV-4_gB1 [29] and modified BACs EHV-1_gD4 and EHV-4_gD1 [36]. Several viruses had been generated here in this study such as UL56 viruses (EHV-1delUL56, EHV-1_UL56.4, EHV-1UL56 Revert, EHV-4delUL56, EHV-4_UL56.1, EHV-4UL56 Revert), complete set of marker viruses in the small capsid VP26 protein, the one used here in our study is EHV-1^{RFP} and finally, recombinant vector vaccine RacH carrying FMD-P1 (rH-P1) and their wild-type parental RacH (rH). The restored gp2 viruses of rH and rH-P1 were also generated. Equine dermal (ED) cells were used for growing viruses except rH and rH-P1 that were grown on RK13. Some viruses express the enhanced green fluorescent protein (EGFP) to rapidly identify the infected cells.

5.2.1.2 Cells.

Primary equine carotid artery endothelial cells (EC) used in the study were a kind gift from Ilka Slosarek, Institut für Veterinär-Anatomie, Freie Universität Berlin). 293T cells were used for reconstituting EHV-4 mutants. RK13 was used for rH and rH-P1 reconstitution and all methods performed for them. Equine dermal (ED) cells, kindly provided by CCLV-RIE 1222, Friedrich-Loeffler-Institut Bundesforschungsinstitut für Tiergesundheit, Federal Research Institute for Animal Health. were used for growing EHV-1 strain Ab4 and all their mutants. Equine PBMC were isolated from heparinized healthy horses blood by using Ficoll-paque density gradient centrifugation. Blood collection was carried out under the rules and guidelines of Institutional Animal Care and the Committee of Berlin (Landesamt für Gesundheit und Sociales, L 0294/13). Equine PBMC were negative for Equine herpesviruses by qPCR and ELISA tests.

5.2.1.2.1 PBMC isolation

Equine blood samples were gently homogenized in their vacutainer. The blood was then collected in 50 ml conical tubes and let to sit for 30 min for separating clear plasma. In a new 15 ml conical tubes containing 5 ml of Biocoll L 6715, 7 ml of clear plasma was added slowly and gently over the top of bicoll. The falcon tube was then centrifuged for whole blood separation with brake off. The buffy coat region was aspirated using 1 ml micropipette over 15 ml conical falcon tube containing 10 ml chilled PBS which then centrifuged for pelleting PBMC. The PBMC was then washed twice with brake with 1x PBS. Resuspension of PBMC in PBS

then counting with the CASY counter was achieved. The Cells were then frozen in cold freezing media including FCS with 10 % DMSO at a density of 5X 10^6/mL in a Mr Frosty freezing container filled with isopropanol for 24 h at -80 ^{0}c and subsequently stored in liquid nitrogen for later use.

5.2.1.3 Plasmids

For assessing fusion assay by luciferase activity measurement, two plasmids were utilized: plasmid expressing the reporter gene encoding firefly luciferase under the control of T7 promoter (pT7EMCLuc) and the plasmid pCAGT7 expressing T7 RNA polymerase. The previous two plasmids were kindly provided by Richard Longnecker. A Plasmid expressing mRFP under human cytomegalovirus promoter (pEP-CMVmRFP-in) [353, 354] was used for assessment of fusion assay by immunofluorescence testing. pEPmRFP1-in carrying kanamycin mRFP cassette had been used as a template for generating EHV-1 strain Ab4-mRFP-VP26 (EHV-1^{RFP}). The recombinant plasmids pKS_UL56-4 and pCDNA3_UL56-1 were constructed by using the vector pCDNA3 for EHV-1 strain Ab4 and pBluescript II KS for EHV-4. The transfer plasmid (pCDNA3 UL56-1-KanR and pKS UL56-4-KanR) were constructed to be used as a template for two-step Red-mediated mutagenesis.

5.2.2 *Cloning procedures used in our assays*

5.2.2.1 Preparation of chemically competent E.coli

For chemically competent E. coli cell preparation, single bacterial colony was used to inoculate 5 ml LB medium for overnight (o/n) incubation at 37 °C with 220 rpm with shaking. 1 ml from the o/n culture was used to inoculate 100 ml LB medium containing 500 ml Erlenmeyer flask at incubator with shaker (37°C) until logarithmic growth phase of the bacteria reaching 0.45-0.6 $O.D_{600}$. Collection of the bacterial culture in pre-chilled 50ml falcon tubes for 5 min incubation. The bacterial culture was pelleted at 1000rpm/2min/4°C and resuspended at 10 ml 50 mM ice-cold CaCl2. Following resuspension and centrifugation, the supernatant was then removed, and the bacterial pellet was resuspended again in 10 ml ice-cold 50 mM CaCl2 for 30 min incubation on ice. The resuspended cells were then centrifugated at 400g/5 min/4°C. The bacterial pellet was resuspended in 2 ml 100 mM ice-cold CaCl2-15% glycerol. The competent cells were either used immediately or stored at -80°C until further use.

5.2.2.2 DNA digestion and de-phosphorylation

Restriction enzyme digestion was performed utilizing multiple restriction endonucleases enzymes for plasmid screening and verification their correct construction. DNAs digestion was carried out according to the manufacturers' instructions. Alkaline Phosphatase, Calf Intestinal

(CIP) or Antarctic Phosphatase (AP) or Shrimp Alkaline Phosphatase (rSAP) (New England Biolabs, UK) were used to dephosphorylate DNA prior to ligation which prevents the plasmid re-ligation. Briefly, the digested plasmid DNA was incubated with AP or CIP or rSAP enzyme at the recommended buffer for 1 hour at 37 °C followed by inactivation step according to the used enzyme.

5.2.2.3 DNA Ligation

Cloning sequence of interest into plasmids via cloning steps was performed using T4 DNA ligase or quick ligase (New England Biolabs, UK) according to manufacturer recommendations. NEBioCalculator (http://nebiocalculator.neb.com) was used for calculating the molarity ratio of vectors and inserts. The digested vector and insert were mixed together with a molar ratio 1:3 vector to insert respectively and incubated with quick ligase for 5 minutes at room temperature or T4 ligase for o/n on 16°C in their appropriate buffer. Only digested vector ligation without insert was used as a parallel control. The ligation mixture was then chilled on ice and transformation of the reaction (1-5 µl) into competent cells (50 µl) acheived.

5.2.2.4 Cloning steps for generating transfer plasmid of UL56 project

Transfer plasmids that encode either EHV-1 or EHV-4 *UL56* with a kanamycin resistance gene (KanR) were constructed. We amplified EHV-1 and EHV-4 *UL56* genes by PCR using primers P1, P2, P3 and P4 in **Table 9.** The PCR products were then digested with the two restriction enzymes EcoRI and BamHI and inserted into the vector pBluescript II KS for EHV-4 and pCDNA3 for EHV-1 strain Ab4, resulting in recombinant plasmids pKS UL56-4 and pCDNA3 UL56-1. To construct pCDNA3 UL56-1-KanR and pKS UL56-4-KanR, the kanamycin resistance gene (KanR) was amplified by PCR from the pEPkan-S plasmid using primers P5, P6, P7 and P8 **(Table 9).** It was then digested with the appropriate restriction enzymes and inserted into pKS UL56-4 and pCDNA3 UL56-1. Correct amplification, insertion, and orientation were confirmed by PCR, Restriction digestion and Sanger sequencing (LGC Genomics, Germany).

5.2.3 *The red recombination system and two-step en passant mutagenesis.*

Although the genetic modifications including insertion of sequences, deletions, point mutations and other modifications of herpesviruses as a large DNA microorganism are complex and difficult, red recombination system facilitates this process and acts as a superior and advantageous technique than other techniques [355]. This system is originated from Enterobacteria phage λ (lambda phage) which contains the machineries and equipments necessary for the recombination to occur. It contains three major proteins that have essential roles for this system to succeed. All of them are activated, induced and expressed at 42°C.

Gam protein maintains and keeps the inserted linear DNA from degradation by inhibiting E. coli RecBCD helicase-nuclease complex. The free 3' single strand overhangs in the DNA template was generated by the action of 5'-3' exonuclease of the homotrimer Exo protein. Beta protein has two roles: the first one is protecting the generated free 3' single strand overhangs and the second role is its achievement of homologous recombination with the target sequence to occur via its assistance in the annealing process of the single-stranded substrate to their complementary sequences. The obtained modification is site-specific and scarless.

Expression of the I-SecI gene in GS1783 occurs under an arabinose-inducible promoter.

The I-SecI gene expresses to a Saccharomyces cerevisiae homing endonuclease which makes specific cleavage to the mutated area more prone and possible. It cleaves a very large and seldom found restriction site (18 base pairs (bp)). This cleavage is very important in removal of kanamycin cassette from the mutated sequence region during the final recombination event.

5.2.3.1 Preparation of electrocompetent bacteria

The *E. coli* strain GS 1783 harboring the EHV was left for o/n growing in 2 ml LB medium containing cam antibiotic at 32°C. 100 ul from the o/n bacterial culture was used for inoculating 5 ml fresh LB media containing chloramphenicol at 32 incubation for 2-4 hrs until OD_{600} reaching 0.5-0.6. The bacterial culture was then transferred to water bath incubator 42^0C with shaker (220) for 15 min. The cultures were then chilled in a water-ice bath at 220 rpm for 20 min. Centrifugation of the bacterial culture for bacteria pelleting was done at 5000 rpm for 2 min at 4°C. Bacterial pellets were then resuspended and washed with ice-cold 10% glycerol in sterile Millipore water and finally resuspended in 50 µl of 10% glycerol. Now, the electrocompetent cells are ready to be electroporated.

5.2.3.2 Generation of mutants by two-steps en passant mutagenesis

Generally, electrocompetent GS1783 bacteria, in the first recombination step, were thawed on ice. One hundred (100) ng of the PCR product, *Dpn*I treated and purified, was added the recombination and electrocompetent bacterial cells. This DNA/bacteria mixture was then transferred to chilled electroporation cuvettes and immediately subjected to electroporation at 1.25 kV, 200 Ω and 25 µF. Afterward, the removed electroporated bacteria from the electroporation cuvette by 1 ml of pre-warmed LB-medium without antibiotics were followed by incubation for 2 h at 32 °C with 220 rpm shaking. Bacterial cells were then centrifuged for pelleting. The bacterial pellet was resuspended in residual media (~100 µl) after discarding the supernatant and then streaked out on LB agar plates with chloramphenicol (Cam) and kanamycin (Kana) for 24-48 h incubation at 32 °C. Afterward, several clones were screened

by RFLP for correct insertion at the specific locus and correct restriction pattern. For removal of the kanamycin cassette, positive co-integrates clones were cultured o/n at 32 °C in 2 ml LB containing Cam and Kana for second-step Red recombination or resolution steps. The next day, 100 µl of the o/n culture was used to inoculate 1 ml LB with Cam for 2 h with shaking at 32 °C. For inducing the I-SceI expression, 1 ml of LB containing Cam with 2 % arabinose was added and incubated for another 1 hr at 32 °C with 220 rpm shaking. Bacteria were transferred and incubated for 42 °C with shaking at 220 rpm for 30 min for activation the red recombination system. For recombination to occur, the culture was subsequently left to grow for another 3 h at 32 °C. Few µl of serial dilutions of the bacterial culture that was done in LB was distributed on LB agar plates containing Cam with 1 % arabinose and incubated at 32 °C for 48hr. Picking of single colonies and replica plating on plates containing LB agar plates with Cam and other LB agar plate with Cam & Kana. Final clones exhibiting sensitivity to Kana were further screened by RFLP, PCR and sequencing.

Table 11: Phusion® High-Fidelity DNA Polymerase protocol for two-step mutagenesis and cloning PCR protocol

PCR step	Temperature (°C)	Time	Cycles
Initial denaturation	98°C	30 sec-5 min	
Denaturation	98°C	10 s	
Annealing	Various	30 s	30 cycles
Extension	72°C	Various	
Final extension	72°C	10 min	

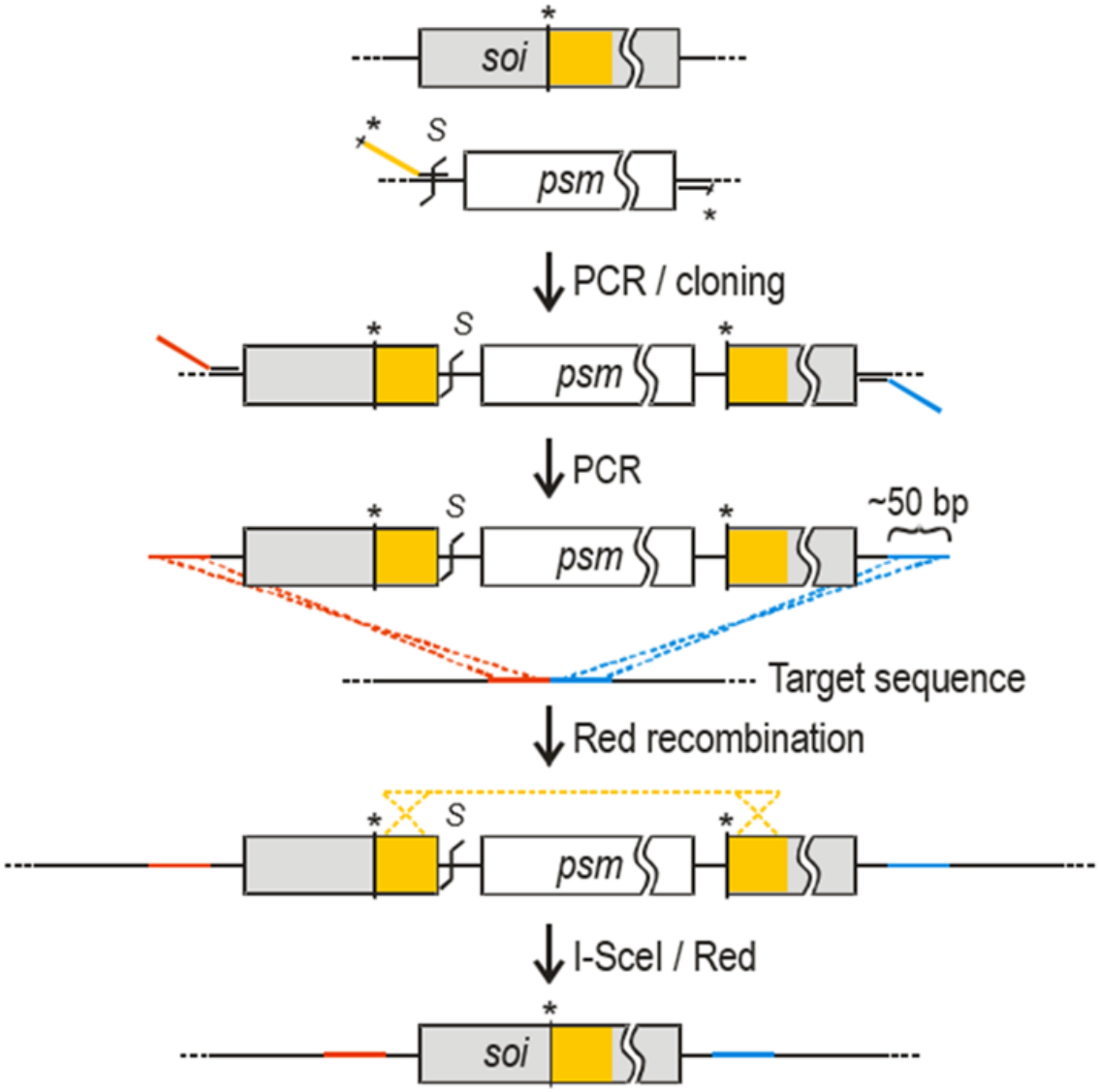


Figure 15: Schematic illustration of the two-step Red-mediated recombination for insertion of the sequence of interest (SOI). This technique has been used to insert UL56.4 into EHV-1 and UL56.1 into EHV-4 and also for revertant viruses (UL56.1 into EHV-1 and UL56.4 into EHV-4). The transfer plasmid has been constructed by cloning the PSM gene the vector containing the sequence of interest (SOI). Mutagenesis PCR has been achieved to amplify the SOI with PSM and HRs. This PCR product is then used to electroporate E.coli GS1783 to get the cointegrate clones. The correct cointegrate clones undergo a second round of mutagenesis to excise the PSM gene resulting in the final clones. Adapted from Tischer et al. [353].

5.2.3.3 BAC Mutagenesis (The role of UL56 in virus transfer from PBMC to endothelial cells project.)

All generated BACs were kept in *E. coli* GS1783, which harbor the Red-recombination system and the endonuclease I-SceI encoding gene in its genome. Two-step recombination method was employed to delete UL56 gene in EHV-1 and EHV-4. Briefly, PCR was done using primers P9-P12 (**Table 9**) to generate 50 nucleotides homology arms to substitute *UL56* by KanR. We digested PCR products with *Dpn*I and purified them to get rid of any residual template DNA. In the first step of recombination, the PCR product fragments of KanR were then electroporated into GS1783 cells harboring the BACs. Kanamycin-resistant colonies were picked and screened by PCR and RFLP to identify the correct clones exhibiting the right and correct restriction pattern and integrity. Positive clones were subjected to the second round of Red-mediated recombination, removing the KanR gene, to obtain the constructs pEHV-1delUL56 and pEHV-4delUL56. The transfer UL56-1Kan and UL56-4Kan sequences were amplified by PCR using pKSUL56-4-KanR and pCDNA3UL56-1-KanR as templates and primers P13-P16 were used. PCR products were then transformed by electroporation into GS1783 containing pEHV-1delUL56 or pEHV-4delUL56. After selection on LB agar plates containing Cam and Kana, resistant colonies were purified and screened by PCR and RFLP to detect *E. coli* containing recombinant pEHV-1_UL56-4Kan and pEHV-4_UL56-1Kan. Positive kanamycin-resistant colonies were subjected to the second round of Red recombination to obtain the final constructs pEHV-1_UL56-4 and pEHV-4_UL56-1. The UL56-1 from EHV-4 and UL56-4 from EHV-1 were deleted again by using primers that used before for the deletion (P9-P12) to insert again UL56-1 into EHV-1 and UL56-4 into EHV-4 for generating revertant mutants by using the primers P17-P20. We confirmed the genotypes of all the mutants and revertants by RFLP, PCR, and Sanger sequencing (primers P21, P22, P23 and P24…). The primers used for the construction of plasmids (pKSUL56-4-KanR, pCDNA3UL56-1-KanR, pKSUL56-4 and pCDNA3UL56-1) are listed in the **table (9)** as (P1-P8).

5.2.3.4 BAC mutagenesis in construction of Equine herpesvirus type 1 vector vaccine against FMD project

Two-step en passant mutagenesis was used to achieve insertion of the P1 cassette and subsequent excision of kanamycin (aphA1) gene into established RacH strain of EHV-1 (pH) under the control of the HCMV IE strong promoter. Briefly. Firstly, the synthetically codonly-optimized FMDP1 gene derived from FMDV strain O1 Campos/Bra/58 was cloned into pEp_CMVin transfer plasmid resulting in pEp_P1. The expression cassette involving FMDP1 under HCMV IE promoter was released from pEP_P1 and then subcloned into pUC19_ORF1/2 generating pUC19_ORF1/2-P1 as a transfer plasmid. pUC19_ORF1/2-P1 was then digested

with I-CeuI to release the fragment containing the P1 gene expression cassette, two flanking sequences, and a kanamycin resistance gene (aphAI) which afterward inserted in the location of ORF1/2 of pRacH1 via two-step en passant recombination. In the first recombination step, the P1 cassette and the aphA1 gene is inserted in the ORF1/ORF2 locus of the RacH genome, which results in transfer of kanamycin resistance into rH and formation of an intermediate recombinant rH_P1 DNA that showed a different restriction pattern from those obtained by wild-type pH BAC DNA. In the second recombination step results in the removal of the aphA1 gene generating the final construct harboring FMD-P1. Together with RFLP mapping analysis, PCR and Sanger sequencing were performed for confirmation the correct insertion of P1 gene without any mutations in the pRacH clone.

5.2.3.5 BAC mutagenesis in Virus cell-to-cell transmission mechanisms project. (mRFP1-labeled virus construction).

Insertion of monomeric red fluorescent protein (mRFP1) into VP26 of EHV-1 strain Ab4 was carried out as previously depicted [54, 353]. Briefly, mRFP1 insertion cassette containing KanR gene was amplified by PCR using pEPmRFP1-in as a template [353]. All of the used primers were listed in **Table (8).** The resulting PCR products were subjected to electroporation step into GS1783 cells harboring the corresponding Ab4 BAC ensuing the first recombination step. Kanamycin-resistant colonies were picked, purified and screened by PCR and RFLP with EcoRV and BamHI restriction enzymes. Positive clones were subjected to a second round of Red-mediated recombination to obtain the final constructs after removal of the KanR gene. The final clones were confirmed by RFLP, PCR, and sequencing.

5.2.4 *Sequencing protocols for the generated plasmids and mutants.*

After amplification of the area of interest with the specific primers using **Phusion-High fidelity DNA polymerase** following their manufactures instruction of the cycling conditions. The PCR product was then visualized on agarose gel electrophoresis (1%) stained with ethidium bromide. The PCR product was then purified using GF-1 Nucleic acid extraction Kit or HiYield Plasmid mini DNA extraction kit. According to the LCG Genomics recommendation (Berlin), the appropriate amount of PCR product was mixed with the suitable amount of primer 1 and sent for sequencing. The obtained result was then analyzed using Vector NTI 9.1 software. In case of plasmids, they were also sent for sequencing following their isolation with HiYield Plasmid mini DNA extraction kit and sent for sequencing according to manufacturer's instruction (LCG genomics).

Nanodrop: Quality and quantification of obtained DNA were assessed with a Nanodrop spectrophotometer.

5.2.5 *DNA mini, midi and large construct DNA isolation*

DNA mini-, midi, and large construct preparation. Viral and plasmid DNAs isolation from the E. coli cultures were carried out **using alkaline lysis procedures**. Generally, bacterial culture was incubated o/n at 32°C, BAC and 37°C in case of plasmids in 2 ml LB broth containing the proper antibiotics. The next day, we centrifuged the bacterial cells for their pelleting for 10 min at 5000 rpm and discarded the supernatant. The bacterial pellets were re-suspended in 100 µl of P1 buffer, lysed by vigorous inversion of 150 µl P2 buffer for 5 min at RT and neutralized by 150 µl of chilled buffer P3 on ice for 10 min. The bacterial samples were then centrifugated at 10,000 rpm for 5 min to get rid of the proteins and cell debris. The supernatant was transferred into a 2 ml new Eppendorf tube containing 1 ml of 100% absolute ethanol solution for ensuring the removal of the bacterial genomic DNA and any residual proteins. After gently mixing the supernatant with the absolute alcohol by inverting several times up and down, centrifugation steps for 10 min at 10000 rpm at 4°C for DNA precipitation were done. The precipitated DNA was then washed with 70 % ethanol. The samples were then incubated for 5 min at 37 °C and/or Eppendorf spinning to completely remove any residual ethanol. DNA was then dissolved in 1x TE-buffer containing RNase A (a final concentration 100 µg/ml).

Midiprep DNA isolation and preparation. For EHV-1 wt and its mutant viral DNA isolation and plasmid preparation, DNA midiprep and maxiprep were used for obtaining large quantity and high-quality plasmid and viral DNA according to manufacturer instructions.

Qiagen large construct kit for EHV-4 BAC isolation. For EHV-4 wt and its mutant viral DNA isolation, I used QIAGEN® Large-Construct Kit according to manufacturer instructions.

5.2.6 *Methods used for both mutagenesis and cloning steps in our study*

5.2.6.1 Colony PCR

To verify the presence or absence of our sequence of interest in plasmid or BAC constructs, colony PCR had been performed. The initial single colony transformant had been either directed used as a template in PCR reaction or used in water for short heating step. The initial heating step lysed the single transformant releasing plasmid or DAC DNA in the PCR reaction tube containing the specific primers targeting specific sequence. The amplification PCR products were then analyzed using agarose gel electrophoresis. **N.B** Viral genomic DNA was isolated for PCR confirmation of some genes from the virus stock using DNeasy blood and tissue DNA extraction kit with some modification for confirmation the inserted gene within the reconstituted virus.

5.2.6.2 DNA extraction or clean up from agarose gel

For DNA recovery from agarose gel or DNA clean-up, GF1 DNA extraction kit was used. This kit contains specially treated glass filter membrane fixed into a column for efficient binding of DNA in the presence of high salt. Briefly, after excision of target DNA fragment from the agarose gel using scalpel blade under UV light, the cut target DNA was placed into a pre-weighed microcentrifuge tube and proceeded as the manufacturer instructions (Vivantis, Malaysia).

5.2.6.3 Agarose gel electrophoresis

Visualization of PCR products and pattern of restriction digestion analysis for plasmid were done using agarose gel electrophoresis. Briefly, the agarose gel was used either 1% or 0.8 % (w/v) for small gel and big gel preparation in TAE respectively. The agarose gel was left to be completely dissolved in a microwave and then left to slightly cooled before adding ethidium bromide stock solution, 5µl (10 mg/ml). The gel was then poured into gel blocks/trays with combs and settled in the electrophoresis chamber filled up with 1x TAE buffer. BACs DNA, PCR products or plasmid DNAs mixed with 6x DNA loading buffer were loaded and analyzed on the gel. The electrophoresis conditions were 60 V for o/n gels (18/20hr) in case of BAC DNAs and 100-130 v for 20-40 min for the small gels. The 1 kb-plus DNA ladder (Invitrogen) or 1 kb DNA ladder (Solis BioDyne) was loaded in the gels as a DNA marker. Gels were visualized and photographed using UV light trans-illumination system (PeqLab, Erlangen).

5.2.6.4 Preparation of bacterial glycerol stocks

Bacteria containing the correct BACs or plasmids were stored and kept in glycerol at - 80°C for long-term use. A single correct plasmid or BAC bacterial colony was inoculated in 2 ml of LB with the proper antibiotic. Bacterial cultures were incubated o/n at 37°C or 32°C incubator with 220 rpm shaking. 600 µl of o/n grown bacterial culture were mixed with 300 µl sterile 100% glycerol in a 1.6 ml cryovial and then kept at - 80°C.

5.2.7 Reconstitution, propagation and virus titration for generated viruses

5.2.7.1 Reconstitution steps

The o/n culture from the correct PCR, RFLP and Sanger sequencing was subjected to DNA isolation using miniprep or midiprep or large construct BAC extraction kit according to the target mutant. Transfecting of the 2µg BAC DNA into the 293T cells using lipofectamine (Invitrogen) or PEI (Polysciences) then overlaying the ED by the transfected 293T cells. In case of EHV-1 mutant, direct transfection of RK13 with the 2µg mutant BAC had been

performed using PEI (Polysciences) or Lipofectamine (Invitrogen) according to the manufacturer instructions. In case of white viruses generation and restoring gp2 expression in the reconstituted viruses, we co-transfected 2 µg BAC DNA and 10 µg plasmid p71 in RK13 cells.

5.2.7.2 Virus propagation

Confluent RK13 or ED cells in a 10 cm tissue culture plate were infected with EHV-1 strains (Ab4 and pRacH and their respective mutants) or ED cells only for EHV-4 and their mutants. The infection was left until complete CPE Infection rate. The cells were then subjected to two freeze-thaw cycles.

5.2.7.3 Virus titration

For virus titration on confluent RK13 or ED, cells were infected and incubated with six viral serial dilutions followed by overlaying semi-fluid methyl cellulose. After 72 hours, plaque numbers were calculated using a standard light microscope and /or crystal violet staining and titers calculated in consequence.

5.2.8 In vitro growth properties (plaque size and growth kinetics)

5.2.8.1 Plaque size and growth kinetics (the role of viral protein UL56 in virus transfer project)

To characterize the growth properties of the generated recombinant and mutant viruses and compare them with EHV-1/4 wild-type viruses, we performed in vitro plaque sizes and growth kinetics assays. With respect to plaque size assays, they were carried out by infecting confluent monolayers ED cells with an MOI of 0.01 in case of EHV-4 and 0.001 in case of EHV-1 for 1 h at 37 °C. Afterward, the medium/virus suspension were then removed. The infected cells were then overlaid with DMEM containing 1.5% methylcellulose (Sigma). After 3 d.p.i, we captured and scanned 50 fluorescent plaques for each virus and their diameter was then measured using NIH ImageJ software. We calculated, normalized and compared the measured values to those of parental viruses, which were set to 100%. Average percentage of plaque sizes and standard deviations were calculated from three independent experiments. For determination of growth kinetics of all generated viruses compared to their wild-types, ED cells were seeded in 24-well plates, infected with MOI 1 for EHV-1 and 0.01 for EHV-4 and incubated for 1 h at 37°C. After washing twice with PBS, the infected cells were treated with citrate buffered saline (pH 3.0) for 30 secs to get rid of unbound residual viruses present on the surface of the cells. At different time points (0, 6, 12, 24, 30, 48 and 72 hr p.i.) for EHV-4 and time points (0, 6, 12, 24, 30 hr p.i.) for EHV-1, both supernatants

and infected cells were collected together as a combination at every indicated timepoint for EHV-4 or collected separately for EHV-1 and then stored at −20 °C. Viral titers were measured on ED cells. Data are from three independent experimental measurements and expressed as means ± standard deviations (error bars).

5.2.8.2 In vitro growth properties of generating Equine herpesvirus type 1 vector vaccine against FMD.

To characterize the growth properties of the recombinant virus compared to EHV-1 parental virus, we performed in vitro plaque sizes and growth kinetics assays. With respect to plaque size assays, they were carried out by infecting confluent monolayer RK13 cells with the recombinant rH-P1 and the wild-type rH viruses with dilution 10^{-5} and overlaid with 1.5% methylcellulose. Three days p.i, for each virus, 150 plaques for each virus were photographed. Mean plaque sizes were measured and analyzed using ImageJ software. For evaluating the single-step growth kinetics of the recombinant rH-P1 virus compared to wild-type rH, RK13 cells were seeded in 24-well plates, infected at MOI of 1 and then incubated for 1 h at 37°C. After washing twice with PBS, the infected cells were subjected to citrate buffered saline, CBS treatment (pH 3.0) for 30 sec to remove unbound and residual viruses. Supernatants and infected cells were collected separately at different time points (0, 6, 12, 24, 30 h p.i.). Viral titers were measured on RK13 cells. Data are from three independent experimental measurements and expressed as means ± standard deviations (error bars).

5.2.9 *Microscopy section*

5.2.9.1 Confocal microscopy and immunofluorescence for the component of extracellular carbohydrate-rich matrix

For detection of the extracellular carbohydrate-rich matrix and biofilm-like structures, confocal assay was used. $2*10^6$ PBMC were infected with EHV-1^{RFP}, MOI 0.5 for 5 minutes followed by ice-cold citrate buffer pH 3 for 1.5 min to get rid of unbound viruses. The cells were then washed three times with PBS and fixed with 4% Paraformaldehyde (PFA) for 20 min. For staining of the carbohydrate-rich extracellular matrix, Fluorescein isothiocyanate (FITC) labeled lectins ConA and lectin from Triticum vulgaris were used with a dilution 1:250 and 1:1000 respectively. The collagen, agrin and ezrin detection and colocalization were also stained by Rabbit anti-Human Collagen I/II/III/IV/V, Agrin Antibody (D-2): sc-374117 and Ezrin Antibody (3C12): sc-58758 with dilutions 1:100,1:50,1:50 respectively. Hoechst 33342 (Invitrogen; Thermo Fisher Scientific (Eugene, OR97402) were used to stain the nucleus. The image stacks were photographed using VisiScope Confocal microscope (Visitron

Systems GmbH, Germany). The images were processed and analyzed by using the NIH Image J software and the Metamorph software.

5.2.9.2 Overlay experiment

In order to test the process of transferring viruses between the two cells via confocal microscopy through the extracellular carbohydrate-rich matrix way, $1*10^6$ PBMC were infected with EHV-1^{RFP} with MOI 1. All the infected cells were treated with ice-cold citrate buffer pH 3 and virus neutralizing antibodies to get rid of any cell-free viruses. The cells were then overlaid on the endothelial cells for 2hr then followed by washing three times to remove non-adherent PBMC. Fixation of cells was done by 4%PFA followed by 3 times washing steps. FITC labeled ConA and lectin was used to stain carbohydrate-rich extracellular matrix with dilutions (1:250 and 1:1000 respectively). Image acquisition, processing, and analysis were done as before.

5.2.9.3 Heparinase and heparin treatments.

We treated infected PBMC with heparinase III (10 U ml^{-1}) for 1 h at 37 °C in serum-free medium. The cells were also treated by heparin for 30 min at 37°C in serum-free medium. The cells were then prepared for immunofluorescence confocal microscopy analysis against carbohydrate-rich extracellular matrix.

5.2.9.4 Live cell imaging microscopy experiment

For live cell imaging, equine PBMC were infected with EHV-1^{RFP} for 5 minutes then the cells were treated with ice-cold citrate buffer and virus neutralizing antibody. The infected cells were then overlaid over ibidi slide coated collagen endothelial cells. Cells were imaged in phenol red-free cell culture media (Biochrom, Berlin, Germany), but supplemented with 20% (v/v) fetal calf serum (PAN-Biotech Ltd, Aidenbach, Germany), 1% (v/v) of penicillin and streptomycin and 1% non-essential amino acid (Biochrom, Berlin, Germany). Images were acquired using a confocal laser-scanning microscope (VisiScope Confocal microscope for the first few hours of overlaying. During time-lapse sessions, the temperature on the microscope stage was held stable using an electronic temperature-controlled airstream incubator. The field area was chosen displaying red EHV-1^{RFP} viral particle on PBMC overlaying the EC. Images were captured in time-lapse every 5 minutes time series. Images and movies were generated and analyzed using the NIH image j software and the Metamorph software. Long time-lapse experiments were accomplished applying the autofocusing function toned in with the advanced time series macro set.

5.2.9.5 Electron microscopy methods

For TEM analysis, endothelial cells were grown on Correlative Microscopy Coverslips LCMC34A (Plano GmbH, Germany). After 5 min infection of equine PBMC with EHV-1^{RFP} followed by citrate buffer PH3 treatment and virus neutralization by virus neutralizing antibodies for few minutes. The infected PBMC was used to overlay the endothelial cells for different time points. After the time incubation, the cells were washed with PBS 3 times followed by 4%PFA for 30 min at room temperature. Before imaging the fluorescence signals and grid patterns at a CellDiscoverer7 equipped with an Axiocam 506 camera (Carl Zeiss, Germany), cell nuclei were stained with 2µg/ml Hoechst (Thermo Fischer Scientific, Germany) in PBS for 10 min at room temperature and kept in PBS after staining. Images were then recorded using a 20x/0.7NA Plan-Apochromat objective in combination with a 1x tube lens and a HE91 triple-band filter set (The HE91 provides a beam splitter at 450, 538 and 610 nm and emission filter at 467/24, 555/25 and 687/145 nm, respectively). After imaging specimens were post-fixed in 2.5% glutaraldehyde (Grade I, Sigma, Germany), freshly prepared in PBS from a 25% stock solution. Coverslips were then kept in glutaraldehyde for 1 night at 4°C and then washed 3 times in PBS and incubated in 0.5 % (v/v) osmium tetroxide (Science Services GmbH, Germany) in PBS for 1.0 h at room temperature. Specimens were washed 4 times in distilled water for 20 min. Samples were then incubated for 30 min in 100 mM Hepes buffer containing 0.1 % (w/v) tannic acid (Science Services, Germany), washed 3 times for 10 min in distilled water, contrasted in 2 % (w/v) uranyl acetate (Sigma-Aldrich, Merck, Germany) for 1.5 h at room temperature and washed once in distilled water followed by dehydration through a series of increasing ethanol concentrations (5 min in 30 %, 10 min in 50 % and 70%, 90%, 96%, 15 min each and finally 3 times 10 min in absolute ethanol, respectively). Samples were then incubated o/n at 4°C in 100 % SPURR (Low Viscosity Spurr Kit, Ted Pella, CA, USA). Spurr's resin mixture was replaced 2 times, 2h each, before embedding. For polymerization, coverslips were transferred between two peaces of Aclar Embedding Film (Science Services, Germany) and placed into an oven (Memmert, Germany) for one day at 60°C. Some resin dummies were prepared in embedding molds and also polymerized. In order to mount the cells in correct cutting orientation, grid pattern areas were isolated and mounted on the dummies in a second step. 70 nm sections were cut using a Leica UC7 ultramicrotome equipped with a 3 mm diamond knife (Diatome, Biel, Switzerland) and placed on 3.05 mm Formvar Carbon coated TEM copper slot grids (Plano GmbH, Germany). Sections were post contrasted using 2% (w/v) uranyl acetate and reynolds lead citrate. In order to visualize ultrastructural details, sections were imaged on a Tecnai Spirit transmission electron microscope (FEI) operated at

120 kV, which was equipped with a 4kx4k F416 CMOS camera (TVIPS). Micrographs were recorded at nominal magnification between 2100x and 21000x applying a defocus of xxx µm.

5.2.9.6 Immunofluorescence for staining of FMD-P1

For immunofluorescence (**IFA),** confluent monolayer RK13 cells in a six-well plate were infected with rH_P1 FMDV and parental virus rH with the dilution 10^{-5}. One hr p.i, media with viruses were removed and infected cells were then overlaid with 1.5% methylcellulose (Sigma). After 72 h of incubation at 37°C, the methylcellulose was removed, and cells were washed with 1× PBS and fixed in 4%PFA for 15 min at room temperature (RT), followed by two to three washing steps with PBS. The cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed twice, blocked with 2% bovine serum albumin (BSA) for 1 hr at RT and then incubated with Polyclonal antibody anti-FMD (Rabbit), Kindly supplied by martin beer, FLI, Germany, with dilution 1:1000 in blocking buffer for 1 hr at RT. Afterward, the cells were then extensively washed 3 times for 10 min with PBS, the secondary goat anti-rabbit lgG conjugated with Alexa Fluor 568, Invitrogen was added at 1: 2,000 dilution in 2.5 % BSA and incubated at RT for 1 h. After washing, we inspected and examined the fluorescence signal using an inverted fluorescence microscope with a CCD camera (Axiovert 25 and Axiocam, Zeiss).

5.2.10 *Virus transfer assays section*

5.2.10.1 Flow chamber assay

To address virus cell-to-cell transmission between PBMC and EC at the dynamics state, flow chamber assays were performed as previously described [29]. After parental (EHV-1 strain Ab4 or EHV-4) virus inoculation as described above, PBMC were subjected to ice-cold citrate buffer pH 3 treatment to get rid of any cell-free viruses. The PBMC ($1*10^6$) were infected with MOI 0.1 after 5 min infection at 37°C and on ice. The infected cells were then washed two times and resuspended in medium containing virus neutralizing antibody (VNA) with a titer 1:2.028 with a dilution 1:100 at 37°C for few minutes. Endothelial cells (ECs) were grown to confluence in 0.4 collagen IV-coated cell flow chambers (ibidi GmbH, Martinsried, Germany) that were connected to a perfusion system by Luer locks (ibidi GmbH, Martinsried, Germany). The flow chamber set was incubated at a temperature 37°C. This permitted and enabled the introduction of infected PBMC at a flow rate of 0.5 mm/s, which lies within the mammalian physiological range of 0.34 to 3.15 mm/s [356]. The velocity was calculated according to the velocity in mammalian brain capillaries and the size of the chamber and was produced by a NE-4000 double-syringe pump (New Era Pump Systems Inc., NY USA). The non-contact cells were then washed three times. After 24 h of incubation, GFP viral plaques on the EC

monolayer representing virus transfer from PBMC to EC were counted (excluding and omitting the inlet/outlet areas) using an inverted fluorescence microscope (IX-81; Olympus).

In case of studying the role of viral protein UL56 in virus transfer from PBMC to EC. After parental (EHV-1 and EHV-4) and UL56 mutant's viruses (EHV-1delUL56, EHV-1_UL56.4, EHV-4delUL56, EHV-4_UL56.1, EHV-1UL56 Revert and EHV-4UL56 Revert) inoculation, PBMC were incubated for 24 hr at 37°C, 5% CO2. The infected cells were sorted using BD FACSARIA III. $1*10^4$ GFP sorted infected cells were treated with ice-cold citrate buffer pH 3 for 1.5 min to get rid of any cell-free viruses and subjected to procedures as previously mentioned.

5.2.10.2 Contact assay

To address the transfer of EHV-1 strain Ab4, contact assay after different time points 5 min, 1 hr and 6 hrs was performed. Briefly, $5*10^5$ equine PBMC were infected with EHV-1 strain Ab4 wt and EHV-4 with MOI 0.1. All the infected cells were treated with ice-cold citrate buffer pH 3 for 1.5 min to get rid of any cell-free viruses. The infected cells were then washed two times and resuspended in medium containing VNA with a titer 1:2.028 with a dilution 1:100 [347]. EHV-4 was used as a control in our study. Overlaying the treated cells over EC was done for co-culturing for 2 hr. The non-adherent cells were then removed by extensive and gentle washing of endothelial cells three times with PBS. Methyl cellulose 1.5% was then overlaid on endothelial cells for 24 hr. The transfer of the virus from infected PBMC to endothelial cells were then assessed by counting the green fluorescent viral plaques using an inverted fluorescence microscope (IX-81; Olympus).

5.2.10.3 Contact assay for assessing the role of viral protein UL56 in virus transfer in EHV-1/EHV-4

After parental (EHV-1 and EHV-4) and UL56 mutant's viruses (EHV-1delUL56, EHV-1_UL56.4, EHV-4delUL56, EHV-4_UL56.1, EHV-1UL56 Revert and EHV-4UL56 Revert) inoculation, PBMC were incubated for 24 hr at 37°C, 5% CO2. The infected cells were sorted using BD FACSARIA III. $1*10^4$ GFP sorted infected cells were subjected to ice-cold citrate buffer pH 3 treatment for 1.5 min to get rid of any cell-free viruses. Infected cells were then washed twice and resuspended in medium containing VNA with a titer 1:2.028 with a dilution 1:100 at 37°C. Overlaying the treated cells over EC was done for co-culturing for 2 hr. The non-adherent cells were then removed by extensive and gentle washing of endothelial cells three times with PBS. Methyl cellulose 1.5% was then overlaid on endothelial cells. After 24 hr incubation, the transfer of the virus from infected PBMC to endothelial cells were then

assessed by counting the green fluorescent viral plaques using Carl Zeiss Axiovert S100 microscope.

5.2.10.4 Detachment of extracellular virus assemblies from infected cells

For destruction and disruption of carbohydrate-rich extracellular matrix, virus inoculation of $5*10^5$ equine PBMC with EHV-1 strain Ab4 GFP wt MOI 0.1 for 5 minutes. Citrate buffer pH3 and VNA with a titer 1:2.028 with a dilution 1:100 were used for the infected cells for 1.5 min and 30 minutes respectively to get rid of any cell-free viruses. The cells were then treated by several methods for observing their effect on these structures and consequently their effect on the transmission of the virus between two cells. Vigorous pipetting, heparin treatment and combination of both methods were tested. The cells had been treated with heparin for 30 min with a concentration 50ug/ml (Sigma-Aldrich). The infectious capacity of the cells and their supernatant to transfer the EHV-1 to endothelial cells were assessed by overlaying them over endothelial cells (target cells) for 2 hr. The cells were washed then three times and overlaid by methyl cellulose. The infectious capabilities of each cellular and supernatant part for every treatment were compared by the number of viral plaques after 24 hr incubation.

5.2.10.5 Transwell assay

The infected PBMC were placed into a Transwell insert (Costar; Corning, NY) representing the upper chamber of the transwell assay without direct contact between infected PBMC and EC ("no contact"). In the "no contact" situation, both cells (infected PBMC and EC) shared the same environment through 0.4 µm pores insert (Costar; Corning, NY), but PBMC were physically isolated and separated from the EC monolayer. The "no contact" situation was used to appraise the efficacy of the citrate treatment of PBMC and virus neutralization by VNA to inactivate and neutralize non-penetrated viruses. Endothelial cells were placed on the bottom chamber and cultured for 24-48 hr. Endothelial cells were examined for viral plaques using Carl Zeiss Axiovert S100 microscope.

5.2.11 Flow cytometry for assessment the expression of Adhesion molecules

Flow cytometry analysis was conducted to assess the expression levels of two specific adhesion molecules (lymphocyte function-associated antigen-1, LFA-1 and very late antigen-4, VLA-4 on the cell surface of PBMC using cytoflex S, Beckman Coulter, Inc). PBMC were infected with either parental (EHV-1 or EHV-4) and mutants (EHV-1delUL56, EHV-1_UL56.4 and EHV-1UL56 Revert) viruses or (EHV-4delUL56, EHV-4_UL56.1 and EHV-4 UL56 Revert) respectively. After twenty-four hours post-infection, cells were incubated for 1 hr with anti-VLA-4 antibodies or anti-LFA-1 (5 µg/mL; Abcam, Cambridge, UK) at 37^0C. Cells were then subjected to three times washing steps with PBS followed by 1 h incubation with goat

anti-mouse Alexa-Fluor-647 (1:400; Thermo Fisher Scientific, Darmstadt, Germany) at 37^{0}C. Finally, the cells were washed, and the expression level of cell surface adhesion molecules on PBMC was quantified among the infected GFP-expressing cells population.

5.2.12 Western blot for in vitro expression of P1 of FMD in recombinant virus (rH_P1)

To confirm transgene expression, western blot was carried out. RK13 cells were infected with the parental or recombinant EHV-1 viruses. Twenty-four hours p.i, cells were collected by cell scraper and micropipette tips and lysed using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1%SDS; 1 mMEDTA) containing benzonase (Novagen) and Complete® mini protease inhibitor cocktail (Roche). Sample buffer (1 M Tris-HCl (pH 6.8), 0.15% β-mercaptoethanol; 0.4% glycerol; 0.8% sodium dodecyl sulfate (SDS); 0.004% bromophenol blue) was added to cell lysates, the mixture was heated to 95 °C for 5 min and cell lysates proteins were separated using reducing conditions via sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) for 20 min at 60V and approximately 90 min at 130V and transferred to a PVDF membrane using the Biorad blot system. After blocking the PVDF membrane with 5% non-fat dry milk in 1X PBS-T (PBS with 0.05% [vol/vol] Tween 20), the membrane was incubated with polyclonal antibody anti-FMD (Rabbit) with dilution 1:500 in blocking buffer o/n at 4^{0}C. The secondary Goat anti-Rabbit IgG, HRP antibody with dilution 1:10000 incubated at room temp for 1hr. Reactive proteins (bands) were visualized using an enhanced chemoluminescence (ECL) detection reagent (Amersham ECL plus, GE healthcare).

5.2.13 *Cell-to-cell fusion assays*

5.2.13.1 Cell-to-cell fusion assessment by luciferase activity measurement

Luciferase activity for fusion detection between PBMC and endothelial cells was assessed according to previous publications [205, 357, 358] with modifications. Equine PBMC were nucleofected with 2µg plasmid expressing T7 RNA polymerase using Amaxa® Cell Line Nucleofector® Kit V (Lonza) according to the manufacturer instructions. Briefly, the adherent cells (endothelial cells) were trypsinized and were then pelleted by centrifugation at 90xg for 10 minutes at room temperature. The suspension cells (PBMC) were also pelleted and the supernatant part was carefully removed. The cellular pellet was then resuspended in 100 µl room-temperature Nucleofector® Solution (82 µl of Nucleofector® Solution plus 18 µl of supplement) per sample. The 100 µl of cell suspension was then combined with 2 µg DNA and transferred to nucleofection cuvette that closed after that with their cap. The cuvette with cell/DNA suspension was then inserted in their holder and appropriate program for every cell

had been applied. Immediately after nucleofection electroporation, ~500 µl of the warmed culture medium was added to the cuvette for transferring the sample into the prepared plates. Next day of nucleofection, the nucleofected PBMC were infected with EHV-1 strain Ab4 with MOI 1. Endothelial cells were also nucleofected at the same day with 2 µg of a plasmid expressing luciferase gene downstream to T7 promoter (pT7EMCLuc) and seeded in collagen-coated 24 well plate. Nucleofected infected PBMC in the third day were washed and overlaid on the nucleofected endothelial cells. The activation of the reporter luciferase gene results from fusion of nucleofected infected PBMC to nucleofected endothelial cells after 24 hr. This luciferase activity was measured and quantified by Luciferase Assay System kit (E1500, Promega, USA) using TriStar LB 941 Multimodal plate reader (Berthold Technologies) for luminescence measurement according to the manufacturer's recommendations and instructions (Promega). Briefly, after the removal of growth medium from the endothelial cells. The cells were carefully and gently washed and rinsed with 1X PBS three times for removal of non-adherent PBMC.100 µl of 1X lysis reagent was added for cell lysis in 24 well plates with scrapping the attached cells and leaving them on ice on a shaker for 15 min for complete lysis. 20µl of cell lysate was taken to be examined in 96 well plates to be assessed for luciferase activity using TriStar LB 941 Multimodal plate reader (Berthold Technologies) by which 100µl of Luciferase Assay Reagent was added to the 20µl cell lysate. Only nucleofected PBMC and only infected PBMC were overlaid on the nucleofected endothelial to serve as control negatives. As a control positive, PBMC were electroporated with both plasmids (pCAGT7 and pT7EMCLuc).

5.2.13.2 Cell-to-cell fusion by exchange of cytoplasmic contents (cell-to-cell fusion by immunofluorescence)

PBMC were nucleofected with 2 ug of plasmid expressing mRFP using Amaxa® Cell Line Nucleofector® Kit V (Lonza), Briefly pelleted PBMC were washed twice by PBS followed by resuspension in 100 µl room-temperature Nucleofector® Solution. The cell suspension was combined with 2 µg of plasmid expressing mRFP and electroporated using Nucleofector® Program (U24). The next day, nucleofected PBMC were infected by Ab4 GFP wt for another 24hr. Overlaying and co-culturing the nucleofected infected PBMC on the top of endothelial cells was at 37^0C for 3.5 hr. Endothelial cells were washed and fixed with 4% formaldehyde in PBS (4%PFA) for 25 min and then washed and examined under VisiScope Confocal microscope ×40 magnification. Images were pseudocolored according to their respective emission wavelengths, merged and processed using the NIH Image J software and the Metamorph software.

5.2.14 *Statistical Analysis*

Statistical analyses were performed using SPSS 23 and GraphPad Prism 7. Data sets were analyzed for the normal distribution using the Shapiro-Wilk normality test. Normally distributed Data sets were evaluated with one-way analysis of variance (ANOVA) for more than two samples followed by multiple comparison tests for the groups and Student's t-test for two samples. Two-way ANOVA test was used for analyzing the normally distributed grouped samples. Non-normally distributed data sets were subject to the Kruskal-Wallis test for one-way analysis of variance or with the Mann-Whitney U test for two-way analysis of variance for more independent samples followed by multiple comparison tests =*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

6 Results

6.1 Virus embedding in the carbohydrate-rich extracellular matrix structures

6.1.1 *Viral clustering and embedding in carbohydrate-rich extracellular matrix structures.*

One of the main factors in viral pathogenesis and virulence is their ability to spread from cell to cell. Direct cell-to-cell contact spread has been established for several viruses to overcome the innate and acquired immunity. The role of ECM in HTLV spread and transmission between lymphocytes has been described [228]. HTLV particles were shown to be transiently stored in a virus-induced ECM mechanism on the surface of infected lymphocytes. The mechanism of EHV-1 cell-to-cell spread is still unknown and has not been addressed. Here, confocal microscopy analyses were carried out to localize virus particles with respect to ECM and plasma membrane of PBMC.

For EHV virus tracking, I generated a set of marker viruses by two en-passant mutagenesis. The one that I used here in my study is EHV-1^{mRFP} in which insertion of monomeric red fluorescent protein (mRFP1) into to the small capsid protein VP26 of EHV-1 strain Ab4 was achieved. Generally, the extracellular matrix has been included in virus attachment and entry. It has an additional role in clustering of viral particles. To test the glycan rich carbohydrate component of this extracellular matrix, FITC labelled plant lectins, ConA and lectin from Triticum vulgaris (WGA) were used for staining PBMC after 5 min infection at 37°C, citrate treatment to get rid of unbound viruses and fixation with 4% PFA (**Figures 16**A and **16**B). Interestingly, our confocal immunofluorescence data showed viral signals (either single or clusters) colocalizing with these ECM components. ECM detection, expression and spatial organization had been investigated for infected cells and uninfected cells (**Figures 18**A and **18**B). The 3D image with surfacing after 5 min infection of PBMC (**Figures 17**D1-**17**D6) showed colocalization and embedding of EHV-1 viral particles in these structures in different views. Our data showed no significant redistribution or overexpression among infected and uninfected cells (**Figures 19**A, **19**B and **19**C). Surprisingly, after 1 hr and 24 hr of infection, the EHV-1 viral particles were imaged colocalizing on theses ECM structures on the surface of infected PBMC **(Figures 17**A and **17**C). In all time points, viral particles were observed outside the cells, even after 24 hr infection the viral particles were shown outside the infected PBMC or even inside and outside PBMC colocalizing with ECM (**Figures 17**B and **17**C). To assess their distribution, reorganization and even overexpression after timepoint infections, different time points (5min, 1 hr and 24 hrs) had been tested.

6.1.2 *Components of extracellular matrix*

We next looked for the proteinous component of extracellular matrix and their coclustering with EHV-1 viral particles. We carried out confocal microscopy analyses after 5 min infection of PBMC and staining against several surface proteins including collagen, agrin and ezrin. Collagen is one of the most structural protein components of extracellular matrix. This structural protein showed colocalization with virus particles (**Figure 16**C). The heparan sulphate agrin protein has a role in neural, viral and immunological synapses. Coclustering of agrin and viral community particles had been shown in (**Figure 16**D). Ezrin showed weak colocalization with viral particles (**Figure 16**E). Non-infected PBMC, stained against several surface proteins including collagen, agrin and ezrin, were shown in **Figures 18C**-**18**E. Heparinase enzyme treatment of the 5 min infected PBMC to hydrolyze heparan sulphate or other extracellular matrix proteins resulted in destruction and fractionation of extracellular matrix with their embedded viral particles indicating their involvement as an extracellular matrix component.

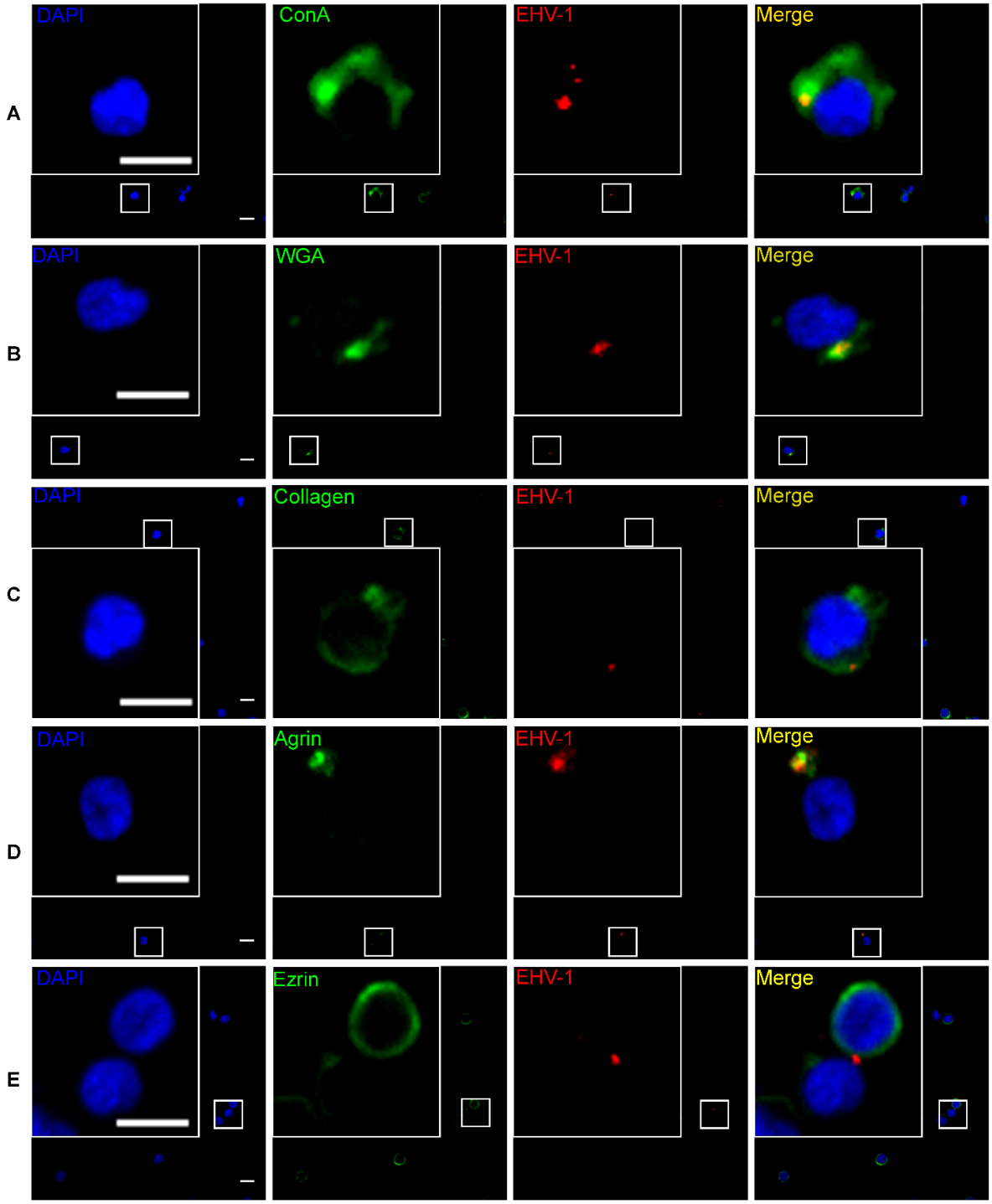


Figure 16: Colocalization of virus particles with the carbohydrate-rich extracellular matrix. Equine PBMC were infected with EHV-1^{RFP} (MOI=0.5) for 5 min. Cell surface glycoproteins of the ECM was stained green with ConA **(A)**, lectin from Triticum Vulgaris (WGA; **B**), anti-collagen **(C)**, anti-agrin **(D)**, or anti-ezrin **(E)**. PBMC nucleus was stained with DAPI (Blue). Data are representatives of three independent experiments. Scale bar =10 µm and Scale bar of magnification =7 µm.

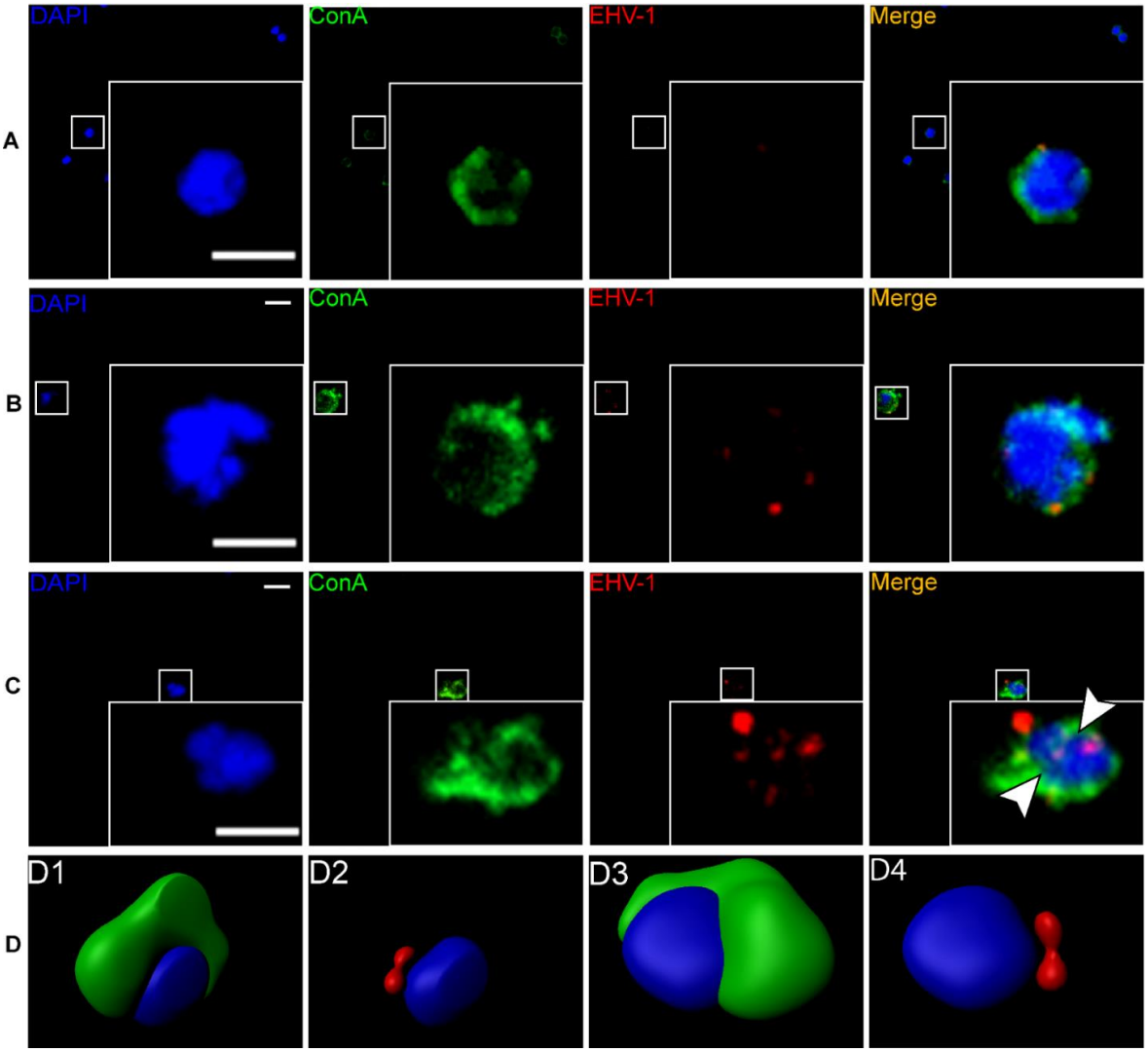
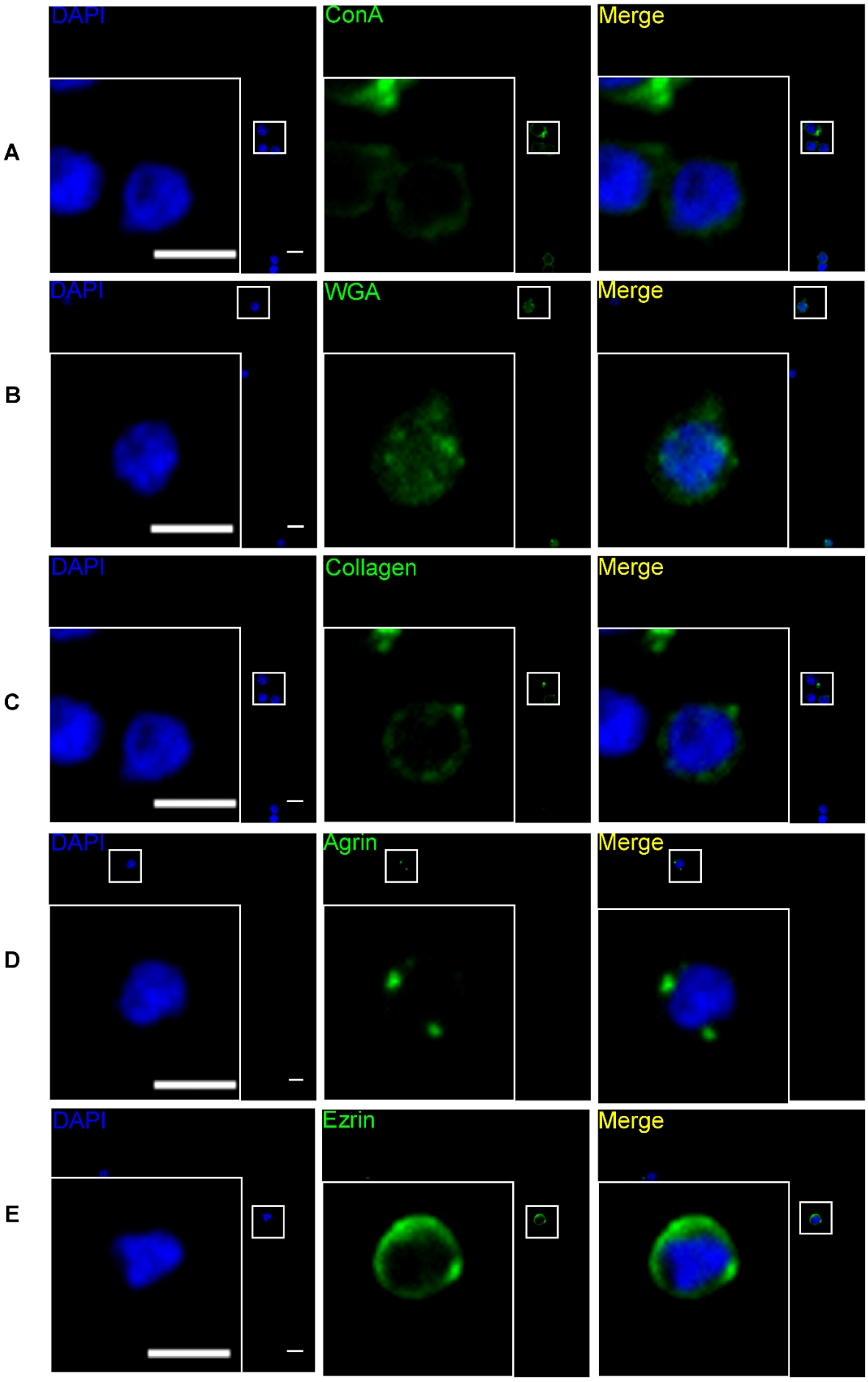


Figure 17: Colocalization of virus particles with the carbohydrate-rich extracellular matrix at different time points. Equine PBMC were infected with EHV-1^{RFP} (MOI=0.5) for 1 hr and 24 hr. Cell surface glycoproteins of the ECM were stained green with ConA **(A).** PBMC nucleus was stained with DAPI (Blue). Data are representatives of three independent experiments. Scale bar =10 µm and Scale bar of magnification =7 µm. 3D overview with surfacing of EHV-1 infected PBMC stained with ConA for extracellular carbohydrate-rich matrix at different overviews, side overview **(D1 and D2)** and back overview **(D3 and D4)**, showing embedding of viral particles (red) in extracellular matrix stained with ConA (green).


A
DAPI
ConA
Merge
B
DAPI
WGA
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C
DAPI
Collagen
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D
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Agrin
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E
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Ezrin
Merge

Figure 18: Non-infected equine PBMC extracellular matrix component staining. Cell surface glycoproteins of the non-infected equine PBMC. ECM were stained green with ConA **(A)**, lectin from Triticum Vulgaris (WGA) **(B)**, anti-collagen **(C)**, anti-agrin **(D)**, or anti-ezrin **(E)**. PBMC nucleus was stained with DAPI (Blue). Data are representatives of three independent experiments. Scale bar =10 µm and Scale bar of magnification =7 µm.

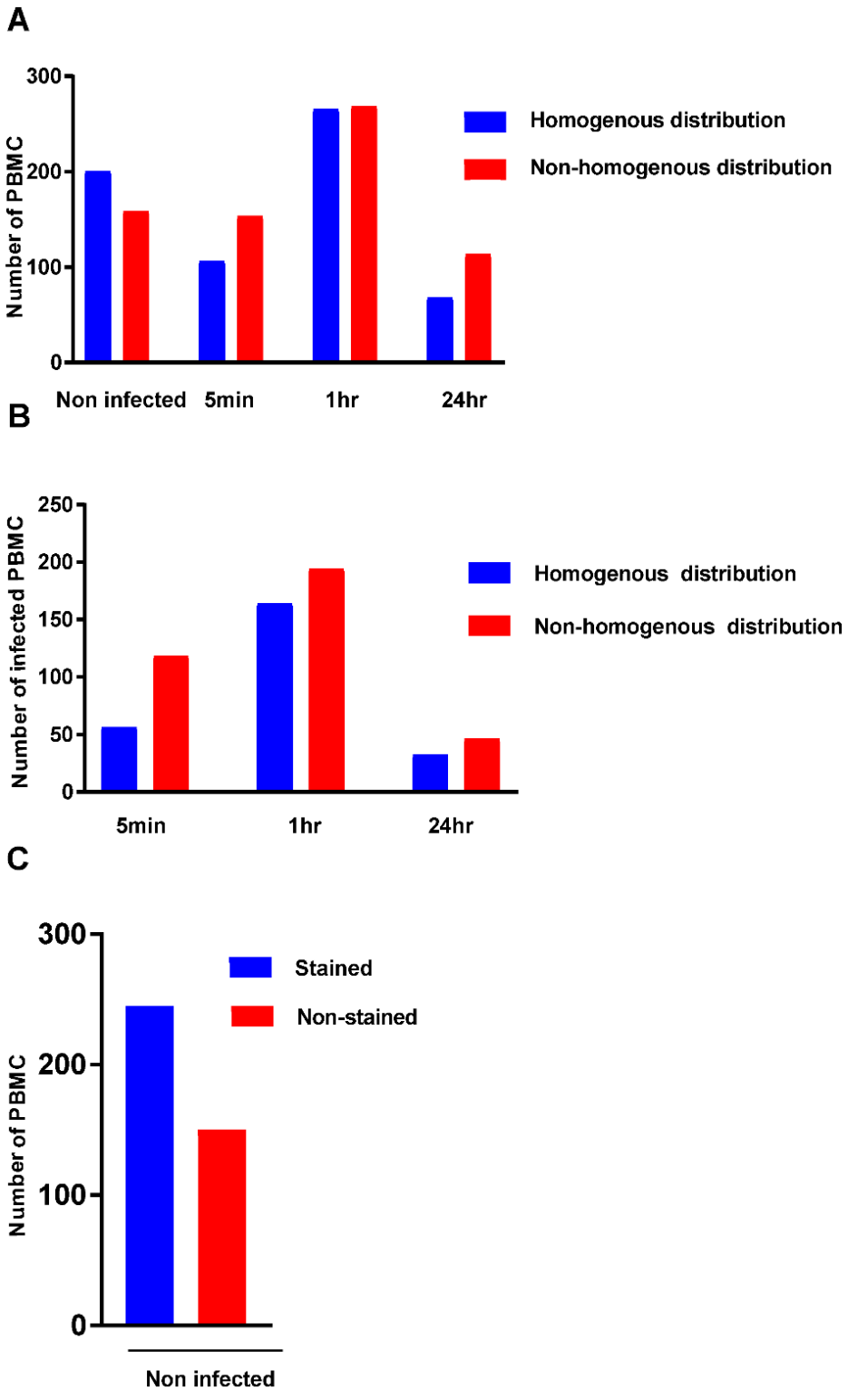

Figure 19: ECM reorganization in infected and non-infected equine PBMC: Assessment and screening for the homogeneity and non-homogeneity distribution among non-infected PBMC and different time points infected PBMC (5min, 1 hr and 24 hrs) stained with ConA **(A)** and lectin from Triticum Vulgaris (WGA) **(B and C)** for assessing viral effect on reorganization of this extracellular matrix.

6.1.3 *Spread of ECM-associated viruses from infected PBMC to endothelial cells.*

Although seeing is believing, combining imaging experiments with infectivity experiments makes the data more convincing. Although static conditions were represented by contact assay between infected PBMC and EC, dynamic conditions were employed representing the flow conditions in between the two cells. In both cases, the non-contact conditions between the infected PBMC and EC were represented by transwell assay for excluding cell-free viruses by citrate treatment and virus neutralizing antibody. For testing the role of extracellular matrix structure in virus transmission from infected PBMC to endothelial cells (EC), these overlay assays were done (contact assays, flow chamber assays and transwell assays). To assess virus spread from PBMC to endothelial cells via ECM mechanism, PBMC were infected with EHV-1-GFP for different time points (5 min, 1 hr, or 6hr). Citrate treatment and virus neutralization by VNA were done to get rid of any cell-free viruses. The cells were then overlaid endothelial cells for 2 hr followed by several washing steps to remove unbound PBMC and methyl cellulose overlay was achieved. Interestingly, EHV-1 transmission from infected PBMC to endothelial cells occurs at all time points in the presence of neutralizing antibodies involving the early 5min infection as shown in **Figure 20**D. This demonstrates that ECM-embedded viruses on the surface of PBMC were able to transmit, spread and replicate in the target EC without previous PBMC entrance **(Figure 16).** As contact assay showing the static condition for virus transmission from PBMC and EC (**Figures 20**B **and 20**D), we tested our investigation using flow chamber assays representing the dynamic conditions (**Figures 20**C and **20**E). Early rolling 5 min EHV-1 infected PBMC (4°C or 37°C) after citrate treatment and virus neutralization over the confluent monolayer endothelial cell at the physiological conditions could transmit the virus from the infected PBMC to EC in presence of virus neutralizing antibodies. Our data showed that infected-PBMC could transfer the virus to EC under flow condition (**Figure 20**E). In all cases, no virus transfer occurred in the non-contact condition in the transwell assay **Figures 20**D-E**.** To check and visualize virus transfer from infected PBMC, we did overlay assays using confocal microscopy. PBMC were infected form 5 min, subjected to citrate treatment and virus neutralization, overlaid on EC for 2hr and then subjected to three times washing steps, PFA fixation and extracellular matrix staining to check virus spread. Our resulting immunofluorescence confocal data came in lines with other biological data (infection assays) showing the viral particles on the surface of the infected PBMC overlaying the endothelial cells that were also infected through viruses spread to endothelial cells via extracellular matrix structures (**Figures 20**F and **20**F). Their 3D image with surfacing for the overlay immunofluorescence **Figure 20F** had been shown in (**Figures 21**A.1-A.3) showing embedding and transfer of viral particles in these structures in different views. This data

strongly displays the relevance of extracellular carbohydrate-rich extracellular matrix in herpesviruses cell-to-cell contact transmission.

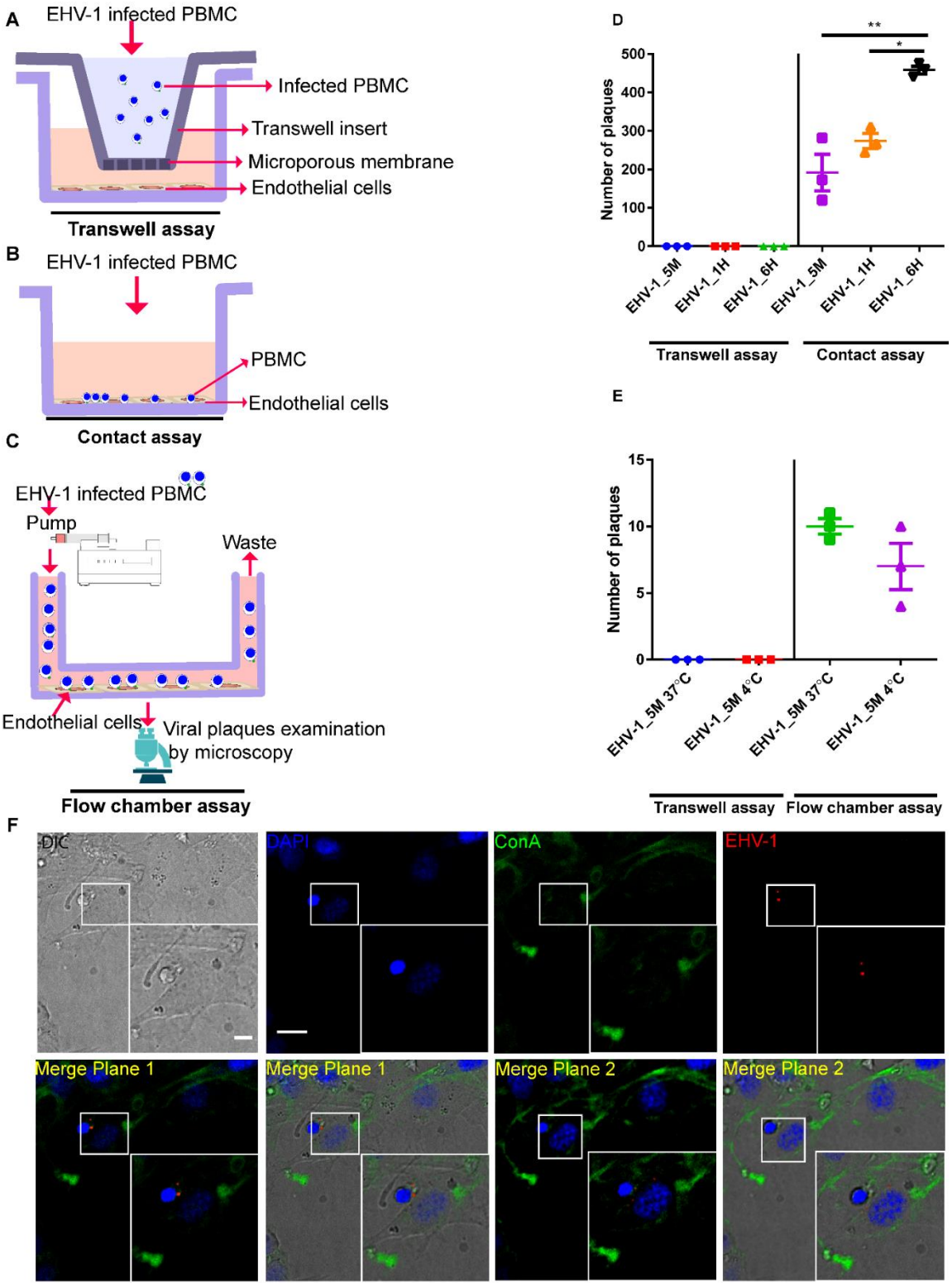

Figure 20: Virus transmission from infected PBMC to EC under "static" and "dynamic" state.

Schematic depiction of transwell- **(A)**, contact- **(B)**, and flow chamber- assays **(C)** is shown. PBMC were infected with EHV-1 (MOI=0.1) for different time points (5 min, 1 hr, and 6 hr). Infected PBMC were overlaid on EC under "static" conditions **(D)** or allowed to flow over EC "dynamic" **(E)** in the presence of neutralizing antibodies. Under dynamic conditions, PBMC were infected for 5 min either at 37°C or 4°C **(E).** After 24 h, virus spread was assessed by counting the plaques on EC excluding the inlet and outlet. As a control, infected PBMC were placed into a Transwell insert without direct contact between PBMC and EC "no contact". The data represent the mean ± standard deviations (SD) of three independent and blinded experiments. Significant differences in plaque numbers were seen between the different infection points under "static" conditions (*) (n=3; One-way ANOVA Test followed by multiple comparisons tests; $P<0.05$). **(F)** Confocal microscopy of overlaid infected equine PBMC on endothelial cells for 2hr, fixed, and immunostained with ConA (green), EHV-1 viral particles (red) showing viral particles on PBMC and endothelial cells in two different planes within the same stack. Data are representative of three experiments for checking viral transfer after 2 hrs between infected PBMC and endothelial cells. Scale bar =15 µm and Scale bar of magnification =7 µm.

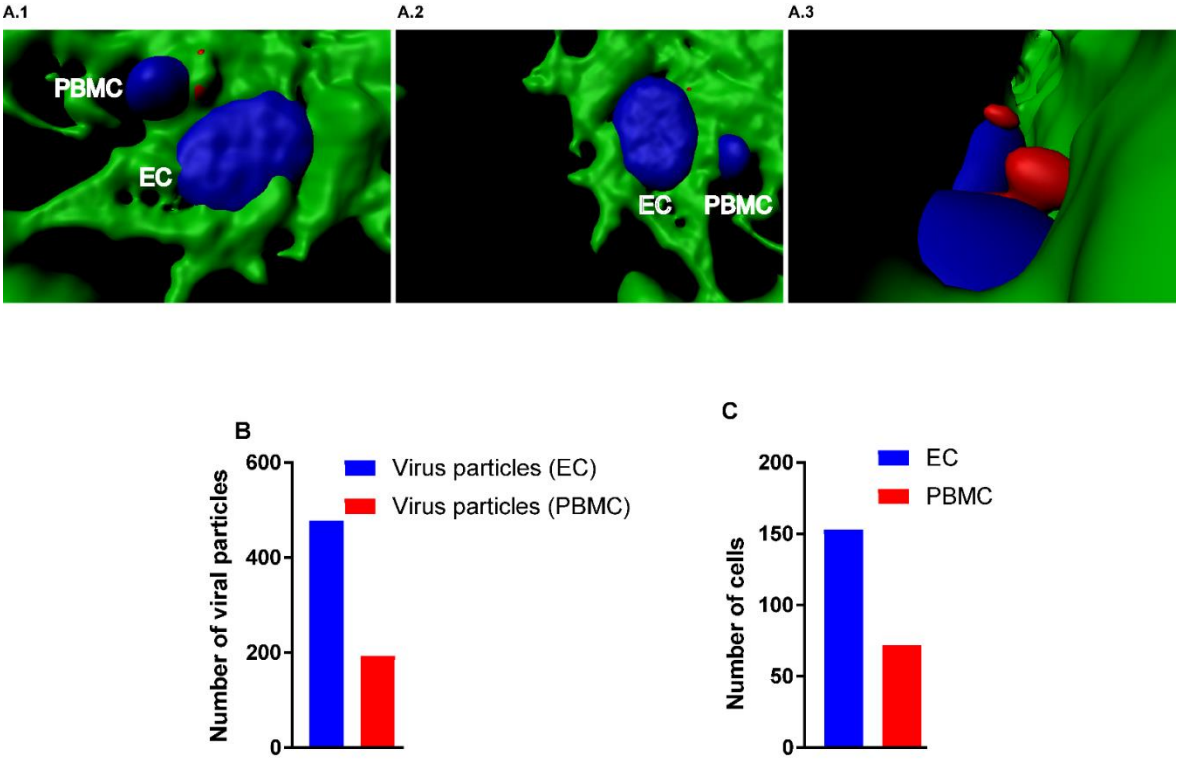

Figure 21 Virus transmission from infected PBMC to EC. 3D overview with surfacing of overlaid infected equine PBMC on endothelial cells for 2hr, fixed, and immunostained with ConA (green), EHV-1 viral particles (red). Virus transfer to

endothelial cells and their embedding within extracellular matrix is shown at different overviews (anterior overview **(A.1)**, side interior overview **(A.2)** and back overview **(A.3). (B and C**) showing viral particles embedded in PBMC extracellular carbohydrate-rich matrix and also the transferred one to endothelial cells following their overlaying on endothelial cells for 2hr followed by fixation.

6.1.4 *Time-lapse live cell imaging for tracking EHV-1 spread from PBMC to EC*

To track EHV-1^{RFP} transmission and spread from the surface of infected-PBMC to EC, we performed time-lapse live cell imaging. After 5 min infection of PBMC with EHV-1^{RFP}, citrate treatment and virus neutralization by VNA, the cells were overlaid EC and visualized by time-lapse live cell imaging confocal microscopy. Surprisingly, EHV-1 movement from infected PBMC to EC was monitored at the early time points and virus transfer and spread to endothelial cells was visualized after 3 hr post overlaying (**Figure 22 and movie 1**). These data strongly display the relevance of extracellular carbohydrate-rich extracellular matrix in herpesviruses cell-to-cell contact transmission.

Taking all previous results together, they reveal the role of extracellular carbohydrate-rich matrix in virus PBMC-EC transmission and physically protect the virus against virus neutralizing antibodies. This carbohydrate-rich extracellular matrix not only has a role in virus transmission between PBMC and EC but also likely has a role in viral transfer between PBMC themselves because of the high tendency of PBMC aggregation together in the infection- than the non-infection state **(Figure 23)** [169, 359].

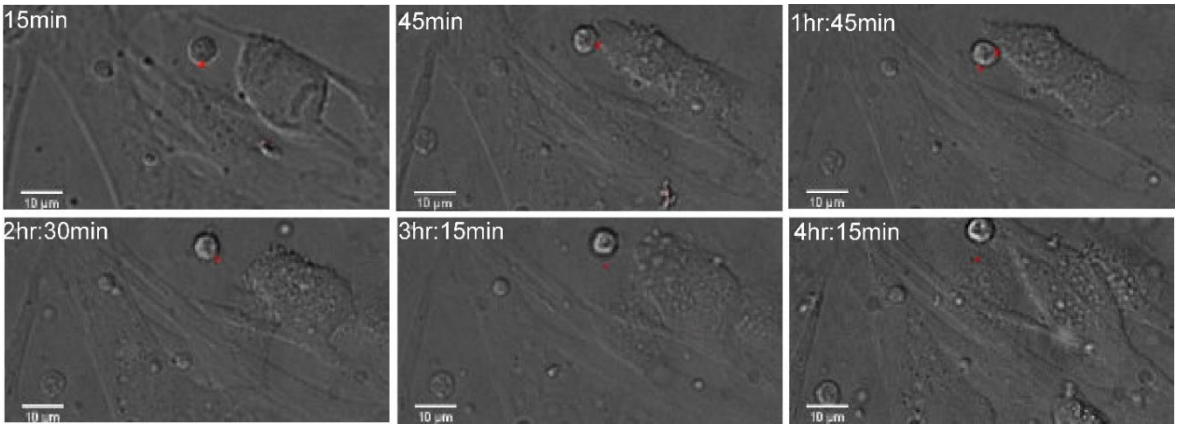

Figure 22: Live cell imaging of virus transmission from infected PBMC to endothelial cells. Time-lapse live cell imaging confocal microscopy showing EHV-1^{RFP} viral particles transfer from the overlaid 5 minutes infected PBMC to the endothelial cells. The viral particles had been visualized transferring following PBMC overlaying on the endothelial cells. Interestingly the viral particle had been shown transferring at

early time points approximately after 3 hr overlaying. The different time points of virus PBMC-EC transmission have been demonstrated and their movements are tracked and visualized in the supplementary video.

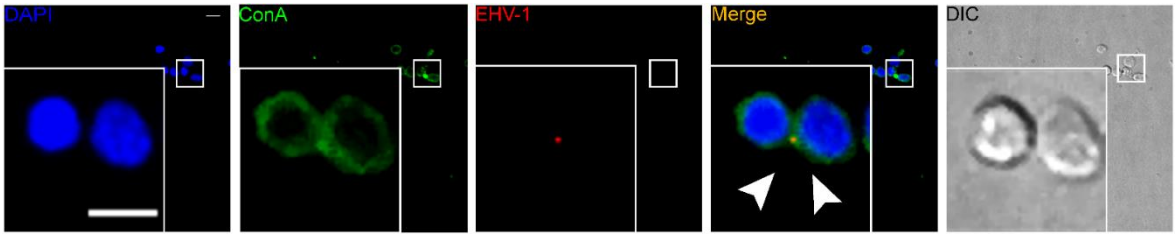

Figure 23: EHV-1 transfer in between the aggregated PBMC. PBMC infected with EHV-1^{RFP} (red) for 1 hr and stained with ConA for ECM surface glycoproteins (green) displaying aggregates of two PBMC together with the likelihood of virus transfer in between. Scale bar =10 µm and Scale bar of magnification =7 µm.

6.1.5 *Destruction of ECM-associated virus clusters reduces virus spread.*

To reveal the prominence of extracellular carbohydrate-rich matrix in EHV-1 virus transmission from PBMC to EC, extracellular viral assemblies were destructed and removed by mechanical method via vigorous pipetting, chemical method via heparin treatment for competing and eluting extracellular matrix that was removed by several washing steps and finally, by combining both methods together**.** Infected PBMC were followed by mechanical and/or chemical treatment, washed several times and added to EC monolayers. Interestingly, the infectious capability of the infected cells after chemical competing and destruction or combining mechanical and chemical methods together was decreased to the endothelial cells which represents virus spread and transfer manifested as a decrease in the infection rate (viral plaques number in endothelial cells) (**Figure 24**A). The treated infected cells were still infectious because the extracellular viral assemblies structure or ECM weren`t completely removed by heparin treatment or extensive pipetting and the remaining ECM-virus clusters on the surface of PBMC can still transfer the virus as shown in immunofluorescence data to (**Figure 24**D). Cell supernatants containing extracellular virus assemblies structures as displayed in figure (**Figure24**) obtained after cell washing were able to infect the endothelial cells and showed a significant increase in infectivity rate in the presence of virus neutralizing antibodies (**Figure 24**B). These results indicate that integrity of extracellular matrix is crucial in virus transmission from PBMC to endothelial cells.

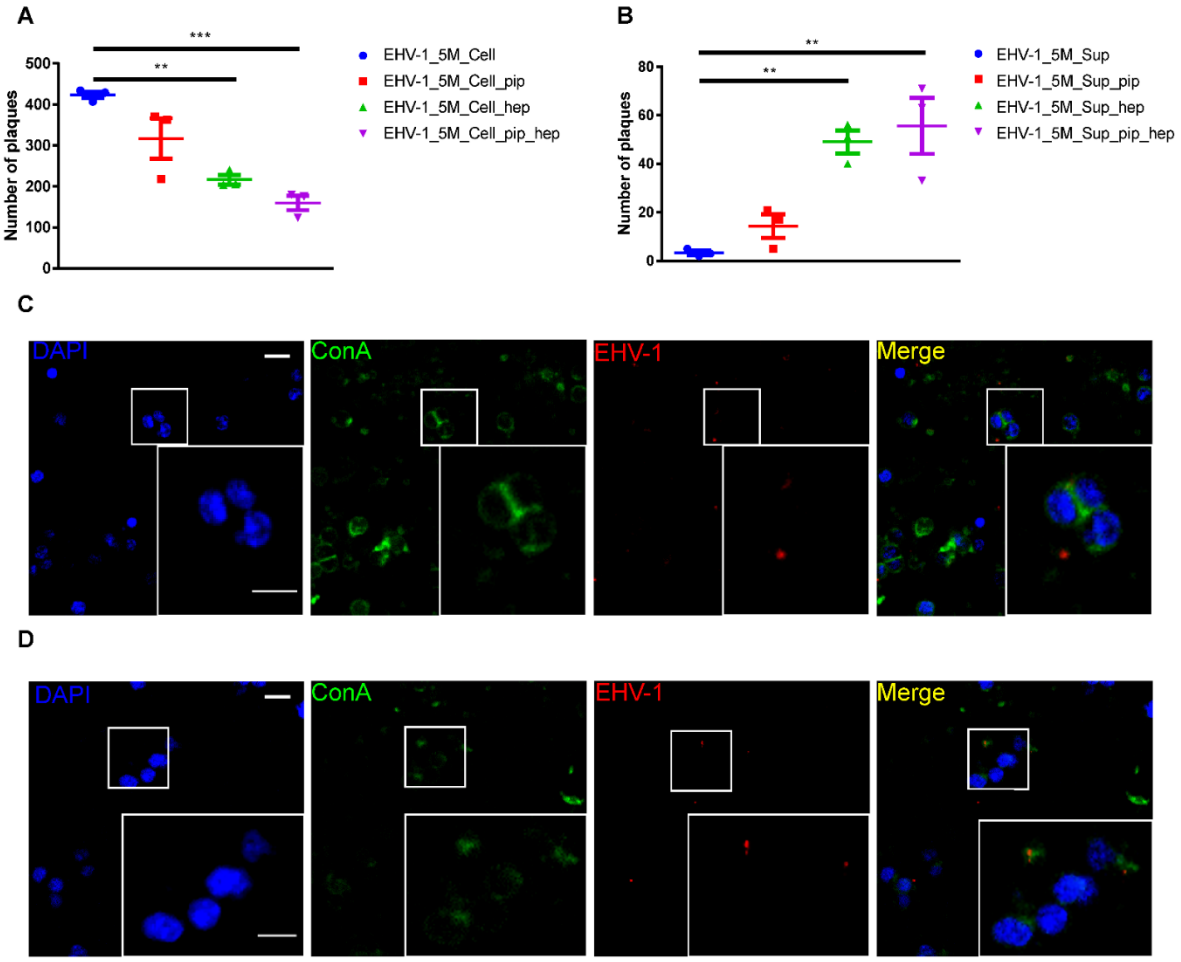

Figure 24: Relevance of extracellular carbohydrate-rich matrix in EHV-1 cell-to-cell spread. PBMC were infected with EHV-1 (MOI=0.1) for 5 min. The ECM were disrupted mechanically (extensive pipetting; EHV-1_5M_Cell_pip), chemically (heparin treatment; EHV-1_5M_Cell_hep), or both (EHV-1_5M_Cell_pip_hep). The disrupted cells **(A)** or the supernatant (EHV-1_5M_Sup_pip, EHV-1_5M_Sup_hep, EHV-1_5M_Sup_pip_hep; **B**) were added to EC. As a control, the ECM was left undisrupted (EHV-1_5M_Cell or EHV-1_5M_Sup). The data represent the mean ± standard deviations (SD) of three independent and blinded experiments. Significant differences in plaque numbers on EC were seen between the different treatment procedures. (*) (n=3; One-way ANOVA Test and Dunnett's multiple comparisons test; $p < 0.05$). **(C and D)** Treatment with heparinase III or heparin fractionates and elutes ECM-viral assemblies. Confocal microscopy showing infected PBMC with EHV-1^{RFP} (red) treated with heparinase III (C) or heparin (D) and stained for cell surface carbohydrate-rich matrix with ConA (green). PBMC nucleus was stained with DAPI (Blue). Scale bar =10 µm and Scale bar of magnification =7 µm.

6.1.6 Transcellular migration of PBMC within the endothelial cell body.

Following the PBMC-EC contact interface, some PBMC could do some migration events through the endothelium. Leucocyte Transmigration through endothelial cells can be paracellular (between the cells at cell junction) or transcellular (through the cell body itself) [143, 360]. PBMC were infected for 5 min with EHV-1 and added to EC monolayers. As paracellular PBMC migration events happen in between the endothelial cells, we could interestingly capture a transcellular endothelial migration event through the endothelial cell body itself by EM **(Figure 25).** This migration represents a secure, efficient and safe way of EHV-1 transmission through the body itself. This transcellular migration represents a novel strategy and mechanism of direct cell-to-cell spread of viruses.

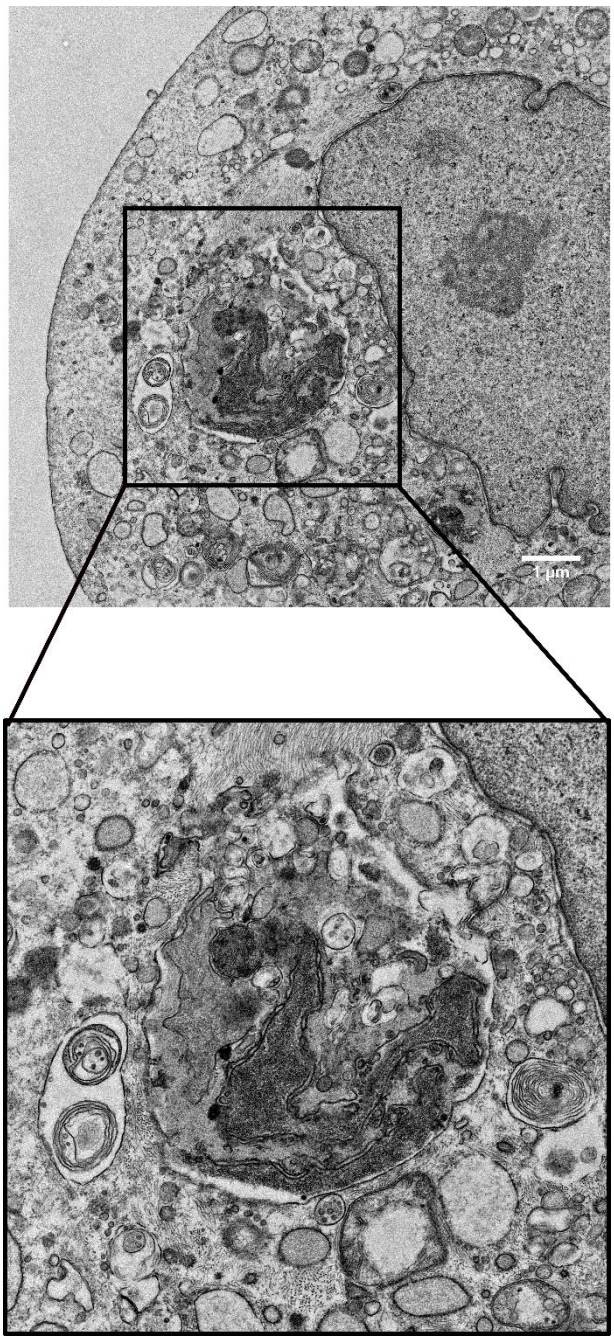

Figure 25: Electron microscopy showing transcellular PBMC migration into endothelial cells. PBMC were infected for 5 min followed by overlaying the endothelial cells.

6.1.7 *Cell-to-cell fusion events between infected PBMC and EC.*

To visualize cell-cell fusion events between PBMC and EC, PBMC were transfected with m-cherry red fluorescent protein (mRFP)-expressing plasmid and then infected with GFP-expressing EHV-1 for 24 hr. PBMC were cocultured with target uninfected endothelial cells for 3 hrs. Upon adhesion of late infected PBMC, it fuses to the underlaying uninfected endothelial cells in presence of neutralizing antibodies. Following their fusion to endothelial cells, red

cytoplasm from the EHV-1 GFP late infected PBMC transferred to the overlaid endothelial cells. Fluorescence microscopy revealed fusion events, identified by transfer of virus cytoplasmic material to the target cell (**Figure 26**A). Furthermore, another luciferase activity assay was done to test cell-to-cell fusion. This luciferase activity induced and recorded as due to virus cell-to-cell fusion occurrence. PBMC were transfected with a plasmid expressing T7 RNA polymerase then infected for 24 hr with EHV-1 or as negative controls (transfected PBMC only without infection or infected PBMC only without transfection), overlaid the target endothelial cells transfected with plasmid expressing luciferase gene downstream to T7 promoter. As a positive control, PBMC were transfected with both plasmids (plasmid encoding T7 RNA polymerase and the other encodes luciferase under a T7 promoter). We could see PBMC-EC fusion events only in the setting at which PBMC were transfected and infected then overlaid on transfected EC. Both plasmids transfection in PBMC gave the same luciferase activity (**Figure 26**B)

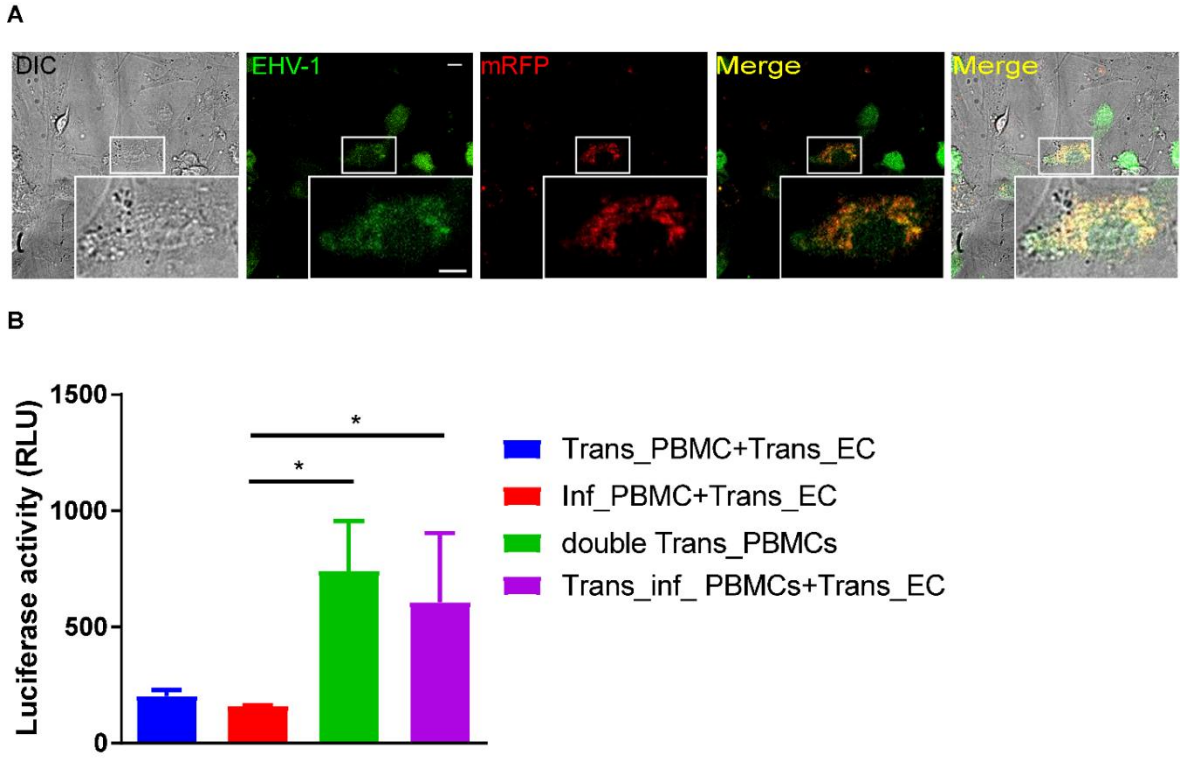

Figure 26: Cell-cell membrane fusion induced by EHV-1. **(A)** Fusion between PBMC and EC was visualized using confocal laser scanning microscopy. Expression of m-Cherry RFP protein is indicated by (red) and EHV-1 replication is represented by (green) due to GFP expression. Colocalization of both colors is shown in the merge panel. Scale bar =10 µm and Scale bar of magnification =7 µm. **(B)** Fusion between infected PBMC and EC was further detected by luciferase activity. Results are shown as means of three independent and blinded experiments with standard errors and are presented as luciferase luminescence Unit (RLU). *, $P < 0.05$ using Kruskal-Wallis

test_Dunn`s test for multiple comparisons. Infected PBMC only (Inf_PBMC+Trans_EC) and transfected PBMC only (Trans_PBMC+Trans_EC) were used as negative controls. Control positive is represented by transfected PBMC with both plasmids (double Trans_PBMC).

6.1.8 *No transmission events for ECM-clustered EHV-4.*

We next examined the closely relative to EHV-1, EHV-4 to check if these ECM-associated viruses mechanism is occurring spontaneously or not. The pathogenesis of EHV-4 is mainly restricted to upper respiratory tract infection. Here I show that EHV-4 like EHV-1 embedded and clustered in the ECM of PBMC showing extracellular viral assemblies (**Figure 27**A). Interestingly, EHV-4 associated ECM isn`t able to spread to uninfected target cells either on static condition of PBMC on EC (contact assay) (**Figure 27**B) or even flowing the EHV-4 associated ECM of PBMC over EC (flow chamber assay) (**Figure 27**C).

To check if destructing these extracellular carbohydrate-rich viral assemblies matrix could enhance or affect EHV transfer, we destructed them by both chemical and mechanical methods together (extensive pipetting and heparin treatment together). Both the cellular part or the destructed extracellular matrix released to the external environment media after destruction (supernatant part) weren`t able to transfer EHV-1 to endothelial cells **(Figures 27D.1 and 27D.2)** despite the EHV-4 free infectious viruses are able to infect EC which is permissive to EHV-4 infection leading to plaque formations **(Figure 27E).** A possible explanation is that EHV-4 is limited in its spread by ECM as occurred in Newcastle disease virus or the virus was trapped and inactivated in these structures hindering them from spreading and infecting EC.

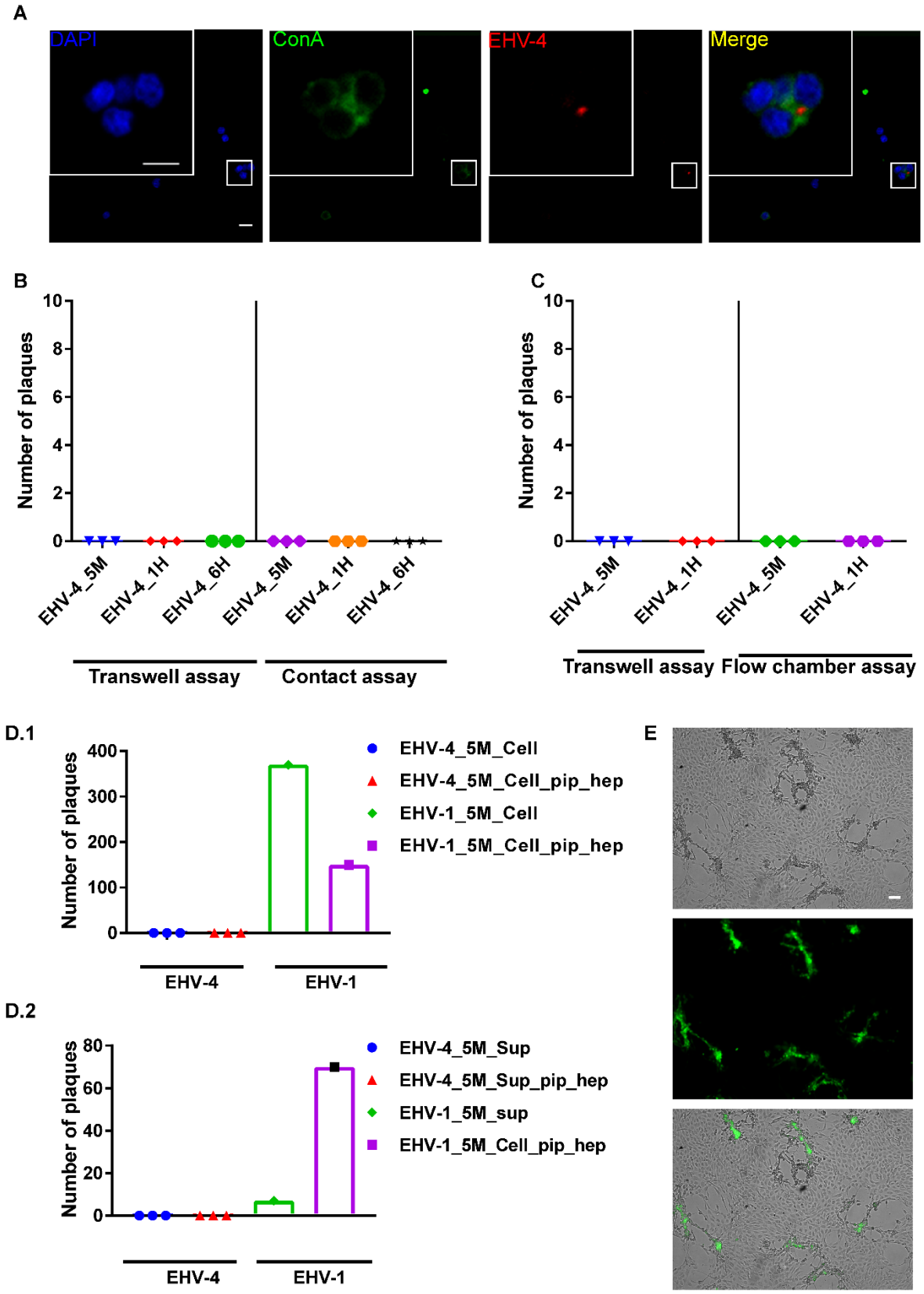

Figure 27: Relevance of extracellular carbohydrate-rich matrix in EHV-4 cell-to-cell spread. (A) Colocalization of EHV-4 virus particles with the carbohydrate-rich

extracellular matrix. Equine PBMC were infected with EHV-4 (MOI=0.5) for 5 min. Cell surface glycoproteins of the ECM were stained green with ConA (A). Viral proteins immunostaining against EHV-4 glycoprotein D (Red). PBMC nucleus was stained with DAPI (Blue). Data are representatives of three independent experiments. Scale bar =10 µm and Scale bar of magnification =7 µm.

(B and C) EHV-4 transmission from infected PBMC to EC under "static" and "dynamic" state. PBMC were infected with EHV-4 (MOI=0.1) for 5 min. Infected PBMC were overlaid on EC under "static" conditions (B) or allowed to flow over EC "dynamic" (C) in the presence of neutralizing antibodies. After 24 h, virus spread was assessed by counting the plaques on EC. As a control, infected PBMC were placed into a Transwell insert without direct contact between PBMC and EC "no contact". The data represent the mean ± standard deviations (SD) of three independent and blinded experiments.

(D.1 and D.2) PBMC were infected with EHV-4 (MOI=0.1) for 5 min. The ECM was disrupted with both mechanical (extensive pipetting) and chemical (heparin treatment) together (EHV-4_5M_Cell_pip_hep). The disrupted cells (D.1) or the supernatant (EHV-4_5M_Sup_pip_hep; D.2) were added to EC. As a control, the ECM was left undisrupted (EHV-4_5M_Cell or EHV-4_5M_Sup). As a control of pipetting and heparin treatment, EHV-1 was used as a control. The data represent the mean ± standard deviations (SD) of three independent and blinded experiments. **(E)** Immunofluorescence data shows that the permissiveness of the endothelial cells to EHV-4 cell-free viruses. EHV-4 couldn`t be transmitted from infected PBMC either on static or dynamic conditions to the endothelial cells and interestingly even after disruption of the extracellular matrix. Scale bar =70 µm.

6.1.9 Influence and impact of Viral gB and gD on ECM-associated virus cell-to-cell transmission

Two important enveloped viral glycoproteins (gB and gD) have a major and important role in virus cell-to-cell spread. To assess their role in ECM associated viruses as a mechanism of cell-to-cell spread in EHV-1 and EHV-4 as previously shown data in our study and also that ECM associated EHV-1 could spread to EC but EHV-4 not, different recombinant viruses in which the two viral glycoproteins were swapped, for EHV-1 WT(EHV-1_gB4 and EHV-1_gD4) and EHV-4 WT (EHV-4_gB1 and EHV-4_gD1) were used [29, 36]. To assess their role in static and dynamics conditions, contact assays and flow chamber assays were done after 5 min infection with the mutant viruses. Interestingly, the infectious transmissibility and spread were interestingly decreased for the EHV-1 mutant containing gD4 or gB4 (EHV-1_gD4 and EHV-1_gB4) in the static and dynamic conditions (**Figures 28**A and **28**C). On the other hand, the

infectious capabilities and transmissibility were gained for EHV-4 containing gB1 or gD1 (EHV-4_gD1 and EHV-4_gB1) on the static conditions only compared to EHV-4 which shows no spread or transfer to EC (**Figures 28**B and **28**D). Taken together, swapping of the two genes (gB and gD) significantly affected ECM-associated virus spread from infected PBMC to EC highlighting their role in direct virus cell-to-cell transmission.

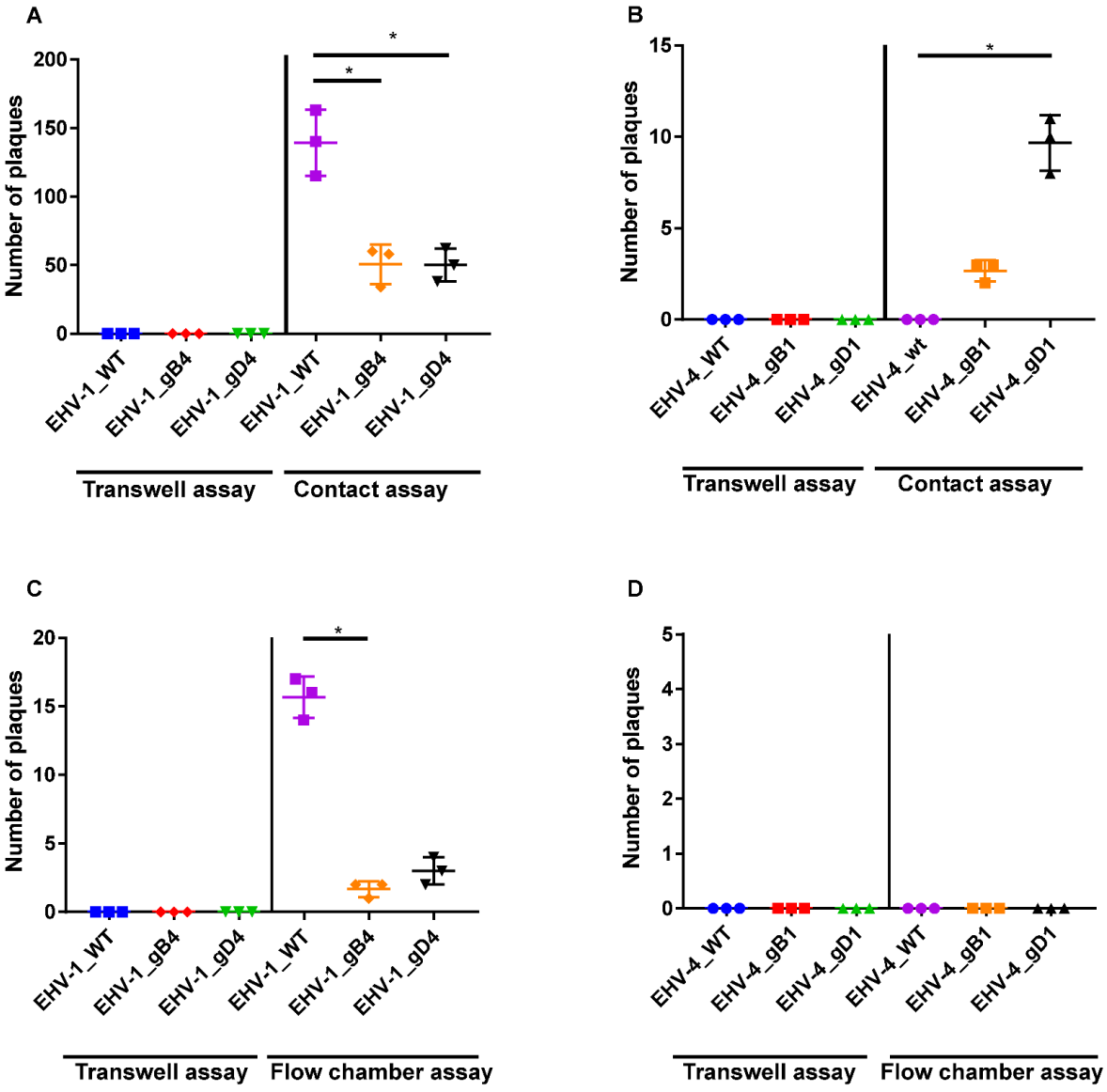

Figure 28: Role of viral gB and gD in virus transmission. PBMC were infected with the indicated viruses (MOI=0.1) for 5 min. Infected PBMC were overlaid on EC under "static" conditions **(A and B)** or allowed to flow over EC "dynamic" **(C and D)** in the presence of neutralizing antibodies. After 24 h, virus spread was assessed by counting the plaques on EC. As a control, infected PBMC were placed into a Transwell insert without direct contact between PBMC and EC "no contact". The data represent the mean ± standard deviations (SD) of three independent and blinded experiments.

Significant differences in plaque numbers were seen between the different viruses as indicated. One-way ANOVA test _ Dunnett's multiple comparisons test (B) or Kruskal-Wallis test followed Dunnett's multiple comparisons test (A and C), *, $p < 0.05$).

6.1.10 *Microvesicle and tight junction events; Two events first time to be shown here and need further and deep investigations to assess their role in the viral life cycle and spread.*

Two events had been visualized and observed by transmission electron microscopy of 24 hr infected EC that needs further and deep investigations to address their role in EHV-1 life cycle and spread from cell to another. The first event (**Figure 29)** is the presence of EHV-1 viral particle inside a microvesicle outside the cells. This is the first time to show EHV-1 viral particle inside the microvesicle. This microvesicle containing the EHV-1 viral particle is originated from infected cells and their role in viral spread to target uninfected cell needs to be investigated. The second important event is the role of tight junction in viral spread between the infected (left one) and uninfected cells (right one) in an antibody resistant way. The viruses seem to be sorted toward the tight junction where they are ready to be transmitted or enter the uninfected cells more likely via receptor-mediated or fusion pathway as a lateral spread of viruses **(Figure 30).**

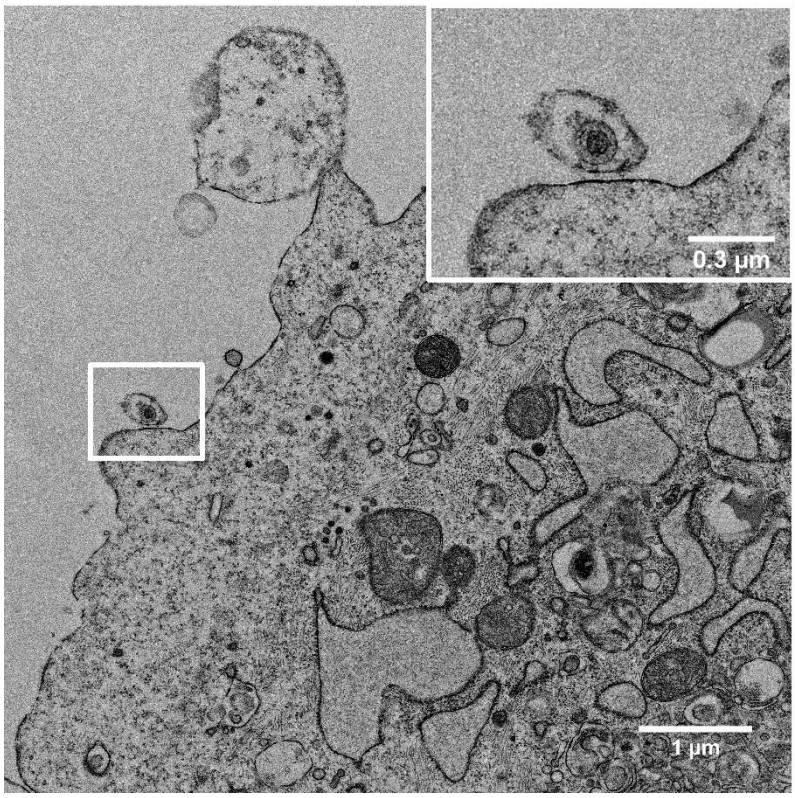

Figure 29: Electron microscopy of endothelial cells showing microvesicle outside the cells containing EHV-1 viral particles.

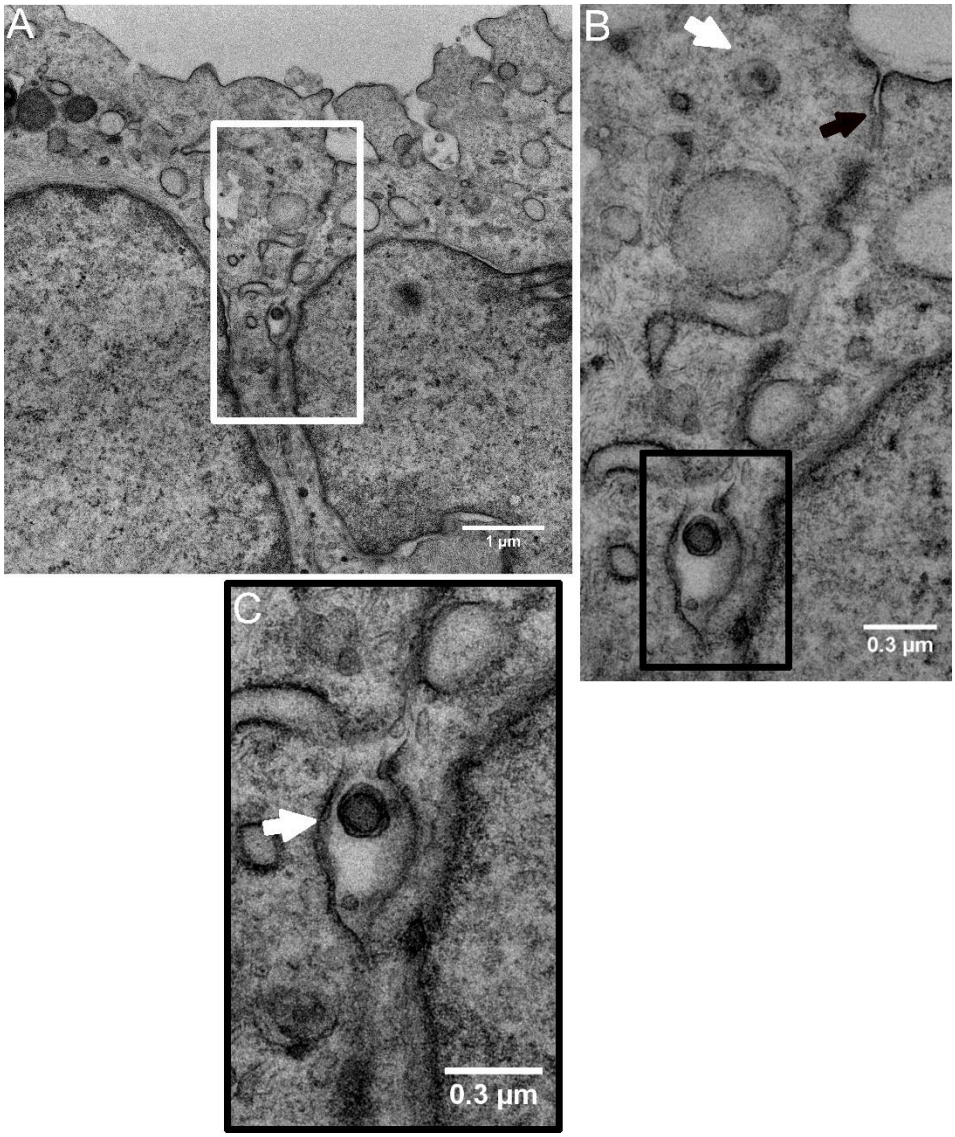

Figure 30: Electron microscopy of 24 hr infected endothelial cells (A) showing EHV-1 infected endothelial cells (white arrow, **B**) closely adherent to uninfected target cells with tight junction (Black arrow, **B** and white arrow, **C**) in between the two cells as a mean for direct likelihood cell-to-cell transmission.

6.2 The role of UL56 in virus transfer between PBMC and EC in EHV-1 & EHV-4

6.2.1 Generation of the mutant viruses using multiple cloning and two-step en passant mutagenesis steps.

To reveal the role of UL56 in EHV-1 strain Ab4 and EHV-4 pathogenesis through investigating their effect on virus transfer from infected PBMC to endothelial cells, we initially deleted UL56 from EHV-1 and EHV-4 resulting in EHV-1delUL56 and EHV-4delUL56 respectively. The

deletion steps were followed by subsequently inserting the vice-versa UL56.4 into EHV-1 and UL56.1 into EHV-4 which were followed again by their deletion to generate, finally, their revertants by re-inserting UL56.1 into EHV-1 and UL56.4 into EHV-4 employing multiple cloning and two-step en passant mutagenesis system **Figures (31 and 32).** Briefly, PCR products using pEPkan-S as a template and primer with homologous sequence for amplification of kanamycin resistant gene were achieved. During the first recombination step, the kanamycin resistance gene (aphAI) cassette was introduced in EHV-1 strain Ab4 or EHV-4 generating intermediate co-integrate clones with inserted kanamycin (aphAI) with removal of the UL56 exhibiting the expected DNA restriction pattern changes. During the second recombination event, resulted in aphA1 gene removal, the final clones with UL56 deleted appeared compared to parental wild types. Transfer plasmids including the insertion cassette of UL56 for EHV-1/4 harboring the kanamycin cassette inside the gene were constructed via several cloning steps confirmed by PCR, restriction digestion, and sequencing. For the swapped mutants, during the first recombination step, the insertion UL56/kanamycin cassette was done generating cointegrate clones with inserted UL56/kanamycin (aphAI). These intermediate clones were subjected to a second recombination event characterized by removal of aphAI generating final clones with the swapped UL56 in between the two viruses. Another two-step en passant mutagenesis was employed for deletion of the UL56.4 from EHV-1 or UL56.1 from EHV-4 followed by two en-passant mutagenesis for generating revertant viruses. All generated mutants were confirmed by PCR, by RFLP, by using the restriction enzymes EcoRV and KpnI for EHV-1 mutants and EHV-4 mutants, for correct restriction pattern and band shift as predicted using VNTI software and finally, by sanger nucleotide sequencing (data not shown) with for no mutation in their sequences (**Figure 33).**

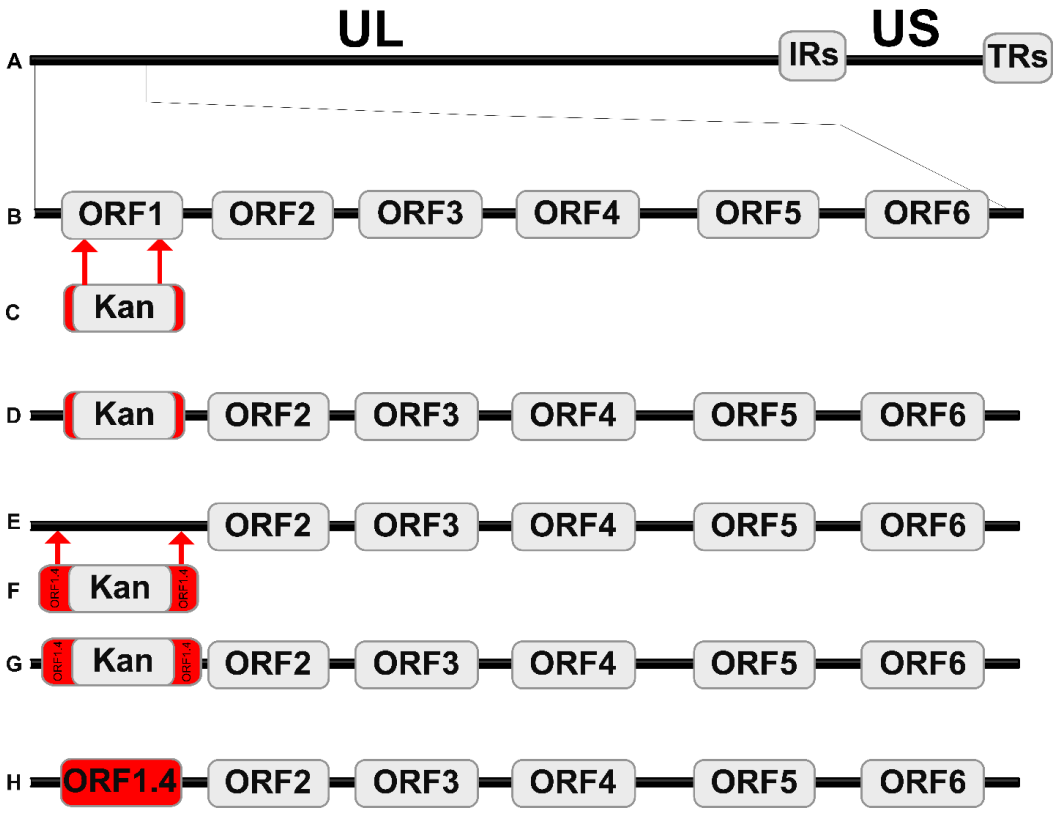

Figure 31: Schematic illustration of the processes of generating mutant genomes (EHV-1_ORF1.4 (UL56_4) as a model). A) The schematic depiction of EHV-1 genome showing unique regions (UL and US) as well as repeat sequences (terminal repeat sequence, TRS and internal repeat sequence, IRS) flanking the US are shown. The inserted mini-F cassette with the organization of genes as demonstrated in **(B). C)** Kanamycin resistance (KanR, aphAI) was amplified form pEPkan-S. PCR product was then electroporated into GS1783 harboring the EHV-1 BACs indicating occurrence of the first step of Red recombination method **(D)**. **E)** The KanR gene was removed, in the second step of Red recombination, thereby achieving the EHV-1delU56 deletion construct. The PCR-amplified ORF1.4Kan, namely UL56.4 Kan **(F)** was then electroporated into GS1783 containing EHV-1delUL56 **(E)** generating kanamycin resistance intermediates clone **(G)** followed by resolution steps (second step of Red recombination) to get the final construct clone with EHV-1_ORF1.4 (EHV_UL56.4), **H.**

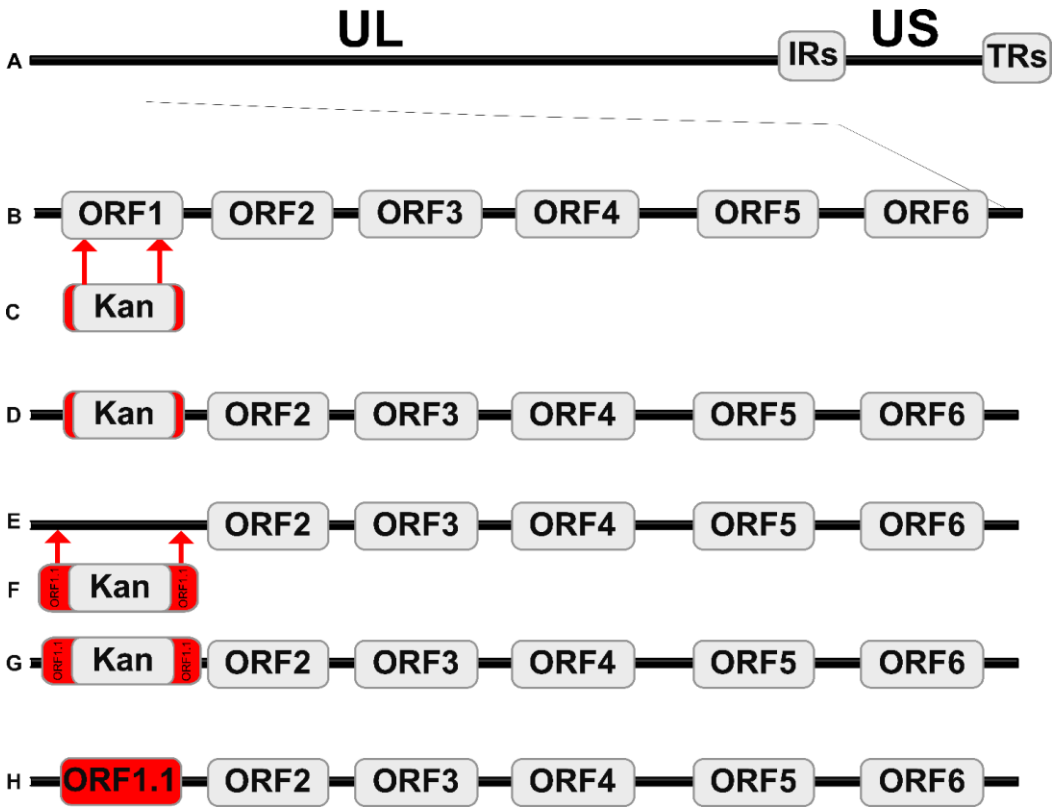

Figure 32 Schematic illustration of the processes of generating mutant genomes (EHV-4_ORF1.1 (UL56_1) as a model). A) The schematic depiction of EHV-4 genome showing unique regions (UL and US) as well as repeat sequences (terminal repeat sequence, TRS and internal repeat sequence, IRS) flanking the US are shown. The inserted mini-F cassette the organization of genes as demonstrated in **(B)**. **C)** Kanamycin resistance (KanR, aphAI) was amplified form pEPkan-S. PCR product was then electroporated into GS1783 harboring the EHV-1 BACs indicating that occurrence of the first step of the Red recombination method **(D)**. **E)** The KanR gene was removed, In the second step of Red recombination, thereby achieving the EHV-4delU56 deletion construct. The PCR-amplified ORF1.1Kan, namely UL56.1 Kan **(F)** was then electroporated into GS1783 containing pEHV-4delUL56 (**E)** generating kanamycin resistance intermediates clone **(G)** followed by resolution steps (second step of Red recombination) to get the final construct clone with EHV-4_ORF1.1 (EHV_UL56.1), **H**.

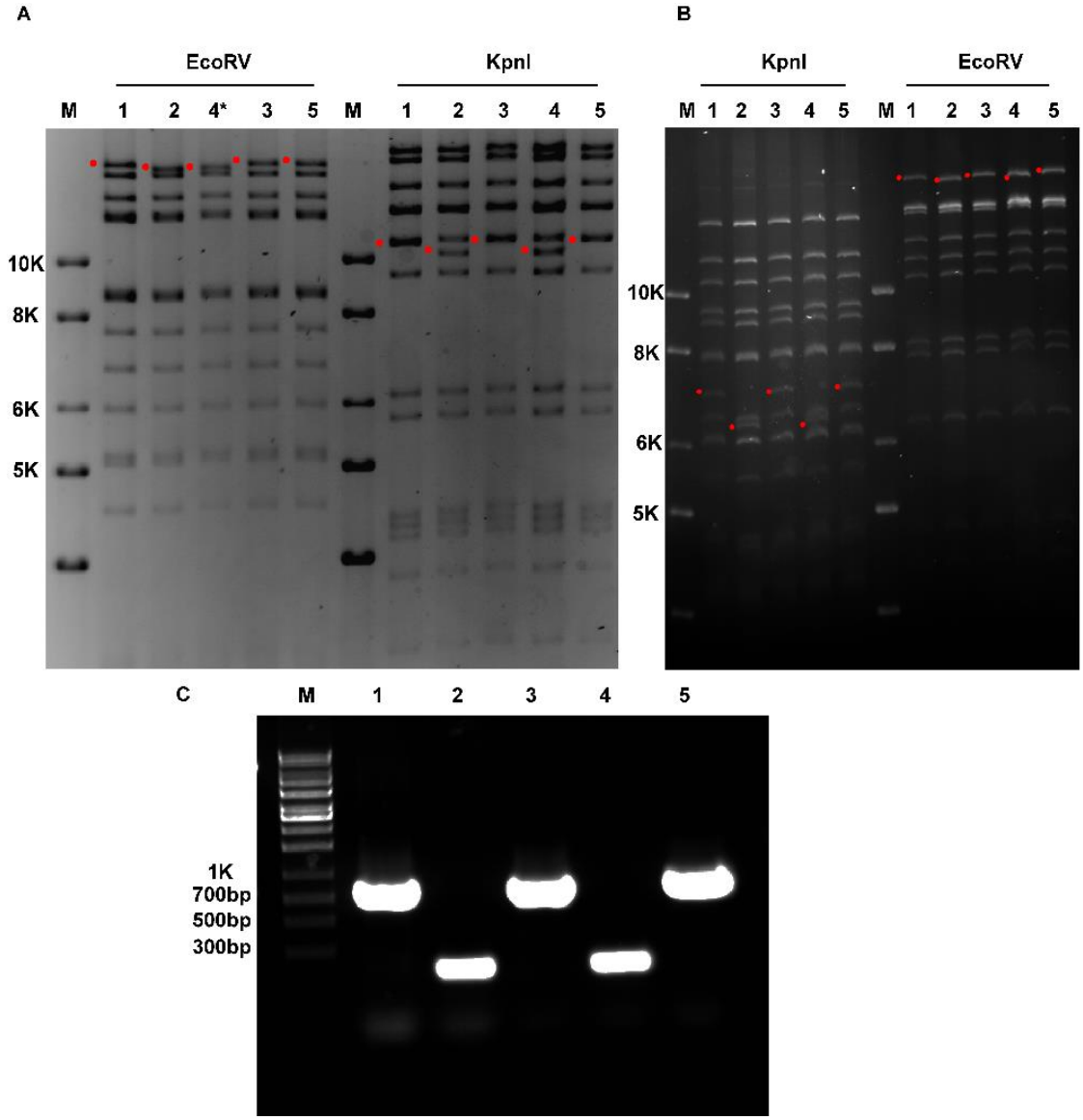

Figure 33: **Restriction analysis and polymerase chain reaction confirmation for UL56 mutants (A)** Restriction fragment length polymorphism (RFLP) of UL56 mutants of EHV-1 showing the final construct using KpnI and EcoRV restriction enzymes. The ethidium bromide o/n stained agarose gel showing the expected corrected restriction pattern change as in silico predicted, 1 kb DNA ladder (Solis BioDyne), M, EHV-1 (Lane 1), EHV-1delUL56 (Lane 2), EHV-1_UL56.4 (Lane 3), EHV-1delUL56.4 (Lane 4), and EHV-1UL56 Revert (Lane 5).

(B) Restriction fragment length polymorphism (RFLP) of UL56 mutants of EHV-4 showing the final construct using KpnI and EcoRV restriction enzymes. The ethidium bromide o/n stained agarose gel showing the expected corrected restriction pattern change as in silico predicted, 1 kb DNA ladder (Solis BioDyne), M, EHV-4 (Lane 1), EHV-4delUL56 (Lane 2), EHV-4_UL56.1 (Lane 3), EHV-4delUL56.1 (Lane 4), and EHV-4UL56 Revert (Lane 5).

(C) Polymerase chain reaction (PCR) for all EHV-1 mutants (UL56) using specific primer for pre and post primers specific to specific region in EHV-1 genome before and after UL56 (P21 and P22 in **Table (9)).** The ethidium bromide-stained agarose gel showing the UL56 band in EHV-1 (Lane 1), EHV-1delUL56 (Lane 2), EHV-1_UL56.4 (Lane 3), EHV-1delUL56.4 (Lane 4), and EHV-1UL56 Revert (Lane 5), 1 kb plus gene ruler DNA ladder (thermoFisher).

6.2.2 *Growth kinetics and plaque size of UL56 mutants*

To evaluate the growth properties of viral mutants compared to parental wild types (EHV-1 and EHV-4), plaques sizes and growth kinetics were performed. The average diameter size of viral mutant's plaques, formed by infection of ED after 3 days PI, displayed no statistical significance ($p >$ 0.05) in all viral mutants, deleted and swapped one in comparison with parental viruses **(Figures 34 and 35)**. On the other side, all viruses, the parental and mutant viruses exhibited comparable virus titers and replication, indicated by single-step growth kinetics, during the 30 h observation period for EHV-1 and 72h observation period for EHV-4, with respect to separated extracellular and the cell-associated titers in EHV-1 and both extracellular and intracellular collected together in EHV-4 (**Figures 34 and 35**). Virus titers were nearly identical between the analyzed viruses. We concluded, based on these results, that the swapping or deletion mutants had negligible non-significant effects on viral growth in *vitro* compared to parental wild types.

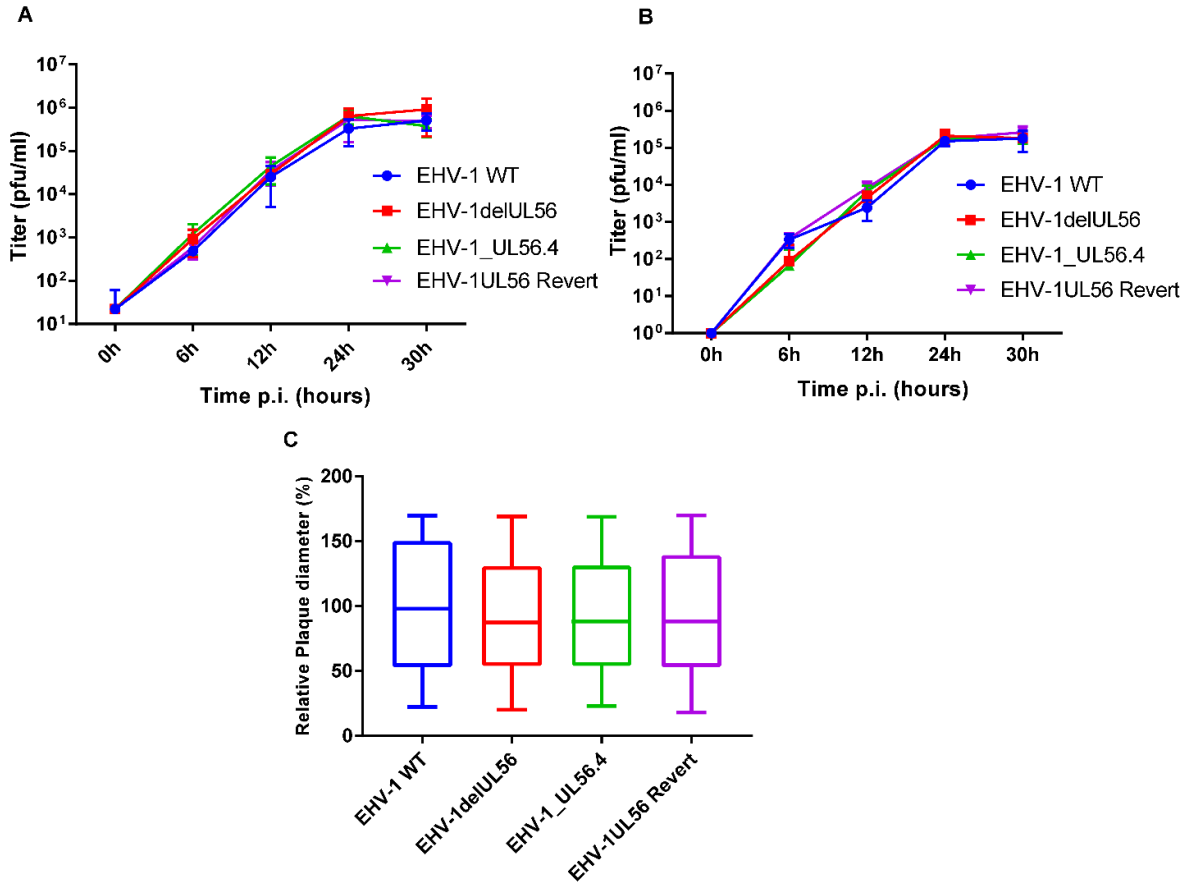

Figure 34: Plaque size and growth kinetics of EHV-1 mutants: Growth properties of mutant and recombinant viruses. Equine dermal cells were grown to confluency and then infected with the different generated viruses as displayed in the figure. Infected cells, cellular **(A)** and the supernatant, extracellular **(B)** were harvested separately at these indicated time points p.i (0, 6, 12, 24 and 30 h pi.) and standard plaque assay was used for virus titers calculations. The three independent experiments data expressed are means ± standard deviations (SD). There were no statistical differences ($p > 0.05$) retrieved from the single-step growth kinetics analyses (Friedman test followed by Dunn's multiple comparison tests). EHV-1: equine herpesvirus type 1 strain Ab4; EHV-1delUL56: EHV-1 where UL56 has been deleted; EHV-1UL56.4: EHV-1 where UL56.4 replaces UL56.1; EHV-1UL56 Revert, EHV-1 with UL56.1 reverted and re-inserted. **For determination of average size diameter by plaque size assay (C).** ED cells were infected with all mutants and recombinant viruses at MOI of 0.001. After 3 days, fifty plaques per virus were measured. The data presented as box plot and the central line in box plot shows the median of the data and 25–75 percentile with whiskers illustrating minimal and maximal values. The plaque diameter of the wild-type EHV-1 virus was set as 100% and then relatively the plaque diameter of other mutant or recombinant was calculated. Three independent experiments data were shown. No

significance level had been detected (one-way ANOVA test followed by multiple comparison tests; p > 0.05).

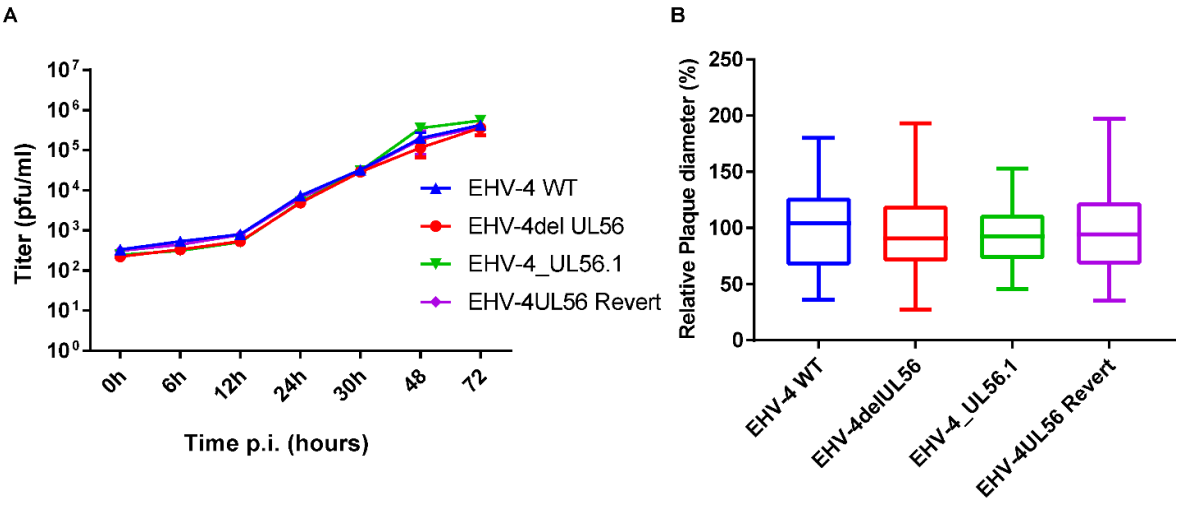

Figure 35: Plaque size and growth kinetics of EHV-4 mutants. in vitro growth kinetics of all generated viruses. Equine dermal cells were grown to confluency and then infected with different generated viruses as displayed in the figure. Infected cells, cellular and the supernatant, extracellular were harvested together **(A)** at these indicated time points p.i (0, 6, 12, 24, 30, 48 and 72 pi.) and standard plaque assay was used for virus titers calculations. The three independent experiments data expressed are means ± standard deviations (SD). There was no statistical differences (p >0.05) retrieved from the single-step growth kinetics analyses. No Significance levels had been detected (Friedman test followed by Dunn's multiple comparison tests). EHV-4: equine herpesvirus type 4; EHV-4delUL56: EHV-4 where UL56 has been deleted; EHV-4UL56_1: EHV-4 where UL56_1 replaces UL56_4; EHV-4UL56 Revert, EHV-4 with UL56.4 reverted and re-inserted. **Determination of average size diameter by plaque size assay**. Equine dermal cells were infected with various viruses (parental, mutant and recombinant viruses) at MOI of 0.01. After 3 days, fifty plaques per virus were measured. The data presented as box plot and the central line in box plot shows the median of the data and 25–75 percentile with whiskers illustrating minimal and maximal values. The plaque diameter of the wild-type EHV-4 virus was set as 100% and then relatively the plaque diameter of other mutant or recombinant was calculated. Three independent experiments data were shown. No significance level had been detected (one-way ANOVA test followed by multiple comparison tests; p > 0.05).

6.2.3 *Spread of viruses from infected PBMC to endothelial cells*

To test virus transfer from infected-PBMC to endothelial cells (EC) and the role of UL56 in virus transfer in between, contact assays (under static conditions) were performed. PBMC were infected with EHV-1/4 GFP mutants viruses for 24 hr. The infected cells were then sorted to introduce the same number of infected cells followed by citrate treatment and virus neutralizing antibodies to get rid of any cell-free viruses. The cells were then overlaid on EC in "contact" model for 2 hr in the presence of neutralizing antibodies, followed by several times extensive washing steps to get rid of unbound PBMC. The "no-contact" model was used as a control to exclude cell-free virus transmission. Our data showed UL56 had no significant role in virus transfer in EHV-1, even UL56 swapping in between from infected PBMC to EC in the static conditions **(Figure 37).** In case of EHV-4 mutants, no virus transfer was detected for the parental virus besides all mutants, which concentrate that UL56 had no role in enhancing or assisting EHV-4 transfer from PBMC to endothelial cells. As was shown in the stationary setup, we further expanded our investigation to test EHV-1 transfer under dynamic conditions using microfluidic setup (flow chamber assay). To test virus transfer from infected-PBMC to endothelial cells (EC) and the role of UL56 in virus transfer in between in the dynamic conditions, flow chamber assays were performed. PBMC were infected with EHV-1 GFP virus for 24 hr. The infected cells were then sorted to introduce the same number of infected cells followed by citrate treatment and virus neutralizing antibodies to get rid of any cell-free viruses. In this assay, the infected sorted PBMC were allowed to flow over the confluent EC monolayer in the presence of virus-neutralizing antibodies. Our data showed that UL56 also had negligible but no significant effect on virus to transfer to EC under flow condition **(Figure 36).** The "no-contact" model was used as a control to exclude cell-free virus transmission. Taken together, we showed that UL56 had no significant role in virus transfer in EHV-1 or EHV-4, even UL56 swapping in between from infected PBMC to EC in the static conditions or dynamic conditions **(Figure 36 and 37).**

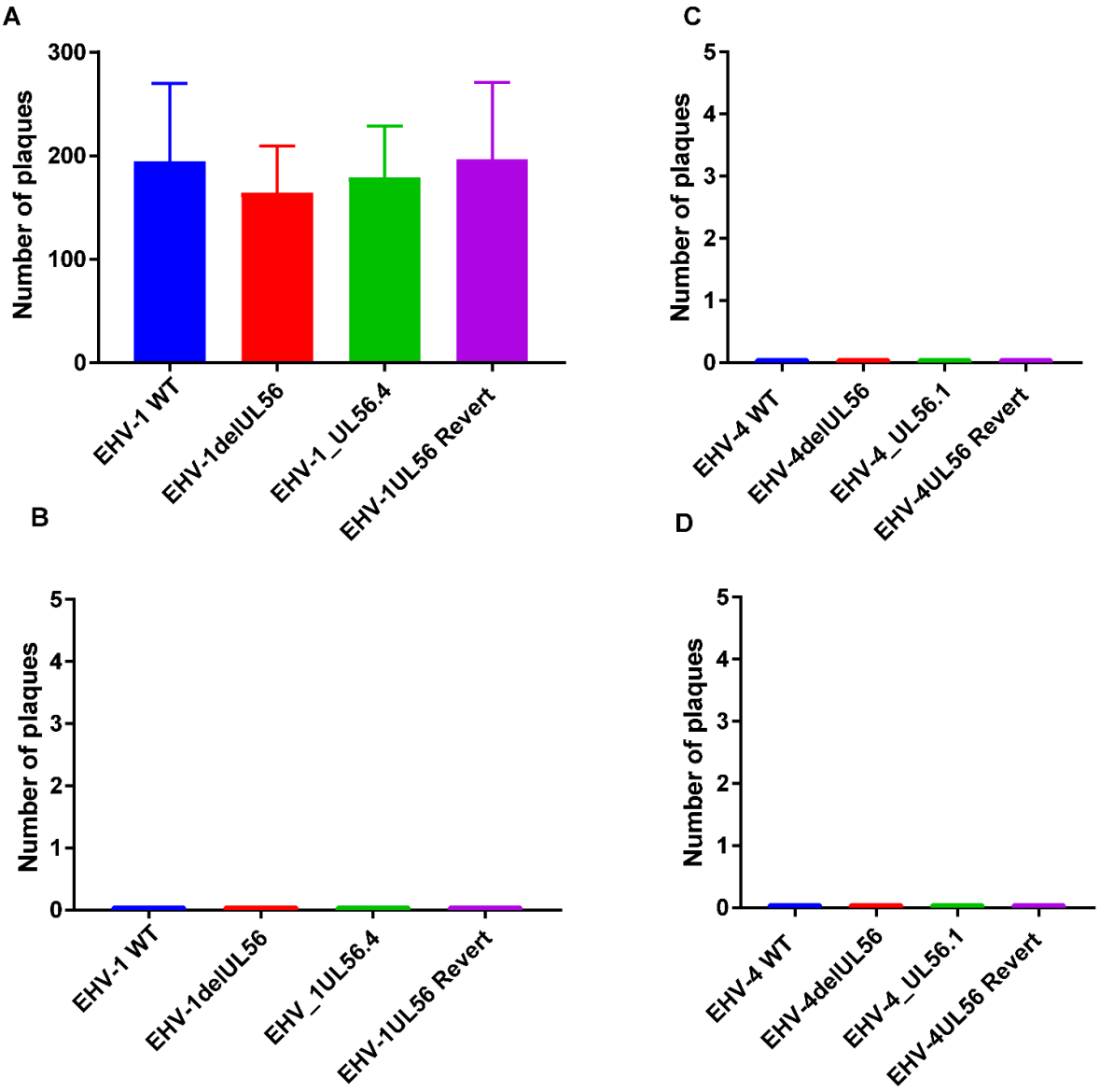

Figure 36: Contact assay to study the effect of UL56 on virus transfer from infected PBMC to endothelial cell. $1*10^4$ infected PBMC from all EHV-1 **(A)** or EHV-4 **(C)** mutants were overlaid on EC under "static" conditions in the presence of neutralizing antibodies for 2hr. After 24 h, virus spread was assessed by counting the viral plaques on EC. As a control, infected PBMC were placed into a Transwell insert without direct contact between PBMC and EC "no contact", (EHV-1 mutants), **B** and (EHV-4 mutants), **D**. Results are shown as means of three independent experiments with standard error (error bars). Statistically, no significant differences in number of plaques were observed between the viruses; wild-type EHV-1 with its mutant viruses and wild-type EHV-4 with its mutant viruses under "static" conditions (n=3; One-way ANOVA Test followed by multiple comparisons tests; P **>** 0.05).

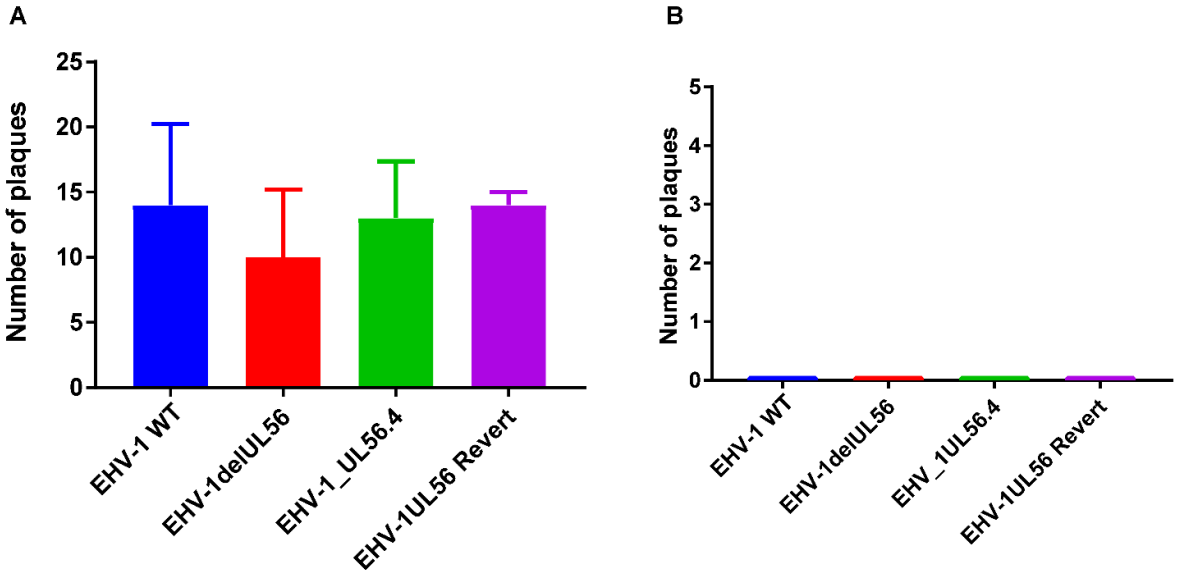

Figure 37: Flow chamber assay to assess the role of UL56 on the virus transfer from infected PBMC to endothelial cell. (A) $1*10^4$ infected PBMC from each mutant were allowed to flow over EC "dynamic " in the presence of neutralizing antibodies. After 24 h, virus spread was assessed by counting the plaques on EC excluding the inlet and outlet. **(B)** As a control, infected PBMC is placed into a Transwell insert sharing the same environment but without physical contact between the two cells "no contact". The data represent the mean with standard deviations of three independent experiments. For the viral number plaques, no significant differences were detected between parental EHV-1 and all mutant viruses under "dynamic" conditions indicating that UL56 has no role in virus transfer (*) (n=3; One-way ANOVA Test followed by multiple comparisons tests; P>0.05).

6.2.4 Effect of UL56 on adhesion molecules expression (VLA-4 and LFA-1) on infected PBMC.

Cell surface adhesion molecules on PBMC adhere to their ligands on endothelial cell surfaces. Two of them, leucocytes function associated antigen (LFA-1) and very late antigen 4 (VLA-4), are responsible to interact with ICAM and VCAM respectively. After establishing and mediation of firm adhesion done by the aid of these two adhesion molecules, virus transfer occurs. For assessing their up or down-regulation compared to parental viruses, flow cytometry assay (FACS) was performed to investigate the effect of UL56 on these adhesion molecules. After PBMC infection, staining with anti-VLA4 and anti-LFA-1 was performed followed by Alexa Four secondary antibody staining. The results showed no significant effect of UL56 in both EHV-1 and EHV-4 mutants compared to their wild types **(Figure 38).**

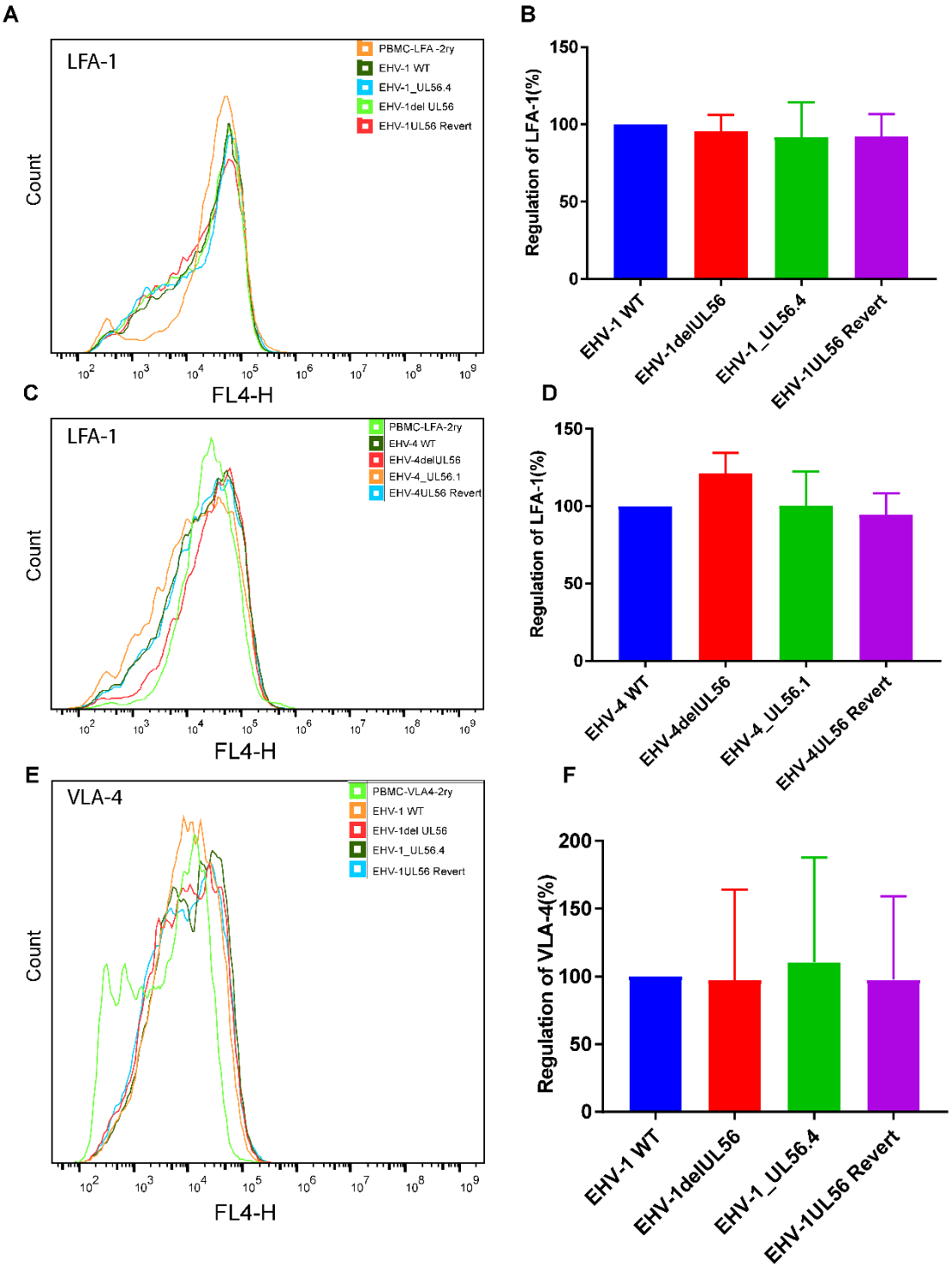

Figure 38: The role of UL56 in cell surface adhesion molecules (VLA-4 & LFA-1) expression on the infected PBMC. (A and B) Surface expression of lymphocyte function-associated antigen-1 (LFA-1) on the surface of infected PBMC with EHV-1 WT and the mutants (EHV-1delUL56, EHV-1_UL56.4 and EHV-1UL56 Revert) to check the effect of the UL56 deletion and swapping mutants compared to parental type on LFA-1 expression. **(C and D)** Surface expression of LFA-1 on the surface of infected PBMC

with EHV-4 WT and the mutants (EHV-4delUL56, EHV-4_UL56.1 and EHV-4UL56 Revert) to check the effect of the UL56 deletion and swapping mutants compared to parental type on LFA-1 expression. **(E and F)** Surface expression of very late antigen-4 (VLA-4) on the surface of infected PBMC with EHV-1 WT and the mutants (EHV-1delUL56, EHV-1_UL56.4 and EHV-1UL56 Revert) to check the effect of the UL56 deletion and swapping mutants compared to parental type on VLA-4 expression.10,000 viable cell (event) were analyzed for each sample. For the bars, the expression level of adhesion molecules (VLA-4 or LFA-1) of wild-type parental viruses EHV-1 and EHV-4 was set to 100%. All data represent the mean ± SD of three independent experiments for LFA-1 and two independent experiments for VLA-4. There was no any significant effect down or up-regulation of all mutants in comparison to the parental wild types in VLA-4 or LFA-4. Histograms and bars of LFA-1 and VLA-4 expression are shown. Data (histogram) represent one experiment out of three. FL4: fluorescence detector.

6.3 EHV-1 viral vector construction to protect against FMD.

6.3.1 *Generation of a vectored RacH vaccine expressing FMD_P1 (rH_P1)*

By using two-step en passant mutagenesis **(Figure 39)**, pRacH was manipulated for insertion of the codonly optimized sequence of the FMDP1 gene derived from foot-and-mouth disease virus strain O1 Campos/Bra/58 into the infectious clone under the HCMV IE promoter/enhancer. Firstly, the synthetically codonly-optimized P1 gene was cloned into transfer plasmid pEp_CMVin and two-step en passant mutagenesis was then applied for viral vector vaccine development [353]. During the first recombination event, the insertion of the amplified FMD P1 cassette into pRacH BAC clone resulted in kanamycin-resistant intermediates clones with the expected changes in the digestion profile than the wild-type pRacH DNA restriction pattern as anticipated *in silico*. The second recombination event resulted in the excision of the KanR gene, which led to the reduction in size **(Figure 40).** Together with RFLP mapping analysis, PCR **(Figure 41)** and Sanger sequencing were performed for confirmation the correct insertion of P1 gene without any mutations in the pRacH clone. From these results, the constructed recombinant rH BAC harboring the FMD P1 gene in the targeted locus had been confirmed. Reconstitution of parental rH and the recombinant rH_P1 viruses were achieved on RK13 after the transfection of heir BAC DNAs using PEI reagent (Poly sciences).

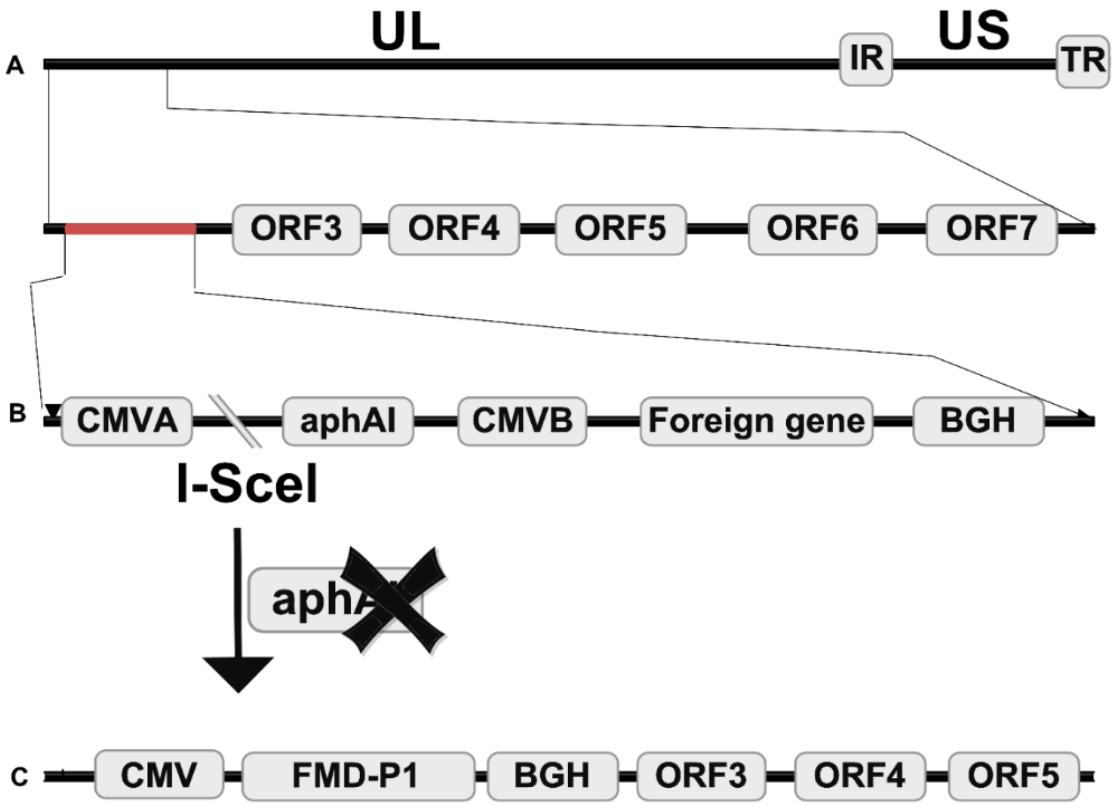

Figure 39: Schematic overview of the construction steps of rH_P1 vaccine vector based on pRacH1. (A) Representation of the left terminus of the UL region of EHV-1 strain RacH infectious BAC genome showing the absence of ORF1 and ORF2 **(B**) A P1-expressing cassette fragment which was obtained from transfer vector plasmid pUC19_P1 by *I-CeuI* restriction enzyme digestion was utilized to recombine with RacH genome, resulting in incorporation of P1 cassette of FMDV, kanamycin resistance gene and HCMV promoter in the ORF1/ORF2 locus of the RacH genome **(C)** Kanamycin resistance gene was removed by *I-SceI* digestion in the second step of en- passant mutagenesis to produce the final arrangement of rH_P1 construct.

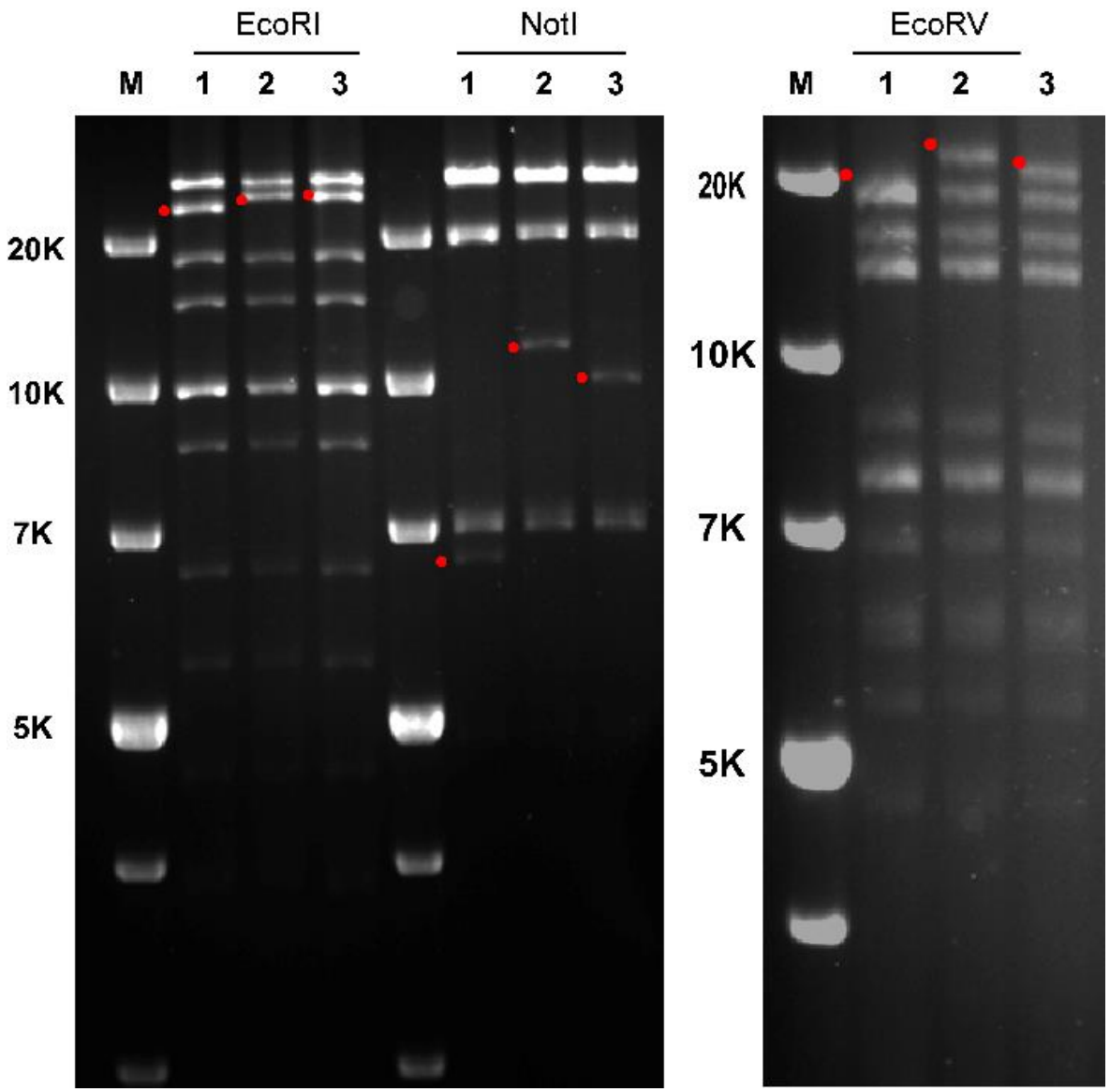

Figure 40: Restriction fragment length polymorphism of rH_P1 showing the mutagenesis cointegrate of rH_P1 and the final construct using EcoRI, NotI, and EcoRV restriction enzymes. The ethidium bromide o/n stained agarose gel showing the expected corrected restriction pattern change as in silico predicted, kanamycin resistant intermediate (lane 2), 1 kb plus gene ruler DNA ladder (thermoFisher), (M), rH(lane 1), rH_P1 (lane 3)

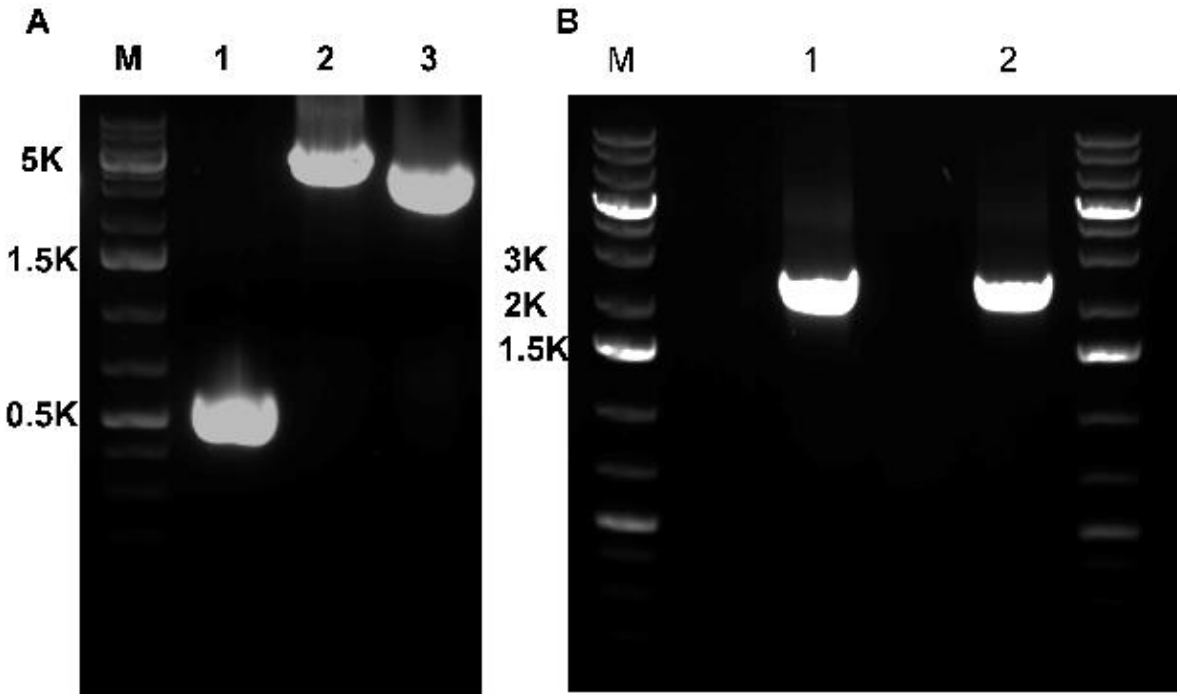

Figure 41: Polymerase chain reaction for P1 confirmation in both BAC and viral genome of rH-P1 (A) Polymerase chain reaction for the rH, rH_P1 cointegrate (Kanamycin+P1) and final rH_P1 construct containing P1 by using pre and post primers specific to the RacH genome showing expected band. The ethidium bromide stained agarose gel showing the P1 band, 1 kb plus gene ruler DNA ladder (thermoFisher)(M), rH (lane 1), rH_P1 intermediate (lane 2), rH_P1 final (lane 3) **(B)** Polymerase chain reaction for the genomic viral DNA to check P1 in rH_P1 using specific primer for P1 genome showing expected band. The ethidium bromide-stained agarose gel showing the P1 band, 1 kb plus gene ruler DNA ladder, M (thermoFisher).

6.3.2 In vitro *expression of* P1 (immunofluorescence and western blot)

For evaluation of P1 gene expression by the recombinant virus, IFA on rH_P1 infected RK13 cells for demonstrating the expression of P1 gene was done. rH_P1 virus plaques were reactive with polyclonal anti-FMD-specific antibodies, whereas those generated by parental rH virus were not. The expression of FMDP1 was also confirmed by western blot. A specific reactive protein band of approximately 81-kDa in size with the anti-P1 antibody was detected in infected cell lysates with the P1 expressing rH_P1 but was absent in both mock cells or parental virus-infected cell lysates **(Figure 42).** We concluded from these results that the FMD-

P1 protein had been integrated and efficiently expressed *in vitro* in the generated recombinant rH_P1.

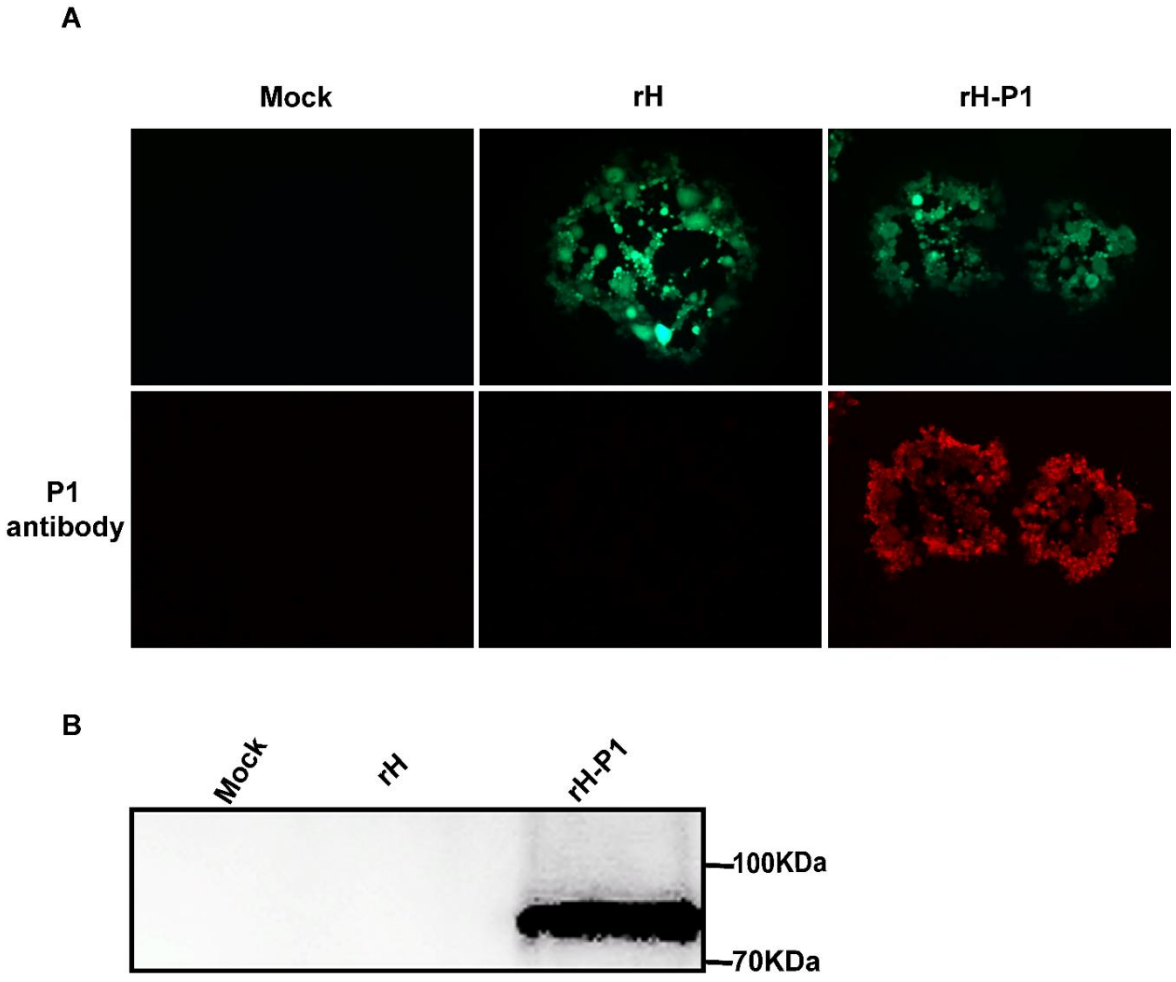

Figure 42: P1 expression visualization for the rH_P1 virus. (A) Immunofluorescence analysis of RK13 cells infected with rH or rH_P1 virus for assessing P1 expression in the recombinant viral vector rH_P1. Viral plaques were immunostained with an anti-FMD P1 polyclonal rabbit antiserum. Bound rabbit antibodies were detected with the use of secondary conjugated goat anti-rabbit Alexa 568 antibody and visualized by fluorescence microscopy. **(B)** Western blot analysis of mock, rH and rH_P1 infected cells. The proteins were separated by 10% SDS-PAGE blotted onto PVDF membrane. A specific reactive band of approximately 81 kDa was detected and visualized by ECL detection system for only rH-P1, not for mock-infected or rH-infected.

6.3.3 *In vitro growth properties (Plaque size and growth kinetics)*

To investigate whether the insertion of P1 or their expression affects viral growth *in vitro*, growth kinetics and plaque sizes of rH_P1 were assessed and compared to those of the parental rH virus. The average diameter size of rH_P1 plaques, following plaque formation on RK13 after 3 days incubation, displayed an approximate 20% reduction in their size when compared to those of parental virus, which were set to 100% **(Figure 43)** shows a statistical significance ($p < 0.05$). On the other side, both viruses, the parental and recombinant virus exhibit comparable virus titers, for viral replication evaluation by single-step growth kinetics, during the 30 h observation period, with respect to the extracellular and cell-associated titers **(Figure 43)**. Virus titers at the end of the observation period were virtually identical between the analyzed viruses. Based on these results, we concluded that the insertion of P1 had negligible and inconsiderable effects on viral growth in *vitro*.

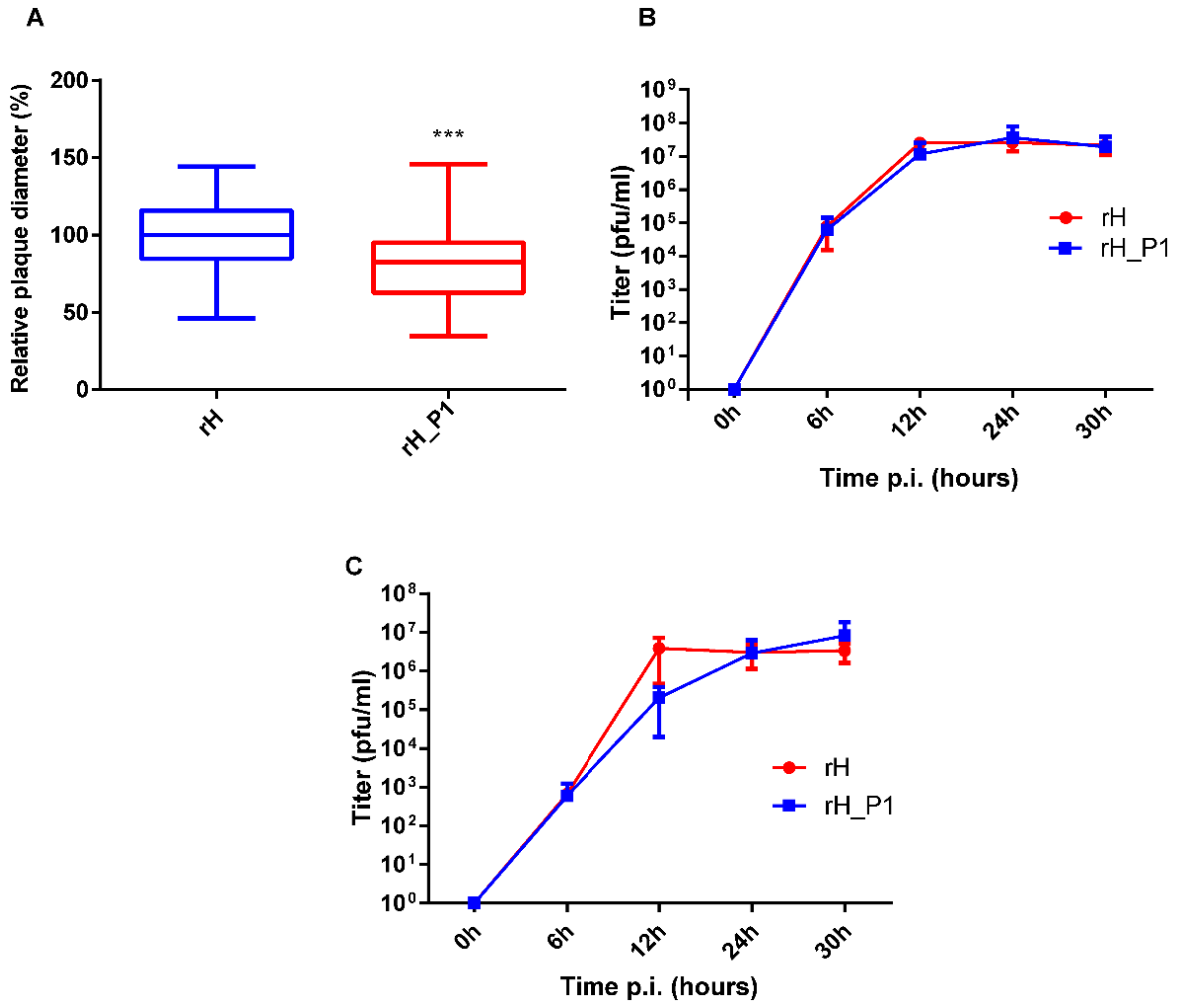

Figure 43: *In vitro* growth properties of the recombinant rH_P1 compared to parental rH virus. (A) Determination of average size diameter by plaque size assay. 150 plaques were measured after 3 days incubation for each virus. The three

independent experimental data presented as box plot and the central line in box plot shows the median of the data. The plaque diameter of the wild-type parental virus was set to 100% and then relatively the plaque diameter of recombinant virus was calculated. Significance level had been detected for the rH_P1 in comparison to rH (Student's t-test; $p < 0.001$). *In vitro* growth kinetics of rH_P1 compared to those of the parental rH virus **(B**). There were no statistical differences retrieved from the single-step growth kinetics analyses which displayed no major differences in growth properties of recombinant and parental virus on intracellularly **(B)** or extracellularly **(C).** These presented data are representative of three independent experiments with error bars representing standard deviations.

6.3.4 *Generation of gp2 restored viruses (white non EGFP expressing plaques viruses)*

nonfluorescing virus plaques were reconstituted on RK13 after cotransfection of plasmid DNA p71 expressing the full-length glycoprotein gp2 and rH_P1 DNA or rH together followed by their picking and transferring to fresh RK13 cells where several rounds of plaque purification were achieved. The expression of gp2 restoration had been confirmed by IF using specific antibodies targeting gp2 with goat anti-mouse IgG conjugated with Alexa Fluor 568 as a secondary antibody **(Figure 44).**

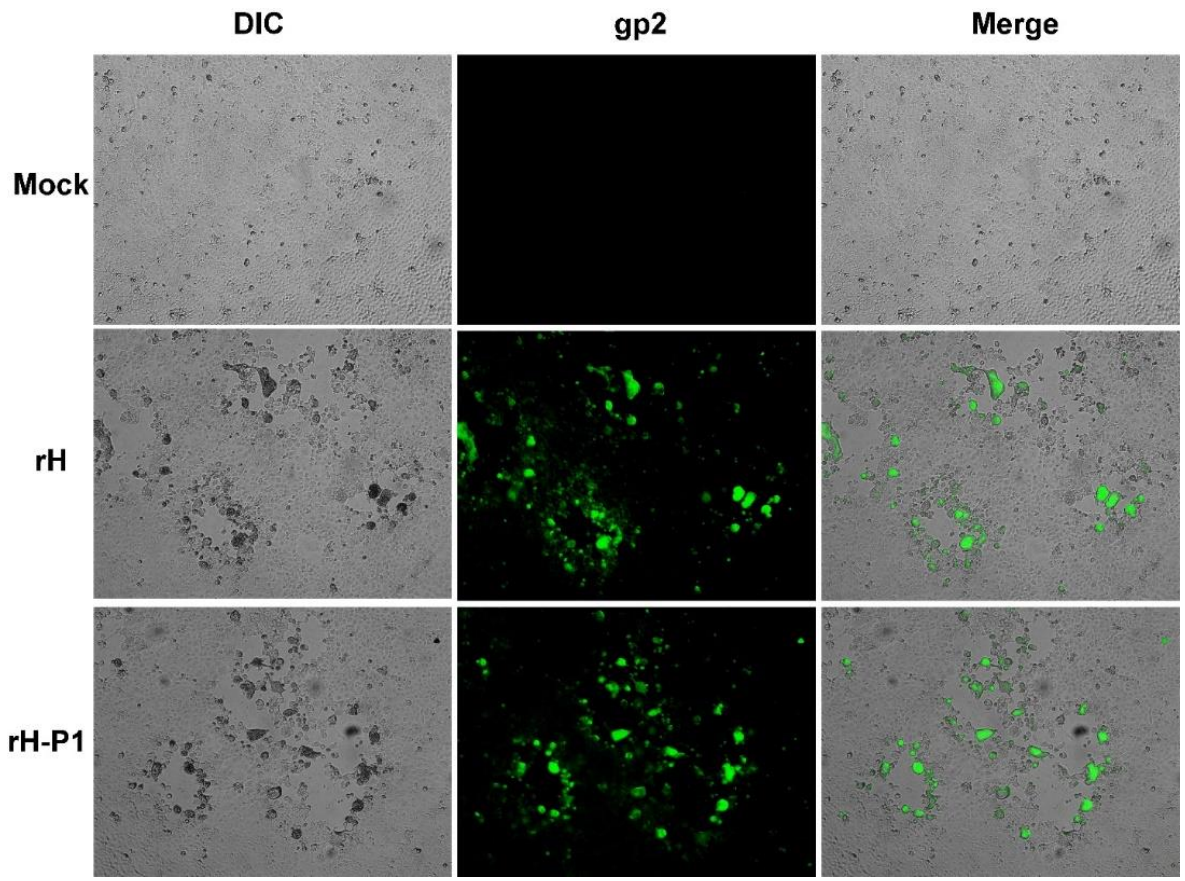

Figure 44: Immunofluorescence of gp2 restored viruses. Recombinant EHV-1 restoring gp2 had been produced via homologous recombination through co-transfection between plasmid expressing gp2 (ORF71) together with RacH BAC DNA by three rounds of plaques picking and purification for the non-fluorescence ones (non EGFP expressing plaques).

7 Discussion

7.1 Cell-to-cell transmission: Raveling and unveiling mechanisms behind EHV transmission from PBMC to EC.

In general, viruses spread from cell to another through two distinct modes either via cell-free viruses (diffusion) or through cell-associated viruses (cell-to-cell contact spread). Each route of virus spread has its own advantages and disadvantages. Although the viruses can spread via cell-free transmission by egressing to the extracellular environment, throughout the host and to other hosts more faster and to long distances within the host, they have to be released in large numbers, resistant to be cleared by immune cells and virus neutralizing antibodies, stable, and have the ability to bind and infect the target cells. Viruses cell-to-cell contact spread in which the viruses released without contacting the external environment. It is more efficient than cell-to-cell transmission by cell-free viruses transmission. It could bypass phagocytic cells, neutralizing antibodies and complement cascades and most obstacles facing cell-free viruses' transmission including face-off engagement with host defenses, involving innate and adaptive immunity. Several mechanisms of virus cell-to-cell transmission in several cells had been evolved by viruses which equipped with the necessary tools and information to facilitate its cell-to-cell dissemination in a secure and rapid way. Virus cell-to-cell transmission can occur through membrane-to-membrane contact as synapses (viral, immunological and neurological), fusion between the cells as a direct cell-to-cell contact virus spread or occur through distance between both cells (filopodia, nanotube and cellular conduits) as seen in HTLV and other viruses [181, 200, 210, 212, 361-364] . Direct cell-to-cell contact can be prevented in vitro by coculturing the infected and target cells utilizing porous transwell that permit only virus diffusion to the target cells [175, 176].

Several herpesviruses reach to their target cells to cause the diseases or even to become latent [365, 366]. One of their ways to spread and infect other cells despite the presence of cell-mediated and humoral immunity is cell-to-cell transmission [365, 366]. To achieve this goal even in the presence of cell-mediated and humoral immunity, they establish various mechanisms of direct cell-to-cell spread involving synapse formation, cell-cell fusion, or the most recent tunneling nanotubes (TNT) [178, 185, 186]. Herpesviruses establish infection and replication within the producer cells that transmit infectious particles to the target next cell via induced cell contacts. Equine herpesvirus type 1 (EHV-1) causes massive economic and social disasters through their reaching to the endothelial lining of blood vessels in central nervous system, pregnant uterus and eye via cell-associated viremia (PBMC). Its pathogenic lesion in endothelial cells leads to vasculitis, ischemia, and thrombosis which subsequently induces nervous manifestations, abortions and chorioretinopathy [105, 106, 113, 118, 367]. EHV-1, as

several herpesviruses, uses several strategies to evade the host immune system. One of them is PBMC associated viremia and cell-to-cell spread which probably through cell-cell fusion to escape from phagocytic cells, virus neutralizing antibodies and complement cascades [169, 218, 368]. The mechanisms behind the how-to-do are still unknown. Understanding the how-to-do this transmission through cell-to-cell contact is very crucial for controlling and even drug therapy development. EHV replication in PBMC is mainly restricted without obvious viral protein production or expression on the cell surface as almost 70% of all EHV-1-infected PBMC do not show viral envelope proteins expression on their surface and more than 98% of infected PBMC, only immediate early and at least some early proteins are expressed in the cell nuclei. The expression of the late envelope glycoproteins in equine leukocytes was decreased. This was in striking contrast to EHV-1 infected epithelial cells. Although seeing is believing approach was convincingly revealed in imaging and time-lapse live cell imaging to monitor virus transmission from infected to another uninfected cell at their cell-to-cell contact area [175, 369, 370], combining these imaging approaches together with functional assays are very crucial and needed to build a convincing argument [176, 228, 370, 371]. The most surprising finding of our results is the virus trapping, concentration, clustering in the extracellular carbohydrate-rich matrix that has efficiently transmitted the virus to endothelial cells. Our findings also reveal that the virus behaves classically as all herpesviruses in lately infected PBMC by the direct cell-to-cell fusion. Lately, trafficking of the leucocyte transcellularly inside the body of the endothelial cell is interestingly shown to happen.

These results, for the first time, provide a mechanism not only for herpesviruses but also for other viruses at which the virus carried on PBMC associating with the ECM transmitting them to the endothelial cells. Infection of EC via infected-PBMC has been publicized for members of several virus families, comprising Bunya-, Filo-, Herpes-, Toga-, Flavi-, and Arena-viruses [159-168]. The associated viruses were fully protected from the surrounding neutralizing antibodies in the environment that neutralize cell-free viruses and prevent their spreading [372, 373]. Direct cell-to-cell spread and transfer from viruses associated ECM infected PBMC to EC could occur on either static or dynamic conditions.To check if this happens spontaneously and any stuck viruses to PBMC surface can be transmitted. A closely relative to EHV-1, EHV-4 which has a restricted infection and pathogenesis to the upper respiratory tract couldn`t spread to the endothelium, despite its stuck to the EMC of PBMC. This indicates that EHV-1 uses a specific mechanism by which it embeds to be protected from the action of neutralizing antibodies until reaching the endothelium where egressing and transmission to EC happens. This doesn`t happen only between infected PBMC to EC but can happen between infected PBMC and uninfected one in the secondary lymphoid organs or blood circulation. As dendritic cells contract the infection from the respiratory epithelium [140], aggregation and clustering of

virus particles on infected PBMC could be easily transmitted to another uninfected one especially during cell aggregates. Similar concept but different mechanism occurs in HTLV. The infected lymphocytes produce the viruses, clustering, concentrating them after budding in a cocoon-like appearance, and attaching them in cell surface extracellular components with increasing their infectious ability to infect and spread to other cells (extracellular virus assemblies). The carbohydrate-rich component composition or spatial organization is affected by the HTLV [228].

EHV-1 concentrates and stores on this extracellular matrix representing the infectious capacity to endothelial cells. The high concentration of viral particles near the target cells making these structures look like a viral reservoir as the virus looks like a community in this structure that are ready to be transmitted. More little to be known about the entry of the virus after their transmission but it is more speculated to be through fusion than endocytosis but in our case in endothelial cells is more likely to happens via fusion. Another explanation of how transmission occurs via this extracellular matrix is that, after infected PBMC adhesion to target cells, surface-attached viruses on the top infected cells were driven toward target cell viral entry receptors with high specificity and affinity gradient in phenomena called directional spreading of surface-associated viruses from low-affinity infected cells [374].

The extracellular matrix (ECM) consists of well-organized complex tissue-specific networks of polysaccharides and proteins including proteoglycans, fibrous proteins, and glycoproteins [220, 375, 376]. Glycosaminoglycans (GAGs) are long, linear, negatively charged heteropolysaccharides including heparan sulfate (HS), keratan sulfate (KS), chondroitin sulfate (CS) or dermatan sulfate (DS) covalently attached to their core proteins to form proteoglycans. This HS proteoglycan (HSPG) provides structural frameworks to mediate cell signaling, adhesion, cell-cell communication and growth factor storage. GAGs represent the important component of the extracellular matrix [220]. Heparan sulfate interactions have been shown with several viruses, including herpesviruses which are a charge-based and a relative non-specific manner [377-381]. Herpesviruses gC and gB interact in unstable and reversible way with HS, which result in virus clustering, concentration and accumulation at the cell surface [28, 72] and this can explain the embedding and clustering of EHV-1 within the ECM. We further showed and detected that colocalization of virus particles with collagen and agrin, which are the major proteins of the ECM. Heparan sulfate proteoglycan agrin plays a significant role in efficient communication at cell-cell contact sites [382]. The synergism between integrin and agrin aggregating activity had been displayed to play an important role in stabilizing lipid raft glycosphingolipid-enriched microdomains in the immune cells at the immunological synapses [383, 384]. Agrin had also an important role in virological, neural

synapses and viral cell-to-cell transmission [228, 382, 385]. It is worth to mention that the role of EHV-1 viral synapse formation as a way for virus cell-to-cell spread has not yet been addressed and investigated. Collagen which is one of the main predominant fibrous and structural component of extracellular matrix was also shown to colocalize and co-cluster with HTLV virions in the extracellular matrix [228] which are in accord with our results. We could only see weak colocalization of ezrin with the viral particles which is a member of the ERM protein family and plays a prominent key role in cell surface adhesion, migration, organization, structure, and act as dynamic connector between plasma membrane and the actin cytoskeleton [386].

Our results indicate that integrity of ECM is essential in virus transmission from PBMC to endothelial cells. Heparin treatment with extensive pipetting or alone could only remove, elute and compete with ECM that markedly decreased the infectious transmissibility of the infected cells to endothelial cells. Although the treated cells were significantly less infectious, there were still infectious as ECM wasn`t completely removed by heparin treatment or extensive pipetting as displayed by our immunofluorescence data. On the other hand, the infectious capabilities of supernatant part were increased and able to infect EC compared to the non-treated one. Extracellular matrix structures were resistant to be removed or destructed by shear flow extensive pipetting, indicating their crucial role in resistance of physiological fluids dynamics during PBMC movement in circulation and physically protects the embedded viruses until reaching the endothelial cells of different systems in vivo. It was previously shown that bacterial biofilms resist shear flow [387]. By enzymatic treatment, heparinase enzyme, we could show the disassembly and destruction of the extracellular matrix with their embedded viruses as removal of heparan sulphate from the cell surface leading to virus detaching with ECM [374]. We showed that the supernatant of destructed extracellular viral assemblies could infect the target endothelial cells which agree with Alais et al [388] who found that the purified biofilm-like structures were infectious to the primary T lymphocytes and Dendritic cells (DCs).
Our immunofluorescence data shows EHV-4 detection and presence associating with the ECM. This detection indicates ECM_EHV-4 interaction or, at least, it`s capture by the gelatinous texture and structure of the ECM. Despite this detection, EHV-4 failed to be transmitted to endothelial cells via static or dynamic conditions. This failure may be due to its genetic makeup which couldn't support its release and escape from ECM and transmission. It`s possible to be inactivated after its stuck to the ECM surface of the PBMC [389]. Introducing the more powerful genes of EHV-1 (gB or gD) enhanced and supported their genetic makeup to be escaped, released, transmitted and attached to EC.
Several viral glycoproteins have been implicated in equine herpesvirus cell-to-cell spread [15, 26, 28, 36, 40, 88]. These viral glycoproteins include gB, gD, gE, gH, gI, gK and gM that have

been shown to be necessary for cell-to-cell spread. Two candidates of them (gB and gD) have essential roles in EHV pathogenesis and virus-host interaction. Glycoprotein B is highly conserved enveloped fusogenic glycoprotein throughout the members of Herpesviridae family playing an important role in cellular penetration and in the direct cell-to-cell spread of virions via interacting with HS or other specific cell receptors [26, 28]. The other envelope glycoprotein, EHV-1 gD, is one of the glycoproteins that are involved and necessitated for virus entry, which mainly occurs through membrane fusion and/or endocytosis [35, 36, 40]. Glycoprotein D (gD) has also another essential roles in cell penetration, cell-to-cell spread, cell tropism and host cell specificity [36, 40, 88]. By swapping gB and gD between EHV-1 and EHV-4, the virus transmissibility was affected and dramatically changed. A significant decrease in cell-to-cell spread was shown in gD4 or gB4 containing ECM-associated EHV-1. On the contrary, EHV-4 with gB1 or gD1 gained, for the first time, the capability of transmission and spread from infected PBMC to EC. The EHV-1 seems to attach and interact with ECM of PBMC via HS or unidentified cell surface receptor until reaching and adhesion of infected PBMC to endothelial cells leaving and breaking this interaction in an unknown mechanism until now which more likely that virus interacts to EC. The interaction of EHV with EC seems to be stronger with higher affinity in EC than the PBMC that have lower temporal affinity. The different transmissibility outcomes could also be explained by the difference between the fusogenic functions and activities of EHV-1 and EHV-4 gBs [29]. We can`t exclude the interaction of EHV-1 gD with new cell surface receptors on EC.

During viremia, the majority of infected mononuclear immune cells doesn`t show viral envelope proteins on their surface [48, 113, 118, 390] which is very noteworthy in immunoevasion to escape antibody-dependent lysis of infected cells. A possible explanation of masking their viral envelope proteins on the cell surface that antibody leads to aggregation of viral glycoproteins and subsequently fast and efficient internalization of glycoprotein–antibody aggregates to protect the circulating infected PBMC from antibody-dependent cell lysis. This phenomenen of glycoprotein–antibody aggregates internalization speculations come in line with previous results in PRV [391]. Upon arrival and adhesion of lately infected PBMC (24hr infection), when the virus entered the cells and produced the fusion machinery, to endothelial cells, cell-to-cell fusion starts to happen. Our results show the transfer EHV-1 cytoplasmic material from the infected PBMC to uninfected target cells via membrane cell-to-cell fusion events. These results come in line with other previously published herpesviruses like HCMV and PRV [185, 207, 215] who showed the direct cytoplasmic materials transfer from the infected cells to the uninfected target cells in a way called microfusion events between the infected and uninfected cells. Cell-to-cell fusion was also shown for other viruses as measles [392]. A possible explanation for this is that the formation of a cytoplasmic bridge between infected PBMC and uninfected endothelial cells establishes a way for EHV-1

cytoplasmic material transfer. Generally, the herpesviruses with their enveloped glycoproteins fusion machinery (gB, gD, gH, gL) could efficiently establish classical membrane fusion to the cell or membrane cell-to-cell fusion which either complete syncytium or microfusion [214, 357, 358, 393]. We speculate that these viral glycoproteins are expressed on the mononuclear cells upon reaching and firmly adhesion by the sets of adhesion molecules on the surface of both cells to the target endothelial cells inducing fusion between both cells. During the fusion between infected PBMC and endothelial cells, the transfer of the cytoplasmic content occurs. It is more likely that virion or cellular surface proteins polarized unilaterally toward contact area between the infected leukocytes and contact cells as previously delineated by [394]. As a conclusion, the lately infected PBMC cells efficiently adhere through cellular adhesion molecules and afterward fuse through viral glycoproteins that expressed on their surface upon the contact to the endothelial cells to transmit the viral material to them in the presence of virus neutralizing antibody.

One of the most interesting data is the transcellular migration of PBMC inside endothelial cells as a way of virus transmission. The PBMC had been imaged inside the endothelial cells body highlighting that the infected cells could migrate throughout the endothelial cells and transmit the bearer viruses to endothelial cells. The entire process of trafficking leucocyte through the endothelium requires four basic steps: rolling, adhesion, firm adhesion and transmigration (or diapedesis) [143, 144, 170]. After firm adhesion, transmigration of leucocyte might occur. It was previously shown in our lab that EHV-1 infected PBMC slow their rolling and adhere to EC for virus transmission [169], here we document the last step of trafficking (transmigration). Although PBMC-endothelial cells interface is generally very crucial for viral spread through cell-to-cell contact, viruses could be transmitted directly to endothelial cells through transcellular migration of leucocytes themselves through the endothelial cell body. The mechanisms underlying transcellular migration are less well established [144]. As our results show leukocytes transcellular migration through the endothelium body (transcellular), paracellular route (between the cells) through the cell borders couldn`t be excluded. After endothelial cells got infected, the likelihood of paracellular and transcellular leucocyte migration is increasing owing to the disruption of endothelial cells barriers making the gap between cells or the cell body more prone to for PBMC to migrate through. Several viruses (HIV-1, feline immunodeficiency virus, simian immunodeficiency virus (SIV-1), and WNV had been reviewed in [395-397] and thought to invade the brain parenchyma by utilizing a 'Trojan horse' mechanism via diapedesis of infected cells that paracellularly cross the blood-brain barrier (BBB). On the other hand, other viruses as herpes simplex, yellow fever, and measles viruses and HIV are only thought to be carried by infected PBMC to cross the endothelial cells in a process called diapedesis paracellular transmigration but not transcellular. Further

investigation is needed to determine the trigger of this pathway which is considered safe, and possible alternative pathway to the others.

Two events had been recorded and captured, for the first time, by the electron microscopy. The first one is seeing what`s called microvesicle (MV) containing the EHV-1 viral particles outside the cells. This observation triggers the question, are they included in virus life cycle, entry and spread?. The microvesicles not only transport or contain lipid, proteins, mRNA, miRNA, DNA but can also contain the virus particle [398-400]. HSV particle had been recently captured and seen in these MVs and their role in virus spread has been documented [401]. The presence of EHV-1 in these microvesicles speculate their role in virus spread to the uninfected target cells which need further and deep investigations. These MVs are a type of extracellular vesicle ranging from 50 to 1000 nm in diameter and found in many types of body fluids [402, 403]. It has been reported to play a role in cell-to-cell communication. Their release from the cells has three mechanisms as follows; exocytosis from multivesicular bodies leads to exosomes formation, budding directly of shedding from a plasma membrane and finally apoptotic blebs formed by outward plasma membrane protrusion or budding [404, 405]. After releasing of MVs to the extracellular space, interacting with specific target cells occurs via fusion or endocytosis or transcytosis to deliver bioactive molecules as miRNA, mRNA, DNA, proteins and even virus particles in between [406, 407]. This observation needs further investigations to address their role in EHV-1 life cycle and/or spread. The second observed event is the presence of EHV-1 viral particles between the infected endothelial cells to the uninfected one in what`s called tight junction. The tight junction formed between cells makes the two cell membranes of cells particularly of the adherent cells are more tightly adhered together by the adhesion proteins especially claudin making them impermeable to fluids and small particles. This tight junction prevents the cell-free viruses from apical surfaces [188] and responsible for cell-to-cell spread in immunity or antibody protected way.

There are several examples and methods for immunoevasion that EHV-1 does for escaping both humoral and cellular host immunity to avoid its elimination. These methods and mechanisms are as follow: downregulating MHC class I expression in the infected cells [81, 229], interfering viral peptide transport to MHC class I molecules in the ER by repressing transporter associated with antigen processing (TAP) protein [230]. inhibiting antibody cell-mediated cell lysis by inhibition or downregulation of cell surface expression of viral envelope proteins or viral antigens [231], and finally, the viruses establish direct cell-to-cell transmission through protecting themselves physically from antibodies by embedding in extracellular carbohydrate-rich matrix or direct cell-to-cell fusion or transmigration to the endothelial cells.

In conclusion, we can infer that herpesviruses use several ways for direct cell-to-cell transmission between PBMC and endothelial cell, three of them, discussed here, are new way, PBMC-hitching (by using extracellular matrix component to attach, embed, protected from neutralizing antibody and finally spread to other cells or EC), Pass-through (via transcellular migration throughout the endothelial cell body in a secure way) and finally classical cell-to-cell fusion transmission between both cells (**Figure 45)**. These findings have important implications for understanding viruses PBMC to EC transmission in general and herpesviruses specifically which will assist in their treatment and control. Owing to the conserved nature of cell-associated viremia and virus direct cell-to-cell transmission among several herpesviruses, these obtained results may be very pertinent for viruses such as pseudorabies virus, varicella-zoster virus, human cytomegalovirus, and others regarding virus cell-to-cell spread (PBMC-EC).

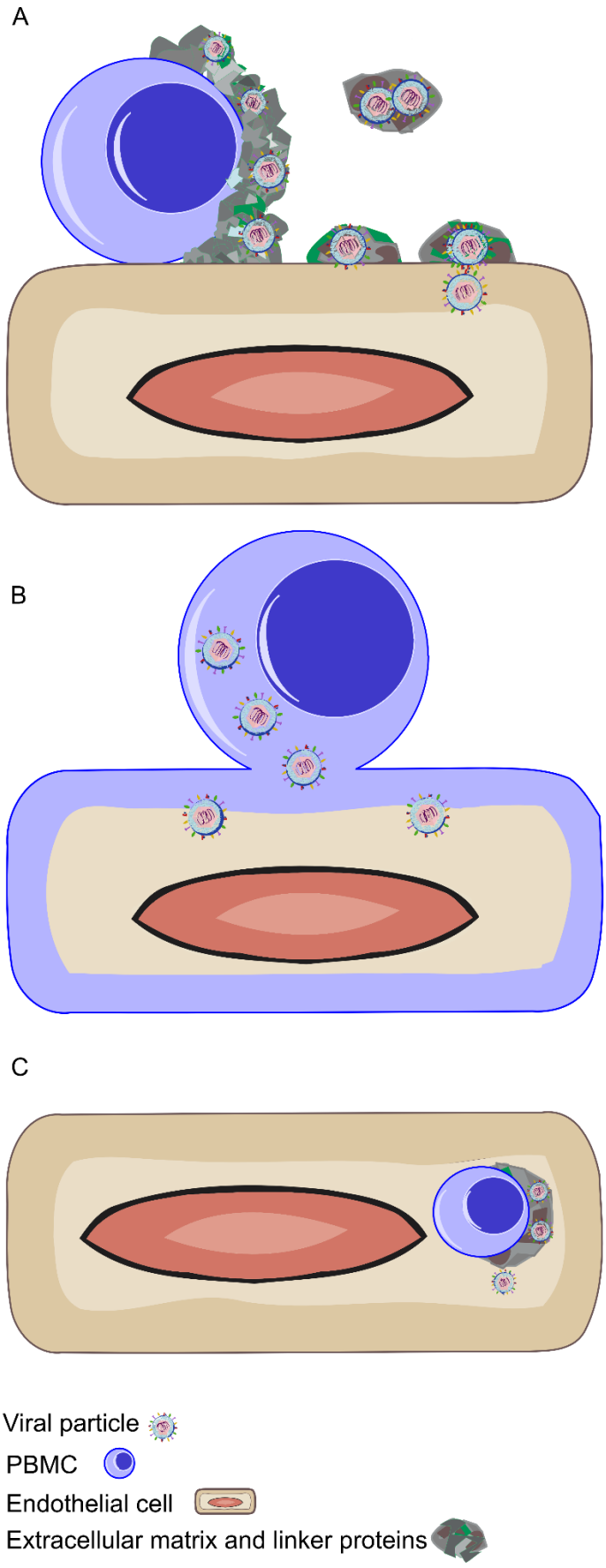
A
B
C
Viral particle
PBMC
Endothelial cell
Extracellular matrix and linker proteins

Figure 45: Three ways of herpesviruses cell-to-cell transmission had been described: (A) Virus transmission through extracellular carbohydrate-rich matrix from infected PBMC to EC (B) Virus transmission from infected PBMC to endothelial cells occurs through microfusion between the two cells (C) Transcellular migration of PBMC within the body of the endothelial cell.

7.2 The role of UL56 in virus transfer between PBMC and EC in EHV-1 and EHV-4

As mentioned before that virus transmission from infected PBMC to endothelial cells is a very crucial step for virus pathogenesis and disease outcomes exhibited as nervous manifestations and abortion. The molecular mechanism of leucocyte recruitment steps had been investigated over several decades [142, 152, 408, 409]. For effective leucocyte recruitment, a complex cascade of events between PBMC and endothelial cells occurs [142, 152, 408, 409]. Each of these events (tethering, rolling, slow rolling, arrest and firm adhesion and transmigration) is essential for effective PBMC recruitment and is controlled by several sets of adhesion molecules. The initial tethering and rolling are mainly initiated and mediated by E and P selectins on endothelial cells with their ligands on leucocytes. Rolling, mainly reversible, has to be followed by leucocyte arrest that is achieved via interaction of activated β1 integrins as very late antigen-4 (VLA4) and β2 integrins as lymphocyte function-associated antigen 1 (LFA-1) with their corresponding ligands, vascular cell adhesion molecule, VCAM-1 and intercellular adhesion molecule-1, ICAM-1, respectively. This activation is mainly either through endothelial-presented chemoattractant and/or chemokines activating VLA-4, LFA-1, Mac-1 [142, 410]. It was previously shown that EHV-1 could maintain capture and establish rolling of infected PBMC over EC monolayer more efficiently than EHV-4 and two genes US3 and gB were demonstrated to reduce virus transfer from PBMC to EC [169].

Equine herpesviruses transmission from infected PBMC to endothelial cells is hypothesized to be affected by UL56 gene. UL56 is an immunomodulatory phosphorylated type II membrane early protein. It was reported to play a key role in several cell surface protein downregulation and immunoevasion in both EHV-1 and EHV-4 [75-78, 81, 82]. Its function for efficient MHC class I interference is achieved together with other proteins (one or more cellular partners induced by the virus or the viral partners) [76, 83]. The role of UL56 is not only restricted to in vitro experiment but also in vivo assessment as together with ORF2 resulted in cytokine responses modulation and disease outcome [79].

To address UL56 role in virus transmission from infected PBMC to endothelial cells, several mutants were constructed. With the fact that had been demonstrated before that EHV-1 is

transmitted from PBMC but EHV-4 not on the static “no-contact” conditions or dynamic conditions, we swapped UL56 gene to investigate their effect on virus transmissibility within the genetic makeup of the vice-versa virus. Both the swapped and deleted UL56 EHV-1 or EHV-4 mutants showed no significant difference in their growth kinetics and plaques size properties compared to parental wild-types viruses. Interestingly, our results revealed that Ul56 had no effect on virus transfer from infected PBMC to endothelial cells either on the static condition or even on dynamic condition (flow chamber setups). EHV-4 wasn`t also induced, following UL56 swapping, to be transmitted in between. The more likely explanation is that the UL56 may be with other genes could affect virus transfer in between [79, 83]. Downregulation of MHC class I was documented to be mainly induced by other viral proteins that assist their role in this downregulation.and it is mainly observed in the virus level, not in the plasmid levels. Double deletion of ORFl/2 resulted in cytokine immunomodulation and the diseases outcomes [79]. Another two genes, UL49.5 or UL43, were displayed to do cell surface molecules downregulation together with UL56 by affecting MHC class I downregulation by affecting TAP [76, 83]. The second possible explanation of the non-role of Ul56 in virus transfer is that not all the observed role of UL56 in cell surface downregulation is the same in different cell types [76, 78, 81-83]. Taken together, UL56 may need the help of other genes to make their effect on virus cell-to-cell transmission which could be through affecting cell surface adhesion molecules or other surface molecules affecting virus cell-to-cell transmission.

The rolling and adhesion between PBMC and endothelial cells are controlled by cell surface adhesion molecules that interact with their ligands on EC. We addressed the two important adhesion molecules (LFA-1 and VLA-4). The expression of these two molecules hadn`t been significantly affected between the mutants and their parental types which come in line with the obtained result for virus transfer in both static and dynamic conditions. The more likely explanation is that each cell type behaves differently than the other one. As previously shown that pUL56 has no role or effect on MHCI down-regulation in mesenchymal cells but it has a significant effect in ED [77]. EHV-1 UL56 suppressed the interferon-alpha and IL-10 induction in ERECs, but no effect or response was observed on PBMC [78]. As a conclusion, these data show that UL56 has no role in virus transfer between infected PBMC and endothelial cells and no effect on cell surface adhesion molecules (VLA-4 and LFA-1) compared to parental viruses.

7.3 EHV-1 viral vector construction to protect against FMD.

The wide species tropism, high infectious capabilities, wide genetic diversity, increase in international trade, animal and human movement, rapid change of environment, the population growth, epidemiological complexity problems, fast replication rate and remarkable transmissibility make foot and mouth disease virus (FMDV) harder to be controlled and make their re-emergence chances are even high and fast. Virus introduction into naïve animals makes severe economic losses and high financial losses to eradicate and control as previously happens in England [300]. The most commonly used control measures are either eradication or vaccination campaigns and strict hygienic biosecurity and control.

Although most of the available commercial and used vaccines are inactivated vaccines either monovalent or bivalent or polyvalent ethyleneimine with aqueous based or oil emulsion based or aluminum-based adjuvant, there were some molecular biological based experimental vaccines which had been addressed [313, 315, 316, 318, 324, 331, 333]. This currently used chemically inactivated virion vaccines against FMDV, which is economically the most important and the highly contagious viral disease of cloven-hoofed livestock (buffaloes, goats, sheep and wild ruminants), have a number of difficulties in their production and applications such as the risk of incomplete virus inactivation and the need to use the circulating serotype or even the subtype to produce the vaccine as there is no cross-immunity between FMDV serotypes and subtypes [339]. Other disadvantages of these currently used vaccines include the need for high–biosecurity production facilities, inability to induce immediate protection, short-lived immunity and the inability to distinguish between infected and vaccinated animals [339]; so the development and construction of a vectored Equine Herpesvirus Type 1 (EHV-1) vaccine against FMDV is very important to be addressed.

As the vaccines are the primary tools in health intervention programs for both humans and animals, this part from the study showed the process for using pRacH as a live universal viral vector vaccine as a vector for FMD P1 polyprotein to hopefully protect animals against several FMD serotypes. Integration and expression of the capsid precursor polypeptide (P1) in RacH were confirmed by immunofluorescence and western blot. The results showed that it is more likely to be good candidate vaccine for protection against FMD. Although most of the available FMD vaccines needs cold chain containment, our viral vector vaccine is stable as it could grow in the normal conditions (37^0C) which is very important in field vaccination. The wide range cell culture infection of pRacH including ruminant cells with no preexisting immunity or antibodies in these animals (ruminants) makes them more suitable than any homologous live viral vector candidates. Another advantage besides that is the non-preexisting immunity makes

Differentiating Infected from Vaccinated Animals (DIVA) is much easier than the homologous lived vector vaccination. The expected humoral and cellular immunity produced by this intracellular live vector besides its more likelihood mucosal immunity produced in the upper respiratory tract may protect against foot and mouth disease. Strong cellular and humoral immune response had been elicited and protected challenged animals completely or partially against several diseases in several studies [281-287]. One of the most momentous factors to consider in developing a viral vaccine vector is the safety. Generally, pRacH is safe as it is attenuated more than 250 continuous/ serial passages in primary porcine kidney cells of the field strain (Rac) isolated in Poland. This attenuation resulted in losing their virulence for baby hamsters as well as lacking clinical symptoms in horses [411]. It produces no clinical signs in animals. It has been utilized as a live vaccine for equine. It didn`t induce or cause any side effects or clinical signs in ruminant animals even during their challenge in several experimental studies [281-287]. RacH could accommodate large sequences, the whole P1 gene, as the strategy aimed to maximize immunogenicity to provide complete protection of animals against FMD by inserting the maximum FMDV protective coding sequence into the RacH (P1). RacH could produce stable and effective expression of the immunogenic proteins and could also provoke both cellular and humoral immune responses. Further outlook animal experimental studies on the immunogenicity and the challenge of recombinant vector will be carried out in the future.

8 Summary

Equine herpesvirus-1 is an alphaherpesvirus affecting equid species resulting in severe clinical outcomes manifested clinically as respiratory infection, abortion and nervous manifestations. EHV-1 establishes cell-associated viremia, following respiratory infection, reaching the target endothelial lining of blood vessel of reproductive tract and nervous system, resulting in vasculitis and thrombosis exhibited as clinical outcomes. Although PBMC-EC interface is very crucial in virus pathogenesis and clinical outcomes advent, the mechanisms underlying virus transfer from infected PBMC to EC are still unknown. To investigate these mechanisms, two-step en passant mutagenesis, functional assays (contact assays, transwell assays and flow chamber assay) together with confocal immunofluorescence data and laser scanning live cell imaging and electron microscope were executed. The single viral particle or viral cluster was found to colocalize with the extracellular matrix components. Viral particle or viral clusters embedding within these extracellular matrices are protected from the neutralizing antibodies and efficiently transmitted to endothelial cells. Destruction of this extracellular matrix from infected PBMC surface affects the infectious capability of PBMC. EHV-4, although found colocalized with ECM, is not transmitted from infected PBMC either in the static or dynamic conditions conjecturing their trapping and inactivation in these structures. Two genes, gB and gD have been implicated their role in virus transfer between PBMC and EC. Another two important mechanisms were assessed for the first time, membrane fusion between infected PBMC and endothelial cell as a mode of cell-to-cell transmission and the other one is the transcellular migration of PBMC into the endothelial cells transferring the virus to them. Two important events had been detected and caught by electron microscopy and require further and deep investigation. The first event is the first-time detection of microvesicle including equine herpesvirus-1 viral particle augmenting their role in viral life cycle and virus spread. EHV-1 was also found in a tight junction between infected and uninfected endothelial cells raising their role in viral cell-to-cell between endothelial cells.

UL56 is a phosphorylated type II membrane early protein which has a function in downregulating MHC class I and other surface molecules. To assess their role in virus transfer between PBMC and endothelial cells in EHV-1 and EHV-4, different set of mutants were generated by two-step en passant mutagenesis. The deletion mutants and swapping mutants of UL56 in EHV-1/4 showed no significant difference in the in vitro growth properties compared to their parental viruses. The deletion mutants and swapping mutants of UL56 between the two viruses had no role in virus transfer in both static or dynamic conditions. The expression levels of adhesion molecules of the surface of mutant-infected PBMC very late antigen (VLA-4) and leucocyte function-associated antigen (LFA-1) showed no statistical significant difference

compared to their parental viruses (EHV-1 or EHV-4 WT). The results manifested that UL56 alone or /swapping in or between EHV-1 and EHV-4 has no effect in virus transfer between PBMC and endothelial cells raising the questions of the other cellular and viral partner that could help UL56 to affect virus transfer as happens with MHC-1 downregulation.

As FMD is one of the most contagious and highly economically viral disease affecting cloven-hoofed animals, it causes severe economic losses and social consequences. The most available used inactivated vaccines for FMD are short-lived humoral immunity, need cold chain containment, can`t protect against different serotypes and even sub serotypes and have difficulties in production. Construction of rH-P1 of FMD was performed and confirmed by PCR, RFLP, and sequencing for future using against FMD to differentiate between vaccinated and humoral immunity following natural infection (DIVA capability), not need for cold chain containment and finally, production of cellular and humoral immunity. Recombinant growth properties (plaque size and growth kinetics) were compared to their parental virus pRacH, P1 integration and expression were assessed by immunofluorescence and western blotting. The virus had been prepared and sent for future animal experiment challenge.

9 Zusammenfassung

Entwicklung und molekulare Charakterisierung eines Equinen Herpesvirus Typ 1-basierten Impfstoffes gegen das Maul-und-Klauenseuche-Virus

Das Equine Herpesvirus Typ 1 (EHV-1) ist ein Alphaherpesvirus, welches Equiden befällt und schwere klinische Symptome wie Infektionen des Respirationstraktes, Aborte und Nervenleiden hervorrufen kann. EHV-1 etabliert nach Infektion über die Atemwege eine zellassoziierte Virämie und erreicht so die Endothelien der Gefäße im Reproduktionstrakt und im Nervensystem. Dies führt zu Vaskulitiden und Thrombosen, welche sich im klinischen Bild widerspiegeln. Obwohl die Schnittstelle zwischen den mononukleären Zellen des peripheren Blutes (PBMCs, *peripheral blood mononuclear cells*) und den Endothelzellen (EZ) immens wichtig für Pathogenese und Klinik der Erkrankung ist, sind die zugrundeliegenden Mechanismen zum Transfer des Virus von PBMCs zu EZs nicht bekannt. Um diese zu untersuchen, wurden neben *Two-step en passant* Mutagenese und funktionalen Assays (Kontaktassays, Transwell Assays und Durchflusskammer Assays) auch konfokale Immunofluoreszenzmikroskopie, Laser-Scanning Live Cell Mikroskopie und Elektronenmikroskopie durchgeführt. Einzelne Viruspartikel oder Viruscluster kolokalisierten mit Komponenten der extrazellulären Matrix. Viruspartikel oder Viruscluster, die in dieser extrazellulären Matrix eingebettet sind, sind dadurch vor neutralisierenden Antikörpern geschützt und können effizient an EZs übertragen werden. Abbau der extrazellulären Matrix auf der Zelloberfläche von PBMCs beeinflusst deren Infektionsfähigkeit. EHV-4 hingegen, welches auch in der extrazellulären Matrix lokalisiert ist, wird nicht durch PBMCs übertragen; weder in statischen noch in dynamischen Konditionen. Dies lässt vermuten, dass die Haftung von EHV-4 in diesen Strukturen zu einer Virusinaktivierung führt.

Zwei EHV-Gene, gB und gD, spielen eine Rolle im Transfer des Virus von PBMCs zu EZs. Zwei weitere wichtige Mechanismen wurden hier zum ersten Mal beschrieben: Membranfusionen zwischen PBMCs und EZs als ein möglicher Weg der Zell-zu-Zell-Übertragung, sowie die transzelluläre Migration der PBMCs in die EZs. Zwei wichtige Ereignisse wurden via Elektronenmikroskopie detektiert und müssen weiterverfolgt und genauer beschrieben werden. Das erste Ereignis ist der erste Nachweis von EHV-1 Viruspartikel enthaltenden Mikrovesikeln, der damit deren Bedeutung sowohl im Virus Replikationszyklus als auch in der Virusausbreitung erweitert. EHV-1 wurde außerdem in einer *tight junction* zwischen infizierten und uninfizierten EZs detektiert, was deren Rolle in der Zell-zu-Zell-Übertragung zwischen EZs unterstreicht.

UL56 ist ein phosphoryliertes Typ II Membranprotein mit früher Kinetik, welches eine Rolle in der Herabregulation von MHC-Klasse I und anderen Oberflächenmolekülen spielt. Um die Rolle von UL56 im Virustransfer zwischen PBMCs und EZs in EHV-1 und EHV-4 zu bestimmen

wurden verschiedene Virusmutanten per *two-step en passant* Mutagenese generiert. Die Deletionsmutanten, sowie Austauschmutanten von UL56 in EHV-1 und EHV-4 zeigten keine signifikanten Unterschiede in *in vitro* Wachstumseigenschaften im Vergleich zu den parentalen Viren. Die Deletionsmutanten, sowie Austauschmutanten von UL56 in beiden Viren spielen keine Rolle bei der Virusübertragung, weder in statischen noch in dynamischen Konditionen. Expressionslevel von Adhäsionsmolekülen auf der Zelloberfläche von PBMCs welche mit den Virusmutanten infiziert wurden, das *very late antigen* (VLA-4) und das *leucocyte function-associated antigen* (LFA-1), zeigten keinerlei statistisch signifikanten Unterschiede im Vergleich zu den parentalen Viren (EHV-1 und EHV-4 Wildtyp). Diese Ergebnisse festigen die Annahme, dass UL56 allein und/oder Austauschmutanten in EHV-1 und EHV-4 keinen Effekt auf den Virustransfer zwischen PBMCs und EZs hat und wirft Fragen zu anderen zellulären oder viralen Interaktionspartnern auf, die mit UL56 bei der Beeinflussung des Virustransfers durch MHC-1 Herabregulation zusammenwirken.

Maul- und Klauenseuche (*Foot-and-mouth disease*, FMD) ist eine der ansteckendsten Krankheiten von Paarhufern, führt zu hohen wirtschaftlichen Verlusten und hat soziale Auswirkungen. Die am meisten genutzte inaktivierte Vakzine gegen FMD ruft eine kurzzeitige humorale Immunität hervor, benötigt eine geschlossene Kühlkette, schützt nicht gegen verschiedene Serotypen und Sub-Serotypen und ist schwierig herzustellen. Die Konstruktion von FMD rH-P1 wurde per PCR, RFLP und Sequenzierung bestätigt. Diese Vakzine ist für den späteren Gebrauch gegen FMD bestimmt und differenziert zwischen geimpften Tieren und Tieren, die eine humorale Immunität durch eine natürliche Infektion erlangt haben (DIVA). Außerdem benötigt sie keine geschlossene Kühlkette und führt zu einer zellulären und humoralen Immunität. Die Wachstumseigenschaften des rekombinanten Virus (Plaque Größen und Wachstumskinetiken) wurden mit dem parentalen Virus pRacH verglichen. P1 Integration und Expression wurden per Immunfluoreszenz und Westernblot bestätigt. Das Virus wurde hergestellt und für Testungen in Tierversuchen verschickt.

10 References

1. Wolf, K. and R.W. Darlington, Channel catfish virus: a new herpesvirus of ictalurid fish. Journal of Virology, 1971. 8(4): p. 525-533.
2. Davison, A.J., et al., The order herpesvirales. Archives of virology, 2009. 154(1): p. 171-177.
3. Murphy, F.A., et al., Virus taxonomy: classification and nomenclature of viruses. Vol. 10. 2012: Springer Science & Business Media.
4. Roizman, B. and J. Baines, The diversity and unity of Herpesviridae. Comparative immunology, microbiology and infectious diseases, 1991. 14(2): p. 63-79.
5. Ackermann, M., Pathogenesis of gammaherpesvirus infections. Veterinary microbiology, 2006. 113(3): p. 211-222.
6. Roizmann, B., et al., The familyHerpesviridae: an update. Archives of virology, 1992. 123(3): p. 425-449.
7. Davison, A.J., Evolution of the herpesviruses. Veterinary microbiology, 2002. 86(1-2): p. 69-88.
8. Tischer, B.K. and N. Osterrieder, Herpesviruses—A zoonotic threat? Veterinary microbiology, 2010. 140(3): p. 266-270.
9. TANIGUCHI, A., et al., Pathogenicity of a new neurotropic equine herpesvirus 9 (gazelle herpesvirus 1) in horses. Journal of Veterinary Medical Science, 2000. 62(2): p. 215-218.
10. Ma, G., W. Azab, and N. Osterrieder, Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)—masters of co-evolution and a constant threat to equids and beyond. Veterinary microbiology, 2013. 167(1-2): p. 123-134.
11. Reed, S.M. and R.E. Toribio, Equine herpesvirus 1 and 4. Veterinary Clinics: Equine Practice, 2004. 20(3): p. 631-642.
12. Ostlund, E.N., The equine herpesviruses. Veterinary Clinics of North America: Equine Practice, 1993. 9(2): p. 283-294.
13. Ehlers, B., et al., Detection of new DNA polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and deoxyinosine-substituted primers. Virus genes, 1999. 18(3): p. 211-220.
14. Telford, E.A., et al., The DNA sequence of equine herpesvirus-1. Virology, 1992. 189(1): p. 304-316.
15. O'Callaghan, D. and N. Osterrieder, Encyclopedia of virology. 2008.
16. Telford, E., et al., The DNA sequence of equine herpesvirus-4. Journal of General Virology, 1998. 79(5): p. 1197-1203.

17. Brown, J.C. and W.W. Newcomb, Herpesvirus capsid assembly: insights from structural analysis. Current opinion in virology, 2011. 1(2): p. 142-149.
18. Fauquet, C.M., et al., Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. 2005: Academic Press.
19. Pellet, P., The family Herpesviridae: a brief introduction. Fields' virology, 2007: p. 3137-3166.
20. Owen, D.J., C.M. Crump, and S.C. Graham, Tegument assembly and secondary envelopment of alphaherpesviruses. Viruses, 2015. 7(9): p. 5084-5114.
21. Roizman, B. and D. Furlong, The replication of herpesviruses, in Reproduction. 1974, Springer. p. 229-403.
22. Osterrieder, N. and G.R. Van de Walle, Pathogenic potential of equine alphaherpesviruses: The importance of the mononuclear cell compartment in disease outcome. Veterinary Microbiology, 2010. 143(1): p. 21-28.
23. Turtinen, L.W. and G.P. Allen, Identification of the envelope surface glycoproteins of equine herpesvirus type 1. Journal of General Virology, 1982. 63(2): p. 481-485.
24. Ahmed, S., S. Broad, and N. Edington, Immunoprecipitation of viral polypeptides of equid herpesvirus 1 and 4 by serum from experimentally infected ponies. Veterinary microbiology, 1993. 34(3): p. 205-219.
25. Spiesschaert, B., et al., Glycoprotein B of equine herpesvirus type 1 has two recognition sites for subtilisin-like proteases that are cleaved by furin. Journal of General Virology, 2016. 97(5): p. 1218-1228.
26. Wellington, J., D. Love, and J. Whalley, Evidence for involvement of equine herpesvirus 1 glycoprotein B in cell-cell fusion. Archives of virology, 1996. 141(1): p. 167-175.
27. Eisenberg, R.J., et al., Herpes virus fusion and entry: a story with many characters. Viruses, 2012. 4(5): p. 800-832.
28. Neubauer, A., et al., Analysis of the contributions of the equine herpesvirus 1 glycoprotein gB homolog to virus entry and direct cell-to-cell spread. Virology, 1997. 227(2): p. 281-294.
29. Spiesschaert, B., N. Osterrieder, and W. Azab, 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, 2015. 7(2): p. 522-542.
30. Audonnet, J.-C., et al., Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. Journal of general virology, 1990. 71(12): p. 2969-2978.

31. Elton, D.M., et al., Identification of the equine herpesvirus type 1 glycoprotein 17/18 as a homologue of herpes simplex virus glycoprotein D. Journal of general virology, 1992. 73(5): p. 1227-1233.
32. Flowers, C. and D. O'Callaghan, Equine herpesvirus 1 glycoprotein D: mapping of the transcript and a neutralization epitope. Journal of virology, 1992. 66(11): p. 6451-6460.
33. Whalley, M., et al., Identification and comparative sequence analysis of a gene in equine herpesvirus 1 with homology to the herpes simplex virus glycoprotein D gene. Virus Genes, 1991. 5(4): p. 313-325.
34. Azab, W., et al., Equid herpesvirus type 4 uses a restricted set of equine major histocompatibility complex class I proteins as entry receptors. Journal of General Virology, 2014. 95(7): p. 1554-1563.
35. Van de Walle, G.R., et al., Equine herpesvirus 1 entry via endocytosis is facilitated by αV integrins and an RSD motif in glycoprotein D. Journal of virology, 2008. 82(23): p. 11859-11868.
36. Azab, W. and N. Osterrieder, Glycoproteins D of equine herpesvirus type 1 (EHV-1) and EHV-4 determine cellular tropism independently of integrins. Journal of virology, 2012. 86(4): p. 2031-2044.
37. Nicola, A.V., et al., Herpes simplex virus type 1 enters human epidermal keratinocytes, but not neurons, via a pH-dependent endocytic pathway. Journal of virology, 2005. 79(12): p. 7609-7616.
38. Nicola, A.V. and S.E. Straus, Cellular and viral requirements for rapid endocytic entry of herpes simplex virus. Journal of virology, 2004. 78(14): p. 7508-7517.
39. Frampton, A.R., et al., Equine herpesvirus 1 utilizes a novel herpesvirus entry receptor. Journal of virology, 2005. 79(5): p. 3169-3173.
40. Csellner, H., et al., EHV-1 glycoprotein D (EHV-1 gD) is required for virus entry and cell-cell fusion, and an EHV-1 gD deletion mutant induces a protective immune response in mice. Archives of virology, 2000. 145(11): p. 2371-2385.
41. Spear, P. Entry of alphaherpesviruses into cells. in Seminars in virology. 1993. Elsevier.
42. Lazear, E., et al., Engineered disulfide bonds in herpes simplex virus type 1 gD separate receptor binding from fusion initiation and viral entry. Journal of virology, 2008. 82(2): p. 700-709.
43. Connolly, S.A., et al., Fusing structure and function: a structural view of the herpesvirus entry machinery. Nature reviews Microbiology, 2011. 9(5): p. 369-381.
44. von Einem, J., et al., In vitro and in vivo characterization of equine herpesvirus type 1 (EHV-1) mutants devoid of the viral chemokine-binding glycoprotein G (gG). Virology, 2007. 362(1): p. 151-162.

45. Bryant, N.A., et al., Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. The EMBO journal, 2003. 22(4): p. 833-846.
46. Van de Walle, G.R., et al., Herpesvirus chemokine-binding glycoprotein G (gG) efficiently inhibits neutrophil chemotaxis in vitro and in vivo. The Journal of Immunology, 2007. 179(6): p. 4161-4169.
47. Thormann, N., et al., 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 research, 2012. 169(1): p. 203-211.
48. Van de Walle, G.R., et al., A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. The Journal of infectious diseases, 2009. 200(1): p. 20-25.
49. Favoreel, H.W., H.J. Nauwynck, and M.B. Pensaert, Role of the cytoplasmic tail of gE in antibody-induced redistribution of viral glycoproteins expressed on pseudorabies-virus-infected cells. Virology, 1999. 259(1): p. 141-147.
50. Schumacher, D., et al., Glycoproteins E and I of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. Journal of virology, 2001. 75(23): p. 11307-11318.
51. Matsumura, T., et al., An equine herpesvirus type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses. Virology, 1998. 242(1): p. 68-79.
52. Frampton Jr, A.R., et al., Contribution of gene products encoded within the unique short segment of equine herpesvirus 1 to virulence in a murine model. Virus research, 2002. 90(1-2): p. 287-301.
53. Tsujimura, K., et al., Equine herpesvirus type 1 mutant defective in glycoprotein E gene as candidate vaccine strain. Journal of Veterinary Medical Science, 2009. 71(11): p. 1439-1448.
54. Azab, W., M.J. Lehmann, and N. Osterrieder, Glycoprotein H and α4β1 integrins determine the entry pathway of alphaherpesviruses. Journal of virology, 2013. 87(10): p. 5937-5948.
55. Chowdary, T.K., et al., Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL. Nature structural & molecular biology, 2010. 17(7): p. 882-888.
56. Azab, W. and A. El-Sheikh, The Role of Equine Herpesvirus Type 4 Glycoprotein K in Virus Replication. Viruses, 2012. 4(8): p. 1258-1263.
57. Derbigny, W.A., et al., The ElCP22 protein of equine herpesvirus 1 physically interacts with the immediate-early protein and with itself to form dimers and higher-order complexes. Journal of virology, 2000. 74(3): p. 1425-1435.

58. Kim, S.K., D.E. Bowles, and D.J. O'Callaghan, The γ2 late glycoprotein K promoter of equine herpesvirus 1 is differentially regulated by the IE and EICP0 proteins. Virology, 1999. 256(2): p. 173-179.
59. Neubauer, A. and N. Osterrieder, Equine herpesvirus type 1 (EHV-1) glycoprotein K is required for efficient cell-to-cell spread and virus egress. Virology, 2004. 329(1): p. 18-32.
60. Stokes, A., et al., The expression of the proteins of equine herpesvirus 1 which share homology with herpes simplex virus 1 glycoproteins H and L. Virus research, 1996. 40(1): p. 91-107.
61. Roop, C., L. Hutchinson, and D.C. Johnson, A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. Journal of virology, 1993. 67(4): p. 2285-2297.
62. Rudolph, J. and N. Osterrieder, Equine herpesvirus type 1 devoid of gM and gp2 is severely impaired in virus egress but not direct cell-to-cell spread. Virology, 2002. 293(2): p. 356-367.
63. Osterrieder, N., et al., 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. Journal of virology, 1996. 70(6): p. 4110-4115.
64. Osterrieder, N., et al., Synthesis and processing of the equine herpesvirus 1 glycoprotein M. Virology, 1997. 232(1): p. 230-239.
65. Rudolph, J., et al., The gene 10 (UL49. 5) product of equine herpesvirus 1 is necessary and sufficient for functional processing of glycoprotein M. Journal of virology, 2002. 76(6): p. 2952-2963.
66. Verweij, M.C., et al., Inhibition of mouse TAP by immune evasion molecules encoded by non-murine herpesviruses. Molecular immunology, 2011. 48(6): p. 835-845.
67. Smith, P.M., et al., 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. Journal of virology, 2005. 79(8): p. 5105-5115.
68. Hansen, J.E., et al., Prediction of O-glycosylation of mammalian proteins: specificity patterns of UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase. Biochemical Journal, 1995. 308(3): p. 801-813.
69. Wellington, J.E., et al., The highly O-glycosylated glycoprotein gp2 of equine herpesvirus 1 is encoded by gene 71. Journal of virology, 1996. 70(11): p. 8195-8198.
70. Azab, W., et al., Glycoprotein C of equine herpesvirus 4 plays a role in viral binding to cell surface heparan sulfate. Virus research, 2010. 151(1): p. 1-9.

71. Sugahara, Y., et al., Adaptation of equine herpesvirus 1 to unnatural host ledto mutation of the gC resulting in increased susceptibilityof the virus to heparin. Archives of virology, 1997. 142(9): p. 1849-1856.
72. Osterrieder, N., Construction and characterization of an equine herpesvirus 1 glycoprotein C negative mutant. Virus research, 1999. 59(2): p. 165-177.
73. Huemer, H.P., et al., gp13 (EHV-gC): a complement receptor induced by equine herpesviruses. Virus research, 1995. 37(2): p. 113-126.
74. Andoh, K., et al., The haemagglutination activity of equine herpesvirus type 1 glycoprotein C. Virus research, 2015. 195: p. 172-176.
75. Ma, G., et al., Identification and characterization of equine herpesvirus type 1 pUL56 and its role in virus-induced downregulation of major histocompatibility complex class I. Journal of virology, 2012. 86(7): p. 3554-3563.
76. Said, A., et al., Equine herpesvirus type 4 UL56 and UL49. 5 proteins downregulate cell surface major histocompatibility complex class I expression independently of each other. Journal of virology, 2012. 86(15): p. 8059-8071.
77. Claessen, C., et al., Equid herpesvirus 1 (EHV1) infection of equine mesenchymal stem cells induces a pUL56-dependent downregulation of select cell surface markers. Vet Microbiol, 2015. 176(1-2): p. 32-9.
78. Hussey, G.S., et al., Equine herpesvirus type 1 pUL56 modulates innate responses of airway epithelial cells. Virology, 2014. 464: p. 76-86.
79. Hussey, G.S., et al., Evaluation of immune responses following infection of ponies with an EHV-1 ORF1/2 deletion mutant. Veterinary research, 2011. 42(1): p. 23.
80. Claessen, C., et al., Equine herpesvirus type 1 (EHV1) induces alterations in the immunophenotypic profile of equine monocyte-derived dendritic cells. The Veterinary Journal, 2016. 210: p. 85-88.
81. Huang, T., et al., Major histocompatibility complex class I downregulation induced by equine herpesvirus type 1 pUL56 is through dynamin-dependent endocytosis. Journal of virology, 2014. 88(21): p. 12802-12815.
82. Claessen, C., et al., Equid herpesvirus 1 (EHV1) infection of equine mesenchymal stem cells induces a pUL56-dependent downregulation of select cell surface markers. Veterinary microbiology, 2015. 176(1-2): p. 32-39.
83. Huang, T., G. Ma, and N. Osterrieder, Equine herpesvirus 1 multiply inserted transmembrane protein pUL43 cooperates with pUL56 in downregulation of cell surface major histocompatibility complex class I. Journal of virology, 2015. 89(12): p. 6251-6263.

84. Harty, R., et al., Mapping the termini and intron of the spliced immediate-early transcript of equine herpesvirus 1. Journal of virology, 1989. 63(12): p. 5101-5110.
85. Yalamanchili, R.R. and D.J. O'Callaghan, Sequence and organization of the genomic termini of equine herpesvirus type 1. Virus research, 1990. 15(2): p. 149-161.
86. Hasebe, R., et al., Infectious entry of equine herpesvirus-1 into host cells through different endocytic pathways. Virology, 2009. 393(2): p. 198-209.
87. Kurtz, B.M., et al., Equus caballus major histocompatibility complex class I is an entry receptor for equine herpesvirus type 1. Journal of virology, 2010. 84(18): p. 9027-9034.
88. Sasaki, M., et al., Equine major histocompatibility complex class I molecules act as entry receptors that bind to equine herpesvirus-1 glycoprotein D. Genes to Cells, 2011. 16(4): p. 343-357.
89. Geraghty, R.J., et al., Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science, 1998. 280(5369): p. 1618-1620.
90. Granzow, H., B.G. Klupp, and T.C. Mettenleiter, Entry of pseudorabies virus: an immunogold-labeling study. Journal of virology, 2005. 79(5): p. 3200-3205.
91. Spear, P.G. and R. Longnecker, Herpesvirus entry: an update. Journal of virology, 2003. 77(19): p. 10179-10185.
92. Sodeik, B., M.W. Ebersold, and A. Helenius, Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. The Journal of cell biology, 1997. 136(5): p. 1007-1021.
93. Zaichick, S.V., K.P. Bohannon, and G.A. Smith, Alphaherpesviruses and the cytoskeleton in neuronal infections. Viruses, 2011. 3(7): p. 941-981.
94. Nicoll, M.P., J.T. Proença, and S. Efstathiou, The molecular basis of herpes simplex virus latency. FEMS microbiology reviews, 2012. 36(3): p. 684-705.
95. LaBoissière, S. and P. O'Hare, Analysis of HCF, the cellular cofactor of VP16, in herpes simplex virus-infected cells. Journal of virology, 2000. 74(1): p. 99-109.
96. Paillot, R., et al., Functional and phenotypic characterization of distinct porcine dendritic cells derived from peripheral blood monocytes. Immunology, 2001. 102(4): p. 396-404.
97. Lehman, I. and P.E. Boehmer, Replication of herpes simplex virus DNA. Journal of Biological Chemistry, 1999. 274(40): p. 28059-28062.
98. Mettenleiter, T.C., B.G. Klupp, and H. Granzow, Herpesvirus assembly: a tale of two membranes. Current opinion in microbiology, 2006. 9(4): p. 423-429.
99. Pomeranz, L.E., A.E. Reynolds, and C.J. Hengartner, Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiology and molecular biology reviews, 2005. 69(3): p. 462-500.

100. Frampton, A.R., et al., Equine herpesvirus 1 enters cells by two different pathways, and infection requires the activation of the cellular kinase ROCK1. Journal of virology, 2007. 81(20): p. 10879-10889.
101. Allen, G., et al., Equid herpesvirus 1 and equid herpesvirus 4 infections. Infectious diseases of livestock, 2004. 2: p. 829-859.
102. Carvalho, R., et al., Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction. Archives of virology, 2000. 145(9): p. 1773-1787.
103. Borchers, K., et al., Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR. Journal of general virology, 1997. 78(5): p. 1109-1114.
104. Slater, J., et al., The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. Journal of General Virology, 1994. 75(8): p. 2007-2016.
105. Gibson, J., et al., Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation. Archives of virology, 1992. 123(3): p. 351-366.
106. Edington, N., C. Bridges, and J. Patel, Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: equine stroke. Archives of virology, 1986. 90(1): p. 111-124.
107. Patel, J. and J. Heldens, Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review. The Veterinary Journal, 2005. 170(1): p. 14-23.
108. Welch, H.M., et al., Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. Journal of General Virology, 1992. 73(2): p. 261-268.
109. Edington, N., H. Welch, and L. Griffiths, The prevalence of latent equid herpesviruses in the tissues of 40 abattoir horses. Equine veterinary journal, 1994. 26(2): p. 140-142.
110. Borchers, K., P. Thein, and A. Sterner-Kock, Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation. Equine veterinary journal, 2006. 38(3): p. 283-287.
111. Said, A., Development of a vectored equine herpesvirus type 1 (EHV-1) vaccine against pandemic influenza A virus (09/H1N1) 2013, Freien Universität Berlin.
112. Kydd, J.H., et al., Distribution of Equid herpesvirus-1 (EHV-1) in respiratory tract associated lymphoid tissue: implications for cellular immunity. Equine Veterinary Journal, 1994. 26(6): p. 470-473.

113. Gryspeerdt, A.C., et al., Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Veterinary microbiology, 2010. 142(3): p. 242-253.
114. Heldens, J.G., et al., Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn EHV 1, 4). Vaccination/challenge experiments in foals and pregnant mares. Vaccine, 2001. 19(30): p. 4307-4317.
115. Van Maanen, C., Equine herpesvirus 1 and 4 infections: an update. Veterinary Quarterly, 2002. 24(2): p. 57-78.
116. Vandekerckhove, A., et al., In vitro culture of equine respiratory mucosa explants. The Veterinary Journal, 2009. 181(3): p. 280-287.
117. Wilsterman, S., et al., Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia. Veterinary microbiology, 2011. 149(1-2): p. 40-47.
118. Kydd, J.H., et al., Distribution of Equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine veterinary journal, 1994. 26(6): p. 466-469.
119. Goodman, L.B., et al., A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS pathogens, 2007. 3(11): p. e160.
120. Wilsterman, S., et al., Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia. Veterinary microbiology, 2011. 149(1): p. 40-47.
121. Vandekerckhove, A.P., et al., Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. Journal of General Virology, 2010. 91(8): p. 2019-2028.
122. Gosztonyi, G., K. Borchers, and H. Ludwig, Pathogenesis of equine herpesvirus-1 infection in the mouse model. Apmis, 2009. 117(1): p. 10-21.
123. Slater, J., et al., Report of the equine herpesvirus-1 Havermeyer Workshop, San Gimignano, Tuscany, June 2004. Veterinary immunology and immunopathology, 2006. 111(1-2): p. 3-13.
124. Smith, K. and K. Borchers, A study of the pathogenesis of equid herpesvirus-1 (EHV-1) abortion by DNA in-situ hybridization. Journal of comparative pathology, 2001. 125(4): p. 304-310.
125. Smith, D., A. Hamblin, and N. Edington, 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 veterinary journal, 2001. 33(2): p. 138-142.
126. Smith, D., A. Hamblin, and N. Edington, Equid herpesvirus 1 infection of endothelial cells requires activation of putative adhesion molecules: an in vitro model. Clinical & Experimental Immunology, 2002. 129(2): p. 281-287.

127. Lunn, D., et al., Equine Herpesvirus-1 Consensus Statement. Journal of Veterinary Internal Medicine, 2009. 23(3): p. 450-461.
128. Allen, G. and J. Bryans, Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. Progress in veterinary microbiology and immunology, 1986. 2: p. 78-144.
129. Smith, K., J. Mumford, and K. Lakhani, A comparison of equid herpesvirus-1 (EHV-1) vascular lesions in the early versus late pregnant equine uterus. Journal of comparative pathology, 1996. 114(3): p. 231-247.
130. Edington, N., B. Smyth, and L. Griffiths, The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions. Journal of comparative pathology, 1991. 104(4): p. 379-387.
131. Corner, A., D. Mitchell, and E. Meads, Equine virus abortion in Canada. I. Pathological studies on aborted fetuses. The Cornell veterinarian, 1963. 53: p. 78.
132. Machida, N., et al., Cardio-histopathological observations on aborted equine fetuses infected with equid herpesvirus 1 (EHV-1). Journal of comparative pathology, 1997. 116(4): p. 379-385.
133. Murray, M.J., et al., Neonatal equine herpesvirus type 1 infection on a thoroughbred breeding farm. Journal of Veterinary Internal Medicine, 1998. 12(1): p. 36-41.
134. Pusterla, N., et al., Equine herpesvirus-1 myeloencephalopathy: a review of recent developments. The Veterinary Journal, 2009. 180(3): p. 279-289.
135. Nugent, J., et al., Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. Journal of virology, 2006. 80(8): p. 4047-4060.
136. Jackson, T., et al., Equine herpesvirus 1 infection of horses: studies on the experimentally induced neurologic disease. American journal of veterinary research, 1977. 38(6): p. 709-719.
137. Maanen, C.v., et al., Neurological disease associated with EHV-1-infection in a riding school: clinical and virological characteristics. Equine Veterinary Journal, 2001. 33(2): p. 191-196.
138. Hussey, G.S., et al., Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. Veterinary research, 2013. 44(1): p. 118.
139. Sprayberry, K.A. and N.E. Robinson, Robinson's Current Therapy in Equine Medicine-E-Book. 2014: Elsevier Health Sciences.
140. Vandekerckhove, A.P., et al., Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect mononuclear cells during early steps of infection in nasal mucosal explants. Veterinary microbiology, 2011. 152(1-2): p. 21-28.

141. Barreiro, O. and F. Sánchez-Madrid, Molecular basis of leukocyte–endothelium interactions during the inflammatory response. Revista Española de Cardiología (English Edition), 2009. 62(5): p. 552-562.
142. Kelly, M., J.M. Hwang, and P. Kubes, Modulating leukocyte recruitment in inflammation. Journal of Allergy and Clinical Immunology, 2007. 120(1): p. 3-10.
143. Muller, W., Getting leukocytes to the site of inflammation. Veterinary pathology, 2013. 50(1): p. 7-22.
144. Muller, W.A., Mechanisms of leukocyte transendothelial migration. Annual Review of Pathology: Mechanisms of Disease, 2011. 6: p. 323-344.
145. Muller, W.A., Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol, 2003. 24(6): p. 327-34.
146. Ley, K., The role of selectins in inflammation and disease. Trends in molecular medicine, 2003. 9(6): p. 263-268.
147. Vestweber, D. and J.E. Blanks, Mechanisms that regulate the function of the selectins and their ligands. Physiological reviews, 1999. 79(1): p. 181-213.
148. Kuwano, Y., et al., Rolling on E-or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils. Blood, 2010. 116(4): p. 617-624.
149. Nandi, A., P. Estess, and M. Siegelman, Bimolecular complex between rolling and firm adhesion receptors required for cell arrest: CD44 association with VLA-4 in T cell extravasation. Immunity, 2004. 20(4): p. 455-465.
150. Schmidt, S., M. Moser, and M. Sperandio, The molecular basis of leukocyte recruitment and its deficiencies. Molecular immunology, 2013. 55(1): p. 49-58.
151. Hynes, R.O., Integrins: versatility, modulation, and signaling in cell adhesion. Cell, 1992. 69(1): p. 11-25.
152. Herter, J. and A. Zarbock, Integrin regulation during leukocyte recruitment. The Journal of Immunology, 2013. 190(9): p. 4451-4457.
153. Williams, M.R., et al., Emerging mechanisms of neutrophil recruitment across endothelium. Trends in immunology, 2011. 32(10): p. 461-469.
154. Petri, B., M. Phillipson, and P. Kubes, The physiology of leukocyte recruitment: an in vivo perspective. The Journal of Immunology, 2008. 180(10): p. 6439-6446.
155. Lawrence, M.B., et al., Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L, P, E). The Journal of cell biology, 1997. 136(3): p. 717-727.
156. Goehring, L., et al., Infection of central nervous system endothelial cells by cell-associated EHV-1. Veterinary microbiology, 2011. 148(2): p. 389-395.
157. Joseph, J., F.D. Lublin, and R.L. Knobler, Modulation of T cell–endothelial adhesion by astrocyte conditioned medium. Glia, 1997. 21(4): p. 408-412.

158. Proft, A., et al., The Role of the Equine Herpesvirus Type 1 (EHV-1) US3-Encoded Protein Kinase in Actin Reorganization and Nuclear Egress. Viruses, 2016. 8(10): p. 275.
159. Charrel, R.N. and X. de Lamballerie, Zoonotic aspects of arenavirus infections. Vet Microbiol, 2010. 140(3-4): p. 213-20.
160. Gowen, B.B. and B.T. Hickerson, Hemorrhagic fever of bunyavirus etiology: disease models and progress towards new therapies. J Microbiol, 2017. 55(3): p. 183-195.
161. Keller, T.T., et al., Infections and endothelial cells. Cardiovasc Res, 2003. 60(1): p. 40-8.
162. Long, S.Y., E.M. Latimer, and G.S. Hayward, Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease. ILAR J, 2016. 56(3): p. 283-96.
163. Ma, G., W. Azab, and N. Osterrieder, Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)--masters of co-evolution and a constant threat to equids and beyond. Vet Microbiol, 2013. 167(1-2): p. 123-34.
164. Parkman, P.D., Togaviruses: Rubella Virus, in Medical Microbiology, th and S. Baron, Editors. 1996: Galveston (TX).
165. Kunz, S. and J.C. de la Torre, Breaking the Barrier: Host Cell Invasion by Lujo Virus. Cell Host Microbe, 2017. 22(5): p. 583-585.
166. Mackow, E.R. and I.N. Gavrilovskaya, Hantavirus regulation of endothelial cell functions. Thromb Haemost, 2009. 102(6): p. 1030-41.
167. Chu, H., et al., Middle East Respiratory Syndrome Coronavirus Efficiently Infects Human Primary T Lymphocytes and Activates the Extrinsic and Intrinsic Apoptosis Pathways. J Infect Dis, 2016. 213(6): p. 904-14.
168. Goldstein, S.A. and S.R. Weiss, Origins and pathogenesis of Middle East respiratory syndrome-associated coronavirus: recent advances. F1000Res, 2017. 6: p. 1628.
169. Spiesschaert, B., et al., Role of gB and pUS3 in equine herpesvirus 1 transfer between peripheral blood mononuclear cells and endothelial cells: a dynamic in vitro model. Journal of virology, 2015. 89(23): p. 11899-11908.
170. Mamdouh, Z., A. Mikhailov, and W.A. Muller, Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. J Exp Med, 2009. 206(12): p. 2795-808.
171. Muller, W.A., Getting Leukocytes to the Site of Inflammation. Veterinary pathology, 2013. 50(1): p. 7-22.
172. Chen, Y.-L., et al., Activation of Peripheral Blood Mononuclear Cells by Dengue Virus Infection Depotentiates Balapiravir. Journal of Virology, 2014. 88(3): p. 1740-1747.

173. Mangada, M.M., et al., Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. The Journal of Infectious Diseases, 2002. 185(12): p. 1697-1703.
174. Muller, W.A. and G.J. Randolph, Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. J Leukoc Biol, 1999. 66(5): p. 698-704.
175. Hübner, W., et al., Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. Science, 2009. 323(5922): p. 1743-1747.
176. Martin, N., et al., Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition. Journal of virology, 2010. 84(7): p. 3516-3527.
177. Sweet, C., The pathogenicity of cytomegalovirus. FEMS microbiology reviews, 1999. 23(4): p. 457-482.
178. Saksena, M.M., et al., Herpes simplex virus type 1 accumulation, envelopment, and exit in growth cones and varicosities in mid-distal regions of axons. Journal of virology, 2006. 80(7): p. 3592-3606.
179. Dingwell, K.S., et al., Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. Journal of virology, 1994. 68(2): p. 834-845.
180. Cole, N.L. and C. Grose, Membrane fusion mediated by herpesvirus glycoproteins: the paradigm of varicella-zoster virus. Reviews in medical virology, 2003. 13(4): p. 207-222.
181. van Leeuwen, H., G. Elliott, and P. O'Hare, Evidence of a role for nonmuscle myosin II in herpes simplex virus type 1 egress. Journal of virology, 2002. 76(7): p. 3471-3481.
182. Favoreel, H.W., et al., Cytoskeletal rearrangements and cell extensions induced by the US3 kinase of an alphaherpesvirus are associated with enhanced spread. Proceedings of the National Academy of Sciences of the United States of America, 2005. 102(25): p. 8990-8995.
183. Ch'ng, T., et al., Glycoprotein D-independent spread of pseudorabies virus infection in cultured peripheral nervous system neurons in a compartmented system. Journal of virology, 2007. 81(19): p. 10742-10757.
184. Reske, A., et al., Understanding HSV-1 entry glycoproteins. Reviews in medical virology, 2007. 17(3): p. 205-215.
185. Gerna, G., et al., Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. Journal of virology, 2000. 74(12): p. 5629-5638.

186. Jansens, R.J., et al., Pseudorabies Virus US3-Induced Tunneling Nanotubes Contain Stabilized Microtubules, Interact with Neighboring Cells via Cadherins, and Allow Intercellular Molecular Communication. Journal of virology, 2017. 91(19): p. e00749-17.
187. Panasiuk, M., et al., Tunneling Nanotubes as a Novel Route of Cell-to-Cell Spread of Herpesviruses. Journal of virology, 2018. 92(10): p. e00090-18.
188. Schneeberger, E.E. and R.D. Lynch, The tight junction: a multifunctional complex. American Journal of Physiology-Cell Physiology, 2004. 286(6): p. C1213-C1228.
189. Wisner, T., et al., The extracellular domain of herpes simplex virus gE is sufficient for accumulation at cell junctions but not for cell-to-cell spread. Journal of virology, 2000. 74(5): p. 2278-2287.
190. Dingwell, K.S. and D.C. Johnson, The herpes simplex virus gE-gI complex facilitates cell-to-cell spread and binds to components of cell junctions. Journal of virology, 1998. 72(11): p. 8933-8942.
191. Trimble, W.S. and S. Grinstein, Barriers to the free diffusion of proteins and lipids in the plasma membrane. J Cell Biol, 2015. 208(3): p. 259-271.
192. Niessen, C.M., Tight junctions/adherens junctions: basic structure and function. Journal of Investigative Dermatology, 2007. 127(11): p. 2525-2532.
193. Aijaz, S., M.S. Balda, and K. Matter, Tight junctions: molecular architecture and function. International review of cytology, 2006. 248: p. 261-298.
194. Gerdes, H.-H., N.V. Bukoreshtliev, and J.F. Barroso, Tunneling nanotubes: a new route for the exchange of components between animal cells. FEBS letters, 2007. 581(11): p. 2194-2201.
195. Gerdes, H.-H. and R.N. Carvalho, Intercellular transfer mediated by tunneling nanotubes. Current opinion in cell biology, 2008. 20(4): p. 470-475.
196. Gurke, S., J.F. Barroso, and H.-H. Gerdes, The art of cellular communication: tunneling nanotubes bridge the divide. Histochemistry and cell biology, 2008. 129(5): p. 539-550.
197. Kumar, A., et al., Influenza virus exploits tunneling nanotubes for cell-to-cell spread. Scientific reports, 2017. 7: p. 40360.
198. Marzo, L., K. Gousset, and C. Zurzolo, Multifaceted roles of tunneling nanotubes in intercellular communication. Frontiers in physiology, 2012. 3: p. 72.
199. Önfelt, B., et al., Long-distance calls between cells connected by tunneling nanotubules. Sci. STKE, 2005. 2005(313): p. pe55-pe55.
200. Sowinski, S., et al., Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. Nature cell biology, 2008. 10(2): p. 211.

201. Eugenin, E., P. Gaskill, and J. Berman, Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking. Cellular immunology, 2009. 254(2): p. 142-148.
202. Kimura, S., K. Hase, and H. Ohno, The molecular basis of induction and formation of tunneling nanotubes. Cell and tissue research, 2013. 352(1): p. 67-76.
203. Peralta, B., et al., Mechanism of membranous tunnelling nanotube formation in viral genome delivery. PLoS biology, 2013. 11(9): p. e1001667.
204. Hernandez, L., et al., Virus-cell and cell-cell fusion. Annual review of cell and developmental biology, 1996. 12(1): p. 627-661.
205. Pertel, P.E., et al., Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. Virology, 2001. 279(1): p. 313-324.
206. Waldman, W.J., et al., Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: an in vitro model. Journal of Infectious Diseases, 1995. 171(2): p. 263-272.
207. Van de Walle, G.R., et al., Transmission of pseudorabies virus from immune-masked blood monocytes to endothelial cells. Journal of general virology, 2003. 84(3): p. 629-637.
208. Iwasaki, Y. and H. Clark, Cell to cell transmission of virus in the central nervous system. II. Experimental rabies in mouse. Laboratory investigation; a journal of technical methods and pathology, 1975. 33(4): p. 391-399.
209. Tomishima, M., G. Smith, and L. Enquist, Sorting and transport of alpha herpesviruses in axons. Traffic, 2001. 2(7): p. 429-436.
210. Mettenleiter, T.C., Pathogenesis of neurotropic herpesviruses: role of viral glycoproteins in neuroinvasion and transneuronal spread. Virus research, 2003. 92(2): p. 197-206.
211. Jolly, C., I. Mitar, and Q.J. Sattentau, Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells. Journal of virology, 2007. 81(24): p. 13916-13921.
212. Aubert, M., et al., The virological synapse facilitates herpes simplex virus entry into T cells. Journal of virology, 2009. 83(12): p. 6171-6183.
213. Dixit, R., V. Tiwari, and D. Shukla, Herpes simplex virus type 1 induces filopodia in differentiated P19 neural cells to facilitate viral spread. Neuroscience letters, 2008. 440(2): p. 113-118.
214. Sattentau, Q., Avoiding the void: cell-to-cell spread of human viruses. Nature Reviews Microbiology, 2008. 6(11): p. 815.

215. Digel, M., et al., Evidence for direct transfer of cytoplasmic material from infected to uninfected cells during cell-associated spread of human cytomegalovirus. Journal of clinical virology, 2006. 37(1): p. 10-20.
216. Chowdhury, S., G. Kubin, and H. Ludwig, Equine herpesvirus type 1 (EHV-1) induced abortions and paralysis in a Lipizzaner stud: a contribution to the classification of equine herpesviruses. Archives of virology, 1986. 90(3-4): p. 273-288.
217. Kydd, J.H., H.G. Townsend, and D. Hannant, The equine immune response to equine herpesvirus-1: the virus and its vaccines. Veterinary immunology and immunopathology, 2006. 111(1): p. 15-30.
218. Laval, K., et al., Equine herpesvirus type 1 enhances viral replication in CD172a+ monocytic cells upon adhesion to endothelial cells. Journal of virology, 2015. 89(21): p. 10912-10923.
219. Drebert, Z., et al., Equid herpesvirus type 1 (EHV-1) disrupts actin cytoskeleton during productive infection in equine leukocytes. Polish journal of veterinary sciences, 2015. 18(1): p. 107-112.
220. Stavolone, L. and V. Lionetti, Extracellular Matrix in Plants and Animals: Hooks and Locks for Viruses. Frontiers in microbiology, 2017. 8: p. 1760.
221. Bonnans, C., J. Chou, and Z. Werb, Remodelling the extracellular matrix in development and disease. Nature reviews Molecular cell biology, 2014. 15(12): p. 786.
222. Humphrey, J.D., E.R. Dufresne, and M.A. Schwartz, Mechanotransduction and extracellular matrix homeostasis. Nature reviews Molecular cell biology, 2014. 15(12): p. 802.
223. Dimitrov, D.S., Virus entry: molecular mechanisms and biomedical applications. Nature Reviews Microbiology, 2004. 2(2): p. 109.
224. DiGiuseppe, S., et al., Cruising the cellular highways: How human papillomavirus travels from the surface to the nucleus. Virus research, 2017. 231: p. 1-9.
225. Richards, K.F., et al., Human papillomavirus species-specific interaction with the basement membrane-resident non-heparan sulfate receptor. Viruses, 2014. 6(12): p. 4856-4879.
226. Hidari, K.I. and T. Suzuki, Dengue virus receptor. Tropical medicine and health, 2011. 39(4SUPPLEMENT): p. S37-S43.
227. Akhtar, J. and D. Shukla, Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. The FEBS journal, 2009. 276(24): p. 7228-7236.
228. Pais-Correia, A.-M., et al., Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses. Nature medicine, 2010. 16(1): p. 83-89.

229. Rappocciolo, G., J. Birch, and S.A. Ellis, Down-regulation of MHC class I expression by equine herpesvirus-1. Journal of General Virology, 2003. 84(2): p. 293-300.
230. Ambagala, A.P., R.S. Gopinath, and S. Srikumaran, Peptide transport activity of the transporter associated with antigen processing (TAP) is inhibited by an early protein of equine herpesvirus-1. Journal of General Virology, 2004. 85(2): p. 349-353.
231. van der Meulen, K.M., H.J. Nauwynck, and M.B. Pensaert, Absence of viral antigens on the surface of equine herpesvirus-1-infected peripheral blood mononuclear cells: a strategy to avoid complement-mediated lysis. Journal of general virology, 2003. 84(1): p. 93-97.
232. Ellis, R.W., New technologies for making vaccines. Vaccine, 1999. 17(13-14): p. 1596-604.
233. Plotkin, S., History of vaccination. Proceedings of the National Academy of Sciences, 2014. 111(34): p. 12283-12287.
234. Leitner, W.W., H. Ying, and N.P. Restifo, DNA and RNA-based vaccines: principles, progress and prospects. Vaccine, 1999. 18(9-10): p. 765-777.
235. Saade, F. and N. Petrovsky, Technologies for enhanced efficacy of DNA vaccines. Expert review of vaccines, 2012. 11(2): p. 189-209.
236. Ulmer, J.M., et al., Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. Vaccine, 1994. 12(16): p. 1541-1544.
237. Osorio, J., et al., Immunization of dogs and cats with a DNA vaccine against rabies virus. Vaccine, 1999. 17(9-10): p. 1109-1116.
238. Hall, R.A., et al., DNA vaccine coding for the full-length infectious Kunjin virus RNA protects mice against the New York strain of West Nile virus. Proceedings of the National Academy of Sciences, 2003. 100(18): p. 10460-10464.
239. Davis, H.L., M.-L. Michel, and R.G. Whalen, DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. Human Molecular Genetics, 1993. 2(11): p. 1847-1851.
240. San Zhao, L., et al., DNA-based vaccination induces humoral and cellular immune responses against hepatitis B virus surface antigen in mice without activation of C-myc. World journal of gastroenterology, 2000. 6(2): p. 239.
241. Ura, T., K. Okuda, and M. Shimada, Developments in viral vector-based vaccines. Vaccines, 2014. 2(3): p. 624-641.
242. Rollier, C.S., et al., Viral vectors as vaccine platforms: deployment in sight. Current opinion in immunology, 2011. 23(3): p. 377-382.

243. Sutter, G., et al., A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. Vaccine, 1994. 12(11): p. 1032-1040.
244. Paoletti, E., Applications of pox virus vectors to vaccination: an update. Proceedings of the National Academy of Sciences, 1996. 93(21): p. 11349-11353.
245. van der Meulen, K., et al., The protective properties of vaccination against equine herpesvirus 1-induced viremia, abortion and nervous system disorders. Vlaams diergeneeskundig tijdschrift, 2007. 76(3): p. 186-194.
246. Prymus, T., et al., Foetal and neonatal foal losses in equine herpesvirus type 1 (EHV-1) infected farms before and after EHV-1 vaccination. Archivum Veterinarium Polonicum, 1986.
247. Bresgen, C., et al., Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines. Veterinary microbiology, 2012. 160(1): p. 9-16.
248. Becker, W., Preventative vaccination against EHV (equine herpesvirus) abortion. Tierarztliche Praxis, 1987. 16(1): p. 61-63.
249. Goodman, L.B., et al., Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1). Vaccine, 2006. 24(17): p. 3636-3645.
250. Goehring, L., et al., Control of EHV-1 viremia and nasal shedding by commercial vaccines. Vaccine, 2010. 28(32): p. 5203-5211.
251. Bürki, F., et al., Viraemia and abortions are not prevented by two commercial Equine Herpesvirus-1 vaccines after experimental challenge of horses. Veterinary Quarterly, 1990. 12(2): p. 80-86.
252. Matsumura, T., et al., Lack of virulence of the murine fibroblast adapted strain, Kentucky A (KyA), of equine herpesvirus type 1 (EHV-1) in young horses. Veterinary microbiology, 1996. 48(3-4): p. 353-365.
253. Patel, J., et al., Derivation and characterisation of a live equid herpes virus-1 (EHV-1) vaccine to protect against abortion and respiratory disease due to EHV-1. Veterinary microbiology, 2003. 91(1): p. 23-39.
254. Patel, J., et al., Equid herpesvirus (EHV-1) live vaccine strain C147: efficacy against respiratory diseases following EHV types 1 and 4 challenges. Veterinary microbiology, 2003. 92(1): p. 1-17.
255. Slater, J., J. Gibson, and H. Field, Pathogenicity of a thymidine kinase-deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. Journal of general virology, 1993. 74(5): p. 819-828.

256. Van de Walle, G.R., et al., A vectored equine herpesvirus type 1 (EHV-1) vaccine elicits protective immune responses against EHV-1 and H3N8 equine influenza virus. Vaccine, 2010. 28(4): p. 1048-1055.
257. Minke, J.M., J.-C. Audonnet, and L. Fischer, Equine viral vaccines: the past, present and future. Veterinary research, 2004. 35(4): p. 425-443.
258. Burrows, R., D. Goodridge, and M. Denyer, Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. The Veterinary Record, 1984. 114(15): p. 369-374.
259. Hannant, D., et al., Responses of ponies to equid herpesvirus-1 ISCOM vaccination and challenge with virus of the homologous strain. Research in veterinary science, 1993. 54(3): p. 299-305.
260. Foote, C., et al., Inoculation of mares and very young foals with EHV-1 glycoproteins D and B reduces virus shedding following respiratory challenge with EHV-1. Veterinary immunology and immunopathology, 2006. 111(1): p. 97-108.
261. Minke, J., et al., Use of DNA and recombinant canarypox viral (ALVAC) vectors for equine herpes virus vaccination. Veterinary immunology and immunopathology, 2006. 111(1): p. 47-57.
262. Soboll, G., et al., Vaccination of ponies with the IE gene of EHV-1 in a recombinant modified live vaccinia vector protects against clinical and virological disease. Veterinary immunology and immunopathology, 2010. 135(1): p. 108-117.
263. Gurunathan, S., D.M. Klinman, and R.A. Seder, DNA vaccines: immunology, application, and optimization. Annual review of immunology, 2000. 18(1): p. 927-974.
264. Soboll, G., et al., Antibody and cellular immune responses following DNA vaccination and EHV-1 infection of ponies. Veterinary immunology and immunopathology, 2006. 111(1): p. 81-95.
265. Liu, S.A., et al., Intramuscular immunization of mice with the live-attenuated herpes simplex virus 1 vaccine strain VC2 expressing equine herpesvirus 1 (EHV-1) glycoprotein D generates anti-EHV-1 immune responses in mice. Journal of virology, 2017. 91(12): p. e02445-16.
266. Packiarajah, P., et al., 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. Veterinary microbiology, 1998. 61(4): p. 261-278.
267. Ruitenberg, K.M., et al., A prime-boost immunization strategy with DNA and recombinant baculovirus-expressed protein enhances protective immunogenicity of

glycoprotein D of equine herpesvirus 1 in naïve and infection-primed mice. Vaccine, 2000. 18(14): p. 1367-1373.

268. Ruitenberg, K.M., et al., Equine herpesvirus 1 glycoprotein D expressed in Pichia pastoris is hyperglycosylated and elicits a protective immune response in the mouse model of EHV-1 disease. Virus Research, 2001. 79(1): p. 125-135.
269. Huemer, H.P., B. Strobl, and N. Nowotny, Use of apathogenic vaccinia virus MVA expressing EHV-1 gC as basis of a combined recombinant MVA/DNA vaccination scheme. Vaccine, 2000. 18(14): p. 1320-1326.
270. Munro, K.I., et al., Characteristics of glycoprotein B of equine herpesvirus 1 expressed by a recombinant baculovirus. Veterinary Microbiology, 1999. 68(1): p. 49-57.
271. Shizuya, H., et al., Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proceedings of the National Academy of Sciences, 1992. 89(18): p. 8794-8797.
272. Wagner, M., Z. Ruzsics, and U.H. Koszinowski, Herpesvirus genetics has come of age. Trends in Microbiology, 2002. 10(7): p. 318-324.
273. She, K., So you want to work with giants: the BAC vector. BioTech. J, 2003. 1: p. 69-74.
274. Zhou, G. and B. Roizman, Characterization of a recombinant herpes simplex virus 1 designed to enter cells via the IL13Rα2 receptor of malignant glioma cells. Journal of virology, 2005. 79(9): p. 5272-5277.
275. Roizman, B., The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors. Proceedings of the National Academy of Sciences, 1996. 93(21): p. 11307-11312.
276. Rudolph, J., D. O'CALLAGHAN, and N. Osterrieder, Cloning of the Genomes of Equine Herpesvirus Type 1 (EHV-1) Strains KyA and RacL11 as Bacterial Artificial Chromosomes (BAC). Journal of Veterinary Medicine, Series B, 2002. 49(1): p. 31-36.
277. Trapp, S., et al., Potential of equine herpesvirus 1 as a vector for immunization. Journal of virology, 2005. 79(9): p. 5445-5454.
278. Hübert, P., et al., Alterations in the Equine Herpesvirus Type-1 (EHV-1) Strain RacH During Attenuation. Journal of Veterinary Medicine, Series B, 1996. 43(1-10): p. 1-14.
279. Neubauer, A., A. Meindl, and N. Osterrieder, [Mutations in the US2 and glycoprotein B genes of the equine herpesvirus 1 vaccine strain RacH have no effects on its attenuation]. Berliner und Munchener Tierarztliche Wochenschrift, 1999. 112(9): p. 351-354.

280. Osterrieder, N., et al., The equine herpesvirus 1 lR6 protein influences virus growth at elevated temperature and is a major determinant of virulence. Virology, 1996. 226(2): p. 243-251.
281. Rosas, C.T., et al., Evaluation of the vaccine potential of an equine herpesvirus type 1 vector expressing bovine viral diarrhea virus structural proteins. Journal of General Virology, 2007. 88(3): p. 748-757.
282. Rosas, C.T., et al., Equine herpesvirus type 1 modified live virus vaccines: quo vaditis? Expert review of vaccines, 2006. 5(1): p. 119-131.
283. Rosas, C.T., et al., Protection of Mice by Equine Herpesvirus Type 1–Based Experimental Vaccine against Lethal Venezuelan Equine Encephalitis Virus Infection in the Absence of Neutralizing Antibodies. The American journal of tropical medicine and hygiene, 2008. 78(1): p. 83-92.
284. Rosas, C.T., et al., Live-attenuated recombinant equine herpesvirus type 1 (EHV-1) induces a neutralizing antibody response against West Nile virus (WNV). Virus research, 2007. 125(1): p. 69-78.
285. Said, A., et al., An equine herpesvirus 1 (EHV-1) vectored H1 vaccine protects against challenge with swine-origin influenza virus H1N1. Veterinary microbiology, 2011. 154(1): p. 113-123.
286. Said, A., et al., Recombinant equine herpesvirus 1 (EHV-1) vaccine protects pigs against challenge with influenza A (H1N1) pmd09. Virus research, 2013. 173(2): p. 371-376.
287. Ma, G., et al., An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model. PloS one, 2012. 7(4): p. e34425.
288. Rosas, C., et al., Evaluation of a vectored equine herpesvirus type 1 (EHV-1) vaccine expressing H3 haemagglutinin in the protection of dogs against canine influenza. Vaccine, 2008. 26(19): p. 2335-2343.
289. Pan, Z., et al., The recombinant EHV-1 vector producing CDV hemagglutinin as potential vaccine against canine distemper. Microbial pathogenesis, 2017. 111: p. 388-394.
290. Said, A., et al., An equine herpesvirus type 1 (EHV-1) vector expressing Rift Valley fever virus (RVFV) Gn and Gc induces neutralizing antibodies in sheep. Virology journal, 2017. 14(1): p. 154.
291. Alexandersen, S., et al., The pathogenesis and diagnosis of foot-and-mouth disease. Journal of comparative pathology, 2003. 129(1): p. 1-36.

292. Stenfeldt, C., et al., The pathogenesis of foot-and-mouth disease in pigs. Frontiers in veterinary science, 2016. 3: p. 41.
293. Arzt, J., et al., The Pathogenesis of Foot-and-Mouth Disease II: Viral Pathways in Swine, Small Ruminants, and Wildlife; Myotropism, Chronic Syndromes, and Molecular Virus–Host Interactions. Transboundary and emerging diseases, 2011. 58(4): p. 305-326.
294. Stenfeldt, C., et al., Early events in the pathogenesis of foot-and-mouth disease in pigs; identification of oropharyngeal tonsils as sites of primary and sustained viral replication. PLoS One, 2014. 9(9): p. e106859.
295. Arzt, J., J. Pacheco, and L. Rodriguez, The early pathogenesis of foot-and-mouth disease in cattle after aerosol inoculation: identification of the nasopharynx as the primary site of infection. Veterinary pathology, 2010. 47(6): p. 1048-1063.
296. Salt, J., The carrier state in foot and mouth disease—an immunological review. British Veterinary Journal, 1993. 149(3): p. 207-223.
297. Burrows, R., et al., The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle. Journal of comparative pathology, 1981. 91(4): p. 599-609.
298. Bates, T.W., M.C. Thurmond, and T.E. Carpenter, Direct and indirect contact rates among beef, dairy, goat, sheep, and swine herds in three California counties, with reference to control of potential foot-and-mouth disease transmission. American journal of veterinary research, 2001. 62(7): p. 1121-1129.
299. Paton, D.J., K.J. Sumption, and B. Charleston, Options for control of foot-and-mouth disease: knowledge, capability and policy. Philosophical Transactions of the Royal Society B: Biological Sciences, 2009. 364(1530): p. 2657-2667.
300. Scudamore, J. and D. Harris, Control of foot and mouth disease: lessons from the experience of the outbreak in Great Britain in 2001. Revue scientifique et technique-Office international des épizooties, 2002. 21(3): p. 699-707.
301. Mason, P.W., M.J. Grubman, and B. Baxt, Molecular basis of pathogenesis of FMDV. Virus research, 2003. 91(1): p. 9-32.
302. Carrillo, C., et al., Comparative genomics of foot-and-mouth disease virus. Journal of virology, 2005. 79(10): p. 6487-6504.
303. Upadhyaya, S., et al., Genetic basis of antigenic variation in foot-and-mouth disease serotype A viruses from the Middle East. Vaccine, 2014. 32(5): p. 631-638.
304. Doel, T., FMD vaccines. Virus research, 2003. 91(1): p. 81-99.
305. Parida, S., Vaccination against foot-and-mouth disease virus: strategies and effectiveness. Expert review of vaccines, 2009. 8(3): p. 347-365.

306. Brehm, K., et al., High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. Vaccine, 2008. 26(13): p. 1681-1687.
307. Golde, W.T., et al., Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: use in emergency outbreak response. Vaccine, 2005. 23(50): p. 5775-5782.
308. Commission, I.O.o.E.B.S. and I.O.o.E.I. Committee, Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. Vol. 2. 2008: Office international des épizooties.
309. Stenfeldt, C., et al., Detection of Foot-and-mouth Disease Virus RNA and Capsid Protein in Lymphoid Tissues of Convalescent Pigs Does Not Indicate Existence of a Carrier State. Transboundary and emerging diseases, 2016. 63(2): p. 152-164.
310. Li, P., et al., Evaluation of a 3A-truncated foot-and-mouth disease virus in pigs for its potential as a marker vaccine. Veterinary research, 2014. 45(1): p. 51.
311. Sanz-Parra, A., et al., Recombinant Viruses Expressing the Foot-and-Mouth Disease Virus Capsid Precursor Polypeptide (P1) Induce Cellular but Not Humoral Antiviral Immunity and Partial Protection in Pigs. Virology, 1999. 259(1): p. 129-134.
312. Ma, M., et al., Immune responses of swine inoculated with a recombinant fowlpox virus co-expressing P12A and 3C of FMDV and swine IL-18. Veterinary Immunology and Immunopathology, 2008. 121(1): p. 1-7.
313. Zhang, K., et al., Recombinant pseudorabies virus expressing P12A and 3C of FMDV can partially protect piglets against FMDV challenge. Research in Veterinary Science, 2011. 91(1): p. 90-94.
314. Gullberg, M., et al., A Prime-Boost Vaccination Strategy in Cattle to Prevent Foot-and-Mouth Disease Using a “Single-Cycle” Alphavirus Vector and Empty Capsid Particles. PloS one, 2016. 11(6): p. e0157435.
315. Grubman, M.J., et al., Use of replication-defective adenoviruses to develop vaccines and biotherapeutics against foot-and-mouth disease. Future Virology, 2012. 7(8): p. 767-778.
316. Mayr, G.A., J. Chinsangaram, and M.J. Grubman, Development of Replication-Defective Adenovirus Serotype 5 Containing the Capsid and 3C Protease Coding Regions of Foot-and-Mouth Disease Virus as a Vaccine Candidate. Virology, 1999. 263(2): p. 496-506.
317. Kim, S.-M., et al., Robust protection against highly virulent foot-and-mouth disease virus in swine by combination treatment with recombinant adenoviruses expressing porcine alpha and gamma interferons and multiple small interfering RNAs. Journal of virology, 2015. 89(16): p. 8267-8279.

318. Wong, H., et al., Plasmids encoding foot-and-mouth disease virus VP1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. Virology, 2000. 278(1): p. 27-35.
319. Beard, C., et al., Development of DNA vaccines for foot-and-mouth disease, evaluation of vaccines encoding replicating and non-replicating nucleic acids in swine. Journal of biotechnology, 1999. 73(2-3): p. 243-249.
320. Borrego, B., et al., A DNA vaccine encoding foot-and-mouth disease virus B and T-cell epitopes targeted to class II swine leukocyte antigens protects pigs against viral challenge. Antiviral research, 2011. 92(2): p. 359-363.
321. İz, S.G., et al., Co-expression of the Bcl-xL antiapoptotic protein enhances the induction of Th1-like immune responses in mice immunized with DNA vaccines encoding FMDV B and T cell epitopes. Veterinary research communications, 2013. 37(3): p. 187-196.
322. Nanda, R.K., et al., An effective mannosylated chitosan nanoparticle DNA vaccine for FMD virus. Virologica Sinica, 2012. 27(6): p. 372-375.
323. Bachrach, H.L., et al., Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. The Journal of Immunology, 1975. 115(6): p. 1636-1641.
324. Wang, C.Y., et al., Effective synthetic peptide vaccine for foot-and-mouth disease in swine. Vaccine, 2002. 20(19-20): p. 2603-2610.
325. Cao, Y., et al., A study on mutative scale straightness measurement based on uncertainty analysis. Measurement, 2013. 46(1): p. 145-153.
326. Blanco, E., et al., Full protection of swine against foot-and-mouth disease by a bivalent B-cell epitope dendrimer peptide. Antiviral research, 2016. 129: p. 74-80.
327. Cubillos, C., et al., Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. Journal of virology, 2008. 82(14): p. 7223-7230.
328. Chinsangaram, J., P.W. Mason, and M.J. Grubman, Protection of swine by live and inactivated vaccines prepared from a leader proteinase-deficient serotype A12 foot-and-mouth disease virus. Vaccine, 1998. 16(16): p. 1516-1522.
329. Mason, P., et al., Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. Virology, 1997. 227(1): p. 96-102.
330. Díaz-San Segundo, F., et al., Inoculation of swine with foot-and-mouth disease SAP-mutant virus induces early protection against disease. Journal of virology, 2012. 86(3): p. 1316-1327.
331. Uddowla, S., et al., Characterization of a chimeric foot-and-mouth disease virus bearing a bovine rhinitis B virus leader proteinase. Virology, 2013. 447(1-2): p. 172-180.

332. Diaz-San Segundo, F., et al., Synonymous deoptimization of foot-and-mouth disease virus causes attenuation in vivo while inducing a strong neutralizing antibody response. Journal of virology, 2016. 90(3): p. 1298-1310.
333. Guo, H.-C., et al., Foot-and-mouth disease virus-like particles produced by a SUMO fusion protein system in Escherichia coli induce potent protective immune responses in guinea pigs, swine and cattle. Veterinary research, 2013. 44(1): p. 48.
334. Wigdorovitz, A., et al., Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology, 1999. 255(2): p. 347-353.
335. Wigdorovitz, A., et al., Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology, 1999. 264(1): p. 85-91.
336. Wilson, W.D., Equine herpesvirus 1 myeloencephalopathy. Vet Clin North Am Equine Pract, 1997. 13(1): p. 53-72.
337. Wellington, J.E., D.N. Love, and J.M. Whalley, Evidence for involvement of equine herpesvirus 1 glycoprotein B in cell-cell fusion. Arch Virol, 1996. 141(1): p. 167-75.
338. Birtley, J.R., et al., Crystal structure of foot-and-mouth disease virus 3C protease. New insights into catalytic mechanism and cleavage specificity. J Biol Chem, 2005. 280(12): p. 11520-7.
339. Mason, P.W., et al., Engineering better vaccines for foot-and-mouth disease. Dev Biol (Basel), 2003. 114: p. 79-88.
340. Borrego, B., et al., DNA vaccines expressing B and T cell epitopes can protect mice from FMDV infection in the absence of specific humoral responses. Vaccine, 2006. 24(18): p. 3889-99.
341. Doel, T.R., et al., Heterotypic protection induced by synthetic peptides corresponding to three serotypes of foot-and-mouth disease virus. J Virol, 1990. 64(5): p. 2260-4.
342. Yi, J.Z., et al., Recombinant bivalent vaccine against foot-and-mouth disease virus serotype O/A infection in guinea pig. Acta Biochim Biophys Sin (Shanghai), 2004. 36(9): p. 589-96.
343. Qian, P., et al., An approach to a FMD vaccine based on genetic engineered attenuated pseudorabies virus: one experiment using VP1 gene alone generates an antibody responds on FMD and pseudorabies in swine. Vaccine, 2004. 22(17-18): p. 2129-36.
344. Zheng, M., et al., Construction and immunogenicity of a recombinant fowlpox virus containing the capsid and 3C protease coding regions of foot-and-mouth disease virus. J Virol Methods, 2006. 136(1-2): p. 230-7.

345. Du, Y., et al., Immune responses of two recombinant adenoviruses expressing VP1 antigens of FMDV fused with porcine granulocyte macrophage colony-stimulating factor. Vaccine, 2007. 25(49): p. 8209-19.
346. Trapp, S., et al., Potential of equine herpesvirus 1 as a vector for immunization. J Virol, 2005. 79(9): p. 5445-54.
347. Goehring, L., et al., Infection of central nervous system endothelial cells by cell-associated EHV-1. Veterinary microbiology, 2011. 148(2-4): p. 389-395.
348. Tischer, B.K., G.A. Smith, and N. Osterrieder, , En passant mutagenesis: a two step markerless red recombination system. . Methods Mol Biol,, 2010. . 634: p. 421-30. .
349. Azab, W., et al., Cloning of the genome of equine herpesvirus 4 strain TH20p as an infectious bacterial artificial chromosome. Archives of virology, 2009. 154(5): p. 833-842.
350. Ma, G., et al., An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model. PloS one, 2012. 7(4): p. e34425-e34425.
351. Fan, Q., R. Longnecker, and S.A. Connolly, A Functional Interaction between Herpes Simplex Virus 1 Glycoprotein gH/gL Domains I and II and gD Is Defined by Using Alphaherpesvirus gH and gL Chimeras. Journal of Virology, 2015. 89(14): p. 7159-7169.
352. Fan, Q., et al., Herpes B virus utilizes human nectin-1 but not HVEM or PILRalpha for cell-cell fusion and virus entry. J Virol, 2012. 86(8): p. 4468-76.
353. Tischer, B.K., et al., Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques, 2006. 40(2): p. 191-7.
354. Campbell, R.E., et al., A monomeric red fluorescent protein. Proceedings of the National Academy of Sciences, 2002. 99(12): p. 7877-7882.
355. Tischer, B.K., G.A. Smith, and N. Osterrieder, En passant mutagenesis: a two step markerless red recombination system, in In vitro mutagenesis protocols. 2010, Springer. p. 421-430.
356. Hudetz, A.G., G. Fehér, and J.P. Kampine, Heterogeneous autoregulation of cerebrocortical capillary flow: evidence for functional thoroughfare channels? Microvascular research, 1996. 51(1): p. 131-136.
357. AlHajri, S.M., et al., Ovine herpesvirus 2 glycoproteins B, H, and L are sufficient for, and viral glycoprotein Ov8 can enhance, cell-cell membrane fusion. Journal of virology, 2017. 91(6): p. e02454-16.

358. Fan, Q., et al., Herpes B virus utilizes human nectin-1 but not HVEM or PILRα for cell-cell fusion and virus entry. Journal of virology, 2012. 86(8): p. 4468-4476.
359. van der Meulen, K.M., H.J. Nauwynck, and M.B. Pensaert, Mitogen stimulation favours replication of equine herpesvirus-1 in equine blood mononuclear cells by inducing cell proliferation and formation of close intercellular contacts. Journal of General Virology, 2001. 82(8): p. 1951-1957.
360. Schimmel, L., N. Heemskerk, and J.D. van Buul, Leukocyte transendothelial migration: a local affair. Small GTPases, 2017. 8(1): p. 1-15.
361. Wang, L., et al., Visualization of HIV T cell virological synapses and virus-containing compartments by three-dimensional correlative light and electron microscopy. Journal of virology, 2017. 91(2): p. e01605-16.
362. Sherer, N.M., et al., Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. Nature cell biology, 2007. 9(3): p. 310.
363. Bennett, A.E., et al., Ion-abrasion scanning electron microscopy reveals surface-connected tubular conduits in HIV-infected macrophages. PLoS pathogens, 2009. 5(9): p. e1000591.
364. Igakura, T., et al., Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. Science, 2003. 299(5613): p. 1713-1716.
365. Zhong, P., et al., Cell-to-cell transmission of viruses. Current opinion in virology, 2013. 3(1): p. 44-50.
366. Mothes, W., et al., Virus cell-to-cell transmission. Journal of virology, 2010. 84(17): p. 8360-8368.
367. Stierstorfer, B., et al., Equine Herpesvirus Type 1 (EHV-1) Myeloencephalopathy: a Case Report. Zoonoses and Public Health, 2002. 49(1): p. 37-41.
368. van der Meulen, K.M., H.J. Nauwynck, and M.B. Pensaert, Increased susceptibility of peripheral blood mononuclear cells to equine herpes virus type 1 infection upon mitogen stimulation: a role of the cell cycle and of cell-to-cell transmission of the virus. Veterinary microbiology, 2002. 86(1): p. 157-163.
369. Jin, J., et al., Assembly of the murine leukemia virus is directed towards sites of cell–cell contact. PLoS biology, 2009. 7(7): p. e1000163.
370. Sherer, N.M., et al., Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. Nature cell biology, 2007. 9(3): p. 310-315.
371. Rudnicka, D., et al., Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses. Journal of virology, 2009. 83(12): p. 6234-6246.

372. Humbert, M. and U. Dietrich, The role of neutralizing antibodies in HIV infection. AIDS Rev, 2006. 8(2): p. 51-9.
373. Varmus, H., Retroviruses. Science, 1988. 240(4858): p. 1427-1435.
374. Sherer, N.M., J. Jin, and W. Mothes, Directional spread of surface-associated retroviruses regulated by differential virus-cell interactions. Journal of virology, 2010. 84(7): p. 3248-3258.
375. Schaefer, L. and R.M. Schaefer, Proteoglycans: from structural compounds to signaling molecules. Cell and tissue research, 2010. 339(1): p. 237.
376. Järveläinen, H., et al., Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacological reviews, 2009. 61(2): p. 198-223.
377. O'Donnell, C.D. and D. Shukla, The importance of heparan sulfate in herpesvirus infection. Virologica sinica, 2008. 23(6): p. 383-393.
378. Barth, H., et al., Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. Journal of Biological Chemistry, 2003. 278(42): p. 41003-41012.
379. Chen, Y., et al., Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nature medicine, 1997. 3(8): p. 866.
380. Tyagi, M., et al., Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. Journal of Biological Chemistry, 2001. 276(5): p. 3254-3261.
381. Shukla, D. and P.G. Spear, Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. The Journal of clinical investigation, 2001. 108(4): p. 503-510.
382. Alfsen, A., et al., HIV-1-infected blood mononuclear cells form an integrin-and agrin-dependent viral synapse to induce efficient HIV-1 transcytosis across epithelial cell monolayer. Molecular biology of the cell, 2005. 16(9): p. 4267-4279.
383. Dustin, M.L. and L.B. Dustin, The immunological relay race: B cells take antigen by synapse. Nature immunology, 2001. 2(6): p. 480-483.
384. Khan, A.A., et al., Physiological regulation of the immunological synapse by agrin. Science, 2001. 292(5522): p. 1681-1686.
385. Dityatev, A. and M. Schachner, The extracellular matrix and synapses. Cell and tissue research, 2006. 326(2): p. 647-654.
386. Vaheri, A., et al., The ezrin protein family: membrane-cytoskeleton interactions and disease associations. Current opinion in cell biology, 1997. 9(5): p. 659-666.
387. Mikaty, G., et al., Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. PLoS pathogens, 2009. 5(2): p. e1000314.

388. Alais, S., R. Mahieux, and H. Dutartre, Viral source-independent high susceptibility of dendritic cells to human T-cell leukemia virus type 1 infection compared to that of T lymphocytes. Journal of virology, 2015. 89(20): p. 10580-10590.
389. Yaacov, B., et al., Extracellular matrix constituents interfere with Newcastle disease virus spread in solid tissue and diminish its potential oncolytic activity. Journal of General Virology, 2012. 93(8): p. 1664-1672.
390. van der Meulen, K., et al., Absence of viral envelope proteins in equine herpesvirus 1-infected blood mononuclear cells during cell-associated viremia. Veterinary microbiology, 2006. 113(3): p. 265-273.
391. Favoreel, H.W., et al., Antibody-induced endocytosis of viral glycoproteins and major histocompatibility complex class I on pseudorabies virus-infected monocytes. Journal of general virology, 1999. 80(5): p. 1283-1291.
392. Takeuchi, K., et al., Wild-type measles virus induces large syncytium formation in primary human small airway epithelial cells by a SLAM (CD150)-independent mechanism. Virus research, 2003. 94(1): p. 11-16.
393. Weed, D.J. and A.V. Nicola, Herpes simplex virus membrane fusion, in Cell Biology of Herpes Viruses. 2017, Springer. p. 29-47.
394. Johnson, D.C., et al., Herpes Simplex Virus gE/gI Sorts Nascent Virions to Epithelial Cell Junctions, Promoting Virus Spread. Journal of Virology, 2001. 75(2): p. 821-833.
395. Ivey, N.S., A.G. MacLean, and A.A. Lackner, Acquired immunodeficiency syndrome and the blood-brain barrier. Journal of neurovirology, 2009. 15(2): p. 111-122.
396. Fletcher, N.F., et al., The neuropathogenesis of feline immunodeficiency virus infection: Barriers to overcome. The Veterinary Journal, 2011. 188(3): p. 260-269.
397. Spindler, K.R. and T.-H. Hsu, Viral disruption of the blood–brain barrier. Trends in microbiology, 2012. 20(6): p. 282-290.
398. Ratajczak, J., et al., Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia, 2006. 20(5): p. 847-56.
399. Iglesias, D.M., et al., Stem cell microvesicles transfer cystinosin to human cystinotic cells and reduce cystine accumulation in vitro. PLoS One, 2012. 7(8): p. e42840.
400. Valadi, H., et al., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol, 2007. 9(6): p. 654-9.
401. Bello-Morales, R., et al., Role of Microvesicles in the Spread of Herpes Simplex Virus 1 in Oligodendrocytic Cells. J Virol, 2018. 92(10).
402. Raposo, G. and W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol, 2013. 200(4): p. 373-83.

403. Yuana, Y., A. Sturk, and R. Nieuwland, Extracellular vesicles in physiological and pathological conditions. Blood Rev, 2013. 27(1): p. 31-9.
404. Colombo, M., G. Raposo, and C. Thery, Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol, 2014. 30: p. 255-89.
405. Akers, J.C., et al., Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol, 2013. 113(1): p. 1-11.
406. Meckes, D.G. and N. Raab-Traub, Microvesicles and Viral Infection. Journal of Virology, 2011. 85(24): p. 12844-12854.
407. Camussi, G., et al., Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int, 2010. 78(9): p. 838-48.
408. Springer, T.A., D.B. Teplow, and W.J. Dreyer, Sequence homology of the LFA-1 and Mac-1 leukocyte adhesion glycoproteins and unexpected relation to leukocyte interferon. Nature, 1985. 314(6011): p. 540-542.
409. Laudanna, C. and R. Alon, Right on the spot. Thrombosis and haemostasis, 2006. 95(1): p. 5-11.
410. Leick, M., et al., Leukocyte recruitment in inflammation: basic concepts and new mechanistic insights based on new models and microscopic imaging technologies. Cell and tissue research, 2014. 355(3): p. 647-656.
411. A., M., et al., Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde1). Zentralblatt für Veterinärmedizin Reihe B, 1968. 15(6): p. 406

12 Publications

Talks

Mononuclear cells transport equine herpesvirus type 1 (EHV-1) to the endothelium and transfer virus by cell-to-cell spread Fourth Havemeyer Equine Herpesvirus Workshop, Beaufort, North Carolina, USA; 7th – 11th October 2018.

Mononuclear cells transport equine alphaherpesvirus type 1 (EAHV-1) to the endothelium and transfer virus by cell-to-cell spread The 42th international herpesvirus workshop. Belgium, Ghent; July 29- Aug 2, 2017.

Equine herpesvirus type 1 (EHV-1) conquers and reprograms mononuclear cells to ensure transfer to endothelial cells. 10th international equine infectious diseases conference. Buenos Aires, Argentina; April 4-8, 2016.

Posters

Mononuclear cells transport equine alphaherpesvirus type 1 (EAHV-1) to the endothelium and transfer virus by cell-to-cell spread The 42th international herpesvirus workshop. Belgium, Ghent; July 29- Aug 2, 2017.

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Thanks a lot
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Selbständigkeitserklärung

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

Berlin, den 16.10.2018 Mohamed Kamel

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